Proceedings of the 2nd Fungal Genomics, 3rd Fumonisin Elimination and 15th Aflatoxin Elimination Workshops 2002

October 23 - 25, 2002
San Antonio, Texas

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SAN ANTONIO, TEXAS
OCTOBER 23-25, 2002

Mycotoxin contamination of food and feeds is a food safety problem in the U.S., and throughout the world. Many countries, especially in the developing world, experience contamination of domestic-grown commodities to alarmingly greater degrees than does the U.S. A recent study revealed a strong association between exposure to aflatoxin in West African children and both stunting (a reflection of chronic malnutrition) and being underweight (a reflection of acute malnutrition). People are chronically exposed to high levels of aflatoxins in this region, beginning in *utero* and continuing through life.

Among mycotoxin research, aflatoxin investigations have occupied "center stage" for many years, due in no small part to their regulation by the U.S. Food and Drug Administration (FDA). The U.S. export market also is potentially affected by the fact that over 50 other nations have established or proposed regulations for controlling aflatoxins in food and feeds, some being more stringent than FDA limits. Recent publication of the FDA Final Guidance for Industry, and recent recommendations of the 56th Joint Expert Committee on Food Additives (JECFA) for maximum daily intake of fumonisins, is elevating fumonisin research to a status similar to that of aflatoxins. The attendance and participation at the 2002 workshop by researchers from Japan, Mexico and Venezuela, and presentations of research involving international collaborations certainly offer hope that worldwide resources, both human and otherwise, can be maximally exploited to develop the means of eliminating aflatoxins and fumonisins from food and feeds and that the research can have global impact.

2nd Fungal Genomics Workshop. The current pre-eminence of genomics and genomic tools in biological research provide invaluable assets for understanding host plant-mycotoxigenic fungus interactions. We have the capability through these tools, of identifying fungal traits and regulatory elements involved in host invasion and the production of aflatoxins or fumonisins. Likewise, plant traits and regulatory factors affecting fungal invasion of host tissue and toxin production may also be discovered. Presentations in this 2nd Fungal Genomics Workshop report findings of investigations involving *Aspergillus* or *Fusarium*, using different genomic tools: Expressed Sequence Tag (EST) sequencing, microarray analysis, proteome analysis and bioinformatics. This cutting-edge methodology can significantly enhance plant breeding through the rapid identification and characterization of genes implicated as necessary for virulence/toxigenesis and/or for host resistance. Keeping in mind the relationship between aflatoxin elaboration and drought and high temperatures, interesting and critical observations should result from the investigation of traits expressed under different environmental conditions.

3rd Fumonisin Elimination Workshop. Fumonisin studies are proceeding on many different 'fronts.' Investigations seeking an understanding of the genetics and biochemistry of fumonisin production using genomic approaches may illuminate targets that can be exploited to control fumonisin expression. Results of investigations of the role of pH in fumonisin production, however, may highlight stress as important not only in

aflatoxin production, but in fumonisin production as well.

It appears that corn lines that reduce aflatoxins levels can be developed, and since aflatoxins are the most studied among mycotoxins, knowledge gained in this area may, in some cases, be applicable to fumonisins as well. Corn genotypes effective in reducing aflatoxin levels should be high on the list of those to be investigated for fumonisin accumulation. Since *A. flavus* is a saprophyte/wound pathogen, resistance found in nature may in most cases be expected to have a generic rather than specific effect. Many West African lines, in fact, shown to possess aflatoxin-resistance were originally selected for ear rot resistance (against *F. verticillioides* and/or other fungi). Likewise, it is important to test proteins and other inhibitory factors against *F. verticillioides*, characterized as efficacious against *A. flavus* (e.g. maize trypsin inhibitor). Success in these trials may facilitate the simultaneous inhibition of these fungi, since they often co-exist in corn kernels. This achievement might have a profound impact on the corn industry.

Special Session: Novel Technologies for Aflatoxin Detection. One of the unique features of this year's workshop was the inclusion of a special paper session reporting research on novel methods for rapid detection of aflatoxin in commodities. These methods could play a role in pre-harvest detection/predictive strategies or post-harvest detection strategies. Protocols for grain sampling to determine aflatoxin levels have been available for several years for most affected commodities, however, they are expensive and time consuming in both the amount of product used and the labor and material expenses of testing. In addition, the number of false positives and false negatives may be unacceptably high, particularly when large volumes of product are involved. Thus, too much unacceptable product may be passed, or conversely, valuable commodity may be unnecessarily declared unacceptable for human use. Non-destructive yet rapid techniques creatively deployed during assessment might significantly enhance accuracy, even to the satisfaction of buyers, growers, and the public alike.

15th Aflatoxin Elimination Workshop. Crop management and handling strategies can make important contributions reducing aflatoxin contamination of commodities, especially in tree nuts and figs. However, significant control of the aflatoxin problem also requires the use of other strategies such as conventional breeding, genetic engineering, or biological control. Large-scale trials in Arizona employing *A. flavus* atoxigenic strains to competitively exclude toxigenic strains in cotton fields have resulted in drastic and significant reduction of aflatoxin levels in cotton. Success using this strategy may also extend to South Texas on both cotton and corn, and to other locations and crops as well. Chronic yearly contamination and the ability of growers to support additional costs associated with deployment of the biocompetitive strategy are conditions that favor its use.

The development of host resistance is a core strategy for effective control of aflatoxin elaboration. The identification of resistant germplasm can lead to an investigation of factors underlying resistance. Subsequent identification and characterization of these factors 1) can provide researchers with an opportunity to decipher nature's requirements for resistance, 2) provide markers for marker-assisted breeding, and/or 3) provide candidate genes for plant transformation. Genetically engineered lines can, in turn, facilitate confirmation of gene function as it relates to resistance, as well as provide germplasm for breeding programs.

The sponsors of the 2002 workshop were the Corn Refiners Association, the

National Corn Growers Association, the North American Millers Association, and the Texas Corn Producers Board. Industry and USDA-ARS cooperation with both ARS and university scientists has provided the foundation for the tremendous research progress made thus far, and is, undoubtedly, the basis for any future work leading to the successful resolution of the aflatoxin and fumonisin problems.

DR. JANE ROBENS
National Program Leader
Food Safety and Health
Beltsville Agricultural Research Center
Agricultural Research Service, USDA
Beltsville, MD

DR. ROBERT L. BROWN
Research Plant Pathologist
Food and Feed Safety Research Unit
Southern Regional Research Center
Agricultural Research Service, USDA
New Orleans, LA

AGENDA

2002 AFLATOXIN/FUMONISIN ELIMINATION AND FUNGAL GENOMICS WORKSHOP

October 23-25, 2002

Holiday Inn – Riverwalk, San Antonio, TX

WEDNESDAY, OCTOBER 23, 2002

7:00 – REGISTRATION / POSTER ASSIGNMENT

8:00 – ANNOUNCEMENTS

8:10 – INTRODUCTORY REMARKS: Jane Robens, National Program Leader, Food Safety and Health, USDA, ARS, Beltsville, MD.

2nd ANNUAL FUNGAL GENOMICS WORKSHOP

Moderator: Gerald Donaldson, Grower, Wharton, TX

8:20 - OVERVIEW – Thomas E. Cleveland, Food and Feed Safety Research, USDA-ARS-SRRC

8:35 **The *Aspergillus flavus* EST Project — A Tool for Eliminating Aflatoxin Contamination.** Jiujiang Yu[1], Deepak Bhatnagar[1], Catherine A. Whitelaw[2], Thomas E. Cleveland[1] and William C. Nierman[2]. [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]The Institute for Genomic Research, Rockville, MD.

8:50 **Expressed Sequence Tags (ESTs): A Genomic Approach to Limit Fumonisin Production by *Fusarium verticillioides* and Commodity Losses.** Daren W. Brown[1], Robert H. Proctor[1], Catherine A. Whitelaw[2], Ronald D. Plattner[1] and David Kendra[1]. [1]Mycotoxin Research, USDA-ARS- NCAUR, Peoria, IL; [2]The Institute for Genomics Research, Rockville, MD.

9:05 **Gene Expression Analysis of *Aspergillus flavus* and *A. parasiticus*.** Gary A. Payne, Ahmad Fakhoury and Greg Obrian. Department of Plant Pathology, North Carolina State University, Raleigh, NC.

9:20 **Cooperative Genomic and Bioinformatic Possibilities for Enhancing Strategies for Improving Resistance to Aspergillus Ear Rot and Aflatoxin**

Production in Maize in the U.S. Torbert Rocheford, Chandra Paul, and Donald White. Department of Crop Sciences, University of Illinois, Urbana, IL.

9:35 **Proteomic Analysis of Maize Rachis Tissue in *Aspergillus flavus* Resistant Inbreds.** Olga Pechanova[1], Tibor Pechan[1], W. Paul Williams[2] and Dawn S. Luthe[1]. [1] Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2] USDA-ARS, Corn Host Plant Resistance Unit, Mississippi State, MS.

9:50 – PANEL DISCUSSION
(**Panel Chair:** Daren Brown, USDA-ARS-NCAUR, Peoria, IL)

10:20 – BREAK

2002 AFLATOXIN / FUMONISIN ELIMINATION WORKSHOP

3rd ANNUAL FUMONISIN ELIMINATION WORKSHOP

SESSION 1: **Fumonisin Contamination of Corn and Development of Cellular, Biological, and Environmental Control Strategies**

Moderator: Dewey Lee, University of Georgia, Tifton, GA

10:35 - OPENING REMARKS: Dave Kendra, USDA-ARS-NCAUR, Peoria, IL

10:50 **Fumonisin Biosynthesis: Search for Regulatory Genes in *Fusarium verticillioides.*** Charles Woloshuk, Joseph Flaherty, and Anna Maria Pirttilä. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

11:05 **Functional Analysis of the Fumonisin Biosynthetic Gene Cluster in *Fusarium verticillioides*.** Robert H. Proctor, Robert A. E. Butchko, Ronald D. Plattner, Anne E. Desjardins and Daren W. Brown. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:20 **Sources of Resistance to Fusarium Ear Rot and Fumonisin Production in Corn.** Don White[1], Mike Clements[1], Craig Kleinschmidt[1], and Chris Maragos. Department of Crop Sciences, University of Illinois, Urbana, IL.

11:35 **Reduction of Fumonisin Levels in Bt. Corn.** B. Hammond[1], K. Campbell[1], T. Degooyer[1], A. Robinson[1], J. Richard[2], J. Segueira[3], C. Rubinstein[3], J. Cea[4], M.Plancke[5], L. Pinson[6], C. Radu[7], H. Esin[7], F. Tatli[8], and R. Grogna[9]. [1]Monsanto Company; [2]Romer Labs; [3]Monsanto, Argentina; [4]Laboratorio Tecnologico del Uruguay; [5]Monsanto, France; [6]INRA, France; [7]Monsanto, Turkey; [8]Adana Crop

Protection Research Institute, Turkey; [9]Monsanto, Europe.

11:50 – 1:00 - LUNCH

1:00 **Evidence for a *Fusarium verticillioides* Seedling Pathogenicity Factor: All Roads Traveled Lead to Fumonisin.** A. E. Glenn[1], L.D. Williams[1, 2], and R.T. Riley[1]. [1]USDA, ARS, Toxicology & Mycotoxin Research Unit, Russell Research Center, Athens, GA; [2]College of Agriculture and Environmental Sciences, University of Georgia, Athens GA.

1:15 **Inhibition of Ceramide Synthase in Corn Seedlings Infected with *Fusarium verticillioides* or Exposed Directly to Fumonisin B_1 in Soil.** L.D. Williams[1,2], A.E. Glenn[2], C.W. Bacon[2], J.L. Showker[2], and R.T. Riley[2]. [1]College of Agriculture and Environmental Sciences, University of Georgia, Athens GA; [2]USDA-ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens, GA.

1:30 - PANEL DISCUSSION
(Panel Chair: Dave Kendra)

2:00 – 2:30 POSTER VIEWING

SPECIAL SESSION - Novel Technologies for Aflatoxin Detection

Moderator: Gerald Donaldson, Grower, Wharton, TX

2:30 - SESSION OVERVIEW – Jane Robens

2:45 **The Potential of Hyperspectral Imaging for the Detection of Aflatoxin.** Gavin H. Poole. The Institute for Technology Development, Stennis Space Center, MS.

Overview - Hyperspectral imaging is a non-destructive technique, where an area of interest is imaged with a CCD camera equipped with a hyperspectral scanner. The result is a unique spectral signature for each pixel of the image, which allows for the differentiation of objects within the image. This technique was tested to see if it can detect molds on grain samples, and there is potential to use the same techniques to find the toxins associated with the molds.

3:05 **Hyperspectral Methods to Detect Aflatoxin in Whole Kernel Corn.** David Casasent and Xue-Wen Chen. Department of Electrical and Computer Engineering, Carnegie Mellon University, Pittsburgh, PA.

Overview - Hyperspectral (HS) data is very useful for many agricultural product inspection applications. Initial results indicate that it is of use in detecting aflatoxin in whole corn kernels. HS data represents a high-dimensional data

processing problem requiring feature reduction. We advance new feature extraction and feature selection algorithms and show that they are preferable to other prior algorithms. HS data appears to be much faster, more economical, and preferable to BGYF methods.

3:25 - BREAK

3:40 **Trained Wasps as Chemical Detectors: Application for Aflatoxin-Related Issues.** W. Joe Lewis[1], Glen C. Rains[2], and David M. Wilson[2]. [1]USDA-ARS, Tifton, GA; [2]University of Georgia, Tifton, GA.

Overview - Parasitic wasps optimize foraging efficiency by learning chemicals associated with food and hosts. Data to date show that wasps of the species, *Microplitis croceipes*, trained to associate odors of fungi with sugar water can subsequently: 1) distinguish among different species of aflatoxin-producing fungi, 2) recognize toxin- versus nontoxin-producing strains, 3) recognize species/ strain independent of diet background, and 4) learn individual chemicals known to be associated with the fungi.

4:00 **Detection of Aflatoxin and Fumonisin Contaminated Corn by Near Infrared Spectroscopy.** Tom Pearson[1], [2]Donald Wicklow, and [1]Carrie Schwartz. [1]USDA-ARS-GMPRC, Manhattan, KS; [2]USDA-ARS-NCAUR, Peoria, IL.

Overview - Rapid, non-destructive, detection of aflatoxin contamination and mold infestation in whole kernels of corn will be discussed. Methods investigated were near infrared spectroscopy and visible and near infrared imaging. The near infrared spectra were used to optimize a commercial sorting machine for separating aflatoxin contaminated kernels.

4:20 **Using Cyclodextrins in a Rapid Inexpensive Aflatoxin Screening Method for Field Research Applications.** David M. Wilson[1], Joao Augusto[1], Corley Holbrook[2] and Neil Widstorm[2]. [1]Department of Plant Pathology, University of Georgia; [2]USDA/ARS, Tifton, GA.

Overview – The fluorescence of the aflatoxins can be modified in solution to develop screening as well as quantitative methods. The fluorescence of B1 and G1 can be enhanced by using bromine, beta-cyclodextrin, dimethyl-beta-cyclodextrin or succinylated (2-hydroxy)propyl-beta cyclodextrin. These fluorescence enchantments can be used to develop useful inexpensive screening methods.

4:40 – 5:10 DISCUSSION – (**Discussion Leader:** Tom Schatzki, USDA-ARS-WRRC, Albany, CA)

7:00 – 8:30 - **RECEPTION**

15TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

THURSDAY, OCTOBER 24, 2002

7:00 - REGISTRATION / POSTER ASSIGNMENT

8:00 - ANNOUNCEMENTS

SESSION 2: **Potential Use of Natural Products for Prevention of Fungal Invasion and /or Aflatoxin Biosynthesis in Crops**

Moderator: Paul Bertels, National Corn Growers Association

8:15 **Induction of Atoxigenicity in *Aspergillus flavus* by Walnut Phytochemicals.** Russell J. Molyneux[1], Noreen Mahoney[1], Bruce C. Campbell[1], Ryann Muir[2], and Abhaya Dandekar[2]. [1]Western Regional Research Center, ARS-USDA, Albany, CA; [2]Department of Pomology, University of California, Davis, CA.

8:30 **Limestone Effect upon Aflatoxin in Naturally Contaminated Corn.** Noemi C. Castañeda, Sandra G. Laguna, Yolanda A. Rodríguez, Gloria R. Laura Anguiano, and Doralinda Guzmán-de-Peña . Departamento de Biotecnología y Bioquímica de la Unidad Irapuato. Centro de Investigación y Estudios Avanzados del I.P.N. México.

8:45 **Genetic Analysis of Inhibitory Proteins from Maize Seeds.** Gary A. Payne[1], Ahmad Fakhoury[1], Quincy Gerrald[1], and Rebecca Boston[2], [1]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Department of Botany, North Carolina State University, Raleigh, NC.

9:00 **Structures and Biological Activities of the Inhibitors of Aflatoxin Production, Aflastatin A, Blasticidin A and their Derivatives.** Shohei Sakuda, Hiroyuki Ikeda, Takefumi Nakamura, Makoto Ono and Hiromichi Nagasawa. Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan.

9:15 PANEL DISCUSSION
(**Panel Chair:** Gary A. Payne, North Carolina State University, Raleigh, NC)

9:45 BREAK

SESSION 3: **Crop Resistance – Genetic Engineering**

Moderator: Scott Averhoff, Waxahachie, TX

10:00 **Genetic Engineering of Peanut for Reduction of Aflatoxin Contamination.** Peggy Ozias-Akins[1], Chen Niu[1], Madhumita Joshi[1], Xiang-Yang Deng[1], Corley Holbrook[2], and Robert Lynch[2]. [1]Department of Horticulture, University of Georgia, Tifton, GA; [2]USDA-ARS-Coastal Plain Experiment Station, Tifton, GA.

10:15 **Genetic Engineering of Cotton to Confer Resistance to *A. flavus*: An Update.** Caryl A. Chlan[1], J. Cary[2], K. Rajasekaran[2], and T. E.Cleveland[2]. [1]University of Louisiana, Lafayette, LA; [2]USDA-ARS-SRRC, New Orleans, LA.

10:30 **Expression of the Alpha-Amylase Inhibitor AILP in *E. coli* and Plants.** Charles P. Woloshuk, Burt Bluhm, Gyung-Hye Huh, and Melanie Gustafuson. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

10:45 **Enhanced, Stable Expression of Antifungal Genes in Transgenic Cotton is Vital for the Control of *A. flavus.*** K. Rajasekaran[1], J.W. Cary[1], T.J. Jacks[1], C.A. Chlan[2] and T.E. Cleveland[1]. [1]USDA-ARS-SRRC, New Orleans, LA: [2]University of Louisiana, Lafayette, LA.

11:00 - BREAK

11:15 **Transformation of Virginia and Runner Type Peanuts with Mod 1, a gene encoding an antifungal RIP from Maize**. Arthur Weissinger[1], Minsheng Wu[1], and T.E. Cleveland[2]. [1]Crop Science, North Carolina State University, Raleigh, NC; [2]USDA-ARS-SRRC, New Orleans, LA.

11:30 **Genetic Engineering and Breeding of Walnuts for Control of Aflatoxin.** Ryann Muir[1], 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]UDSA/ARS Western Regional Research Center, Albany, CA.

11:45 – PANEL DISCUSSION
(**Panel Chair:** Kanniah Rajasekaran, USDA-ARS-SRRC, New Orleans, LA)

12:15 – 1:15 LUNCH

SESSION 4: **Microbial Ecology**

Moderator: Betsy Faga, North American Millers Association

1:15 **Vegetative Compatibility and Aflatoxin Production in *Aspergillus*.** Donald T. Wicklow[1] and Bruce W. Horn[2]. [1]USDA, ARS, Mycotoxin Research Unit, National Center for Agricultural Utilization Research, Peoria, IL; [2]USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

1:30 **Dispersal and Overwintering of *Aspergillus flavus* in Arizona and Texas.** Peter J. Cotty, USDA-SRRC-USDA, New Orleans, LA.

1:45 **Advances in the Utilization of Atoxigenic Strain Technology to Manage Aflatoxin in Commercial Cotton.** Larry Antilla[1], and Peter J. Cotty[2]. [1]Arizona Cotton Research and Protection Council; [2]USDA-ARS-SRRC, New Orleans, LA.

2:00 **Towards Implementation of Biological Control Strategy to Reduce Aflatoxin Contamination in Tree-Nuts.** Sui-Sheng Hua. USDA, ARS, Western Regional Research Center, Albany, CA.

2:15 **Effect of Nontoxigenic Strains of *Aspergillus flavus* and *A. parasiticus* Applied Separately and in Combination on Preharvest Aflatoxin Contamination of Peanuts.** J. W. Dorner and B. Horn. USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

2:30 - BREAK

2:45 – PANEL DISCUSSION
(**Panel Chair:** Larry Antilla, Arizona Cotton and Research Protection Council)

3:15 – 4:15 POSTER VIEWING

4:15 – UPDATE - "Aflatoxin Levels 2002"

5:00 – **COMMODITY BREAKOUT SESSIONS**

FRIDAY, OCTOBER 25, 2002

8:30 - ANNOUNCEMENTS

SESSION 5: **Crop Management and Handling, Insect Control and Fungal Relationships**

Moderator: Dee Vaughn, Grower and President-Elect, NCGA, Dumas, TX

8:45 **Aflatoxin Control in Pistachios: Biocontrol and Removal of Contaminated Nuts.** Themis Michailides, and Mark Doster. University of California, Davis/Kearney Agricultural Center.

9:00 **Aflatoxin Control in Figs: Biocontrol and Development of Resistant Cultivars.** Mark Doster, and Themis Michailides. University of California, Davis/Kearney Agricultural Center.

9:15 **Aflatoxin Determination in Pistachios by Dry Grinding and Wet Slurrying.** Thomas F. Schatzki. USDA-ARS-WRRC, Albany, CA.

9:30 **With-In Row Spacing of Maize and its Effects on Yield and Mycotoxin Incidence.** H. Arnold Bruns, and H.K. Abbas. USDA-ARS, Stoneville, MS.

9:45 **Validating a Computer Program that Predicts Mycotoxin Levels in Midwest Corn.** Patrick F. Dowd. USDA-ARS-NCAUR, Peoria, IL.

10:00 – PANEL DISCUSSION
(**Panel Chair:** Patrick Dowd, USDA-ARS-NCAUR, Peoria, IL)

10:30 - BREAK

SESSION 6: **Crop Resistance – Conventional Breeding**

Moderator: Jeff Nunley, Exec. Director, South Texas Cotton and Grain Assoc.

10:45 **Use of Marker Assisted Selection for Improving Commercial Inbred Lines for Resistance to Aspergillus Kernel Rot and Production of Aflatoxin.** Torbert Rocheford and Don White. Department of Crop Sciences, University of Illinois, Urbana, IL.

11:00 **Use of MI82 as a Source of Resistance to Aspergillus Ear Rot and Aflatoxin Production and Utilization of BGYF as a Selection Tool.** Don White, Laura Maupin, Kevin Busboom, Mike Clements, and Torbert Rocheford. University of Illinois, Urbana, IL.

11:15 **Aflatoxin and Fumonisin Distribution in Maize Populations.** Steve Moore[1] and Hamed K. Abbas[2]. [1]Dean Lee Research Station, LSU Agricultural Center,

Alexandria, LA; [2]Crop Genetics and Production Research Unit, USDA-ARS, Stoneville, MS.

11:30 **Aflatoxin Accumulation in Maize Inbreds and Hybrids.** Javier Betrán[1], Tom Isakeit[2], Gary Odvody[3], and Kerry Mayfield[1]. Texas A&M University: [1]Soil & Crop Sciences Department, College Station, TX; [2]Pathology Department, College Station, TX; [3]Pathology Dept., Corpus Christi, TX.

11:45 – 12:45 LUNCH

12:45 **Selection of Peanut Breeding Lines with Resistance to Preharvest Aflatoxin Contamination and Improved Drought Tolerance**. C. Corley Holbrook[1], Baozhu Guo[1], David M. Wilson[2], C. K. Kvien[2], Weibo Dong[2], Diane L. Rowland[3], and Meng Luo[2]. [1]USDA-ARS, Tifton, GA; [2]UGA, Tifton, GA; [3]USDA-ARS, Dawson, GA.

1:00 **Identification and Characterization of Resistance Mechanisms to Preharvest Aflatoxin Contamination in Corn and Peanut.** B. Z. Guo [1], M. Luo [2], A. E. Coy[2], W. Dong[2], C. C. Holbrook[3], R. D. Lee[2], N. W. Widstrom[3], and R. E. Lynch[1]. [1]USDA-ARS, Crop Protection and Management Research Unit; [2] University of Georgia Coastal Plain Experiment Station, Tifton, GA.; [3]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

1:15 **Integrated Seed and Endocarp Based Resistance to Preharvest Aflatoxin Contamination in Almond.** Thomas M. Gradziel, and Abhaya M. Dandekar. Department of Pomology, University of California, Davis, CA.

1:30 **Identification and Characterization of Potential Resistance Markers through Proteome Analysis.** Zhi-Yuan Chen[1], Robert L. Brown[2], Thomas E. Cleveland[2], and Kenneth E. Damann[1]. [1]Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA; [2]USDA-ARS-SRRC, New Orleans, LA.

1:45 - PANEL DISCUSSION
(**Panel Chair:** Thomas Gradziel, University of California-Davis)

2:15 – 2:30 **CLOSING REMARKS** - Jane Robens, National Program Leader Food Safety and Health, USDA, ARS, Beltsville, MD

2nd ANNUAL FUNGAL GENOMICS WORKSHOP

Moderator – *Gerald Donaldson, Wharton Texas*

Fungal Genomics--An Overview

T. E. Cleveland[1], J. Yu[1], Z. Chen[2], R. Brown[1] and D. Bhatnagar[1]. [1]USDA-ARS-SRRC, New Orleans, LA; [2]Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA.

Fungal genomics is a valuable tool in gaining an understanding of the host plant - mycotoxigenic fungus interaction. Understanding the complex interelationships of plant and fungal gene products during the plant-fungus interaction is key in developing strategies to interrupt the mycotoxin contamination process. Fungal processes necessary for invasion of the plant and production of mycotoxins can be broken down into three categories: 1) cell wall degradation (cellulases, pectinases, amylases and proteinases), 2) fungal development (conidiophore and conidial formation), and 3) mycotoxin biosynthesis and regulation. Fungal EST projects have led to a rapid expansion of candidate genes regulating these processes possibly involved in invasion of the plant and mycotoxin contamination.

Plant factors, discovered through the use of proteomics, may influence fungal processes involved in invasion and mycotoxin contamination. These factors also can be divided into three categories: 1) seed proteins/inhibitors of fungal cell wall degrading enzymes, 2) "hormonal" products, a complex family of compounds (some of which are volatile) associated with the lipoxigenase pathway, and 3) stress responsive proteins. If the interaction between the plant and the fungus can be better understood, particularly in how plant factors may influence fungal processes necessary for virulence and mycotoxin contamination, this information could lead to development of breeding and/or gene insertion technologies for enhancing plant processes linked to resistance.

Report On *Aspergillus Flavus EST* Project — A Tool For Eliminating Aflatoxin Contamination

Jiujiang Yu[1], Catherine A. Whitelaw[2], Deepak Bhatnagar[1], Thomas E. Cleveland[1] and William C. Nierman[2]. [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA and [2]The Institute for Genomic Research, Rockville, MD

Study of *Aspergillus flavus* genomics is an innovative strategy and a rapid investigative tool for simultaneous analysis of the biochemical function and genetic regulation of the genes in the fungal system to understand the molecular regulation of aflatoxin biosynthesis. *Aspergillus flavus* Expressed Sequence Tag (EST) technology allows rapid identification of the majority, if not all, of the genes expressed in the fungal genome and helps to better understand the coordinated regulation of gene expression. The *A. flavus* EST program at USDA/ARS, Southern Regional Research Center (SRRC) is aimed at (1) understanding the genetic control and regulation of aflatoxin biosynthesis by potential regulator(s) upstream of *aflR*, (2) the mechanism of toxin production in response to internal and external factors, (3) the relationship between primary and secondary metabolism, (4) plant-fungal interaction and fungal pathogenicity, as well as (5) evolutionary biology. A normalized cDNA library was made and the cDNA clones have been sequenced at The Institute for Genomic Research (TIGR). Currently, over 7,000 unique gene sequences have been identified within 14,000 cDNA sequences obtained. By expectation, sequencing a total of 16,000 - 17,000 cDNAs, could help us to reach our goal of identifying 8,000 unique cDNA gene sequences. Preliminary blast search of the identified unique sequences indicated that all of the known aflatoxin pathway genes have been identified within the EST sequences obtained so far and many rare copy genes potentially involved in regulation have also been identified from this normalized library. A microarray containing these EST sequences, will be made to detect simultaneously a whole set of genes expressed under specific environmental conditions. By applying EST/Microarray technologies, we may be able to screen out those genes that may be responsible for or related to aflatoxin production, signal transduction, plant-microbe interaction, fungal development and pathogenicity.

A. oryzae is a close relative of *A. flavus* except that the industrially significant strain of *A. oryzae* has lost its ability to produce aflatoxins eventhough some of the pathway genes are present. *A. oryzae* EST data (from the Japanese Consortium) will be made available to SRRC scientists through a memorandum of understanding. Comparison of the identified *A. flavus* sequences with *A. oryzae* sequences will also help in identifying these most important factors governing aflatoxin formation. Therefore, the *A. flavus* EST program at SRRC is expected to provide valuable information on turning on and off aflatoxin production in this fungal system. This information will provide vital information for developing new strategies for control of aflatoxin contamination of crops.

Expressed Sequence Tags (Ests): A Genomic Approach To Limit Fumonisin Production By *Fusarium Verticillioides* And Resulting Commodity Losses

Daren W. Brown[1], Robert H. Proctor[1], Catherine A. Whitelaw[2], Ronald D. Plattner[1] and David Kendra[1]. [1]Mycotoxin Research, National Center for Agricultural Utilization Research, ARS, Peoria, IL; [2]The Institute for Genomics Research, Rockville, MD

Fumonisins are mycotoxins produced by the maize pathogen *Gibberella moniliformis* (anamorph *Fusarium verticillioides*). Although *F. verticillioides* can be detected in most healthy maize grown in the US (the fungus can grow in and colonize maize tissues without causing symptoms), under some conditions, the fungus can cause significant ear and stalk rot and can produce fumonisins in diseased kernels. Fumonisins are synthesized from simple primary metabolic building blocks like acetate, alanine, and tricarboxylic acids and are structurally similar to the sphingolipid intermediates sphinganine and sphingosine. In both plants and animals, fumonisins can disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthase. Fumonisin FB_1 is associated with several animal diseases and has been epidemiologically correlated with human esophageal cancer in some regions of the world. Our goal is to reduce the economic and health impact of *F. verticillioides*. Our approach is to first develop a knowledge base of the mechanisms of fungal pathogenesis and of fumonisin biosynthesis and regulation, and from this identify potential control strategies that limit growth of the fungus and/or its ability to produce fumonisins in maize.

The molecular and biochemical pathways that govern endophytic and/or pathogenic infection of corn by *F. verticillioides* as well as mycotoxin production are poorly understood. Gene expression studies using expressed sequence tags (ESTs) are a readily applicable and, in contrast to whole genome sequencing, a reasonably affordable genomics strategy to identify key mechanisms involved in plant/fungal interactions and toxin biosynthesis. We, in collaboration with The Institute for Genomic Research (TIGR), are sequencing the 5' ends of cDNAs from *F. verticillioides* that represent genes expressed under varying fungal growth conditions. At present, TIGR has sequenced two cDNA libraries generating over 17,000 ESTs that represent over 4800 unique sequences (i.e. genes). We are presently examining BLAST analyses of 1900 ESTs that are unique to a cDNA library generated from a fumonisin-producing culture of *F. verticillioides*. When genes of interest are identified, their functions will be evaluated via gene disruption analysis. Genes will be disrupted by standard protocols and the resulting effect on fumonisin production and/or ability to cause ear/stalk rot will be assessed. Information produced from this research is expected to lead to the knowledge necessary to develop effective strategies to minimize or eliminate fumonisin production in maize and to reduce disease caused by *F. verticillioides*.

Gene Expression Analysis Of *Aspergillus Flavus* And *A. Parasiticus*.

Gary A. Payne, Ahmad Fakhoury and Greg OBrian. Department of Plant Pathology, NC State University, Raleigh, NC

A complex network that involves both nutritional and developmental signals regulates the biosynthesis of aflatoxin. Because of the complexity of this network, the use of traditional molecular biology tools to study its regulation has limitations. We are using gene expression analysis to compare the simultaneous expression of hundreds of genes at once. Our initial focus is to identify a set of genes that are differentially expressed during aflatoxin biosynthesis under a number of physiological conditions with the goal of identifying a few key genes for further analysis. We plan to disrupt these genes, examine their aflatoxin phenotype, and compare the gene expression profile of the mutant strains with a wild type strain. To keep the study focused on genes involved in aflatoxin biosynthesis, we started with clones from a cDNA library that was constructed from mRNA isolated from aflatoxin-producing cultures. cDNA clones from this library enriched for transcripts of genes involved in aflatoxin biosynthesis were arrayed on nylon filters. Hybridization of the filter with target cDNA made from transcripts expressed during conducive and non-conducive conditions for aflatoxin production resulted in the identification of over 10000 expressed clones. Quality sequence was obtained for 2200 clones. Further analysis resulted in the identification of 753 unique expressed sequence tags. Many of these ESTs showed sequence similarity to genes known to be involved in metabolic and regulatory pathways; however, no known function could be ascribed to over 50% of the ESTs. These sequences were further analyzed using tblastx against the dbEST database at NCBI. This analysis revealed hits for 14% of the ESTs, 9% to Aspergillus ESTs. After this analysis, only 29% of the *A. flavus* ESTs were orphans. The 753 ESTs were arrayed on glass slides and used to identify differentially expressed genes during the temporal induction of aflatoxin biosynthesis. An analysis of three independent experiments identified 26 genes with relatively higher levels of expression during aflatoxin biosynthesis and 18 genes more expressed prior to aflatoxin biosynthesis. No predicted function could be ascribed to 17 of the 26 genes that were more expressed during aflatoxin biosynthesis. Experiments are in progress to disrupt these genes in the fungus and to characterize their function in aflatoxin biosynthesis.

Proteomic Analysis Of Maize Rachis Tissues In *Aspergillus Flavus* Resistant Inbreds

Olga Pechanova[1], Tibor Pechan[1], W. Paul Williams[2] and Dawn S. Luthe[1].
[1]Department of Biochemistry and Molecular Biology, Mississippi State, MS;
[2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS

We have been using a proteomics approach to identify proteins that may be associated with resistance or susceptibility to *A. flavus* during ear development. We are comparing 2-D gel electrophoresis (2-DE) profiles of proteins extracted from the rachis, silk, pericarp and embryos of ears (both inoculated and control) collected at various intervals after silk emergence. Samples are extracted from each tissue using a phenol-based procedure, and analyzed on large format 2-D gels. Gels are scanned and analyzed using PDQuest software (BioRad). This allows us to compare several gels simultaneously and visualize proteins that are unique to a genotype. Proteins of interest are then excised from gels and identified by MALDI-TOF mass spectroscopy. There were approximately 300 Coomassie blue-stained rachis proteins visualized in both Mp313E (resistant) and SC212M (susceptible). Twenty-five proteins were specific to Mp313E and 20 were specific to SC212M. MALDI-TOF MS analysis of one "spot" that was shifted in position between the two inbreds indicated that it was a member of the RP1-D family of transcription factors. Other proteins that differ between the two genotypes have tentatively been identified.

Cooperative Genomic, Databasing & Bioinformatic Approaches To Facilitating Development Of Commercial Maize Hybrids With Low Levels Of Aflatoxin Production

Torbert Rocheford, Chandra Paul, and Don White. Department of Crop Sciences, University of Illinois, Urbana, IL

There are a number of groups taking diverse research approaches towards the overall goal of developing maize hybrids with resistance to Aspergillus flavus ear rot and low levels of aflatoxin production. Examples include mapping quantitative trait loci for resistance, proteomic evaluation of seeds, and creation of cDNA libraries from developing seeds. These efforts are all aimed at identification of chromosome regions or genes associated with lower levels of ear rot and aflatoxins. Although these efforts are complementary, greater research progress may be achieved if the results could be integrated using database, genomic, and bioinformatic tools.

Recent genomic tools and resources developed through the NSF Plant Genome Program may facilitate integration of different research efforts. One resource is the maize Mo17xB73 intermated mapping population (IBM). A number of quantitative trait loci (QTL) have been mapped in different genetic backgrounds. Frequently different RFLP and SSR markers have been used to map them. Thus it would be most useful to place all of the specific markers used for QTL associations on the same mapping population to examine for presence of common associations, providing supportive evidence for a single QTL, or whether, there may be closely linked QTL, suggesting a cluster of resistance-related genes. The IBM population could be used to map the DNA sequences derived from proteomic analyses. The two efforts could be compared to determine if there are relationships between the proteins associated with resistance and the QTL. Similarly, cDNAs from efforts to identify candidate ESTs that may be associated with resistance could also be mapped onto the IBM population. These new cDNA map locations could be compared with QTL and sequences mapped from proteomic analyses to potentially identify candidate genes for QTL. The new IBM population could also be inoculated with Aspergillus flavus and the QTL for resistance in the IBM population, could be directly related to the common database collected on this genomic resource. The Mo17 and B73 parents of the IBM population represent a historically important heterotic pattern of commercial maize.

Planned developments in databases for maize genetics may serve to better pull together the raw data (actual genotypic and phenotypic scores) from the different QTL mapping population studies. There have been new developments in QTL mapping analyses, and the possibility exists of merging all this raw data together and performing mega-analyses. A challenge is in collecting the raw QTL mapping data for integration into one central database. There is a need to stay abreast of the evolving maize database efforts and work with curators.

Since most research supports a polygenic mode of resistance for the ear rot and aflatoxin production, it is likely that pyramiding of genes for resistance will be necessary. A number of chromosome regions associated with resistance have been identified. Since there are practical limitations to how many chromosome segments can be selected with marker assisted selection (MAS), we need to find the most favorable alleles of genes in

these regions. This could be done with selective genotyping in bulk segregant analyses of crosses of the best sources of resistance, using markers also placed on the IBM population. This will help to identify the best alleles of genes for resistance that can be used in MAS, and to provide results to be integrated into the database.

PANEL DISCUSSION: Fungal Genomics Workshop

Panel Chair: Daren W. Brown
Panel Members: Jiujiang Yu, Gary A. Payne, Torbert Rocheford and Dawn S. Luthe

Summary of Presentations and Panel Discussion. Gerald Donaldson, a Grower from Wharton, Texas, served as moderator for this session. The second Fungal Genomics Workshop met with the goal to extend participants knowledge of and utilization of Genomic Tools for the control and management of mycotoxins and fungal damage of agricultural commodities. The presentations, from 2 ARS laboratories and 3 university laboratories, described four different genomic tools: Expressed Sequence Tag (EST) sequencing (2 with fungi), microarray expression analysis (1 with fungi), proteomic analysis (1 with plants) and bioinformatics (1 with plants). EST projects sequence one or both ends of hundreds to tens-of-thousands of cDNA clones in order to quickly identify as many genes as possible that are expressed during the growth conditions from which the mRNA was isolated. Primary objectives may be to compare levels of gene expression during different growth phases or development or to develop a comprehensive set of genes required for growth and development for microarray expression analysis. Microarrays are generally glass slides that contain hundreds to thousands of DNA fragments representing open reading frames (ORFs) or genes from an organism. Hybridization experiments can then determine alterations in the type and levels of gene expression in response to altered conditions. Proteomic analysis involves the comparison of complex protein extracts from different tissues or growth conditions to identify new or differentially expressed proteins.

Tom E. Cleveland, USDA-ARS-SRRC, started off this session with an overview of how genomics and proteomics will help elucidate the mechanisms governing crop-fungus interactions and toxin contamination. Previous research has provided leads/targets through understanding plant and fungal processes (i.e. genes) that are involved in the disease process and plant/fungal interactions. The task now is to bring genomic and proteomic information together with previous work to meet our goals.

Jiujiang Yu reported on the SRRC *Aspergillus flavus* EST project, in collaboration with The Institute for Genomics Research (TIGR). This project seeks to create a comprehensive collection of *A. flavus* genes for microarray expression analysis. The overall goal is to identify critical gene(s), global regulators and signal transduction pathways involved in plant/microbe interactions and pathogenicity. A normalized cDNA library was created from mRNA extracted from *A. flavus* NRRL 3357 cultured on different media and for different periods of time. TIGR has generated over 17,000 EST representing over 6,400 unique genes. The TIGR Bioinformatics group has created an *A. flavus* Gene Index where each gene is catalogued according to predicted amino acid similarities to proteins in the public protein databases. Preliminary BLAST analysis has identified numerous genes of interest including aflatoxin pathway genes, potential virulence/pathogenicity genes and numerous potential regulatory or signal transduction genes.

Daren Brown reported on the NRRL *Fusarium verticillioides* EST project, in collaboration with TIGR. NRRL is creating separate EST libraries from 8 to 10 cDNA

libraries. An *in silico* comparison of EST sets will immediately identify unique or highly expressed genes. In future, a comprehensive set of genes will be used to conduct microarray expression experiments. BLAST analysis of 1900 ESTs that are unique to a fumonisin-producing culture of *F. verticillioides* has identified over 20 regulatory type genes and all of the fumonisin pathway genes. Sequence analysis of the next three EST libraries should identify genes involved in the plant/fungal interaction.

Gary Payne of the Department of Plant Pathology, North Carolina State University, described microarray experiments to help unravel the complexities of aflatoxin biosynthesis by *Aspergillus flavus* and *A. parasiticus*. They are using a DNA microarray containing 753 expressed sequence tags to identify a common set of genes that are differentially expressed under different culture conditions. The 753 potential aflatoxin related tags were originally derived from a set of 4000 cDNAs. Three experiments with four arrays identified 26 genes that are up-regulated during aflatoxin biosynthesis (including 3 known aflatoxin genes) and 18 that were more highly expressed before aflatoxin biosynthesis. Gary stressed an overall goal shared by the previous speakers which is to carry out functional analysis of targets or lead genes identified by ESTs and microarray experiments. Gary described recent results characterizing one such gene, 14-3-3. Fungal strains deleted in 14-3-3 were inhibited in aflatoxin synthesis, sensitive to oxygen stress, reduced in conidiation and temperature sensitive. Future plans include gene expression profiling experiments with the 14-3-3 deletion strain to look for affected genes.

Dawn Luthe of the Department of Biochemistry and Molecular Biology, Mississippi State University, described efforts using *A. flavus* resistant corn inbred lines and proteomic analysis to identify proteins that may be involved in resistance to fungal infection. The strategy is a systemic analysis of protein populations in tissues, cells or subcellular components using 2-D gels and mass spectroscopy. Experiments using a Gus-tagged *A. flavus* strain suggested that the fungus infects rachis tissue of a Resistant line (RL) differently than rachis tissue of a Susceptible line (SL). 2-D protein gels were able to detect over 300 proteins from each line which were then compared using computer software. Preliminary MALDI-TOF-MS analysis of 5 RL specific proteins and 5 SL specific proteins were described.

Tobert Rocheford of the Department of Crop Sciences, University of Illinois, discussed how genomics, bioinformatics and database tools can help us more efficiently and effectively achieve our goal to protect our food supply. He reminded us that resistance or tolerance to aflatoxin in maize is polygenic and is similar to many other plant diseases. Various states (i.e. research programs) have mapped quantitative trait loci (QTL) for resistance and have successfully moved them into commercially elite germplasm. Tobert emphasized the need to put markers associated with QTL in different genetic backgrounds onto a common maize map. Someone needs to shoulder the responsibility to collect raw data, organize it and share it with the research community.

Panel Discussion: Discussion began with the general comment that the goal of fungal ESTs and microarray analysis is to identify a subset of genes from the thousands of genes in the genome, to conduct functional analysis experiments. The first focus of the discussion was on data availability. Both Daren and Jiujiang stated that the *Aspergillus* and *Fusarium* EST projects are in progress and the data will be available to the public in the future. Torbert was asked to clarify: What is raw data? He described

toxin scores, disease ratings and other issues important to QTL analysis. Ed Cleveland noted that there is a tremendous body of information about factors related to resistance in the literature and asked whether there has been much effort to map these factors. Torbert responded that some factors have been mapped but more work needs to be done. Kay Simmons stated that the ARS is dedicated to the preparation of a crop genome database and that there are efforts to link maize and rice genome data. Torbert cautioned that the database efforts are an excellent start but we still need to figure out how to best use the databases. A portion of the discussion also focused on the tendency of some researchers use specific genotypes while others use aggregates of isolates. The point was made that plants are exposed to numerous isolates over time and focusing on a minimal set is limiting. But, for logistical reasons, initial studies are only manageable with a minimal set of variables and as studies progress, more factors can be examined to address more real-world conditions. Other discussion focused on practical outcomes from the proteomics work. Could promising proteins be used to develop more resistant lines? It is more likely that a marker would be found to move any protein by breeding.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

3rd ANNUAL FUMONISIN ELIMINATION WORKSHOP

SESSION 1: FUMONISIN CONTAMINATION OF CORN AND DEVELOPMENT OF CELLULAR, BIOLOGICAL, ENVIRONMENTAL CONTROL STRATEGIES

Moderator: *Dewey Lee, University of Georgia, Tifton, GA*

Fumonisin Biosynthesis: Search For Regulatory Genes In *Fusarium Verticillioides*

Charles Woloshuk, Joseph Flaherty, and Anna Maria Pirttilä. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN

Fumonisin biosynthesis in *Fusarium verticillioides* is influenced by tissue specific factors in the host, nitrogen and pH. The fungus grows equally well in germ and endosperm tissues, however fumonisin production is greatest in the endosperm. Fumonisin biosynthesis is also repressed by ammonium in culture media (Shim, W-B. and Woloshuk, C. P., FEMS Microbiol. Lett. 177:106-116), and its production requires acidic pH. To further understand the role of pH in fumonisin biosynthesis, we have cloned a *PACC* gene (*Fv-PACC*) from *F. verticillioides*. *PACC* genes encode the major transcriptional regulators of several pH-responsive pathways in filamentous fungi. Growth of a *Fv-PACC* disruption mutant was severely impaired at alkaline pH (8.4) while no adverse effects on growth or conidiation was observed at acidic pH. Furthermore, the *Fv-PACC* mutant produced fumonisin on corn and on a synthetic medium buffered at acidic pH. These data suggest that *Fv-PACC* is not directly involved a positive regulator of fumonisin biosynthesis.

A mutated *FCC1* in *F. verticillioides* was found to disrupt fumonisin biosynthesis and conidiation when grown on cracked corn (Shim, W-B. and Woloshuk, C. P., Appl. Environ. Microbiol. 67:1607-1612). These phenotypic effects are suppressed when the mutant is grown in media with a pH of 3.0. Forward and reverse subtraction expressed sequence tag (EST) libraries were made from RNA isolated from a wild-type strain and the *fcc1* mutant grown on corn. Approximately 800 EST clones from each subtraction library were sequenced and analyzed via BLASTX. The wild-type library (forward) has 302 unique ESTs and the mutant library (reverse) has 365 unique ESTs. The two libraries have 49 clones in common. Clones containing inserts with high similarity to known genes (P value $<10^{-5}$) were further categorized into eight groups: carbohydrate metabolism, protein metabolism, fatty acid metabolism, secondary metabolism, cell differentiation, pH response, stress response and signal transduction.

Among the genes in the wild-type library there are several putative regulatory genes we have selected for disruption experiments. One of these genes, designated *Fv-ZFR*, encodes a protein with a zinc finger domain similar to the transcription factor *GAL4*. The *Fv-ZFR* disruption mutant exhibits growth and conidiation characteristics similar to wild type when grown on cracked corn but produces negligible quantities of fumonisins. Other phenotypes exhibited by the *Fv-ZFR* mutant are currently under investigation.

To further study the relationship between pH and fumonisin biosynthesis the EST libraries were spotted to GAPS-coated glass slides for microarray analysis. We have probed the array with labeled RNA isolated from the wild type strain grown at low and high pH to identify genes involved with fumonisin biosynthesis and pH regulation. The goal of this study is to determine more precisely the genes that are coordinately expressed during fumonisin biosynthesis.

Functional Analysis Of The Fumonisin Biosynthetic Gene Cluster In *Fusarium Verticillioides*

Robert H. Proctor, Robert A. E. Butchko, Ronald D. Plattner, Daren W. Brown and Anne E. Desjardins. National Center for Agricultural Utilization Research, Peoria, IL

Fumonisins are polyketide mycotoxins produced by *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*), a causal agent of maize ear and stalk rot. These toxins cause several fatal animal diseases, including cancer in laboratory rodents, and their consumption is epidemiologically correlated with human esophageal cancer in some regions of the world where maize is a dietary staple. The objective of our research is to eliminate or reduce fumonisin contamination of maize. To achieve this objective, we are studying the biochemistry and genetics of fumonisin production in *F. verticillioides* in order to identify targets that can be exploited to control fumonisin contamination.

Based on the chemical structures of fumonisins, their biosynthesis is predicted to begin with the formation of a linear, 20-carbon polyketide and proceed with the condensation of the polyketide and alanine, carbonyl reduction at carbon 3, hydroxylation at carbons 5, 10, 14 and 15, and esterification of tricarballylic acids to the hydroxyls at carbons 14 and 15. Our understanding of the genes and enzymes required for these reactions improved markedly with the recent identification of a fumonisin biosynthetic gene cluster in *F. verticillioides*. The cluster consists of 15 co-regulated genes (*FUM1* and *FUM6* through *FUM19*) that exhibit patterns of expression that are correlated with fumonisin production. Based on BLAST sequence comparisons, the predicted functions of the proteins encoded by these genes are consistent with activities expected for fumonisin biosynthesis, transport, and self-protection.

To determine the functions of the *FUM* genes, we disrupted each gene. Disruption of four of the genes (*FUM15*, *FUM16*, *FUM17* and *FUM18*) had no apparent effect on fumonisin biosynthesis. Disruption of three of the genes (*FUM1*, *FUM6* and *FUM8*) blocked fumonisin biosynthesis but did not result in the accumulation of detectable fumonisin intermediates. Disruption of the remaining eight genes (*FUM7*, *FUM9*, *FUM10*, *FUM11*, *FUM12*, *FUM13*, *FUM14* and *FUM19*) resulted in the production of altered combinations of fumonisin homologues, including some homologues that had not been previously described. The accumulation of the homologues in the eight latter disruption mutants suggested functions for the corresponding genes, and these functions were consistent with those predicted from the BLAST comparisons. Although the *FUM1*, *FUM6*, and *FUM8* disruption mutants did not accumulate fumonisin homologues, the sequences of these genes strongly suggest their functions in fumonisin biosynthesis: Sequence data indicate that *FUM1* encodes a polyketide synthase, which most likely catalyzes the synthesis of the 20-carbon polyketide, that *FUM8* encode an aminotransferase, which most likely catalyzes the condensation of the polyketide and alanine, and that *FUM6* encodes a cytochrome P450 monooxygenase, which most likely catalyzes hydroxylation(s) of the polyketide. Why *FUM6* and *FUM8* disruption mutants do not accumulate fumonisin intermediates is not clear, but the resulting intermediates may be so similar in structure to fatty acids that they are metabolized by fatty acid metabolic enzymes. Why disruption of *FUM15*, *FUM16*,

FUM17 and *FUM18* did not affect fumonisin production is also not clear, but may result from these genes having redundant functions. Taken together, the results of the disruption analysis of the clustered *FUM* genes suggest a fumonisin biosynthetic pathway.

Sources Of Resistance To Fusarium Ear Rot And Fumonisin Production In Corn

Don White[1], Mike Clements[1], Craig Kleinschmidt[1] and Chris Maragos[2]. [1]University of Illinois, Urbana, IL; [2]USDA ARS, Mycotoxin Research Unit, Peoria, IL

Our research objective is to identify high levels of resistance to Fusarium ear rot and fumonisin production in corn. A search for sources of resistance is possible due to a collection of approximately 1,600 corn inbreds maintained by D.G. White, University of Illinois Department of Crop Sciences. The collection contains inbreds from Canada, Europe, Mexico, northern and central china, India, South Africa and the United States. It also includes a number of lines that were selfed from exotic populations. In 2000 we evaluated F_1 crosses of 1,559 of the inbreds in the collection in crosses with the commercially used inbred FR1064 with inoculation in Urbana, IL and under conditions of natural infection in Ponzer and in Winterville, North Carolina. Ear rot severity of the F_1 crosses ranged from 1 to 59% (mean 8%), 0 to 54% (mean 5%), and 0 to 52% (mean 5%) of the ear rotted in Urbana, Ponzer and Winterville, respectively. Fumonisin concentrations in corn grain ranged from 2 to 180 g/g (mean 23 g/g), 2 to 163 g/g (mean 15 g/g) and 1 to 239 g/g (mean 13 g/g) in Urbana, Ponzer and Winterville, respectively. Twenty-nine of the most resistant F_1's were chosen for further study in 2001. In 2001, 29 resistant selections were evaluated as the resistant parent, the susceptible parent (FR1064), the F_1, the F_2, and the backcross susceptible generation. Evaluations were done at Urbana, IL and Haubstadt, IN. Ears were inoculated twice with 10 ml spore suspension. As expected, several of the F_1 crosses were more susceptible than in 2000 with greater than 4 g/g in grain. These were eliminated from further study. With several, the inbred lines crossed with FR1064 was relatively susceptible even though the F_1 was resistant. This was expected as it is likely that some inbreds rated as susceptible have genes that contribute resistance to FR1064. These inbreds need to be studied sometime in the future, however, have been eliminated from further study at this time. Eleven F_1 crosses of various inbreds with FR1064 have been identified that need to be studied in further detail. With the 11 many had high levels of resistance in the F_1, F_2 and backcrosses. For example, with one source of resistance the fumonisin level averaged over three replicates and two locations was 2.4 g/g for the F_1; 145.9 g/g for FR1064; 1.8 g/g for the resistant inbred in the F_1 cross; 7.8 g/g for the F_2 generation; and 16.6 g/g for the back-cross to FR1064 generation. Because progeny in the F_2 and back-cross rows are segregating the low values in those rows are indicative of numerous resistant individuals and several dominant genes for resistance. Of the 11 inbreds selected for further study, several have flinty endosperms, one has red colored kernels, one is a late-maturing white kerneled inbred, one is a yellow kerneled dent inbred grown in Spain, one is a yellow kerneled inbred developed in Texas, and one is a yellow kerneled dent developed in Iowa. In conclusion, 11 sources of resistance have been identified that conveyed resistance in F_1 crossed with a widely-used commercial inbred grown in up to five different environments, two of which were infected naturally and three of which were inoculated using a severe inoculation technique.

Reduction Of Fumonisin Levels In Grain From Yieldgard® Cornborer

B. Hammond[1], K. Campbell[1], T. Degooyer[1], A. Robinson[1], J. Richard[2], J. Segueira[3], C. Rubinstein[3], J. Cea[4], M.Plancke[5], L. Pinson[6], C. Radu[7], H. Esin[7], F. Tatli[8], and R. Grogna[9]. [1]Monsanto Company; [2]Romer Labs; [3]Monsanto, Argentina; [4]Laboratorio Tecnologico del Uruguay; [5]Monsanto, France; [6]INRA, France; [7]Monsanto, Turkey; [8]Adana Crop Protection Research Institute, Turkey; [9]Monsanto, Europe

Biotechnology has made it possible to develop corn hybrids that are protected against lepidopteran corn boring pests such as the European corn borer (*Ostrinia nubilalis*). The coding sequence for Cry1Ab protein derived from *Bacillus thuringiensis* (*Bt.*) has been introduced into corn plants. Cry proteins are the active insecticidal ingredients of *Bt.* microbial insecticides that have been safely used on agricultural crops around the world for 40 years.

For YieldGard CornBorer hybrids, the Cry1Ab protein is produced throughout corn plant tissues providing season-long protection against corn borers. While season long protection has benefits for the farmer and environment (reduced insecticide use), there is growing evidence of another important health benefit, reduction of fumonisin mycotoxin contamination of corn grain.

Scientists at Iowa State University were the first to report that *Bt.* corn had reduced ear rot and fumonisin contamination when compared to near isogenic, non-transgenic controls. This reduction was attributed to reduced insect damage to the corn kernels caused by corn borers. Insect damage creates ports of entry for *Fusaria* that can infect grain producing ear rot and fumonisins. Reduction in fumonisin contamination was most evident in those hybrids where the Cry protein was expressed in all plant issues throughout the growing season. Field trials with YieldGard CornBorer hybrids have been conducted in the U.S., France, Argentina and Turkey.

In these trials, YieldGard CornBorer hybrids have, on average, reduced fumonisin levels when compared to their non-transgenic controls. When all sites were averaged from 2001 US trials, fumonisin levels were 54% of control levels. Many sites had a 3 fold or greater reduction in fumonisin levels. A few sites had no differences or increased fumonisins in *Bt* hybrids. It is possible that other corn pests that are not controlled by Cry1Ab protein may have affected mycotoxin levels in these locations. In Argentina, a YieldGard CornBorer hybrid tested at over 50 sites had fumonisin levels, on average, 38% of controls. In France, fumonisin levels were decreased several fold in YieldGard CornBorer hybrids. In Turkey, fumonisin levels were decreased 7 fold in grain from a YieldGard CornBorer hybrid.

Reduction in fumonisin levels could have important health implications for corn grown in countries with high fumonisin contamination where corn represents a major portion of the diet. In China, parts of Africa and in Mexico, where corn consumption is high, fumonisin exposures are considerably in excess of the adult PMTDI (2 ug/kg body weight/day).

Protection of corn crops against boring insect pests can reduce ports of entry to fungi that produce fumonisins leading to decreased contamination of corn grain with this mycotoxin. The application of biotechnology has the potential to reduce mycotoxin

contamination of corn, improving its safety for consumption. To more fully evaluate the potential reduction of fumonisin levels in YieldGard CornBorer hybrids in other world areas, additional field trials are being initiated with in South Africa, the Philippines, South America and other countries.

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Evidence For A *Fusarium Verticillioides* Seedling Pathogenicity Factor: All Roads Traveled Lead To Fumonisin

A.E. Glenn[1], L.D. Williams[1, 2] and R.T. Riley[1]. [1] USDA, ARS, Russell Research Center, Toxicology & Mycotoxin Research Unit, Athens, GA; [2] College of Agricultural & Environmental Sciences, University of Georgia, Athens, GA

We have previously reported that *Fusarium verticillioides* produces an apparent seedling pathogenicity factor that results in necrotic leaf lesions and abnormal development. Genetic analysis of field isolates indicated a single locus segregated for ability to cause disease. Strains carrying the non-pathogenic allele did not cause any disease symptoms, yet still infected and endophytically colonized the corn seedlings. We have also utilized mutant strains that were greatly attenuated in their ability to infect corn seedlings yet nonetheless caused severe disease symptoms, suggesting the pathogenicity factor may be a translocated phytotoxin. Fumonisin B1 (FB1) production was assessed among the parental and progeny strains and also was found to segregate as a single locus. Linkage between pathogenicity and fumonisin production was supported since only the pathogenic strains produced FB1; non-pathogenic strains did not produce any detectable FB1. PCR targeting *FUM1* and *FUM9* indicated a possible deletion within the fumonisin biosynthetic gene cluster in non-producing strains, providing further support for the inability of these strains to produce FB1. Watering seedlings with solutions of FB1 showed a stimulatory effect on seedling growth at the 1 ppm concentration, yet showed small leaf lesions and obvious stunting effects on seedling development at the 10 ppm concentration. Thus far, data suggest that fumonisin is the phytotoxin causing the seedling disease symptoms, and that production of FB1 is a *F. verticillioides* pathogenicity factor. Further experiments will examine the dynamics of FB1 production in soils as well as the plant and fungal genes differentially expressed under the various symptomatic and asymptomatic associations between *F. verticillioides* and corn.

Inhibition Of Ceramide Synthase In Corn Seedlings Infected With *Fusarium Verticillioides* Or Exposed Directly To Fumonisin B1 In Soil

L.D. Williams[1, 2], A.E. Glenn[2], C.W. Bacon[2], J.L. Showker[2], and R.T. Riley[2]. [1]College of Agriculture and Environmental Sciences, University of Georgia, Athens GA; [2]USDA-ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens, GA

Fumonisins (FB) are water soluble mycotoxins produced by *F. verticillioides*, which is parasitic to corn plants. FBs are inhibitors of ceramide synthase. While FB1 is not known to cause plant disease, it is found in the ear, roots, and stalks of corn. FB1 in corn debris can be leached by rainfall and can move through soils intact. However, a large amount is bound in certain soils. In sandy loam soils, FB1 can be released under acid conditions. These data suggest that FB1 from corn debris, or produced by *F. verticillioides* in the soil, can accumulate in soil and under certain environmental conditions the FB1 could be released and become biologically available. The objectives of this study were to determine: i) can *F. verticillioides* produce FB1 in soil, ii) is the FB1 in soil biologically active, and iii) is FB1 in soil toxic to corn seedlings. Corn seeds were inoculated with spore suspensions from pathogenic or non-pathogenic strains of *F. verticillioides*. Seedlings were grown for 21 days in potting soil. The roots and soil were analyzed for sphingoid bases (a marker of ceramide synthase inhibition) and FB1, respectively. Only seedlings inoculated with the pathogenic strain had elevated levels of sphinganine and the degree of elevation was correlated with the severity of the pathology. FB1 was only detected in soil from the plants inoculated with the pathogenic strain. In a second experiment surface sterilized seeds were planted and watered for six days with of 1, 5, or 10 ppm FB1 and grown for 15 more days. The FB1 in soil and the sphinganine in roots were closely correlated with the FB1 dosage. Reduced root mass was noted at 10 ppm. These results show that under laboratory conditions i) FB1 can be produced by *F. verticillioides* in soil, ii) the soil FB1 taken up by plants is biologically active, and iii) FB1 in soil can have adverse effects on plants. Thus, providing proof in principle that FB1 in soil can cause corn seedling disease.

Panel Discussion: Fumonisin Contamination of Corn and Development of Cellular, Biological, and Environmental Control Strategies

Panel Chair: David F. Kendra
Panel Members: Anthony E. Glenn, Bruce Hammond, Robert H. Proctor, Ronald T. Riley, Don White and Charles Woloshuk

Summary of Presentations: The Third Fumonisin Elimination Workshop continued the tradition of preceding the Aflatoxin Elimination Workshop. The session moderator was Dewey Lee of the University of Georgia, Tifton. Since the initial report of fumonisin in South Africa in 1988, the role of fumonisins in food safety continues to evolve. The biological data and analytical methodology was recently reviewed in a monograph prepared at the fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on 6-15 February 2001 at the WHO Headquarters in Geneva, Switzerland.

Lack of a comprehensive and systematic survey of fumonisin contamination in crops and food products continues to be a major challenge for developing an assessment of the true impact of fumonisins in global food safety. According to the most recent CAST report on mycotoxins (January 2003) mycotoxin contamination of feeds and foods, including fumonisin, is poorly studied. Plant genotype and environmental conditions along with insect pressure strongly influence fumonisin levels and need to be included as part of the survey results. Such information will are critical for the development of better risk assessment and disease prediction models.

Charles Woloshuk of Purdue University outlined a model describing the influence of several host and fungal factors on fumonisin synthesis. On autoclaved, mature corn kernels, fungal growth is well supported on both endosperm and embryo tissues; however, fumonisin synthesis is more significant in the endosperm. In synthetic media and on cracked corn kernels, fumonisin formation is suppressed by ammonium and stimulated by acidic pH. However, disruption of two genes involved in pH regulation in filamentous fungi (PACC, FCC1) did not suppress fumonisin formation under acidic conditions. Forward and reverse subtraction EST libraries made from RNA isolated from wild-type and fcc1 mutant grown on corn revealed numerous unique sequences. Clones with high similarities to know genes were classified into eight groups based on function. Work is now underway to identify genes involved in both fumonisin biosynthesis and pH regulation and study their coordinated expression profile.

The major objective of the fumonisin research team at the USDA ARS lab in Peoria, IL is to develop strategies to eliminate or reduce fumonisin contamination in maize. The focus of their work is to understand the genetics and biochemistry of fumonisin production in order to identify targets that can be exploited to control fumonisin contamination. Robert Proctor presented an overview of the rationale and history involved in elucidating the fumonisin biosynthetic gene cluster. The cluster consists of 15 co-regulated genes (*FUM*1 and *FUM*6 through *FUM*19) whose expression is correlated with fumonisin formation. Predicted functions of the *FUM* genes are based on sequence similarities and include activities for fumonisin biosynthesis, transport and self-protection. Disrupting specific *FUM* genes resulted in three distinct phenotypes for fumonisin production. Disruption of *FUM1, FUM6* and *FUM8* blocked fumonisin

biosynthesis with no detectable accumulation of intermediates while disruption of *FUM7, FUM9, FUM10, FUM11, FUM12, FUM13, FUM14* and *FUM19* resulted in the production of new and altered fumonisin homologues. Disruption of *FUM15, FUM16, FUM17* and *FUM18* had no noticeable effect on fumonisin production.

For many years, Fusarium ear rot of corn was not considered an important ear rot disease in the United States because kernel damage was relatively minor on most hybrids in most locations. However detection of fumonisin in apparently healthy corn kernels and corn-based products and the publication of the Guidance for Industry: Fumonisin Levels in Human and Animal Feeds issued by the FDA and published in the November 9, 2001 Federal Register [Vol. 66, No. 218, page 56688] stimulated a proactive interest in incorporating natural resistance to Fusarium ear rot and fumonisin contamination into commercial hybrids. Don White presented an update on the evaluation of approximately 1,600 inbred corn lines with a wide geographic distribution and maturity for high levels of resistance to Fusarium ear rot and fumonisin contamination. After two years, of field evaluations in five different environments (two natural infected and three inoculated using a severe inoculation technique), eleven sources of resistance were identified that conveyed resistance in F1 hybrids made with FR1064, a moderately susceptible, widely-used commercial inbred. One interesting observation was that some lines that are by themselves susceptible to Fusarium ear rot and fumonisin formation, are resistant in an F1 hybrid; thereby suggesting that some inbreds classified as susceptible can contribute resistance to FR1064.

The role of insects serving as vectors for Fusarium is well document in the literature. Over the past few years, scientists for Monsanto Company have conducted field trials throughout the world evaluating the impact of transgenic insect control events on controlling ear rot development and mycotoxin contamination. Bruce Hammond presented the results of the Monsanto's 2001 domestic and international field trials comparing Bt hybrids (Cry1Ab) and isogenic non-transgenic controls on fumonisin production. These results supported previously published data showing a general trend for reduced fumonisin levels in Bt hybrids compared to non-transgenic controls. Location and background genetics also influence fumonisin levels.

Fusarium verticillioides can infect maize throughout the growing season and is capable of causing disease at different growth stages, including seed root, seedling blight, root rot, stalk rot and ear rot. As part of the research effort to elucidate factors involved in the seedling rot disease phase, Anthony Glenn from the Toxicology and Mycotoxin Research Unit in Athens, GA presented additional evidence that fumonisin is a seedling pathogenicity factor and is responsible for necrotic leaf lesions and abnormal development in the sweet corn variety "Silver Queen". Only *F. verticilloides* strains capable of producing FB1 were pathogenic in the assay system supporting the idea linking pathogenicity and fumonisin production. Surprisingly, plant growth was stimulated and no necrosis was observed when seedlings were watered with a 1 ppm solution of FB1. Toxic effects (lesions and stunting) were observed when seedlings were watered with a 10 ppm solution of FB1.

Since *F. verticillioides* can infect maize at numerous developmental stages and can colonize several tissue types, there is a very strong likelihood that FB1 will be in corn debris at the end of the growing season and can leach into the soil. Migration in the soil is dependent on soil type and pH. Using a seedling colonization bioassay, Ron Riley and

colleagues showed that under laboratory conditions, after 21 days, only seedlings inoculated with a pathogenic strain of *F. verticilloides* had elevated levels of sphinganine (a maker for ceraminde synthase inhibition) and FB1 and that the degree of elevation was correlated with symptom severity. FB1 was leached into the soil only from plants inoculated with the pathogenic isolate. FB1 was taken up in a dose dependent manner from soil watered for six days with 1, 5 or 10 ppm FB1. Increased sphinganine levels were closely correlated with FB1 dosage. At 10 ppm, FB1 – treated plants had a visibly reduced root mass compared to the un-treated controls.

Panel Discussion Summary:

Kendra commented that the nitrogen metabolite repression model proposed by Woloshuk supports field observations that fumonisin formation is often higher in cornfields exhibiting nitrogen stress symptoms. Nitrogen use efficiency may play a role in regulating fumonisin levels. Under normal field conditions, nitrogen levels may be sufficient to keep the soil pH alkaline enough to keep FB1 bound to the soil. When nitrogen levels decrease, the soil pH may increase and FB1 may become biologically available. This hypothesis supports the conclusions reported by Riley; however, no one was aware of field data testing this hypothesis. A question directed to Glenn asked if the seedling pathogenicity evaluation was done on any other corn germplasm besides the sweetcorn variety "Silver Queen" and if similar results were observed under field conditions. Glenn responded that the assay was done using only "Silver Queen" and in greenhouse/growth chamber conditions but agreed that additional genotypes and growth conditions need to be examined. A discussion ensued about the likelihood of success of introducing *Fusarium* Ear Rot resistance into germplasm with commercial potential. Don White indicated that he was confident that the resistance observed in his two year multi-location screening program was sufficient but cautioned that molecular markers would be needed. Some individuals commented that to ensure superior control, the *Fusarium* Ear Rot trait should be stacked with an insect control trait, like Bt as described by Hammond. A more complete understanding of fumonisin biosynthesis and regulation process may lead to identification of effective targets for suppressing toxin formation in the field using biotechnology or chemical control methods. The potential for biological control is attractive; however, no field data is available to show efficacy and safety of this approach. Continued monitoring and selection of the highest quality corn for food uses is still the most efficient approach to minimize fumonisin contamination. Quick, inexpensive and non-destructive surveillance protocols are critical.

Microarrays To Search For pH-Dependent Genes Involved In Fumonisin Biosynthesis In *Fusarium Verticillioides*

Pirttilä A. M.[1], Payne G. A.[2], and Woloshuk, C. P.[1] [1]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN [2]Department of Plant Pathology, North Carolina State University, Raleigh, NC

Fumonisins, a class of mycotoxins produced by *Fusarium verticillioides* are synthesized under conditions of nitrogen stress and acidic pH. Whereas the structural genes of fumonisin biosynthesis belong to the polyketide pathway (4, 5), little is known about the regulatory genes involved in fumonisin biosynthesis. Mutation in the *FCC1* gene, coding for a C-type cyclin inhibits fumonisin biosynthesis at pH 6, which is also the pH of corn kernels (6). In addition, the mutation affects conidiation at high pH (6). The yeast analog of FCC1, UME3 protein phosphorylates the C-terminal domain of the RNA polymerase II (3). UME3 is downregulated postranslationally through degradation in carbon starvation, aerobic growth, oxidative stress, heat, and ethanol shock (1). UME3 is not regulated through the RAS pathway, but oxidative stress-induced destruction of UME3 requires the phosphatidylinositol-specific phospholipase C and the 26S proteasome (2). Whereas, the effect of pH on UME3 is not known, low pH restores the fumonisin biosynthesis and conidiation in the *fcc1* mutant.

In order to determine why fumonisin biosynthesis is pH-dependent, and how the FCC1 is involved in fumonisin biosynthesis and conidiation, we will perform the following microarray comparisons: 1) wild type at low and high pH to find genes involved with toxin production and pH regulation (current study), 2) wild type and *fcc1* mutant on corn to confirm the differentially expressed genes, 3) wild type and *fcc1* mutant at low pH to determine if the mutant acts like the wild type at low pH, and 4) *fcc1* mutant at low and high pH to find genes involved with condiation and pH regulation.

When gene expression in *F. verticillioides* wild type strain was compared at pH 3 and 9, 28 genes were highly expressed (> 2x) at pH 3. Of these15 were unknown, and the functions of the known genes fell into classes of toxin production, housekeeping, regulation of protein synthesis, ion transportation, and genes of unknown or other function. Since fumonisin biosynthesis is affected by nitrogen stress, high expression of an ammonium transporter at pH 3 is intriguing and the subject of further studies. As more data from the comparison of the wild type and *fcc1* mutant on corn becomes available, we will determine more precisely the genes that are coordinately expressed during fumonisin biosynthesis. In the future, the results from the microarray analyses will be confirmed by Northern blots, and the role of selected genes in fumonisin biosynthesis will be studied by generating knock-out mutants.

(1) Cooper KF, Mallory MJ, Smith JB, Strich R (1997) EMBO J 16: 4665-4675
(2) Cooper KF, Mallory MJ, Strich R (1999) Mol Cell Biol 19: 3338-3348
(3) Liao S-M, Zhang J, Jeffery DA, Koleske AJ, Thompson CM, Chao DM, Ciljoen M, van Vuuren HJJ, Young RA (1995) Nature 374: 193-196
(4) Proctor RH, Desjardins AE, Plattner RD, Hohn TM (1999) Fungal Genet Biol 27: 100-112
(5) Seo J-A, Proctor RH, Plattner RD (2001) Fungal Genet Biol 34: 155-165
(6) Shim W-B, Woloshuk CP (2001) Appl Environ Microbiol 67: 1607-1612

Fumonisin B1-Induced Neural Tube Defects: Disruption Of Membrane Sphingolipids And Folate Transport

J. Maddox[1], L. Starr[1], R. Riley[2], G. Bennett[1], and J. Gelineau-van Waes[1].
[1]University of Nebraska Medical Center, Omaha, Nebraska, [2]USDA-ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens, GA

Fumonisin B1 (FB1) is a mycotoxin produced by *F. verticilliodes*, a common contaminant of corn involved in the etiology of various diseases in different species. FB1 disrupts sphingolipid biosynthesis by inhibiting the enzyme ceramide synthase, and has recently been implicated as a developmental toxicant. The current studies investigated maternal FB1 exposure, sphingolipid depletion, and subsequent disruption of folate transport systems in the induction of neural tube defects (NTDs). FB1 exposure during early stages of gestation caused NTDs (exencephaly) in inbred mice, but its incidence was highly strain-dependent, and the timing of FB1 exposure was critical. The ability of FB1 to cross the placenta was demonstrated by injecting pregnant dams with ^{14}C-labeled FB1 and measuring radioactive uptake in embryos and maternal tissues. In addition, sphinganine levels were shown to be significantly elevated following FB1 exposure in embryos and placenta from the susceptible murine strain. The impact of FB1 on folate transport was investigated *in vivo* by measuring ^{3}H-folate uptake in control and FB1 embryos. In this study, ^{3}H-folate uptake was inhibited by FB1 exposure. The ability of supplemental maternal folate to protect against the teratogenic effects of FB1 was also investigated. Maternal folinic acid supplementation by daily oral gavage was able to reduce the incidence of FB1-induced NTDs by approximately 50%. However, co-administration of the ganglioside GM1 during FB1 exposure was able to almost completely (95%) rescue the phenotype. Since GM1 is co-localized with the GPI-anchored folate receptor in "lipid rafts", it is proposed that GM1 is an important component of the membrane microdomain necessary for proper folate receptor function, and adequate uptake of folate during development to protect against NTDs. It is anticipated that the development of this *in vivo* model system to study FB1-induced teratogenesis will aid in further understanding the mechanism(s) of FB1 toxicology, and the induction of NTDs following maternal FB1 exposure.

Identification Of *Fusarium Verticillioides* Genes Differentially Expressed In Response To A Corn Antimicrobial Compound (BOA)

Anthony E. Glenn. USDA, ARS, Russell Research Center, Toxicology & Mycotoxin Research Unit, Athens, GA

Corn produces DIMBOA and DIBOA, small molecular weight, highly reactive preformed compounds that are implicated in resistance to microbial diseases and insect feeding. Due to the inherent instability of these compounds, they chemically transform into the more stable antimicrobials MBOA and BOA, respectively. *Fusarium verticillioides*, the most common fungal pathogen associated with corn, has the physiological capacity to biotransform MBOA and BOA into non-toxic metabolites. Thus, the antimicrobials are not effective deterrents of this fungal endophyte of corn. While data suggest biotransformation of these compounds is not a major virulence factor, such metabolic capacity may enhance the ecological fitness of *F. verticillioides* in a cornfield environment. Genetic analyses of *F. verticillioides* showed at least two loci, *FDB1* and *FDB2*, are necessary for biotransformation. The biotransformation pathway is suggested to involve hydrolysis of BOA (Fdb1p) to produce 2-aminophenol, which is subsequently modified by addition of a malonyl group (Fdb2p) to produce *N*-(2-hydroxyphenyl) malonamic acid (HPMA). If either gene is mutated, detoxification does not occur and the fungus cannot grow on BOA-amended medium. In an effort to molecularly characterize *FDB1* and *FDB2* as well as other genes involved in biotransformation, we employed suppression subtractive hybridization (SSH), which would target genes up-regulated in response to BOA. The BOA-subtracted cDNA library (384 clones) was sequenced, providing 182 clones with homology to >30 proteins (BLASTX; ≤1E-04). Among the clones identified, those with similarities to amidase and arylamine *N*-acetyltransferase were of particular interest, since these enzymes catalyze chemical modifications similar to those postulated for Fdb1p and Fdb2p. Genomic cosmid clones were identified for each using the respective cDNAs as probes. The putative amidase cosmid clone genetically complemented an *fdb1* mutation, while the putative *N*-acetyltransferase (=*N*-malonyltransferase) cosmid clone complimented an *fdb2* mutation. Thus, the proposed chemical modifications and the putative proteins involved are mutually supported. These results demonstrate the utility of SSH for cloning genes previously characterized by forward genetics.

Sources Of Resistance To Fumonisin Accumulation And Fusarium Ear Rot Of Corn

M.J. Clements[1], C.M. Maragos[2], J.K. Pataky[3] and D.G. White[3]. [1]USDA-ARS Corn Host Plant Resistance Research Unit, Mississippi State, MS; [2]USDA-ARS Mycotoxin Research Unit, Peoria, IL, [3]University of Illinois Dept. of Crop Sciences, Urbana, IL

Fumonisins have been associated with serious toxicoses of animals and humans. Although corn (*Zea mays* L.) inbreds with resistance to kernel infection by *Fusarium verticillioides* (Sacc.) Nirenb. (Syn = *F. moniliforme* J. Sheld.) and/or to Fusarium ear rot have been reported, inbreds with a high level of resistance to fumonisin have not been identified. F_1 crosses of nearly 1600 inbreds with the widely-used inbred, FR1064, were evaluated for a low concentration of fumonisin in grain and resistance to Fusarium ear rot in inoculated and naturally infected plots in 2000. Thirty-five F_1 crosses with a concentration of fumonisin in grain less than or equal to 5µg/g at both locations were selected for further study. The inbreds from which these 35 F_1 crosses were produced form a genetically diverse group, including yellow, white and red kernelled lines; flint and dent lines; and early through late maturing lines. In 2001, a low concentration of fumonisin and low severity of Fusarium ear rot were associated with several of the F_1's and their distinct F_2 and BCP_2 generations. This suggests that several dominant genes are involved in resistance, and that alleles for resistance from these inbreds can be transferred to FR1064.

Characterizing Fumonisin Biosynthesis Through Analysis Of *FUM* Gene Deletion Mutants

Robert A.E. Butchko, Ronald D. Plattner, Anne E. Desjardins and Robert H. Proctor. USDA-NCAUR-ARS, Peoria, IL

Fumonisins are produced by *Gibberella moniliformis*, a causal agent of maize ear and stalk rot, and pose a health risk to humans and livestock alike. Recently, a fumonisin biosynthetic gene cluster was described in *G. moniliformis*. The cluster consists of 15 co-regulated genes (*FUM1* and *FUM6* through *FUM19*) with patterns of expression that are correlated with fumonisin production. In an effort to determine the functions of *FUM* genes and to more fully characterize the biochemical pathway leading to the formation of fumonisins, we used targeted gene disruption to generate individual deletion mutants for *FUM7*, *FUM9*, *FUM10*, *FUM11*, *FUM12*, *FUM13*, *FUM14* and *FUM16*. LC-MS analysis of deletion mutant culture filtrates and extracts revealed that all but the *FUM16* deletion mutants accumulated one or more putative fumonisin pathway intermediates, including some metabolites that that have not been previously described. NMR analysis confirmed the novel structure of some of the compounds. The accumulation of these metabolites suggested functions for the enzymes encoded by the *FUM* genes. These functions were consistent with the functions predicted by BLAST analysis of the genes. From the results reported here and those previously reported for *FUM1*, *FUM6* and *FUM8,* a biochemical pathway leading to the formation of fumonisins is emerging.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

SPECIAL SESSION: NOVEL TECHNOLOGIES FOR AFLATOXIN DETECTION

Moderator: *Gerald Donaldson, Wharton, TX*

Novel Technologies for Aflatoxin Detection – Opening Remarks

Jane Robens, National Program Leader, Food Safety and Health, USDA-ARS, Beltsville, MD

The average levels of aflatoxin or other mycotoxins cannot be measured accurately with current sampling technologies in the large commercial lots of peanuts, corn, cottonseed and tree nuts and any other affected commodities. Aflatoxin as a natural contaminant never occurs evenly distributed, in the field or in a harvested commodity. In addition, commercial lots are in ton amounts for many commodities; a truckload may hold up to 27.5 tons of corn and a railcar may hold 120 tons. It is thus exceedingly difficult to accurately sample for aflatoxin in the field, during transportation, in storage, or at a buying point. To handle large amounts of commodity efficiently and not impede commerce, accurate and rapid technologies are a necessity for the agricultural industries. In addition the sample size needed to achieve a level of accuracy acceptable in today's commerce uses sizable amounts of valuable commodity, and this is costly particularly to the peanut and tree nut industries. Non-destructive on line detection methods that could achieve more accurate estimates of total/average amounts of aflatoxin would speed the commerce of the agricultural industries and particularly enhance their exports. It would also aid the regulatory agencies to assure the public health. Rapid non-destructive methods of measuring mycotoxins hold the promise of being able to be incorporated into harvesting equipment to immediately sideline contaminated crop immediately for non-food uses.

The Potential Of Hyperspectral Imaging For The Detection Of Aflatoxin

G. H. Poole. Institute for Technology Development, Stennis Space Center, MS

Hyperspectral imaging is a non-invasive, non-destructive imaging technique, usually based on reflected energy. The process of taking a hyperspectral image produces a 3 dimensional dataset (called a hypercube) where the x and y dimensions are spatial, and the z dimension is spectral, which means there is a spectral curve for each pixel within the x-by-y image. The spatial size of the pixel in the image is based on the set-up of the imaging system, but can range from acres (if imaged from an airplane), to microscopic (if imaged using a microscope).

There are two primary methods of taking a hyperspectral image. The first, called a "Framing Camera", or band sequential imaging system, takes multiple x-by-y images, with a different wavelength filter each time. This system is easy, cheap and relatively quick, but limited in that it can't image moving targets very well. The second method called a "Pushbroom" or band interleaved imaging system, takes one spatial line of data across the entire spectral range, and then moves to take the next line of data. This produces many x-by-z images, varied by y. This system allows for many more closely stacked wavelength bands, and also allows for the imaging of moving targets. However, this system can be slower than the framing camera, and tends to be more expensive.

The Institute for Technology Development has used a pushbroom style imaging system to see if hyperspectral imaging would be useful to the detection and identification of molds in feedstuffs. Our preliminary study examined several different molds, and indicated that hyperspectral imaging could (a) identify mold on corn samples, and (b) differentiate what type of mold it is. Future research would look at using the already developed visible/near infrared system to find aflatoxin, as well as a UV hyperspectral imaging system currently under development.

Hyperspectral Methods To Detect Aflatoxin In Whole Kernel Corn

David Casasent, Xue Wen Chen and Songyot Nakariyakul. Dept. ECE, Carnegie Mellon Univ., Pittsburgh, PA

Hyperspectral (HS) data is very useful in agricultural product inspection, as it provides fast non-destructive internal detail on chemical and other aspects of products. We consider data for the specific case of inspection of corn. HS data allows inspection of images of many whole corn kernels in parallel; it is thus much faster than present BGYF methods, and can allow more of a shipment to be inspected. This seems needed to locate the small fraction of kernels with large aflatoxin levels. HS methods seem to overcome many BGYF inspection accuracy concerns; one corn kernel with an aflatoxin level in excess of 10,000 ppb was located by HS methods, but not by BGYF methods.

New algorithms for feature extraction and feature selection (choosing the HS wavelengths to use) are the major new contributions of our work. These are solutions to the general problems of high-dimensional data, a training set of inadequate size, and optimal feature selection. A new High-Dimensional Generalized Discriminant (HDGD) feature extraction algorithm is described. Data shows that its performance is preferable to other proposed methods on our database. Optimal feature selection is an N-P complete problem. We advance a new High-Dimensional Branch and Bound (HDBB) algorithm to select the best HS features (wavelengths to use). Our algorithm yields better results than standard SAS forward/backward selection etc methods and its results are found to be comparable to those of optimal methods on our database.

Our results are initial only. A larger database is needed. Similarly, details of the HS imaging system need to be determined and refined. However, initial results are very promising, as our algorithms consistently outperform others and BGYF methods. The use of HS data also seems useful in grain cleaning operations. Our algorithm results are general enough to be of use in many other USDA applications and databases. Similarly, HS data has uses in many other USDA inspection applications.

We thank Dr. Tom Pearson (ARS, Kansas) and Dr. Don Wicklow (ARS, Peoria) for their database and problem definition assistance.

Trained Wasps As Chemical Detectors: Application For Aflatoxin Related Issues

Glen C. Rains[2], W. Joe Lewis[1] and David M. Wilson[2]. [1]USDA-ARS, Tifton, GA; [2]University of Georgia, Tifton, GA

Parasitic wasps optimize foraging efficiency by learning chemicals associated with food and hosts. Recent studies have also indicated that wasps of the species Microplitis croceipes can be trained to odors that they have no known history of encountering in their natural habitat. Additionally, these wasps can respond to trained odors with a distinct behavioral response that can be used to positively identify target odors. Data to date show that wasps trained to associate odors of aspergillus fungi with sugar water can subsequently: 1) distinguish among different species of aflatoxin-producing fungi, 2) recognize toxin-versus nontoxin-producing strains, 3) recognize species/ strain independent of diet background, and 4) learn individual chemicals known to be associated with the fungi. One application of this trained response is in monitoring and/or detecting aflatoxin in post harvest storage and distribution facilities for corn and peanuts. Future work will focus on development of a portable detection system that uses cartridges of trained wasps to indicate the presence of target odors in this and other applications.

Physical And Optical Properties Of Corn Infested With *Aspergillus Flavus*, *Fusarium Verticillioides* And Other Molds

Tom Pearson[1], Donald Wicklow[2], Carrie Schwartz[1]. [1] USDA-ARS-GMPRC, Manhattan, KS [2]; USDA-ARS-NCAUR, Peoria, IL

The goal of this project was to evaluate the physical and optical properties of whole corn kernels infected with one of a variety of molds and to identify features of these kernels that would identify the mycotoxin contaminated kernels from non-contaminated kernels. Corn ears in the late milk to early dough stage of kernel maturity were inoculated with one of the following molds and harvested in the Fall: *Aspergillus flavus, Aspergillus niger, Fusarium verticillioides, Fusarium graminearum, Diplodia maydis, Trichoderma viride, Acremonium zeae, Nigrospora oryzae, Penicillium funiculosum, Penicillium oxalicum, Penicillium variable.* Individual kernels were visually inspected and classified according to symptoms and severity of kernel infection.

The following types of images were collected from all kernels: color, visible and NIR transmittance, fluorescence images under a black light, and x-ray. In addition, NIR reflectance spectra (500 –1700nm) of individual kernels was collected. All images and spectra were taken on both the germ side and opposite side of the kernels. Kernel thickness, weight and volume of individual kernels were also measured.

A neural network was trained to classify kernels into their mold infection and symptom severity categories using the full NIR spectra. Classification results were greater than 95% for controls and kernels infected *Aspergillus* and *Fusarium* fungi. Classification accuracy for kernels infected with other molds was generally greater than 80% and most of the misclassification errors only placed kernels into a different mold species or severity category.

There are commercial high speed sorters capable of measuring reflected light from two different NIR spectral bands. A statistical method was developed to exhaustively search through the NIR spectra for the optimal two spectral bands to remove aflatoxin contaminated corn. It was found that the spectral bands centered at 725 nm and 1175 nm would detect greater than 99% of the kernels with aflatoxin greater than 100 ppb, and correctly classify 100% of the kernels with no detectable aflatoxin. Most kernels with measurable aflatoxin below 100 ppb were classified as non-contaminated. Applying the same decision rule on kernels infested with other molds indicate that kernels with slight symptoms were mostly classified as non-contaminated while kernels with a shriveled appearance would be classified as aflatoxin contaminated.

Data from all the images were combined and used to classify infected kernels into their infecting mold species and disease severity category. The most important images for classification purposes were, in order of importance: blue channel of the color reflectance image, x-ray image, and NIR transmittance image at 780 nm, and UV fluorescence image. Using the mean pixel intensities and standard deviations from all these images, approximately 85% of all kernels could be classified into the correct mold species and severity of damage category.

Using Cyclodextrins In A Rapid Inexpensive Aflatoxin Screening Method For Field Research Applications.

David M. Wilson[1], Joao Augusto[1], Corley Holbrook[2] and Neil Widstorm[2]. [1]Department of Plant Pathology, University of Georgia; [2]USDA/ARS, Tifton, GA

The approach we have taken in developing the screening method is to use beta-cyclodextrins to enhance the fluorescence of aflatoxins after an extraction with 80 % methanol-water. Prior work published using several beta-cyclodextrins with HPLC aflatoxin analysis was verified and we have been able to extend this work to develop a rapid screening method for corn. The samples are extracted in 80 % methanol, filtered, the sample is diluted and the beta cyclodextrin is added to the sample. The resulting mixture is placed in the fluorometer and results read. The total cost for supplies for this procedure is around $1.00 per sample. The results can be used for field screening from 10-100,000 ppb and more expensive quantitative aflatoxin analytical techniques can be used to verify the results when needed. This simple method will make field screening possible with a minimum expense. We need to improve the method and make sure it works with many corn, peanut and millet genotypes.

The use of the cyclodextrins to enhance the fluorescence of aflatoxin is a desirable trait because the solution is very stable. When one reacts the plant extract with bromine or iodine to increase the fluorescence the resulting solution had to be allowed to react before reading and then the fluorescence rapidly declines. The simple method we have developed seems to work quite well with corn, it has not yet been tested on millet and it works well with some peanut genotypes and not others. Therefore we need to improve the preliminary cleanup of the peanut, corn and millet extracts. We can remove the problems in peanut using a purification column made in the laboratory and containing alumina and florisil. The purification steps are very similar to those published by the Dawson lab by Charles Holiday many years ago. However we are not using any benzene or halogenated solvents in our method.

The approach we are using is to mimic the extraction that one would use when the determination step is ELISA or the immunoaffinity column. We will verify our results by correlating the aflatoxin concentrations we get with the numbers obtained by the Vicam fluorometer method and by one of the several AOAC official HPLC methods.

The primary goal is not to replace any current quantitative method with a simple method. The goal is to give a rapid and reliable screening method for use in research that can be complemented by a rigorous analytical assay to serve as the quality check.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

15th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 2: POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS

Moderator: *Paul Bertels, National Corn Growers Association*

Induction Of Atoxigenicity In *Aspergillus Flavus* By Walnut Phytochemicals

Russell J. Molyneux[1], Noreen Mahoney[1], Bruce C. Campbell[1], Ryann Muir[2] and Abhaya Dandekar[2]. [1]Western Regional Research Center, ARS-USDA, Albany, CA; [2]Department of Pomology, University of California, Davis, CA

Walnuts show less propensity for aflatoxin contamination than other tree nuts. In particular, the cultivar 'Tulare' is exceptionally resistant to aflatoxigenesis. The resistance factor(s) are restricted to the seed coat (pellicle) and are not present in the kernel. Chemical analysis of the seed coat has established that the inhibitory activity resides in a complex of hydrolyzable tannins common to all walnut cultivars. The 'Tulare' tannin completely suppressed growth of *A. flavus* at a concentration of 0.5% in the media, with no aflatoxin formed; at a concentration of 0.25%, fungal growth was retarded and aflatoxin was reduced to 0.06% of control.

Aflatoxin biosynthesis appears to be inhibited by gallic acid, produced from the tannin by the action of a tannase known to be present in *A. flavus*. Experiments *in vitro* with pure gallic acid showed that aflatoxin levels were reduced to ca. 12% of control at a concentration of 200 ppm in media consisting of either walnut or pistachio kernel without seed coat. Ellagic acid is concurrently produced and may also have an inhibitory effect, although its comparative activity cannot be determined because of its extreme insolubility. Treatment of walnut seed coat tissue with anhydrous methanolic HCl yields methyl gallate and ellagic acid, the levels of which can be measured by reverse phase HPLC. Gallic acid levels in seed coat of 'Tulare' and the variety 'Chico', which is susceptible to aflatoxin formation, were determined on a biweekly basis throughout the growing season. Levels in 'Tulare' were significantly higher, and were maintained throughout the growing season, whereas those in 'Chico' declined steadily as the nuts matured. At maturity, 'Tulare' had a gallic acid content of 3.3% (dry weight basis) while the level in 'Chico' was only 1.4%.

'Kerman' pistachio seed coat had 0.5% gallic acid, but 'Nonpareil', 'Mission', and six other almond varieties, only had trace levels (<0.1%). Within the group of tree nut seed coats tested so far, gallic acid content correlated inversely with ability to produce aflatoxin. The hydrolyzable tannins in pistachio are structurally similar to those in walnut, differing primarily in that they do not generate ellagic acid on hydrolysis; they therefore consist entirely of gallate esters of polyols. Moreover, the tannin is concentrated mainly in the hull, with low levels in specific parts of the seed coat. However, pistachio hull tannin is a potent inhibitor *in vitro* of *A. flavus* growth, with none being observed at 40 ppm tannin in pistachio kernel/agar media.

The evidence indicates that hydrolyzable tannins are capable of inhibiting growth of *A. flavus* and that atoxigenicity is phytochemically induced by biosynthesis and maintenance of high levels of tannins throughout the growing season. Gallic acid, produced *in situ* by a fungal tannase, is a specific tannin component responsible for suppression of aflatoxin biosynthesis by the fungus and should be amenable to enhancement of levels by conventional breeding or genetic manipulation.

Limestone Effect Upon Aflatoxin In Naturally Contaminated Corn

Noemi Castaneda, Sandra Laguna, Yolanda Rodríguez, Gloria Laura Anguiano y Doralinda Guzmán-de-Peña. Departamento de Biotecnología y Bioquímica. CINVESTAV-IPN, Unidad Irapuato

Corn treated with limestone, in hot water and overnight soak is a process used in Mexico for more than 5000 years, as a treatment to make tortillas. This process known as "nixtamalizacion" can improve the nutritious quality of corn an it could also destroy the aflatoxin present in corn. However, the hypothesis stated by several authors, that the opening of the lactone ring of the aflatoxin occurred during the alkalin treatment could be closed when the dought reached the human stomach, has devalued the use of this ancient technique.

The objective of the present study was to confirm the high effectiveness of the limestone treatment upon aflatoxin in corn and the possible reconstitution of the aflatoxin in the corn dought after acidic treatment. Thus, samples of naturally contaminated corn with aflatoxin (760±ug 1Kg) and no contaminated corn from the state of Tamaulipas were used. Ten grams of natural limestone and 4 lts of water were used for each Kg of corn, the mixture was boiled during 50 min and soaked for more than 12 hrs. Every experiment had one control and two replicates. After alkaly treatment the grain was washed 3 times and grounded to obtain the dought.

For the acidic treatment two different amounts of dough were used (53 and 328 gms) based on the minimal and maximum amount of tortilla Mexicans eat. Concentrated HCl was added to the dought and after 30 min of incubation at 37°C, and aflatoxin extraction was perfomed. Samples of corn flower (AFB+ and AFB-) as well as dought (AFB+ and AFB-) were bioassayed in 8 days old chicks to evaluate toxicity of the product.

The use of limestone treatment as previously described showed an effectiveness around 95% destroying aflatoxin B1 and the aflatoxicol present in naturally contaminated corn. The acidic treatment did not increase the amount of aflatoxin. The remanent product (15%) present in dought was not toxic for 8 days old chicks. Emphasis is made in the importance of maintaining the use this process in corn for human consumption as well as the necessity to evaluate its role in the industrial process to make tortillas and other corn products.

Genetic Analysis Of Inhibitory Proteins From Maize Seeds

Gary A. Payne[1], Ahmad Fakhoury[1], Kirsten Nielsen[2], Mariana Kirst[2] Quincy Gerrald[1], and Rebecca Boston[2]. Departments of [1]Plant Pathology and [2]Botany, NC State University, Raleigh, NC

The focus of our research is to identify corn seed proteins with inhibitory activity against *Aspergillus flavus*. We report here the further characterization of a ribosome inactivating protein (RIP1) and two chitinases (Chit 1 and Chit2) that inhibit the growth of *A. flavus*. Previous research showed that RIP1 is toxic to *A. flavus* in vitro. To examine the effect of RIP1 on infection by *A. flavus* in the field, we screened the maize inbred W64A and its near isogenic mutants *opaque-2* (*o2)*, *o2- Agroceres (o2-Agro)*, *o2-Italian* (*o2-It)*, and *floury-2* (*fl2)* for resistance to infection by an *A. flavus* transformant that expresses a green fluorescent protein reporter gene. Kernels homozygous for the *o2* mutant alleles had maize ribosome-inactivating protein 1 proenzyme (proRIP1) levels 50-100-fold lower than normal W64A kernels whereas W64A *fl2* had proRIP1 levels equivalent to those of normal kernels. Kernels from ears inoculated with GFP-*A. flavus* were cultured for evaluation of infection of the endosperm by GFP-*A. flavus*. Kernels with low levels of proRIP1 had an infection rate by GFP-*A. flavus* of almost 50%. In contrast, kernels with high proRIP1 levels had only a 2% infection rate. These results suggest that maize proRIP1 plays a role in protection of the kernel against invasion by *Aspergillus flavus*.

We recently purified a chitinase from Tex6 with inhibitory activity against *A. flavus*. Primers from a partial peptide sequence of the purified chitinase were used to obtained full-length clones for two Tex6 chitinases. These two chitinases, designated Chit1 and Chit2, were sequenced and their sequences compared to those of known chitinases. Chit1 and Chit2 show 92% identify to each other. These two chitinases also have sequence similarity to two previously identified chitinases from corn, ChitA and ChitB. Chit1 is 90% identical to ChitA, and Chit2 is 96% identical to ChitB. Small differences in sequence can account for large differences in chitinase toxicity. ChitA and ChitB, for example, show 90% similarity yet ChitA is much more toxic to fungi than ChitB. It is not known if the toxicity of the Tex6 chitinases is due to their unique sequences or if they are expressed at a higher level in Tex6 than other corn genotypes. The overall goal of this project is to develop DNA markers that can be used to follow the segregation of resistance to aflatoxin accumulation in marker-assisted breeding programs. We are in the process of designing specific probes for these chitinases to determine if their presence segregates in corn with resistance to *A. flavus*.

Structures And Biological Activities Of The Inhibitors Of Aflatoxin Production, Aflastatin A, Blasticidin A And Their Derivatives

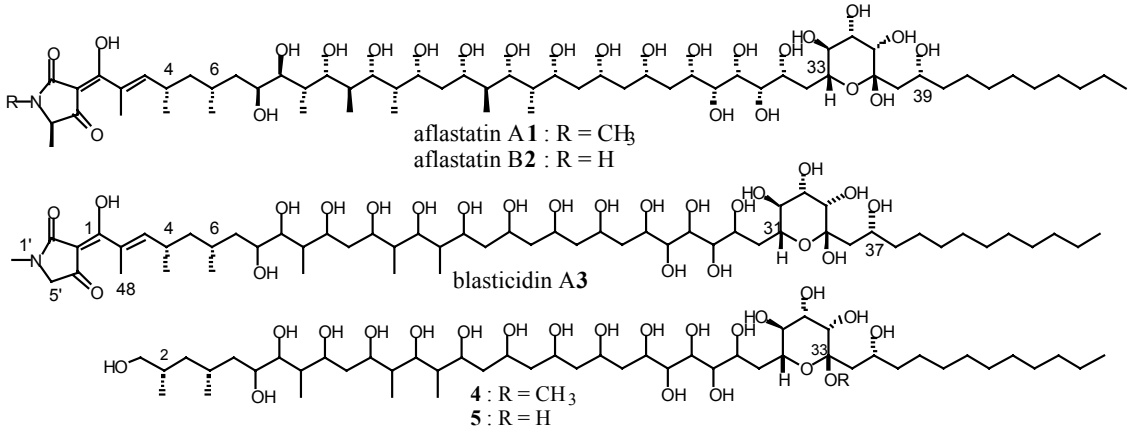
Shohei Sakuda, Hiroyuki Ikeda, Takefumi Nakamura, Makoto Ono and Hiromichi Nagasawa. Department of Applied Biological Chemistry, The University of Tokyo

During the course of our studies on specific inhibitors of aflatoxin production in *Aspergillus parasiticus*, we found aflastatin A (**1**) and B (**2**) produced by *Streptomyces* sp. MRI142.[3] We determined the structures of **1** and **2** by NMR analysis and chemical degradation experiments.[1,4] They are tetramic acid derivatives with a highly oxygenated alkyl side chain. Aflastatin B is *N*-demethyl derivative of **1**. Absolute configurations of 29 chiral centers contained in **1** were chemically determined.[6]

We discovered blasticidin A (**3**), an antibiotics produced by *Streptomyces griseochromogenes*, in the literature. We found its inhibitory activity toward aflatoxin production and determined its structure.[2,7] The structure of **3** is very similar to **1**.

Aflastatin A completely inhibited aflatoxin production by *A. parasiticus* at 0.5 □M.[3] It caused a little growth delay, but mycelial weight of the fungus was not affected after 7 days of cultivation in liquid culture. When **1** was topically applied on raw peanuts and *A. parasiticus* was cultivated on the peanuts, aflatoxin production in the peanuts was inhibited. Aflastatin A showed antimicrobial activity against some microorganisms such as *Saccharomyces cerevisiae*. Aflatoxin production inhibitory activity of **2** and **3** was almost the same as that of **1**. Pentaketide-derived melanin biosynthesis by *Colletotrichum lagenarium* was also inhibited by **1**.

Derivatives **4** and **5** without the tetramic acid moiety of **3** maintained strong inhibitory activity toward aflatoxin production by *A. parasiticus*.[8] They did not cause any growth delay or morphological changes of the fungus. Furthermore, they completely lost antimicrobial activity toward *S. cerevisiae*.



References

1) S. Sakuda *et al*., *J. Am. Chem. Soc*., **118**, 7855-7856, 1996. 2) S. Sakuda *et al*., *Tetrahedron Let*., **38**, 7399-7402, 1997. 3) M. Ono *et al*., *J. Antibiotics*, **50**, 111-118, 1997. 4) M. Ono *et al*., *J. Antibiotcs*, **51**, 1019-1028, 1998. 5) S. Sakuda *et al*., Biologically Active Natural Products: Agrochemicals., Edited by H. G. Cutler, CRC Press LLC, 185-199, 1999. 6) H. Ikeda *et al*., *J. Org. Chem*., **65**, 438-444, 2000. 7) S. Sakuda *et al*., *J. Antibiotics*. **53**, 1265-1271, 2000. 8) S. Sakuda *et al*, *J. Antibiotics*. **53**, 1378-1384, 2000.

Panel Discussion. Potential Uses Of Natural Products For Prevention Of Fungal Invasion And/Or Aflatoxin Biosynthesis In Crops

Panel Chair: Gary A. Payne
Panel Members: Doralinda Guzman-de-Pena, Russell J. Molneux, Gary A. Payne, and Shohei Sakuda

Summary of Presentations: The moderator of this session was Paul Bertels of the National Corn Growers Association. Three presentations in this session examined the inhibitory activity of natural products toward *Aspergillus flavus* and aflatoxin production. A fourth presentation described the effect of limestone used during nixtamalizacion on the inactivation of aflatoxin.

Russell Molyneux presented exciting results on his research with walnut tannins. Walnuts have been observed to be more resistant to aflatoxin contamination than other nut crops. At least part of this resistance is associated with the seed coat, as removal of the seed coat reduces the resistance of the seed to aflatoxin accumulation. The researchers at the Western Regional Research Center and the University of California at Davis showed that the resistance to fungal growth and to aflatoxin production associated with the seed coat is due to tannins. A tannin extract of the walnut variety Tulare completely suppressed growth to the fungus at a concentration of 0.5% in media. At a concentration of 0.25%, fungal growth was retarded and aflatoxin reduced to 0.06% of the control. The most active compound in walnut tannin is gallic acid, which was shown to be in a higher concentration in the variety Tulare than in a susceptible cultivar Chico. Gallic acid is produced in situ from tannins by the fungal tannase. These results show that breeding strategies to elevate the concentration of gallic acid in the seed coat is a viable strategy to reduce aflatoxin contamination.

Shohei Sakuda described another family of inhibitory natural products, not from plants but from Streptomyces species. Chemical characterization of these inhibitory compounds revealed two structures that were given the names Aflastatin A and Aflastatin B. Aflastatin A, the more toxic species, inhibited aflatoxin biosynthesis but had little effect on fungal growth.

Aflatoxin A, which shows structural similarity to Blasticidin A, completely inhibited aflatoxin biosynthesis at 0.5uM. While the compound did slightly delay fungal growth it did not inhibit growth after 7 days. Aflastatin A inhibited norsolorinic acid production at 0.25 ug/ml and RT-PCR analysis showed that it also inhibited the transcription of *pksA*, *ver*-1, *omtA* and *aflR*. Aflastatin A treatment also elevated glucose consumption and ethanol accumulation in *A. parasiticus*. Interestingly, this compound also inhibited biosynthesis of the pentaketide-derived melanin from *Colletotrichum lagenarium*. The compound was shown to also inhibit aflatoxin production when applied to raw peanuts before they were inoculated with *A. flavus*.

Gary Payne described two classes of inhibitory compounds for corn seeds, a ribosome inactivating protein (RIP1) and two chitinases. The effect of RIP1 on infection of corn kernels in the field was examined using near isogenic lines of corn differing in RIP1 concentrations. Kernels with low levels of proRIP1 had an infection rate by GFP-*A. flavus* (a strain expressing the green fluorescent protein) of almost 50%. In contrast, kernels with high proRIP1 levels had only a 2% infection rate. These results suggest that

maize proRIP1 plays a role in protection of the kernel against invasion by *Aspergillus flavus.* Gary Payne also reported on the cloning of two chitinase genes from Tex6. Primers developed from a partial peptide sequence of a chitinase were used to obtain full-length clones of two chitinase genes from Tex6. These two chitinase genes (Chit1 and Chit2) are similar to one another and to two previously described chitinases from corn. Primers specific for these chitinase genes are being designed to determine if their presence segregates in corn with resistance to *A. flavus*.

Doralinda Guzman-de-Pena presented interesting results on the effect of nixtamalizacion, a process used to make tortillas, on the concentration of aflatoxin in tortillas. Controversy has surrounded the efficacy of this treatment. Some detractors have hypothesized that the lactone ring of aflatoxin, which is opened as a result of the nixtamalizacion treatment, is closed again once the treated corn enters the acidic conditions of the stomach. Dr. Guzman and her colleagues showed that the use of limestone destroyed 95% of the aflatoxin B1 and aflatoxicol present in naturally contaminated corn and that acidic treatment did not result in either an increase in detectable aflatoxin or toxicity to 8-day-old chicks. These results support other studies showing that the process, which has been used for 5000 years, is effective in reducing aflatoxin contamination of food.

Summary of Panel Discussion: Presentations in this session generated a number of questions from the audience and panel. When Russell Molyneux was asked if the increased gallic acid concentration affects flavor of the seeds, he stated that taste tests had not shown adverse favors from the higher tannin content of Tulare Walnut. Shohei was asked if he would speculate on the mode of action of Aflastatin A. He said that he knew that it had an effect on transcription but that the mechanism remains unknown. He further stated that there is no evidence to show that the compound inhibits protein synthesis. Blasticidin C is known to inhibit protein synthesis, but the structure of aflastatin A resembles Blasticidin A more than Blasticidin C. Shohei was asked if Aflastatin A could inhibit aflatoxin once its synthesis had begun. He stated that this has not been tested. Gary Payne was asked about the poster of Huiqin Xu showing that high oleic peanuts supported more post harvest aflatoxin contamination than low oleic peanuts. Gary responded by saying that the results for these studies show the importance of testing the effect of peanut fatty acid composition of post harvest susceptibility of the peanut. Gary Payne was asked about the mammalian toxicity of maize RIP. The maize RIP is a type II RIP, and these RIPs in general have low toxicity to mammals and not of concern because they are consumed in corn and wheat with no apparent toxicity. Type I RIPs, such as ricin, however can be very toxic. Doralinda Guzman-de-Pena was commended for her research clarifying the effect of nixtamalizacion on aflatoxin contamination.

Reduction Of Aflatoxin Contamination In Pistachio Kernels By Hydrolyzable Tannins In The Hull

Noreen Mahoney, Russell J. Molyneux, and Bruce C. Campbell. USDA, ARS, Western Regional Research Center, Albany, CA

Pistachio fruits represent an ideal system to study host plant resistance to *Aspergillus flavus* growth and aflatoxin accumulation. Pistachio kernels and hulls retain enough moisture at maturity to support *A. flavus* colonization and the hulls remain fleshy such that they are easily sliced open to expose the kernel though the shell split. Pistachio fruits prepared in this manner were inoculated with *A. flavus* spores, resulting in extensive colonization of both the hulls and kernels. Although the kernels were contaminated with aflatoxin, no aflatoxin was detected in the hulls. Bioassay-directed chemical fractionation of the hulls indicated that gallotannins were source of the aflatoxin inhibition. Gallotannins are hydrolyzable tannins consisting of esters of glucose (or other polyols) and gallic acid. Due to the complexities of gallotannin chemistry, the level of hydrolyzable tannins in plant tissue is determined by measuring the free gallic acid liberated upon chemical hydrolysis. Pistachio hulls were tested to contain 1.6% (dry wt.) gallic acid. A likely mode of action for aflatoxin inhibition by the gallotannins is the liberation of free gallic acid upon hydrolysis with fungal tannase. Gallic acid is a potent inhibitor of aflatoxin production *by A. flavus*; although fungal growth is not affected. However, once isolated from hull tissue, the gallotannins have strong growth inhibiting properties against *A. flavus*. One hull tannin fraction (8.2% gallic acid) completely inhibited *A. flavus* growth at only 0.04% (w/v).

The inoculation of fresh pistachio kernels with and without the hull (50 each) demonstrated the importance of the hull in reducing preharvest aflatoxin contamination in pistachios. The aflatoxin produced on the in-hull kernels was only 92µg, a 99% reduction of the 14.3mg produced on the no-hull kernels. Statistical testing of aflatoxin contaminated pistachio lots indicate that aflatoxin contamination is usually due to a single highly contaminated kernel. If each in-hull kernel was added as the only source of aflatoxin contamination to a 10lb test lot, 100% of such lots would pass the US standard of 20ppb total aflatoxin, 96% would pass the EU standard of 2ppb B1, and 24% would have no measurable aflatoxin. Significantly higher levels of aflatoxin contamination result from the addition of the no-hull kernels to otherwise clean 10lb test lots. Only 24% of these lots would pass the US standard of 20ppb, 2% would pass the EU standard of 2ppb B1; 22% would test out over 100ppb.

This laboratory study is also supported by a field study in which 99% of preharvest aflatoxin contamination was found in early split pistachio kernels with rough, shriveled hulls (Doster and Michailides, Phytopathology, 84:583-590, 1994). These shriveled hulls may have had too low a moisture content to allow colonization by *A. flavus*, resulting in pistachios similar to the no-hull kernels. More likely, since aflatoxin was detected in these hulls, is that abnormal hull development resulted in abnormal gallotannin development, reducing the aflatoxin inhibiting qualities of the hull. Research into increasing the gallotannin content of pistachio hulls could result in not only increased resistance to preharvest aflatoxin contamination, but also a reduction of the *A. flavus* fungal load in the orchard.

Characterization Of Oxygenases Involved In The *Aspergillus*/Seed Interaction

Dimitrios I. Tsitsigiannis, Terri M. Kowieski, and Nancy P. Keller. Department of Plant Pathology, University of Wisconsin

Commercial oil seed crops such as corn, peanut, cotton, and nuts are susceptible to infestation by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus.* These mycotoxigenic fungi colonize the seeds and cause tremendous yield and cash loss through tissue destruction as well as a significant health problem by the contamination of the seed with the mycotoxin aflatoxin (AF), the most potent natural carcinogen known. Because traditional plant protection and breeding methods are not sufficient to prevent this disease, research efforts have turned to deciphering the molecular events regulating the *Aspergillus*/seed interaction as a means to develop effective control measures. The purpose of my research is to identify *Aspergillus* and seed molecules important in the *Aspergillus*/seed interaction which can lead to rational approaches in designing control strategies that can ultimately reduce or eliminate AF contamination. *Aspergillus* spp. activate seed lipid metabolizing enzymes called lipoxygenases (LOXs) that convert linoleic acid into hydroperoxy fatty acid derivatives. In the plant, these metabolites are converted into a wide array of compounds having functions that range from elicitation of plant defenses to generation of volatiles that are released in response of insect feeding and serve as a chemical signal for natural insect enemies. Seed LOX products induce asexual sporulation in *Aspergillus* spp. Our hypothesis is that these seed fatty acids induce sporulation as they have structural and functional similarities to endogenous *Aspergillus* sporogenic factors called psi factor (a mixture of oleic, linoleic and linolenic acid derivatives). We have recently cloned three genes putatively involved in psi biosynthesis*, ppoA*, *ppoB* and *ppoC*. The major objective of our research is to characterize these genes in *Aspergillus* and determine the relative importance of psi factor and seed fatty acids in *Aspergillus* sporulation and mycotoxin production.

The Mode Of Action Of The Aflatoxin Production Inhibitors, Aflastatin A And Blasticidin A

Tatsuhiko Kondo, Tomoya Yoshinari, Hiromichi Nagasawa, and Shohei Sakuda.
Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Aflastatin A and blasticidin A have very similar structures, and they strongly inhibit aflatoxin production by *Aspergillus parasiticus* via an unknown mechanism. We found that aflastatin A clearly inhibited production of norsolorinic acid, an early biosynthetic intermediate of aflatoxin, at a concentration of 0.25 μg/ml. RT-PCR, and real-time quantitative PCR (TaqMan PCR) experiments indicated that the transcription of genes encoding aflatoxin biosynthetic enzymes (*pksA*, *ver-1*, and *omtA*) and a gene encoding a regulatory protein for expression of the biosynthetic enzymes (*aflR*) were significantly reduced by the addition of aflastatin A or blasticidin A. We also found that aflastatin A or blasticidin A elevated the glucose consumption and ethanol accumulation by *A. parasiticus*, and repressed transcription of genes involved in ethanol utilization.[1)] On glucose mineral salt medium, which contains glucose as a sole carbon source, growth of *A. parasiticus* was completely inhibited by addition of 1 μg/ml of blasticidin A. When fatty acid or succinate was added to the medium, such an antifungal effect of blasticidin A was not observed, suggesting that blasticidin A might have some negative effect on glucose metabolism. These results indicated that aflastatin A inhibits a very early step in aflatoxin biosynthesis prior to the transcription of *aflR* and can influence carbon metabolism in the fungus.[2)]

References

1) T. Kondo *et al.*, *J. Antibiotics.* **54**, 650-657, 2001. 2) S. Okamoto *et al*., Microbiology, **147**, 2623-2628, 2001.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

15th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 3: CROP RESISTANCE – GENETIC ENGINEERING

Moderator: *Scott Averhoff, Texas Corn Producers Board*

Genetic Engineering Of Peanut For Reduction Of Aflatoxin Contamination

Peggy Ozias-Akins[1], Chen Niu[1], Madhumita Joshi[1], Xiang-Yang Deng[1], Corley Holbrook[2], and Robert Lynch[2]. [1]Department of Horticulture, University of Georgia, Tifton, GA; [2]USDA-ARS-Coastal Plain Experiment Station, Tifton, GA

Through genetic engineering of peanut, we have focused mainly on two levels of protection against aflatoxin contamination: the entry of spores through insect-damaged tissues and the growth of the fungus after entry, although interfering with the aflatoxin biosynthetic pathway also is of interest. The insecticidal crystalline protein from the bacterium *Bacillus thuringiensis* (Bt *cryIA(c)*) was introduced into peanut (*Arachis hypogaea* L.) several years ago. Field tests have been conducted for five years to assess the level of insect resistance and more recently the level of aflatoxin reduction associated with insect resistance. In 2001 and 2002, both lesser cornstalk borer (LCB) infestation and *Aspergillus* inoculation were carried out in these field tests. Leaves from Bt-expressing plants contained toxin levels that were comparable between the two years (15-20 ng cryIA(c) per ml extract or ~0.03% of total protein). Pods were separated into four categories for aflatoxin analysis - damaged, non-Bt; undamaged, non-Bt; damaged, Bt; undamaged, Bt. There was a significant difference for aflatoxin levels between damaged pods vs. undamaged pods. There also was a highly significant difference in aflatoxin levels between non-transgenic, non-Bt and transgenic, Bt pods when the data were log transformed. The field experiment has been repeated in 2002 with the same experimental design and pods have been harvested. LCB damage and aflatoxin levels will again be quantified.

A bacterial chloroperoxidase (CPO) gene, was obtained from Kanniah Rajasekaran and Jeff Cary (USDA-SRRC, New Orleans). This gene is under the control of the CaMV35S promoter and has been introduced into peanut. Fifty-seven hygromycin resistant lines have been recovered and 24 have been tested by PCR for the presence of the CPO transgene. All are positive, again indicating that hygromycin selection is very effective in peanut. Southern blot analysis of two independent lines demonstrated integration of the transgene with a relatively simple integration pattern. Out of 20 hygromycin-resistant lines analyzed by Northerns, 18 showed expression at the RNA level. Tissue extracts have been tested for activity against *Aspergillus flavus* in in vitro assays. For multiple lines, there is a significant reduction in the number of colony forming units recovered after incubation of pregerminated spores in tissue extract. All cell lines are in some phase of regeneration and several have reached the whole plant stage. Additional molecular analysis will be conducted on whole plants.

A green fluorescent protein (GFP) gene driven by an enhanced double CaMV35S promoter has been introduced into peanut either alone or co-bombarded with CPO and the hygromycin resistance gene. Visual selection has allowed the recovery of several cell lines expressing GFP, but these lines appear to be mixtures of transgenic (GFP-expressing) and non-transgenic cells. More effective recovery of homogeneous GFP-expressing tissues was accomplished in the co-bombardment experiments after selection on hygromycin. The frequency of co-transformation is high enough to be of practical use. GFP allows the early detection of a transgenic line and would be particularly useful in future studies with novel selectable markers.

Genetic Engineering Of Cotton To Confer Resistance To *A. Flavus*: An Update

Caryl A. Chlan[1], Jeffrey Cary[2], Kanniah Rajasekaran[2] and Thomas E. Cleveland[2]. [1]The University of Louisiana at Lafayette, Lafayette, LA; [2] USDA/ARS SRRC, New Orleans, LA

Our goal is to develop cotton with enhanced resistance to the fungal pathogen *Aspergillus flavus* using genetic engineering. Our approach depends upon a consistent, efficient transformation/regeneration system for cotton, and the development of suitable promoter/structural gene constructs to confer resistance to the pathogen. We have made significant progress towards engineering cotton that is resistant to *A. flavus* infection. In past years we have reported on the development and refinement of cotton regeneration systems, implementation of non-cotton test plant transformation systems, and identification of regulatory regions and structural genes that we feel are excellent candidates to confer resistance to *A. flavus in vivo*. The goal is to develop transgenic cotton with modified genetic information that expresses a new pathogen resistant phenotype.

Earlier studies in our laboratories identified several regulatory elements (promoters) that might be useful to drive the expression of anti-fungal genes. In order to obtain regulated gene expression as opposed to constitutive expression, we have focused on elements of a cottonseed storage protein gene promoter. This promoter is derived from a cottonseed vicilin gene. Vicilins are seed storage proteins whose genes are transcribed during embryogenesis, and the proteins accumulate to extremely high levels in the seed. We compared the DNA sequence of a cotton vicilin promoter to other well-characterized plant promoters. Several conserved regulatory elements were identified including a seed storage protein specific element, RY repeats, an A rich response element and a vicilin box. Deletions of this promoter fused to either the native seed storage protein or GUS demonstrated that the region between positions –725 and –535 decreased levels of expression. When that region was deleted, expression of either GUS or the cottonseed storage protein was greatly enhanced. Several positive response elements were functionally identified between the –535 and – 315 region. Based on these promoter studies, we have focused this year on using functionally identified vicilin promoter elements to drive the expression of genes that are effective in inhibiting *A. flavus*.

Although we have tested a variety of candidate structural genes whose products may confer resistance to *A. flavus*, a haloperoxidase gene, and a lytic peptide gene product have been shown to be effective both *in vitro* and *in vivo*. In past years, we have also presented data concerning another potential candidate, a neutral cotton chitinase. Because we have in vivo and vitro data to support the efficacy of the haloperoxidase and the lytic peptide, this year we have focused on generating constructs to express those genes in transgenic cotton.

To regulate the expression of anti-fungal gene expression, we are focusing on combinations of two segments of the cottonseed vicilin promoter that include the previously characterized –725 or –535 bp sequences fused to either the chloroperoxidase gene or the lytic peptide coding sequencein the binary vector pBI 121.

One of these constructs, the–535/chloroperoxidase in the binary vector pBI 121 is complete and currently being used to transform plants. The other 3 constructs (-725/chloroperoxidase, -535/d4E and –725/d4E in pBI 121) are in the process of being verified prior to the initiation of transformation experiments.

Expression Of The α-Amylase Inhibitor AILP In *E. Coli* And Plants

Charles P. Woloshuk, Gyung-Hye Huh, and Burt Bluhm. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN

Our previous research has suggested that the α-amylase of *Aspergillus flavus* facilitates the colonization of maize kernels and promotes aflatoxin production in the endosperm of infected maize kernels (Woloshuk et al., Phytopathology 87:164-169; Fakhoury and Woloshuk, Phytopathology 89: 908-914). A 36 kDa α-amylase inhibitor was purified from *Lablab purpureus* (AILP) and shown to inhibit α-amylases from a number of fungi but not those from animal and plant sources (Fakhoury and Woloshuk, Mol. Plant Microbiol. Interact. 18:955-961). The protein also inhibited conidial germination and hyphal growth of *A. flavus*. Partial amino acid sequence indicates that AILP is similar to members of a lectin-arcelin- α-amylase inhibitor family. This class of inhibitors has been associated with plant resistance to insect pests. AILP is the first of this class of proteins to inhibit a fungal α-amylase and the data indicate that AILP represents a novel variant in the lectin-arcelin-α-amylase inhibitor family, having both lectin-like and α-amylase inhibitory activity.

We have hypothesized that expression of AILP in maize kernels may slow colonization by *A. flavus* and reduce aflatoxin contamination. To test this hypothesis, we are cloning the gene (*LAI*) encoding AILP, and we will express *Lai* in *E. coli* and maize. Southern analysis indicated that *Lai* in *L. purpureus* is a multigenic family containing at least nine genes. We have cloned the upstream and downstream regions of the *LAI* genes by genomic walker PCR. From the DNA sequence analysis of these regions we designed PCR primers to obtain all *Lai* genes. Of these, six genes encoded proteins containing the peptide sequences obtained from the native protein. The six putative *LAI* genes were cloned into the pET28C vector for expression in *E. coli*. All six proteins accumulated as inclusion bodies. Solubilization of the AILP proteins was achieved with non-detergent sulfobetains as solubilizing agents. We have also developed antibodies in rabbit to one of the recombinant proteins. Western analysis indicated that the antibodies recognized all six of the soluble, renatured AILP proteins. Additionally we cloned one of the *LAI* genes into the binary vector pBI121 and transformed the construct into Arabidopsis. Currently we are testing the AILP proteins from *E. coli* for α-amylase inhibitor activity and screening *Arabidopsis* transformants for AILP expression. Our goal is to identify a *LAI* gene that encodes an active α-amylase inhibitor prior to transforming the gene into maize.

Enhanced, Stable Expression Of Antifungal Genes In Transgenic Cotton Is Vital For The Control Of *Aspergillus Flavus*

K. Rajasekaran[1], J.W. Cary[1], T.J. Jacks[1], C.A. Chlan[2] and T.E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]University of Louisiana, Lafayette, LA

Our previous research has demonstrated that the transgenic expression of a bacterial chloroperoxidase (*cpo-p*) or a linear, amphipathic, synthetic, lytic peptide (D4E1) offers the potential for the control of the aflatoxigenic *Aspergillus flavus*. Our presentation at this workshop will be limited to our work on the antifungal peptide, D4E1. So far, we have systematically demonstrated the effectiveness of the synthetic peptide D4E1 in the following ways: 1) purified D4E1 controlled *A. flavus* (IC_{50} = ±10 μM) and several other microbial pathogens, including vascular pathogens of cotton. 2) Crude protein extracts from leaf tissue of transgenic tobacco plants expressing the peptide significantly reduced the fungal colonies arising from germinated conidia of *A. flavus* and *Verticillium dahliae*. 3) Transgenic tobacco plants also showed increased resistance in planta to the anthracnose-causing fungal pathogen, *Colletotrichum destructivum*. 4) Crude extracts from transgenic cotton callus and leaf tissue showed control of *A. flavus* although the results were not significant with leaf extracts. 5) In situ assays with immature cottonseeds, inoculated with a GFP-expressing *A. flavus* strain, showed that the transgenic cottonseeds are capable of delaying and reducing the fungal advance in both seed coat and cotyledons, as measured by the GFP fluorescence. Based on these antimicrobial assays and corresponding molecular assays (PCR, RT-PCR and Southern blot analyses) we selected several transgenic cotton lines for evaluation in planta of the R_1 progeny seedlings to a seedling pathogen, *Thielaviopsis basicola* that causes black root rot in emerging seedlings. The IC_{50} value for this pathogen assayed with the purified peptide was 0.5 μM. The results were compared with control, transgenic seedlings expressing the β-glucuronidase marker gene only. The seedlings were evaluated for black discoloration on hypocotyl and roots, branching of roots and in severe cases, constricted hypocotyl with loss of cortical tissue and lack of vigor. A greater percentage of transgenic progeny seedlings escaped severe damage by *T. basicola* whereas majority of the control seedlings succumbed to the disease. The fresh weight of transgenic seedlings was significantly higher than that of controls. Similar tolerance in planta to *A. flavus* infection is yet to be determined in greenhouse or field trials. Obviously, a higher level of stable transgenic expression is needed to control the saprophytic, aflatoxin-producing fungus, *A. flavus* at the site of entry by the fungus. To achieve this we are currently working on increasing the expression level by the following means:

1. We are exploring the possibility of increased expression of antifungal genes through chloroplast transformation. Several studies in other laboratories with tobacco and tomato have demonstrated that increased expression of up to 50% total soluble cellular protein is possible. Upon fungal infection the lysed chloroplasts at necrotic areas would release the antifungal peptide at the site of attack thus preventing further advance by fungal pathogens. Maternal transmission of the plastid genome prevents escape of transgenes through pollen.

2. With regard to the second antifungal gene that we are evaluating, the codon usage for the bacterial *cpo-p* gene was optimized for expression in cotton. A synthetic, codon-optimized *cpo-p* gene has been generated and subcloned into a plasmid vector and its sequence confirmed. The optimized gene is being placed into a binary vector for expression in tobacco and cotton.
3. Experiments are underway in cotton to study the effects of scaffold attachment regions (SARs) on the enhanced expression and stability of antifungal proteins in cotton. To our knowledge, no reports exist on the role of SARs on cotton transformation efficiency and transgene expression. *Agrobacterium*-mediated transformation of cotton hypocotyl and cotyledon tissue has been performed with a GUS reporter gene alone or flanked by SAR sequences (obtained from Dr. Weissinger, NC State). Callus tissue is currently being subcultured for generation of embryogenic callus. Transformation efficiency, GUS expression levels, and stability of cotton transgenes will be evaluated.
4. Tissue specific expression of the antifungal genes is also one of our major focus areas of research. Using differential display and proteomics, we have identified genes/proteins that were selected based on their apparent seed-coat specific expression. One gene that displays homology to a yeast and *Arabidopsis* cell differentiation gene is currently being characterized for seed coat-specific expression in cotton. Three unique seed coat-specific proteins have been isolated by proteomics and their amino acid sequences are being determined for comparison to sequence databases. From microscopic studies of GFP-expressing *A. flavus* invasion of cottonseed in our laboratory, we have determined that the fungus appears to enter through the chalazal end and ramifies extensively along the inner seed coat prior to invading the cotyledons. Therefore, use of seed coat-specific promoters should be effective in inhibiting fungal invasion of cottonseed and at the same time keep the antifungal compound from being expressed in tissues such as the embryo and endosperm that are components of cottonseed meal used in feeds. We have studied the expression patterns of soybean *lox-3*, a gene that is highly expressed in seed. In collaboration with Dr. Chlan we are focusing on promoters derived from cottonseed storage protein genes (e.g. vicilin).

Transformation Of Virginia And Runner Type Peanuts With Mod 1, A Gene Encoding An Anti-Fungal RIP From Maize

Arthur Weissinger[1], Minsheng Wu[1], and T.E. Cleveland[2]. [1]Crop Science, North Carolina State University, Raleigh, NC; [2]USDA-ARS-SRRC, New Orleans, LA

One of the most difficult aspects of producing transgenic peanut lines with resistance against *Aspergillus* infection and subsequent aflatoxin contamination is the dearth of appropriate antifungal proteins that are effective in preventing growth of the fungus, and yet are harmless to the plant in which they are expressed. For example, although we were able to demonstrate that D5C and other lytic peptides developed by Demegen, Inc., are highly active against *A. flavus in vitro*, it was not possible to produce peanut plants that expressed D5C because the peptide is also strongly phytotoxic. In order to overcome this difficulty, we have focused our present efforts on a native plant protein, RIP 1, that inhibits growth of *A. flavus*, but which has very little activity against plant ribosomes.

RIP 1 is a ribosome inactivating protein from maize which has been shown to inhibit hyphal growth of *Aspergillus flavus in vitro* when spores were exposed to an activated form of the protein. RIP 1 is expressed in maize as an inactive pro-RIP that must be activated by proteolytic cleavage. Mod 1 is a synthetic gene that encodes an active RIP 1 identical to the proteolysis-activated form of the protein found in maize. Expression of this gene to produce the active form of RIP 1 in peanuts could offer significant protection against aflatoxin contamination by retarding fungal growth.

We have also worked to improve the overall efficiency of peanut transformation through the use of matrix attachment regions to enhance gene expression and stability. We have also developed a new procedure in which DNA is transferred to imbibed peanut embryos before somatic embryo formation, in an attempt to reduce the time in culture and to improve fertility of transgenic plants. Finally, we are using co-bombardment to transform peanuts. In this procedure two plasmids are introduced into recipient tissue simultaneously. This simplifies plasmid construction, facilitates substitution of one gene of interest for another, and offers the possibility of removing selectable marker genes from transgenic lines to alleviate fears regarding genes conferring antibiotic resistance in foods.

Earlier, we found that Rb7 matrix attachment regions (MARs) flanking a transgene increases transient expression in peanut, indicating that these element will likely function in peanut as they do in tobacco to suppress transcriptional silencing. Further, in experiments with a gene conferring resistance against tomato spotted wilt virus, presence of flanking Rb 7 MARs significantly increased the proportion of transgenic lines that exhibited resistance against the virus. Accordingly, MARs have been incorporated into all new plasmid vectors for peanut transformation.

A transformation vector (plasmid), pMARUM, was prepared that carries the Mod 1 gene driven by the ubiquitin 3 promoter from potato, flanked with Rb 7 MARs. A second vector was prepared in which a CaMV 35S promoter drives a gene encoding hygromycin phosphotransferase (HPT), which confers resistance against the antibiotic hygromycin in peanut cultures. The HPT cassette was also flanked with Rb 7 MARs.

These constructs have been introduced into embryos of 'NCV 11' (Virginia type)

and 'Georgia Green' (runner type). Transgenic plants have been recovered from both lines that survived rigorous selection on hygromycin, and also carry the mod 1 gene. These lines will be tested for presence of RIP 1 and subsequently subjected to testing for enhanced resistance to infection by *A. flavus.*

Genetic Engineering And Breeding Of Walnuts For Control Of Aflatoxin

Ryann Muir[1], 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]UDSA/ARS Western Regional Research Center, Albany, CA

California nut crops represent a 1.5 billion dollar industry, a significant amount of which is threatened by regulations regarding aflatoxin contamination. Our strategy for reducing aflatoxin contamination has two major goals; 1) develop insect resistance to reduce insect damage 2) identify genes that reduce *A.flavus* growth and or toxin production.

For developing insect resistant walnuts we have focused on the *cry*IAc gene from *Bacillus thuringiensis*. We are currently analyzing field grown transgenic plant material and are comparing the expression of *cry*IAc using two promoters; CaMV35S and Ubi3 (from potato). Several of the transformed lines have flowered and nuts have been harvested. Feeding studies with whole nuts are currently underway and preliminary indications suggest that nuts with the CaMV35S produce better results than those with the Ubi3 promoter. Earlier analysis of vegetative tissues revealed that both constructs, CaMV and Ubi, gave significant results compared to control untransformed walnut tissues. The CaMV35S promoter gave consistently higher mortality and delayed larval development relative to the Ubi3 promoter. Activity was higher in young leaves than in mature leaves for both promoters.

Since walnuts have less aflatoxin contamination than other nut crops, we have begun examining walnut tissues (embryo and seed coat) as potential sources of resistance genes. This year our work emphasized seed coat tissue. Studies conducted at the USDA facility at Albany (Mahoney et al., unpublished) have shown that seed coat extracts from several walnut varieties, and especially from the cultivar Tulare, contain a 'factor' that strongly inhibits aflatoxin production by *A.flavus*. Preliminary results have suggested that this 'factor' is gallic acid (GA), a key component of hydrolysable tannins (HTs). Estimation of free GA by staining with rhodanine and precipitation with boric acid indicates that GA is the component of HTs responsible for the observed suppression of aflatoxin synthesis in Tulare pellicles. Genetic and molecular analysis of HT metabolism is underway to identify candidate genes responsible for the accumulation of free GA in Tulare. The first candidate gene involved in the early stages of GA biosynthesis has been cloned by RACE-PCR. cDNA libraries have been constructed from Tulare pellicle mRNA and currently fifteen thousand clones have been picked and archived. EST sequencing reactions have been initiated at 1000 clone intervals to identify the network of genes involved in GA metabolism.

Panel Discussion: Crop Resistance – Genetic Engineering

Panel Chair: Kanniah Rajasekaran
Panel Members: Arthur Weissinger, Peggy Ozias-Akins, Caryl A. Chlan, Ryann Muir and Charles P. Woloshuk

Summary of Presentations and Panel Discussions: Six presentations on the prevention of preharvest aflatoxin contamination in peanut, cotton, and walnuts were moderated by Scott Averhoff, a grower from Waxahachie, Texas. Transformation methods for these crop species have been well established by the conveners' groups and the presentations mainly centered on the efficacy of several antifungal proteins. For example, the New Orleans group (Rajasekaran, Cary, and Cleveland) summarized their efforts on the antifungal peptide D4E1 in controlling *A. flavus* and other vascular pathogens of cotton. They also presented their current focus on increasing the gene expression through various means, a key point emphasized by other presenters as well. Quantification of antifungal peptides in transgenic crops is still difficult by standard analytical and molecular methods and a concerted effort is necessary to overcome this problem. The efficacy of the bacterial chloroperoxidase gene (*cpo-p*), originally reported by the New Orleans group, on the control of *A. flavus* by crude extracts from transgenic callus or plants was also reproduced by Ozias-Akins and Chlan. Work in Weissinger's lab with another peptide D5C was discontinued because of its phytotoxicity to peanut cultures although it is highly active against *A. flavus*. Such observations are extremely important and need to be established for other antifungal proteins or peptides. For example, non-target effects of D4E1 on nodulating bacterium, *Rhizobium* as well as the possible allergenicity in animals and humans due to the presence of transgenic antifungal proteins are currently unknown. The overall cost of testing transgenic crops for commercial release and approval was a point of concern by several participants. Weissinger is currently focusing on RIP 1, a native plant protein that inhibits growth of *A. flavus*. Woloshuk's laboratory is testing the hypothesis that a fungal α-amylase-inhibiting protein from *Lablab purpureus* may be helpful in slowing the colonization by *A. flavus* and reduce aflatoxin contamination. *Lablab* is used as a human food in several developing counties with no deleterious effects. Other food crops such as corn and wheat produce amylase inhibitors but they don't inhibit fungal amylases. In walnut, the presence of hydrolysable tannins or HTs (e.g., gallic acid) was found responsible for suppression of aflatoxin production in pellicles. Ryann Muir, the presenter of this work from Dandekar's lab, mentioned that they cloned the first candidate gene involved in the early stages of gallic acid biosynthesis, DAHP synthase, using 5' and 3' RACE-PCR. Then they used the computer program MacDNASIS to translate the gene sequence into the protein sequence. In response to a question whether an elevated concentration of hydrolysable tannins affect the taste of food, she replied that in walnut, a high GA/HT content does not seem to increase astringency. Dandekar and Muir emphasized that by limiting tannin synthesis to a single, isolated tissue layer, the purported negative taste effects of tannins could be avoided.

Advantages of plastid transformation (e.g., chloroplast) were outlined by Rajasekaran (for example, increased expression – not limited to green tissues only, release of antifungals at the site of infection, prevention of gene flow through pollen,

lower probability of resistance development among target pathogens), although it was pointed out that the technology is not widely applicable beyond a few crop species. Weissinger cautioned that high production of selection agent or antifungal proteins due to chloroplast transformation may be metabolically taxing to the host plant. Achieving stable expression through the use of matrix or scaffold attachment regions (MARs or SARs) was under investigation by several research groups. In this regard, Weissinger mentioned that a MARs construct is available from his lab for research purpose only. Tissue specific expression of antifungal genes was also a point of discussion among walnut and cotton researchers (UCDavis and ARS New Orleans, respectively); in both crops, the integrity and/or the ability of the seed coat in preventing the insect or fungal entry is well established as an important factor in preventing aflatoxin contamination. Last but not the least concern among all scientists was the wherewithal to achieve successful technology transfer to the stake holders while providing amiable licensing of intellectual property from commercial, private, academic and government laboratories.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

15th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 4: MICROBIAL ECOLOGY

Moderator: *Betsa Faga, North American Millers Association*

Vegetative Compatibility And Aflatoxin Production In *Aspergillus*

Donald T. Wicklow[1] and Bruce W. Horn[2]. [1]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]USDA-ARS, National Peanut Research Laboratory, Dawson, GA

Intraspecific competition is the basis for for biological control of aflatoxins and there is a common interest in understanding the mechanism(s) by which competing strains inhibit toxin production. Evidence is presented which demonstrates a relationship between strength of the vegetative compatibility reaction and aflatoxin production in wild-type isolates of *A. flavus* and *A. parasiticus* using the suspended disc culture method (R.A. Norton, 1995). Combining aflatoxin-producing isolates belonging to different vegetative compatibility groups (VCGs) resulted in a substantial reduction in aflatoxin yield. Pairs of aflatoxin-producing isolates within the same VCG, but showing weak compatibility reactions using complementary nitrate-nonutilizing mutants, also were associated with reduced levels of aflatoxin B_1. In contrast, pairings of isolates displaying a strong compatibility reaction typically produced high levels of aflatoxins. These results suggest that interactions between vegetatively compatible wild-type isolates of *A. flavus* or *A. parasiticus* are cooperative and result in more aflatoxin B_1 than pairings between isolates that are incompatible. Successful hyphal fusions among spore germlings produce a common mycelial network with a larger resource base to support aflatoxin biosynthesis. By comparsion, vegetative incompatibility reactions result in the death of those heterokaryotic cells composed of incompatible nuclei (Leslie, 1993) and may disrupt the formation of mycelial networks at the expense of aflatoxin biosynthesis. The concept of cooperation and scale in fungal morphogenisis and secondary metabolite formation is presented.

Dispersal And Overwintering Of *Aspergillus Flavus* In Arizona And Texas

Peter J. Cotty. Southern Regional Research Center, ARS, USDA, New Orleans, LA

Contamination of cottonseed with more than 20 ppb aflatoxin is a serious concern in the United States because use of such contaminated cottonseed in the lucrative dairy market is prohibited by law. Contamination costs the cotton industries in Texas and Arizona millions of dollars annually. In most cases no options are available to producers to prevent contamination from exceeding acceptable limits.

Isolates of *Aspergillus flavus,* the causal agent of aflatoxin contamination of cottonseed, are divided to many vegetative compatibility groups (VCGs) by a heterokaryon incompatibility system. There is great variability among VCGS in aflatoxin-producing ability. Certain VCGs that do not produce aflatoxins (called atoxigenic strains) can compete with aflatoxin producers during crop development and thus reduce the aflatoxin content of the crop. Atoxigenic strains are being utilized for aflatoxin management in commercial cotton production in Arizona. Selection of effective atoxigenic strains for use in aflatoxin management was based initially on performance in greenhouse tests. Strains that were relatively common in the environment, had stable phenotypes, and were most effective at limiting contamination of wound-inoculated developing cottonbolls were selected for field use. Information is lacking on variability among VCGs in ability to disperse to and colonize crops and in ability to survive between seasons.

In order to assess potential variability among VCGs of *A. flavus* in ability to displace aflatoxin- producing fungi, small-scale field tests (1 acre) were performed at 8 locations in South Texas from the Upper Coast to the Lower Rio Grande Valley. Three tests were performed in 2000 and five were performed in 2001. VCGs were monitored with auxotroph based vegetative compatibility analyses. Eight VCGs of *A. flavus* were isolated for use in these tests from soils and cottonseed in South Texas. For each VCG, sterile wheat seed colonized by that VCG was applied to soil beneath the cotton canopy, at a rate of 10 lb. per acre in mid-May. All VCGs evaluated in a given tests were applied at the same rate to the same area. Thus, the VCGs directly competed with each other. Compositions of the *A. flavus* communities resident in fields (soil) prior to treatment, on the cotton crops at harvest, and in the fields one year after treatments were assessed. In five tests (three in 2000 and two in 2001), three VCGs were compared. In three tests, all in 2001, eight VCGs were compared.

Variability among *A. flavus* VCGs was observed in crop colonization, dispersal from treatment areas, and in overwintering. However, all VCGs demonstrated at least some ability to competitively exclude aflatoxin producers under all test conditions. This indicates that atoxigenic strain technology may have value in aflatoxin management across both irrigated and non-irrigated portions of South Texas. Superior ability to colonize and disperse was not correlated with superior ability to survive between seasons. An isolate from South Texas belonging to the same VCG as the primary atoxigenic strain (AF36) used for aflatoxin management in Arizona was included in all tests. In

competitive ability, this isolate (AF36-TX) was equal or superior to all other VCGs evaluated in 7 of the 8 tests. These data provide a basis for initial evaluation of *Aspergillus flavus* AF36 for aflatoxin management in South Texas. Overall, the relative performance of VCGs differed with test location. This may reflect superior adaptation of certain VCGs to specific environmental conditions and/or ecological niches. Use of mixtures of atoxigenic VCGs in aflatoxin management may provide an improvement over use of individual VCGs both by providing superior displacement of aflatoxin producers and by forming modified fungal communities with increased stability.

Advances In Utilization Of Atoxigenic Strain Technology To Manage Aflatoxin In Commercial Cotton

Larry Antilla[1] and Peter J. Cotty[2]. [1]Arizona Cotton Research and Protection Council; [2]USDA-ARS-SRRC, New Orleans, LA

Atoxigenic strains of *Aspergillus flavus* can be highly effective agents for limiting or preventing aflatoxin contamination of Arizona cottonseed. Field tests in Yuma County, conducted from 1996-1998 established that application of atoxigenic strain AF36 on colonized sterile wheat seed was effective at altering the *A. flavus* community associated with the treated crop so that the fungi associated with the crop had reduced potential to produce aflatoxins. This modification was associated with reductions in the aflatoxin content of the crop. The Arizona Cotton Growers Association through the Arizona Cotton Research and Protection Council (ACRPC) initiated development of a facility to manufacture commercially useful quantities of atoxigenic strain material late in 1998. Design and development of the manufacturing process, required equipment, and facility was undertaken by ACRPC in partnership with USDA/ARS. The facility has gone through several development phases and material produced at the facility has been applied to commercial crops since 1999. Collaborative research between ACRPC and USDA/ARS on the use of atoxigenic strain technology to limit aflatoxin contamination of Arizona cottonseed continues with the goal of developing both a theoretical and practical framework by which area-wide reductions in aflatoxin contamination may be achieved. The year 2001 represents the third season of broad scale commercial utilization of atoxigenic strain technology. The atoxigenic strain used is *Aspergillus flavus* AF36. This report addresses progress in the manufacturing, application and evaluation of atoxigenic strain technology on cotton in Arizona during 2001.

During the 2001 crop year a total of eight organized treatment areas representing eleven cotton gins and fifty-two growers were established in Mohave, La Paz, Yuma, Maricopa and Pinal Counties in Arizona. A combined total of 19,975 acres in all areas received AF36 applications with individual areas ranging from 988 to 4492 acres. Analysis of the 2001 crop revealed cumulative effects of large-scale AF36 treatments. *Aspergillus flavus* communities on crop samples from seventy-one (71) randomly selected treated fields averaged 62.9% AF36 and only 1.9% S strain. Statewide individual areas reflected the effects of multi versus first year treatments on seed samples. Roll/Texas Hill (85.2% AF36; 4.1% S), Stanfield (70.4% AF36; 1.3% S), and Paloma (85% AF36; 3.3% S) showed consistently greater AF36 presence on crop than first year treatment sites in Yuma Gila Valley (55.6% AF36 0% S); Parker (42.7% AF36; 7.8% S) and Buckeye (59.6% AF36, 0% S). The observed fungal community changes are particularly significant when considering the extent of AF36 applications over time and space. 2001 treatments were most extensive to date but represent treatment of only 2% of the total available agricultural land mass in the counties affected.

Positive effects on the aflatoxin content of the crop as indicated by commercial analyses were widespread in 2001. In Parker (La Paz County) a large block of cotton (3,200 acres) received its first treatment of AF36. Even thought this area was treated over 1 month late, at harvest the gin reported 44% clean seed (below 20 ppb). This

represented a significant improvement over the 30-year average of less than 20 percent clean seed. Near Texas Hill (Yuma County) a new treatment area was established in another area with habitually high contamination. Analysis of the harvested crop showed a very high incidence of AF36 and commercial toxin analysis indicated seed aflatoxin content below 10 ppb. In Pinal County, prior to the initiation of AF36 treatments one farm in the Stanfield area had not produced clean seed in 30 years. By the second year of treatments (2000) fourteen of seventeen fields (82%) tested below 20 ppb. In 2001 after a third year of AF36 applications 86% of the fields were below 20 ppb. As a result the grower was able to derive an economic advantage through the sale of clean seed.

Improvements to manufacturing processes and facilities were made in 2002. These improvements resulted in increased product uniformity and improved product quality. Stabilization of incubation procedures were the most significant corrective measure. Incubation conditions will be optimized during 2003. Improvements to facilities for product drying were also designed in 2002 and are currently being fabricated.

Towards Implementation Of Biological Control Strategy To Reduce Aflatoxin Contamination In Tree-Nuts

Sui-Sheng Hua. USDA, ARS, Western Regional Research Center, Albany, CA

Several Saprophytic yeasts isolates were shown to be effective in inhibiting the growth and aflatoxin production by the visual bioassay and were identified as *Pichia anomala, Candida krusei and Debaryomyces hansenii*. The interactions of *P. anomala* and *A flavus* are further investigated. Growth of *A. flavus* was greatly inhibited by this yeast in potato dextrose broth. A vital fluorescent stain, FUN1, was used to demonstrate that the yeast interferes with the metabolic activities of the fungus and retards the hyphal growth. The effect of this yeast to reduce the spore production of *A. flavus* on pistachio flowers, leaves and almond leaves was evaluated for several years. Spore production of *A. flavus* was reduced by 60-80% in plant material sprayed with the yeast. The results suggest that the yeast, *P. anomala,* may be a potential biocontrol agent to reduce population of *A. flavus* in the orchards. The outcome would be a reduction of aflatoxin in edible nuts.

The necessary steps to implement the biocontrol strategy include: 1. production of yeast in sufficient quantity for field application, 2. formulation of the yeast to enhance the biocontrol efficacy in the field and 3. determination of the best time for applying the yeast in the field.

Several media were evaluated for supporting the growth of yeast. The yield of yeast cells in one of the media was $6x10^{12}$ colony forming units (CFU) per liter of culture. The viability of yeast cells stored at 4^0C for three and six month were assayed. Very little decline in CFU was observed over a period of six month.

Effect Of Nontoxigenic Strains Of *Aspergillus Flavus* And *A. Parasiticus* Applied Separately And In Combination On Preharvest Aflatoxin Contamination Of Peanuts

Joe W. Dorner and Bruce W. Horn. USDA-ARS, National Peanut Research Laboratory, Dawson, GA

Application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* to soil around peanut plants has been shown to effectively reduce pre- and post-harvest aflatoxin contamination. Many of our studies conducted over several years demonstrating this effect have utilized inocula consisting of a combination of nontoxigenic *A. flavus* and *A. parasiticus*. For purposes of commercializing this process, it would be preferable to produce inocula containing only one strain. Therefore, the purpose of this study was to determine the effect of nontoxigenic strains of *A. flavus* and *A. parasiticus* applied alone and in combination on preharvest aflatoxin contamination of peanuts.

The study was conducted during two growing seasons in environmental control plots that can ensure optimum conditions for the development of preharvest aflatoxin. Treatments, replicated three times, included an untreated control and formulations of hulled barley coated with conidia of nontoxigenic *A. flavus* (NRRL 21882), nontoxigenic *A. parasiticus* (NRRL 21369), and a mixture of the two formulations. Coated barley was applied at a rate of 20 lb/acre 65 days after planting in 2000, and plots were re-inoculated at the same rate 60 days after planting in 2001. All peanuts were harvested, shelled, and analyzed for the presence of toxigenic and nontoxigenic strains of *A. flavus* and *A. parasiticus* and for aflatoxins.

Results for 2000 showed no significant differences among treatments for total colonization of peanuts by *A. flavus* and *A. parasiticus* with colony forming units (CFU)/g ranging from 5.5×10^5 to 8.2×10^5. However, in 2001 untreated peanuts had a significantly higher total colonization (1.2×10^6 CFU/g) than did treated peanuts (average = 1.5×10^5 CFU/g). The incidence of toxigenic strains was significantly less ($P < 0.05$) in peanuts treated with *A. flavus* alone in both years (19.9% and 24.3% in 2000 and 2001, respectively) than in controls (69.8% and 95.0%, respectively). The incidence of toxigenic strains was significantly reduced by the mixture in 2000 (18.1%) but not in 2001 (47.8%). Treatment with *A. parasiticus* alone did not significantly reduce the incidence of toxigenic strains in peanuts (62.8% and 67.2%, respectively).

In 2000, aflatoxin contamination was significantly reduced ($P < 0.05$) by treatment with *A. flavus* alone (71.3%) and the *A. flavus*/*A. parasiticus* mixture (77.1%), but not by *A. parasiticus* alone. Aflatoxin contamination was significantly reduced ($P < 0.05$) by all treatments in 2001 with reductions ranging from 89.7% for the *A. parasiticus* treatment to 92.8% for treatment with *A. flavus* alone.

Regression analysis of data for both years showed a significant ($P < 0.01$) positive correlation between the incidence of toxigenic strains in peanuts and aflatoxin contamination with an R^2 of 0.70 for 2000 and 0.68 for 2001. As toxigenic strains of *A. flavus* and *A. parasiticus* were displaced in peanuts by nontoxigenic strains, aflatoxin levels were reduced correspondingly. Results of this two-year study indicate that application of *A. flavus* NRRL 21882 to soil was more effective than *A. parasiticus*

NRRL 21369 and equally as effective as a mixture of the two strains in displacing toxigenic strains in peanuts and reducing aflatoxin contamination.

PANEL DISCUSSION: Microbial Ecology

Panel Chair: Larry Antilla
Panel Members: D.T. Wicklow, P.J. Cotty, S. Hua, and J.W. Dorner

Talks in this section discussed biocontrol of aflatoxin contamination and interactions among isolates of *A. flavus* with varying aflatoxin-producing potential. Interest was expressed in the studies of Dorner and Horn in which treatments with a nontoxigenic strain of *Aspergillus flavus* showed significant reductions in the quantity of aflatoxins in peanuts without increasing the overall quantity of *A. flavus* on the crop at harvest. The results suggested producers utilizing the nontoxigenic strain to manage aflatoxins could achieve significant economic gain.

During his presentation, Cotty indicated that there were many strains of *A. flavus* that do not produce aflatoxins and he presented data that showed several of these have potential as agents to limit aflatoxin contamination through competitive exclusion of aflatoxin producers. During the panel discussion Cotty was asked why he would prefer utilizing multiple atoxigenic strains of *A. flavus* rather that the single strain currently used. Cotty indicated that he feels there are at least slight adaptive differences among the diverse *A. flavus* vegetative compatibility groups that reside in agricultural fields and these VCGs may differ in the conditions under which they are most competitive. Conditions vary across regions and fields and Cotty expressed the possibility that by utilizing multiple strains optimal efficacy (competitive exclusion of aflatoxin producers) might be obtained in the broadest number of regions, fields, and within field habitats. Cotty also indicated that some ecological theories suggest more complex communities are more stable and thus he hoped that by utilizing multiple atoxigenic strains that the modified fungal community, with a reduced aflatoxin producing potential, would have increased stability.

Don Wicklow was asked what his thoughts were regarding a potential mechanism of intraspecific competion that could explain the substantial suppression of aflatoxin recorded for mixtures of aflatoxin-producing, but genetically incompatible, strains of *Aspergillus flavus* and *Aspergillus parasiticus* in suspended disk culture (SDC). Wicklow outlined his working hypothesis as follows:

(1) fungal incompatibility reactions in *Aspergillus* are known to produce lethal cell fusions;

(2) each fungus responds by producing new hyphal branches (i.e. compensatory growth with the "barrage zone")

(3) mycelial dry weights were often shown to be high in pairings between incompatible strains when contrasted with weights obtained for each strain cultured separately

(4) aflatoxin biosynthesis is negatively impacted by the metabolic demands of fungal compensatory growth.

Antilla presented information on successful use of atoxigenic strains in large scale treatments (over 19,000 acres were treated in 2001) in Arizona. Producers from Texas, where aflatoxin contamination of both cottonseed and corn was severe in 2002, expressed interest in how such technology might be applied in their state. Antilla indicated that the

aflatoxin management program in Arizona was instigated by the producers themselves and that they retained program oversight .

Antilla was asked about the issue of liability given the fact that aflatoxin control utilizing Dr. Cotty's atoxigenic strain technology had reached commercial scale application in Arizona cotton. Both Antilla and Cotty responded that in any program of this size liability is always a concern. However it was pointed out that extensive testing has clearly shown that while shifts in ratios of toxic versus atoxigenic strains in Arizona do occur, there was no increase in the total amount of fungal presence or propagule levels demonstrated in the fungal community associated with the organized area-wide treatment program. To the contrary, known aflatoxin-producing strains of *Aspergillus flavus* are being progressively replaced by the non-toxic strain AF36. This should result in reduced exposure to aflatoxins that are commonly at high concentrations in both *A. flavus* conidia and the fines of crops.

Saprophytic yeasts that inhibit growth of *A. flavus* were discussed by Hua as potential biological control agents for the management of aflatoxin contamination. Hua indicated that she is currently developing methods to scale up production of the best yeast antagonists for larger scale tests.

Commom Genotypes (Rflp) Within A Diverse Collection Of Yellow-Green Aspergilli Used To Produce Traditional Oriental Fermented Foods

D.T. Wicklow, C.E. McAlpin, and S.W. Peterson. USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL

DNA fingerprinting was performed on 72 strains of *Aspergillus oryzae* and 9 strains of *Aspergillus sojae* isolated from chu (China) or koji (Japan), mold inoculum used in the production of traditional Oriental fermented beverages or foods including soy sauce, miso, and sake. The cultures were deposited with the ARS Culture Collection (NRRL) in the years between 1909 and 2001. PstI digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. All strains of *A. sojae* that we examined produced an identical DNA fingerprint and belong to the same DNA fingerprint group (GTAo-9). Strains of *A. oryzae* were distributed among 41 DNA fingerprint groups, including GTAo-12 represented by eleven strains, GTAo-19 represented by five strains, GTAo-5, GTAo-15 each represented by four strains, and GTAo-8, GTAo-17, GTAo-24 each represented by three strains. Thirty-three single strain isolates of *A. oryzae* produced unique fingerprints. *Aspergillus oryzae* NRRL 30038 (= S-03) and *A. sojae* NRRL 30039 (= S-12) are identified in U.S. Patent No. 6,027,724 as useful fungal biocontrol agents for preventing aflatoxin contamination in agricultural commodities. The DNA fingerprint produced by *A. sojae* NRRL 30039 was identical to fingerprints of *A. sojae* isolates in GTAo-9. Aspergillus *oryzae* NRRL 30038 did not match fingerprints produced by any other strain of *A. oryzae* (<80% band similarity). Our data offers evidence suggesting that (1) the successful domestication of *A. parasiticus* genotypes yielding *A. sojae* occurred far less frequently than among genotypes *of A. flavus* var. *oryzae*; (2) some *Aspergillus* genotypes employed in different fermentations and regions were derived from a common ancestral clonal population.

Phylogenetic And Regulatory Relationships Among Aflatoxin-Producing Species Based On *Aflr* Sequence Comparisons

Kenneth C. Ehrlich, Beverly G. Montalbano and Peter J. Cotty. Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture, New Orleans, LA

Aflatoxin contamination of foods and feeds is a worldwide agricultural problem. *Aspergillus* section Flavi includes the species, *A. parasiticus*, *A. flavus, A. nomius, A. bombycis*, and *A. pseudotamarii,* which under certain conditions produce highly toxic and carcinogenic aflatoxins. Crops can become contaminated with aflatoxins when conditions favor growth of these fungi. Annual costs resulting from crop losses and the need to limit food contamination have been estimated to be more than $100 million. Of the aflatoxin-producing species, *A. flavus* and *A. parasiticus* are the most common species implicated as causal agents of aflatoxin contamination. Roles of agriculture in structuring communities of aflatoxin-producing fungi are unclear. Aflatoxin production requires expression of the biosynthetic pathway regulatory gene, *aflR*, which encodes a Cys_6Zn_2-type DNA-binding protein. Homologs of *aflR* from the above species were compared in order to investigate the molecular basis for variation among aflatoxin-producing taxa in the regulation of aflatoxin production. Variability was found in putative promoter consensus elements and coding region motifs, including motifs involved in developmental regulation (AbaA, BrlA), regulation of nitrogen source utilization (AreA), and pH regulation (PacC), and in coding region PEST domains that are expected to be involved in protein turnover. Compared to the other isolates, *A. flavus* and *A. parasiticus* isolates have an additional AreA-binding site in the *aflJ*-promoter regulatory region that appears to be involved in the expression of *aflJ*, a gene divergently transcribed from *aflR*, that also is required for aflatoxin accumulation. *A. nomius* and *A. pseudotamarii* isolates have consensus BrlA and AbaA sites not found in the *A. flavus* isolates. While variable among the recognized taxa, a PacC site in the promoter regions for *aflR* and *aflJ* may play a role in pH regulation of aflatoxin pathway gene expression both at acid and basic pHs. Differences in the effect of developmental state, nitrate, and pH on aflatoxin accumulation have been observed among the recognized species of aflatoxin-producing fungi. Deduced AflR amino acid sequence indicates that *A. nomius* and *A. bombycis* isolates should have PEST domains near the activation domain of the protein. The presence of such regions could reflect a strategy for maintaining levels of AflR necessary for aflatoxin formation different than that of taxa whose AflRs lack such sites. Comparisons of phylogenetic trees obtained with either *aflR-aflJ* intergenic region sequence or coding region sequence and the observed divergence in regulatory features among the taxa provide evidence that regulatory signals for aflatoxin production evolved to respond to environmental stimuli under differential selective pressures. Marked incongruence between phylogenetic signals for the *aflJ/aflR* intergenic region and the *aflR* coding region was observed with partition homogeneity and Kishino-Hasegawa tests. The latter test indicated that the topology of the intergenic region tree is not a good fit for the coding region data set.

Analyses also indicate that isolates assigned to the *A. flavus* morphotype S_{BG} represent a distinct species and that *A. nomius* is a diverse paraphyletic assemblage likely to contain several species.

Molecular And Phylogenetic Characterization Of The Aflatoxin Biosynthetic Gene Cluster From *Aspergillus Ochraceoroseu*.

J. W. Cary, M. A. Klich, C. A. Bennett and S. B. Beltz. USDA-ARS Southern Regional Research Center, New Orleans, LA

Aflatoxins (AF) are carcinogenic secondary metabolites produced by fungi of the genus *Aspergillus*. *Aspergillus flavus*, *Aspergillus parasiticus* (*A.p.*) and *Aspergillus nomius* are commonly isolated AF-producers. Recently, two new species within section *Flavi*, *A. pseudotamarii* and *A. bombycis*, have also been shown to produce aflatoxin (1,2). *Aspergillus nidulans* (*A. n.*) produces sterigmatocystin (ST), which is the penultimate precursor to AF. *Aspergillus ochraceoroseus* (*A.o.*) has been found to produce both ST and AF. *A.o.* has been placed in Subgenus *Circumdati,* section *Circumdati* based largely on morphological characteristics. The common AF-producers are considered members of Subgenus *Circumdati*, section *Flavi* while the ST-producer *A. n.* is a member of the Subgenus *Nidulantes,* section *Nidulantes*. The objective of this research was to identify genes involved in AF production from *A.o.* and attempt to determine their evolutionary origin based upon sequence homology to AF/ST genes from other AF/ST producing Aspergilli. Previous Southern hybridizations of *A. o.* genomic DNA with AF/ST gene probes only identified the presence of putative *A.p. avn*A and *ver*B homologs. An *A.o.* genomic library was generated and probed with an *A.o. ver*B PCR product and *A.p. avn*A genes. Phage clones that hybridized with these probes were isolated and their DNA purified and analyzed. Southern blots of restricted phage DNAs were hybridized to AF/ST gene probes. DNA from phage isolate C3 hybridized to the *A. o. ver*B PCR probe while that of isolate C1 hybridized to probes for *avn*A/*stc*F, *afl*R and *nor*1/*stc*E genes. Sequence analysis of *A.o.* genomic clone restriction fragments showed amino acid identities of the *verB/stcL, avnA/stcF, nor1/stcE*, and *aflR* homologs of 84/84%, 79/73%, 65/71%, and 34/61% to the *A.p./A.n.* genes, respectively. The apparent order of the *stc*F-*afl*J-*afl*R-*stc*E gene homologs within phage isolate C1 DNA and *stcJ-stcK-stcL-stcN* in phage C3 indicates that the *A.o.* AF gene cluster is arranged similar to that of the *A.n.* ST cluster. Phylogenetic analyses of the *aflR* and *nor-1* genes and non-cluster genes coding for beta tubulin and the 5.8S rRNA-ITS region also indicate that *A.o.* is most closely related non-aflatoxigenic members of the Subgenus *Nidulantes*, section *Nidulantes,* but morphologically *A.o.* appears to be more Subgenus

Vegetative Compatibility Groups And Aflatoxin Production In *Aspergillus Flavus* From A California Orchard

Sui-Sheng Hua[1], Siov Bouy Ly[1], Shirley Hong[1], Cesaria E. McAlpin[2]. [1]USDA, ARS, Western Regional Research Center, Albany, CA, and [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL

Population variability in *Aspergillus flavus* has been examined by sorting isolates into vegetative compatibility groups (VCGs). Many morphological and physiological features, such as sclerotia size, mycotoxin production, and spatial distribution have associated with VCGs in this genus. Complementary nitrate-nonutilizing (*nit*) mutants are commonly used to identify compatible isolates. Genetic complementation of *nit* mutants of *A. flavus* strains from California orchard has not been carried out previously. In the present study, a large number of *nit* mutants were isolated by selecting for chlorate resistance on PDA agar. VCGs were determined by pairing the mutants on minimal nitrate agar medium. Heterokaryon formation occurred between compatible strains.

We examined the ability of the DNA probe pAF28 to distinguish *A. flavus* strains isolated from California. Strains which had identical or similar (similarity index C > 80%) fingerprinting profile were assigned to be in the same VCG. A phenogram was established based on the fingerprinting patterns. Twenty five VCGs were assigned to forty one isolates. The data indicate a very diversified population. A few dominant groups containing three to five strains are found among the VCGs. The S type strains produce abundant small sclerotia and all are toxigenic. The L type of *A. flavu* comprises both toxigenic and atoxigenic isolates. Many isolates do not produce any sclerotia. The VCGs of California isolates determined by complementation of *nit* mutants are in good agreement with the phenogram based on fingerprints in 80% of the cases. Thus pAF28 is a useful tool for predicting VCGs of *A. flavus* isolates from the field.

Reduced Virulence In An *Aspergillus parasiticus* Strain Lacking A Desaturase Enzyme

Richard Wilson, Ana Calvo, and Nancy P. Keller. Department of Plant Patrhology, University of Wisconsin, Madison, WI

Unsaturated fatty acids are important constituents of all cell membranes. In the mycotoxigenic oilseed pathogen *A. parasiticus*, and the model filamentous fungus *A. nidulans*, unsaturated fatty acids and their derivatives are also important developmental signals affecting spore and sclerotial development. To dissect the relationship between lipid metabolism and development, we have generated a Δ12-desaturase mutant in *Aspergillus parasiticus*, *ΔApodeA*. This enzyme is responsible for the biosynthesis of linoleic acid from oleic acid. The resulting mutant is impaired in unsaturated fatty acid biosynthesis, accumulating large amounts of oleic acid but only trace amounts of linoleic acid. The mutation also leads to reduced spore and sclerotial development. We wished to characterize the effect of this mutation on seed colonization. We infected peanut seed of the sunrunner and sunoleic 97R lines with *A. parasiticus* wild type and *ΔApodeA*, and for comparison *A. nidulans* wild type. Compared to sunrunner, sunoleic 97R has increased oleic acid: linoleic acid ratios. An aflatoxin-susceptible corn line (Asgrow 404) and an aflatoxin-resistant corn line (Tx6) were also infected with *A. parasiticus* wild type and *ΔApodeA*. Virulence of the different *Aspergillus* strains was determined by asexual spore counts. Our results were as follows:

1) *A. parasiticus* sporulation is not affected by live or dead peanut seed.
2) *A. nidulans* wild type sporulation is greatly attenuated on live but not dead peanut seed.
3) Altering host seed fatty acid content (ie. to increased oleic acid:linoleic acid ratios) does not affect sporulation of any strain on peanut seed.
4) *A. parasiticus* wild type and *ΔApodeA* are attenuated on live Asgrow 404 corn seed compared to dead seed, but sporulation is not affected by live or dead Tx6 seed.
5) *ΔApodeA* has significantly reduced rates of infection compared to wild type on all substrates except dead corn seed.

Therefore, a possible strategy to reduce sporulation and subsequent dissemination of fungal inocula in the field could be to target *Aspergillus* Δ12-desaturases. Sequence alignment of the *ApodeA* gene with known plant desaturases could reveal areas of the two proteins differing in amino acid sequence. These regions could become targets for fungicides or antibodies that selectively inhibit *ApodeA* activity.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

15th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 5: CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

Moderator: *Dee Vaughn, National Corn Growers Association*

Aflatoxin Control In Pistachios: Biocontrol And Removal Of Contaminated Nuts

Themis Michailides[1], Mark Doster[1], Peter Cotty[2], Walter Bentley[1], Dave Morgan[1], Lorene Boeckler[1], and Dan Felts[1]. [1]University of California/Kearney Agricultural Center, Parlier, CA; [2]Southern Regional Research Center, ARS/USDA, New Orleans, LA

In 2001 we selected two promising atoxigenic strains (A564 and A815) of *A. flavus* for testing as biocontrol agents. On 2 July, wheat seeds infected with these strains were applied at the rate equivalent to 10 lbs/acre in a research pistachio orchard. None of the *A. flavus* isolates from soil collected just prior to application of the wheat belonged to either of the atoxigenic strains. Soil collected on 5 October in the nontreated area had substantially lower density of *A. flavus*/*A. parasiticus* (16 cfu/g) than the area treated with A564 (294 cfu/g) or the area treated with A815 (251 cfu/g). Almost all of the *A. flavus* isolates (99%) from the soil in the area treated with A815 belonged to the A815 strain. Similarly, almost all of the isolates (94%) from the area treated with A564 belonged to the A564 strain. Both A564 and A815 strains were detected outside the area of application. The density of *A. flavus* on leaves did not differ significantly between treatments. The atoxigenic strains were detected on the leaves, although at low levels.

In 2002 we repeated the biocontrol experiment in the same research orchard. On 1 July, wheat seeds infected with three strains (A564, A815, and AF36) were applied. Soil collected just prior to application of the wheat in 2002 had a relatively high density of *A. flavus* in the areas treated in 2001 with A815 (254 cfu/g) or treated with A564 (160 cfu/g), but had a relatively low density in nontreated areas (16 cfu/g). On 23 September, leaf and additional soil samples were taken and are currently being evaluated.

In 2001 a study was initiated that investigated the infestation by navel orangeworm (an important factor in aflatoxin contamination) of nuts with no or little shell staining. The results from 2001 suggested that size might be used in addition to shell stain to remove infested nuts. In order to confirm these results, in 2002 we obtained nut samples for 24 commercial orchards from a processor and are currently evaluating these samples. Because the citrus flat mite can damage the hull (which is possibly favorable for navel orangeworm infestation), nut samples were collected in 2001 from an orchard with mite damage. Preliminary results showed that mite damage decreased nut size and could result in navel orangeworm infestation of the nuts. In 2002 we collected larger samples of mite-damaged nuts from three orchards and are currently evaluating these samples.

In 2001 we initiated a study of the relationship of several factors that might contribute to aflatoxin contamination in commercial pistachio orchards. Soil, leaf, and nut samples were collected from 10 orchards. Orchards with high incidences of early split nuts tended to also have high incidences of nuts with cracked hulls. Pupae and empty pupal cases of navel orangeworm were found in the early split nuts, suggesting that conditions were favorable for navel orangeworm. The density of aflatoxin-producing fungi on leaves was moderately correlated to that in soil. This study was repeated in 2002 using the same 10 orchards, and samples are still being evaluated. After the samples are evaluated, a thorough statistical analysis will be done including data from both years.

Aflatoxin Control In Figs: Biocontrol And Development Of Resistant Cultivars

Mark Doster[1], Themis Michailides[1], Peter Cotty[2], James Doyle[1], David Morgan[1], Lorene Boeckler[1], and Dan Felts[1]. [1]University of California, Davis/Kearney Agricultural Center, Parlier, CA; [2]Southern Regional Research Center, ARS/USDA, New Orleans, LA

In 2001 we selected two promising atoxigenic strains (A564 and A815) of *A. flavus* for testing as biocontrol agents. On 5 July, wheat seeds infected with these strains were applied at the rate of 41.2 g wheat/tree (equivalent to 10 lbs/acre) in a research fig orchard. None of the *A. flavus* isolates from soil collected just prior to application of the wheat belonged to either of the atoxigenic strains. On 17 August, we collected noncaprified figs from the soil in the experimental block. Out of 20 isolates obtained from colonies of *A. flavus* decaying these noncaprified figs, six belonged to A564 strain and 13 to A815 strain. On 3 October, leaf and additional soil samples were collected and evaluated. The soil in the area treated with A815 had more *A. flavus*/*A. parasiticus* (15.4 cfu/g) than the area treated with A564 (7.2 cfu/g) or the nontreated area (5.3 cfu/g). Approximately half of the *A. flavus* isolates from the soil in the area treated with A564 belonged to the A564 strain, whereas 98% of the isolates from the area treated with A815 belonged to the A815 strain. The density of *A. flavus* on leaves only differed slightly between treatments (ranging from 0.012 to 0.018 cfu/cm^2, depending on treatment). The atoxigenic strains were detected on the leaves, although at low levels (2.1-12.4% of the isolates for A564 strain and 0.0-5.1% for A815 strain, depending on treatment).

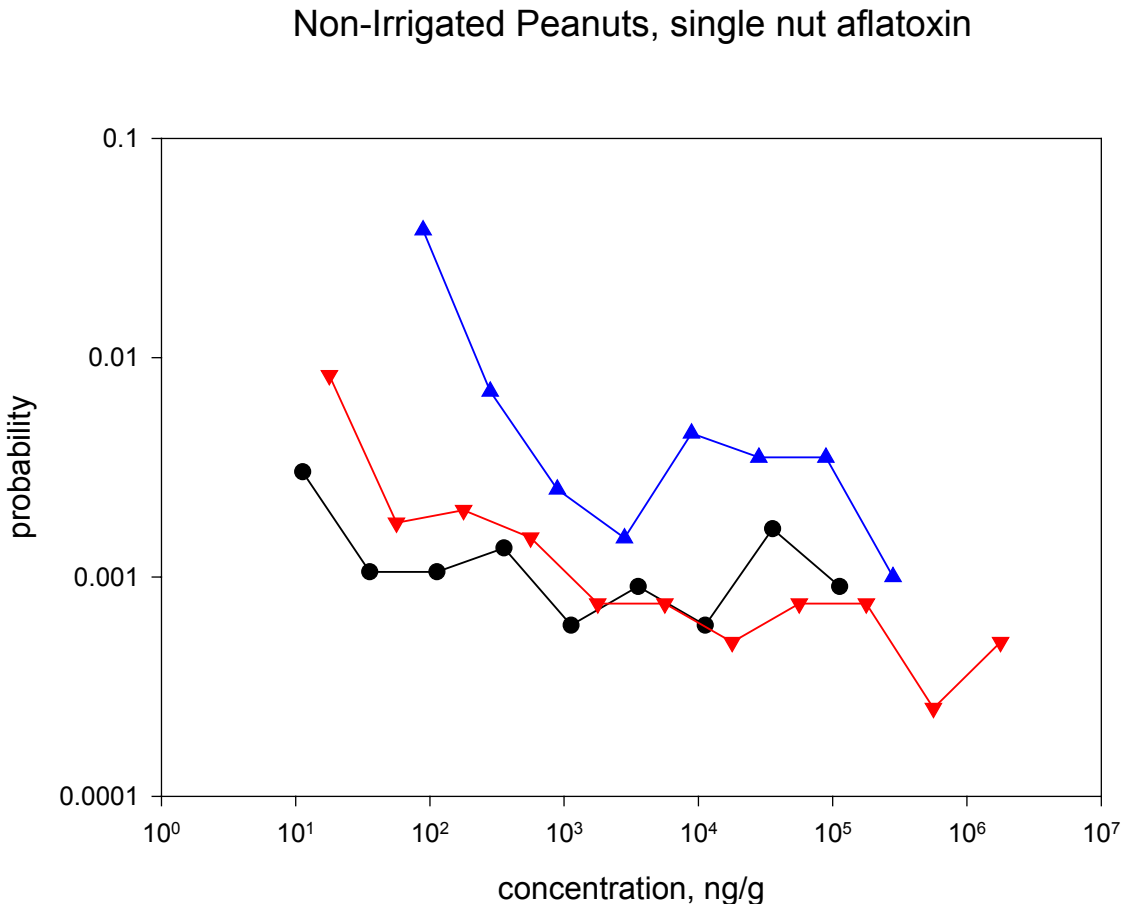
In 2002 we repeated the biocontrol experiment in the same research orchard. An additional atoxigenic strain (AF36) was included after an isolate belonging to this strain was found in a California orchard. On 17 June, wheat seeds infected with these strains were applied using the same methods as in 2001. Soil collected just prior to application of the wheat had a low density of *A. flavus* (ranging from 3.8 to 15.2 cfu/g soil, depending on treatment). On 19 August, we collected noncaprified figs from the soil in the experimental block. More noncaprified figs were colonized by *A. flavus* in the inoculated areas (23.3-46.6%, depending on treatment) than in the noninoculated areas (8.6%). On 2 October, leaf and additional soil samples were taken and are currently being evaluated.

New fig selections have been developed by a breeding program that is attempting to produce a Calimyrna-type fig that has the advantages of Calimyrna figs but not the disadvantages (high levels of decay, insect infestation, and aflatoxin contamination). The four selections remaining in the breeding program all had very low levels of fungal decay for dried fruit harvested from several commercial orchards in 2001. In 2002 the most promising selection, 6-38W, had substantially smaller eye diameter of ripe fruit (0.8 mm) than Adams (3.5 mm) and Calimyrna (3.1 mm) but about same as Conadria (0.6 mm). In addition, several thousand dried figs of 6-38W and more than 1,000 dried figs for each of several commercial fig cultivars (Calimyrna, Conadria, and Adams) have been collected from commercial orchards and are being evaluated for fruit decay. The new fig selections continue to show promise in reducing aflatoxin contamination compared to Calimyrna figs.

Status Of Peanut Research At WRRC

T.F. Schatzki, D. Eastwood and M.S. Ong. USDA-ARS Western Regional Research Center, Albany, CA

Previous work (Schatzki, J. Agric. Food Chem.[JAFC] 43, 1566-1569, 1995) enabled the computation of pistachio (Schatzki, JAFC 46, 2-4, 1998) and almond (Schatzkiand Ong, JAFC 49, 4513-4519, 2001) lot distributions, p(c) = probability, p, that a random kernel from the lot has aflatoxin concentration c. Note that this differs from the sample distribution, P(N,C), of the means of samples of N kernels, which is a strong function of N. P(N,C) can be derived from p(c) and N, but the reverse is possible only under special conditions (Schatzki, JAFC 43, 1561-1565, 1995).



The lot distribution of several sublots of a lot of non-irrigated Florunner farmer stock peanuts has now been obtained. Using 400 samples each from Jumbos (20 nuts/sample), Mediums (10 nuts/sample) and small OK (<14/64 in, 5 nuts/sample) the depicted distributions were obtained. The peanut distributions are of order of 20-50 times higher than those found in unsorted pistachios or even insect infested almonds. Using these lot distributions the sublot mean aflatoxin concentrations are calculated to be 170

ng/g (Jumbos), 370 ng/g (Mediums), and 750 ng/g (small OK). This is in line with a field determination of 920 ng/g for a unknown mix of LSK and DK sublots of this lot. One further computes that 98% of the total aflatoxin (of the measured sublots) is found in the kernels exceeding 5000-8000 ng/g/kernel. In principle, the admittedly poor Jumbo and Medium sublots could be cleaned to the point of being acceptable for human consumption by removing these high aflatoxin nuts, discarding just 2% of product. However, this would require a perfect sorter, producing no false positives or negatives. In practice, cleanup of such sublots does not appear to be economic. The shape of the distributions shown matches rather well the shape of pistachio and almond distributions. This suggests that the main source of aflatoxin in peanuts may be similar, infection by mold during the month or so prior to harvest (Schatzki, JAFC 46, 2-4, 1998). Further work will involve measuring the distribution in a sorted, ready-to-consume lot. In addition, sample distributions will be computed from the presented lot distributions and compared with the results obtained by Whitaker and coworkers.

The dipping technique to obtain intact peanuts has been applied to sublots of the same lot. (Schatzki, T.F. and Haddon, W.F., JAFC 50, 3062-3069, 2002) From a total of 65,000 nuts 24 Jumbos and Mediums and 23 small OK were recovered above the detection limits of 1000 and 25000 ng/g, resp. Along with about 50 nuts of negligible aflatoxin content, these formed a training set which has been examined spectroscopically. Results of this work are described in an abstract by Eastwood and Schatzki in these proceedings.

With-In Row Spacing Of Maize And Its Effects On Yield And Mycotoxin Incidence

H. Arnold Bruns and H.K. Abbas. USDA-ARS, Stoneville, MS

Hybrid maize (*Zea mays* L.) grown in the 1950's and 1960's usually responded to plant densities over 45,000 plants ha-1with high numbers of barren plants ha^{-1} and less grain per plant. Planting recommendations for modern hybrids use populations of over 62,000 plants ha^{-1} to achieve the high yields commonly seen today. Maize grown in the Mid South frequently uses a 101.6 cm row spacing, which is used to produce much of the cotton (Gossypium hirsutum L.) grown in the area. Six maize hybrids, two Bt and four non-GMO, were grown at Stoneville, MS in 2000 and 2001 using a 101.6 cm row spacing and plant densities of 43,000, 48,000, 54,300, 64,000 and 76,500 plants ha^{-1}. Increasing plant density resulted in increased grain yields with no yield plateau or decline observed at 76,500 plants ha^{-1}. Grain bulk densities varied among plant populations. However, no trend was evident. Kernel weights, ear weights, and leaf area $plant^{-1}$ all declined as plant density increased. Declines in kernel and ear weights however did not affect grain yield negatively. Leaf area index increased with increasing plant density thus negating the decline in leaf area $plant^{-1}$. Leaf area index was higher in 2000 than in 2001, probably due to more rainfall. Hybrids differed in LAI, yield, grain bulk density and kernel weight. However, these differences were not correlated to one another. Aflatoxin levels were below the maximum allowable level of 20 mg Mg^{-1}. Fumonisin levels were higher in 2001(5.0-7.9 mg kg^{-1}) than in 2000 (0.5-1.6 mg kg^{-1}) probably due to a more favorable environment for growth of the fungus. Modern maize can be grown in the Mid South using a population of 76,500 plants ha^{-1} without a decline in yield or grain quality.

Validation Of A Mycotoxin Predicting Computer Program For U.S. Midwest Grown Maize In Commercial Fields

Patrick F. Dowd. USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Crop Bioprotection Research Unit, Peoria, IL

A computer program designed to predict relative amounts of fungal inoculum at pollination and mycotoxin levels prior to harvest was developed from intensive data collected from 1993-1997 in central Illinois in the U.S. Temperature, rainfall, and insect presence are the main factors involved. The program could predict fumonisin values close to actual field values when data collected from fields sampled in 1994-1999 was used, which included fumonisin values ranging from ca. 0.1 to 8 ppm. The computer program has now been well validated for fumonisin levels in 3 commercial fields in 2000 and 7 fields in 2001 (most fields were planted with two hybrids) using the generalized values (a version using customized values is under development). In 2000, rainfall was relatively common and insect damage was limited. A predicted level of ca. 1.5 ppm compared well to actual field means of 0.8, 1.3 and 1.6 ppm for prevously studied hybrids. In 2001, the climate was drier during the summer, and insect damage varied according to planting date, with later planted corn being more heavily damaged by European corn borers. For "early" plantings, predicted fumonisin levels (ca. 0.5 ppm) were close to actual means in fields (0.2, 0.3, 0.4, 0.4, 0.6 ppm) except for one field, which ran well below the predicted value. We have a tentative explanation for the lower values of this one field, which was restudied in 2002. Later planted corn encountered more insect damage, but predicted values of near 2.0 ppm compared well with actual values of 2.2, 2.2 and 4.2 for non Bt maize. No aflatoxin was predicted for 2000, and none was found. In 2001, predicted aflatoxin values of circa 10 ppm were higher than mean values found in some fields, although some individual samples had 2-10 ppm. Initially equations used for aflatoxin prediction in the program were based on published values from 1983 and 1987, but we knew this data was probably not detailed enough (no insect damage levels available) to predict values very well (although predictions of negative occurrences have all been correct for years from 1992 through 2000). The new data has allowed for adjustment in the program that will be checked against data collected in 2002, another year that the program has predicted aflatoxin will be present. The program is currently being converted from DOS Basic to a format that will run in Windows, with the intent of producing a version that is commercial quality. The predictive computer program will supply data to farmers that will them to make decisions on whether to control insects from silking to milk stage, or harvest early, in order to help prevent accumulation of undesirable levels of mycotoxins in midwest grown maize in the U.S.

Panel Discussion -Crop Handling, Management, Insect Control

Panel Chair: Patrick Dowd
Panel Members: Themis Michailides, Mark Doster, Thomas Schatzki and H. Arnold Bruns

Presentations covered figs, pistachios, peanuts and corn. T. Michailides reported on initial applications of biocontrol yeasts for control of *A. flavus* in pistachios, where significant reductions in inoculum in soils were reported. Citrus flat mite was also identified as an insect that promoted navel orangeworm damage (which can subsequently lead to aflatoxin problems). New information suggests dust levels on leaves may be useful in predicting aflatoxin levels, and pupal cases of navel orangeworms may be a useful indication of infestation levels at harvest. M. Doster reported on application of atoxigenic strains of *A. flavus* in a fig orchard, which resulted in displacement of other strains by the applied strains. In addition, a fig-breeding program using the cultivar Calimyrna has produced new fig selections that have smaller eyes and less decay than Calimyrna figs. T. Schatzki reported on statistical methods and experiments for determining the probability that any peanut has aflatoxin. Smaller nuts typically have higher toxin levels. Toxin levels for non-irrigated Georgia peanuts tend to run 20-50 times those in tree nuts. Kernels containing more than 5000-8000ng/g of aflatoxin contain 85% of total aflatoxin in the lot. A. Bruns reported on studies where planting densities of corn were compared using several hybrids. Even with plant densities up to 76,500 plants/ha, no plant stress occurred. Although there were no problems with aflatoxins, fumonisins did occur, and were higher in 2001 than 2000, apparently due to late rains in 2001. P. Dowd reported on a computer program designed to predict inoculum levels at silking and fumonisin and aflatoxin levels well before harvest in midwest corn. Back calculations based on the 6 years of data used to design algorithms indicated fumonisin values pretty close to actual ones under varying environmental conditions, and correct yes or no values for aflatoxin (for which less data are available). Tests of several commercial fields in 2000 and 2001 indicated close predictability for most hybrids for fumonisin, even with widely different environmental conditions for the two years. Low levels of aflatoxin were also were also predicted for 2001, and low levels were found in two fields.

During the discussion session, considerable information was made available concerning producer concerns for costs of nut refusal, such as for aflatoxin, with information provided by the industry from R. Klein and M. Jacobs and additional info. by T. Schatzki. For pistachios, the largest growers (which make up the major percentage of growers) are vertically integrated, and there is no penalty based on aflatoxin. There is also currently no penalty for insect damage, although growers are not paid for the insect damaged nuts that are included in a load. Some felt it would be difficult to sort fast enough to eliminate aflatoxin containing nuts, but sorters are available to eliminate aflatoxin containing nuts (Pearson, T., Lebensm.-Wiss. U. Techn. 29, 203-209 (1996). Almonds do have deductions for insect damage. The process of sorting for insect damage removes the aflatoxin containing nuts. It is likely some penalty will be instituted in the

future for insect damaged pistachios. Further discussion centered around application of biocontrol organisms for *A. flavus* control in figs and pistachios. As for the work in cotton and peanuts, the idea is that colonizable sites will be available, and it is better to have these sites colonized by nontoxin producing organisms than by *A. flavus* that produces aflatoxin. Further study should indicate if significant increases in mold or decay do occur when biocontrol organisms are used, but they are not expected. Persistence of the biocontrol organisms may be a function of whether orchards are cultivated or irrigated, and future planned studies should determine this. For peanuts, J. Dorner indicated small nuts generally have more aflatoxin because they are typically more immature, and immature pods are more susceptible to *A. flavus* invasion and growth. When asked about whether asymptomatic infection was common in prior years of high fumonisins, P. Dowd indicated the data was published in J. Econ. Entomol. 92:68-75, which indicates differences from year to year and hybrid to hybrid in the strength of association between visible mold and fumonisin. Some discussion occurred about whether increased corn plant density had led to higher aflatoxin as was published in the mid 1970s, but A. Bruns indicated they had not yet seen this under their study conditions.

Validation Of A New Microtiter Plate ELISA Total Aflatoxin Test (Agraquant®)

[1]Michael Zheng, [1]Craig W. Humphrey, [1]Robin S. King and [2]John L. Richard.
[1]ASCP; [2]Romer Labs, Inc®, Union, MO

An ELISA Microtiter Plate, Total Aflatoxin Test, called AgraQuant®, was developed to measure total aflatoxins in a range from 4-40 ppb using a monoclonal antibody specific for aflatoxins. The test is performed as a solid phase direct competitive ELISA using a horseradish peroxidase conjugate as the competing, measurable entity. For the test method aflatoxins are extracted from ground samples with 70 % methanol and sample extracts plus conjugate are mixed and then added to the antibody-coated microwells. After the plates incubate for 15 min. at room temperature they are washed and enzyme substrate is added and allowed to incubate for an additional 5 minutes. Stop solution is added and the intensity of the resulting yellow color is measured optically with a microplate reader at 450 nm. Results obtained from internal validation studies assessing accelerated stability, accuracy, precision and limit of detection in corn and other grains and grain products, comparison of method to HPLC, ability to detect individual aflatoxins and ruggedness of the test kits determined this test to be a rugged, sensitive precise, accurate and effective test comparable to HPLC for measuring total aflatoxins ranging from 4-40 ppb in several commodities.

Aflatoxin Accumulation In Conventional And Transgenic Corn Hybrids Infested With Southwestern Corn Borer

W. Paul Williams, Gary L. Windham, Paul M. Buckley, and Christopher A. Daves. USDA, ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

Aflatoxin is a potent carcinogen produced by the fungus *Aspergillus flavus*. Aflatoxin contamination of corn greatly diminishes its value and is a major impediment to profitable corn production in the South. Aflatoxin contamination is frequently linked with drought, high temperatures, and insect damage. Southwestern corn borer, *Diatraea grandiosella,* and corn earworm, *Helicoverpa zea,* are major insect pests in the southern United States. Both insects have been linked to high levels of aflatoxin contamination. Genetically engineered corn hybrids expressing genes from *Bacillus* thuringiensis that encode proteins with insecticidal properties provide farmers with new opportunities for insect control. This investigation was conducted to compare aflatoxin contamination levels for Bt and non-Bt corn hybrids following inoculation with *A. flavus* and infestation with southwestern corn borer. In 2000 and 2001, five pairs of commercially available conventional and transgenic Bt corn hybrids were inoculated with an *A. flavus* spore suspension using either an injection into the side of the ear, which wounded kernels, or weekly sprays, which did not wound kernels . An additional treatment consisted of weekly sprays with a spore suspension and infestation of developing ears with southwestern corn borer larvae. An uninfested and uninoculated control was also included. Aflatoxin contamination of Bt and non-Bt hybrids was compared following the four treatments. Larval survival and ear damage were compared in additional plots infested with southwestern corn borer. The side needle, or kernel-wounding, technique resulted in the highest levels of aflatoxin contamination in both 2000 and 2001. In 2000, differences between non-Bt and Bt hybrids were not significant. Aflatoxin contamination was significantly higher in hybrids infested with southwestern corn borer and inoculated with *A. flavus* than in hybrids that were only inoculated with *A. flavus.* The Bt hybrids exhibited less ear damage from insect feeding and lower levels of aflatoxin contamination than non-Bt hybrids. Only 0.1 southwestern corn borer larva per plant was recovered from ears of Bt hybrids 14 days after infestation with 30 southwestern corn borer larvae per plant, but 0.9 larva per plant was recovered from non-Bt hybrids. Although not artificially infested with corn earworm larvae, approximately 0.5 larva per plant was recovered from ears of both Bt and non-Bt hybrids. In areas where southwestern corn borer is a problem, Bt hybrids could play a significant role in reducing aflatoxin contamination.

Determination Of Aflatoxin Production By *Aspergillus* Species From The Mississippi Delta By Various Methods

H. K. Abbas[1], R. M. Zablotowicz[1], B. W. Horn[2], W. Xie[2], and W. T. Shier[3]. [1]USDA-ARS, Stoneville, MS; [2]USDA-ARS, Dawson, GA; [3]University of Minnesota, Minneapolis

This study evaluated several methods to detect aflatoxins in *Aspergillus*. *Aspergillus* isolates (517) were obtained from various Mississippi Delta crops (corn, peanut, rice, cotton) and soils. Cultural methods included fluorescence on *Beta*-cyclodextrin media (FL), yellow pigment (YP), and color change after ammonia vapor exposure (AV) on potato dextrose agar. Aflatoxins in culture extract was confirmed by TLC and LC-MS and quantified by ELISA and HPLC. Of the 517 isolates 314 produced greater than 20 ppb aflatoxin based on ELISA, 89%, 93% and 86% of these isolates gave positive FL, YP and AV responses respectively. Of the 203 isolates producing <20 ppb aflatoxin 27%, 46%, and 25% gave positive FL, YP, and AV responses. This study showed a good agreement between FL and AV methods. However these cultural techniques did not detect aflatoxin in all cultures that were found to produce aflatoxin by ELISA, HPLC and TLC. Based upon LC/APC/MS analysis aflatoxin positive *A. flavus* isolates only produced AFB1 and AFB2, while *A. nomius* and *A. parasiticus* produced four aflatoxins: AFB1, AFB2, AFG1, and AFG2. These methods can be used for quick screening but are not 100% reliable in detecting aflatoxin-producing *Aspergillus* species.

The Saito And Machida Method For Identifying Aflatoxin-Producing Isolates Of *Aspergillus Flavus* Acts By Detecting Aflatoxin Biosynthetic Intermediates

Yanbin Lao[1], Terry W. Steele[1], Hamed K. Abbas[2] and W. Thomas Shier[1].
[1]Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN; [2] USDA-ARS, CG&PRU, Stoneville, MS

Saito and Machida (*Mycoscience*, **40**, 205, 1999) have developed a rapid method for identifying aflatoxin (AF)-producing and non-producing strains of *A. flavus* and *A. parasiticus*, which may provide a useful pre-screen for identifying non-toxigenic strains. In this method, the reverse side of colonies of AF-producing strains on potato dextrose agar (PDA) medium turn from yellow to pink immediately after exposure to ammonium hydroxide vapor. Aflatoxins, among the most potent carcinogens known, are produced by some, but not all, strains of *Aspergillus flavus* and *A. parasiticus*. A higher % toxigenic strains is observed in the soil reservoir of *A. flavus* than in naturally-infected crop plants. This observation lends support to strategies for reducing AF contamination by preinoculation with non-toxigenic strains. *A. flavus* cultures on PDA were lyophilized, extracted with methanol until no more yellow pigment was obtained, and the extracts combined and evaporated. The yellow pigment is a pH indicator dye, which turns from yellow to pink at pH ≥8 by addition of any base, and turns yellow again when the pH is lowered with acid. The methanol extract was dissolved in water, applied to an open C18-silica reversed phase column and eluted with a methanol step gradient. Fourteen pigments which changed color (usually yellow to pink) when spots on TLC plates were exposed to ammonium hydroxide vapor, were isolated by repeated preparative normal phase TLC on silica gel. Seven pigments representing most of the color, were identified by comparing UV spectra, negative ESI-MS/MS and ^{1}H-NMR properties with literature or predicted values. All are known intermediates in the biosynthesis of AF: versicolorin C, versicolorin A hemiacetal, nidurufin, averufin, versicolorin A, norsolorinic acid, and averantin. Seven more pigments with the same UV profile, which could not be identified as known AF biosynthetic intermediates, were isolated in amounts too small to permit structure identification; additional yellow pigments were present, but not obtained pure. Identification of the pigments that predict AF production by *A. flavus* strains as being AF biosynthetic intermediates provides a convenient rationalization for the predictive power of the method. Strains producing pigment amounts below the level of visible detection would be expected to give false negative results. Only strains with a mutation in a biosynthetic enzyme beyond that which makes norsolorinic acid would be expected to give false positive results.

Fluorescence And Near IR Spectroscopy To Characterize Peanuts For Aflatoxin

DeLyle Eastwood and Thomas F. Schatzki. USDA-ARS Western Regional Research Center, Albany, CA

A feasibility study is being conducted to investigate several optical spectroscopy methods for a rapid process control sensor to identify and eliminate high aflatoxin B1 peanuts. UV-Vis fluorescence and near infrared absorption were considered to be the most promising approaches and fluorescence was potentially the most sensitive. Approximately 100 peanuts ranging in aflatoxin level from zero to 8000 ppb by the dip method (T.F. Schatzki and W.F. Haddon, J. Agric. Food Chem. 2002,50, 3062-3069) were measured by front surface synchronous fluorescence and near infrared absorption and diffuse reflectance.

The fluorescence emission from a solid surface such as a peanut (which can be expected to have several fluorescent components in the peanut flesh, the mold (*Aspergillus flavus*) and possibly aflatoxin can be represented as an excitation-emission matrix. Cross sections of this matrix are emission (parallel to x-axis), excitation (parallel to y-axis or synchronous if measured at a 45-degree angle. The emission intensities are represented either along the z-axis or as contours in the x-y plane. The synchronous spectra are achieved by moving both the excitation and emission monochromators at the same speed with a constant offset in wavelength. Several offsets from 15 nm to 120 nm were tested. Of all of these, the 30 nm offset proved the most satisfactory in distinguishing those peanuts with high levels of aflatoxin from those with lower levels of aflatoxin; At 30 nm the offset spectra had the most structure.

For the fluorescence, A Spex Fluorolog 2 research grade spectrofluorometer was utilized. This instrument has double excitation and emission monochromators for better stray light rejection and front surface (used here) or right angle illumination. The source was a 450 W xenon lamp. The detector was a Hamamatsu 928 S-20 photomultiplier tube. The range for the synchronous spectra was from 250 to 750 nm plotting the excitation wavelength. A clean blanched peanut produced a synchronous spectrum with 30 nm offset with at least 3 peaks: One at 285 nm, one at 374 nm and at least one more around 453 nm. The peak ratios 374/285 and 453/285 showed the most discrimination between peanuts with high and low aflatoxin levels.

Near infrared measurements were obtained using a Varian Cary 500i spectrophotometer (from 700 to 1740 nm) by absorption using an InGaAs as detector and from 400 nm to 2500 nm using an integrating sphere and a PbS detector for diffuse reflectance. When a 1725 nm peak (with local baselines subtracted) was contrasted with the 374/285 ratio from fluorescence, discrimination on the basis of aflatoxin was discernable.

These techniques should be rugged enough for a rapid process control method and the fluorescence is intense enough that an expensive laser is not needed. These results show the necessary speed, simplicity and sensitivity. We are continuing to refine these approaches. Other spectroscopic techniques such as FT-mid IR transient emission and near IR-Raman (830 nm excitation) will be used for comparison.

The Association Between Corn Earworm Damage And Aflatoxin Production In Preharvest Maize Grain

Craig A. Abel[1], Hamed K. Abbas[2], Robert Zablotowicz[3], Melanie Pollan[1], and Kenya Dixon[1,2]. [1]USDA/ARS Southern Insect Management Research Unit, Stoneville, MS; [2]USDA-ARS Crop Genetics and Production Research Unit, Stoneville, MS; [3]USDA-ARS Southern Weed Science Research Unit, Stoneville, MS

Aflatoxin is a naturally occurring toxin produced by the fungus *Aspergillus flavus*. In the southeastern U. S., the carcinogenic toxin caused $100 million worth of damage in 1998 and a > 50% reduction in maize acres grown the following year. Maize is a preferred host of *Helicoverpa zea* (corn earworm/ cotton bollworm) that commonly infests the crop in the southeastern U. S. Numerous studies have been conducted to determine the possible association between *H. zea* damage and pre-harvest aflatoxin production in maize with conflicting results. It is unknown if the insect is an important vector of the fungus. Understanding the possible relationship between the fungus, insect, and plant is vital. Once this relationship is understood, appropriate control methods can be designed to disrupt the association between the fungus, insect, and crop, thus, reducing the production of aflatoxin in preharvest maize. The objectives for this study were to: 1) test the association between *H. zea* damage to maize ears and aflatoxin levels in *H. zea* resistant and susceptible maize hybrids, and, 2) study the relationship between *A. flavus* and *H. zea.* Eight maize entries, four insect resistant lines with their susceptible isolines, were grown at Stoneville, MS 2001, in a randomized complete block design with three replicates. Four treatments, nested as sub-plots within each row, were applied at maize silking. The treatments consisted of all combinations of spraying the silks with *A.* flavus and artificially infesting the silks with *H.* zea. At plant maturity, entries were harvested and ppb aflatoxin were determined by ELISA after methanol extraction. Third to fifth instar *H. zea* were collected from *A. flavus* infested silks/ears. Insects were dissected under sterile conditions and *A. flavus* propagules were determined on modified dichloran-rose bengal medium for the exterior of the insect (cuticle) and digestive tract foregut, midgut, and hindgut sections. All log-transformed means were analyzed using REML-ANOVA (PROC MIXED) and separated using LSMEANS at alpha-0.05. There was no interaction between the spray and insect treatments (F = 1.28, df = 1,19, P = 0.2719) because a high percentage (72%, n = 89 ears) of ears from the sprayed only treatment were infested with feral *H. zea.* Most insect resistant maize entries (not DK 626Bt) had less aflatoxin when compared to their susceptible isolines for the sprayed treatments . Silk bioassays confirmed insect resistance and susceptibility in the maize entries. The maize entries from the four sources are genetically similar with the exception of the gene(s) conferring resistance to the insects, thus, this data indicates that: 1) *H. zea* is associated with increased aflatoxin in maize, and; 2) growing *H. zea* resistant hybrids can reduce the incidence of aflatoxin in harvested maize grain. There were significant differences among insect tissue entries for *A. flavus* (F = 25.19, df = 3,22, P = < 0.0001). The foregut and hindgut had significantly more propagules than either the cuticle or

midgut. Digestive enzymes and a high alkaline environment in the midgut (the primary digestive organ) may have destroyed fungal propagules and suppressed spore germination. Some fungal components (e.g. spores) probably moved through the midgut and reproduced in the hindgut, which is primarily a water conservation organ. No propagules were identified in 16 samples taken from the insect haemolymph, indicating the gut may effectively filter *A. flavus* from entering the circulatory system. Future research will examine fungus reproduction within the insect and mechanisms of vectoring.

Geographical Distribution of Aflatoxin Contamination of Commercial Cottonseed in South Texas from 1997 to 2000

Ramon Jaime-Garcia and P. J Cotty, Southern Regional Research Center, ARS, USDA, New Orleans, LA

The aflatoxin content of foods and feeds is limited via regulation throughout most of the world. Aflatoxin is produced by asexual fungi belonging to *Aspergillus* section *Flavi*. Aflatoxin contamination has long been a concern for the cottonseed industry because aflatoxins in contaminated seed can be readily transferred to milk of dairy cows in slightly modified form. Cottonseed is a preferred feed for dairy cows and in the United States regulations prohibit aflatoxin concentrations over 0.5 mg/kg in milk. Milk exceeding that limit may be dumped and the producing dairy placed on quarantine. In order to prevent unacceptable aflatoxin levels in milk, regulations prohibit feeding cottonseed with aflatoxin levels equal to or higher than 20 ppb to dairy cows. Aflatoxin contamination of cottonseed costs millions of dollars annually. In the US, contamination of cottonseed is most severe in the desert production regions of Arizona and in South Texas. The current study assessed the geographic distribution of aflatoxin contamination of commercial cottonseed in South Texas during the period of 1997 to 2000.

Between 4,472 and 9,949 truckloads of cottonseed from 31 to 35 gins in South Texas were analyzed for aflatoxin content each year from 1997 to 2000 upon receipt at the Valley Co-op Oil Mill in Harlingen, TX. Aflatoxin data in ppb was organized by region and gin of origin, and date of receipt at the mill. Each gin analyzed was geo-referenced in the Universal Transverse Mercator (UTM) coordinate system to be used in geostatistical analysis. Geostatistical analyses of both the aflatoxin content and the percent of cottonseed with aflatoxin content equal to or exceeding 20 ppb (percent over 20 ppb) was performed to obtain the patterns of aflatoxin contamination throughout South Texas. The mean values for the entire season for each gin were used in these analyses

Aflatoxin contamination of commercial cottonseed in South Texas is a perennial problem presenting both temporal and spatial variation. Both, the annual average aflatoxin content and the percent over 20 ppb of aflatoxin were significantly different among crop seasons from 1997 to 2000 with the mean aflatoxin content ranging from 24.0 to 112.3 ppb and the mean percent over 20 ppb ranging from 15.5% to 65.7%. The highest levels of contamination occurred in 1999 and the seasons with the lowest levels of contamination were 1997 and 2000. Geostatistical analyses of both the average aflatoxin content and the percent over 20 ppb indicate that the area with the greatest aflatoxin contamination problem during the study period extended from the central part of the Coastal Bend area (Corpus Christi, TX) to the south part of the Upper Coast area (around Port Lavaca and Victoria, TX). The area surrounding and including the Rio Grande Valley had the lowest contamination levels. In general, aflatoxin contamination increased as the ginning season progressed.

Population Structure Of *Aspergillus Flavus* In Soils And Corncobs In South Texas: Implications For Management Of Aflatoxins In Corn-Cotton Rotations

Ramon Jaime-Garcia and Peter J. Cotty. Southern Regional Research Center, ARS, USDA, New Orleans, LA

Organic material in soil favors the survival of *Aspergillus flavus*, the causal agent of aflatoxin contamination in cottonseed and corn. Crop residues that remain in the field after harvest provide important sources of organic matter. Most crop residues decay readily in the soil. However, corncobs do not decay easily and can stay in the soil for years before being totally decomposed. Furthermore, corncobs are already colonized by *A. flavus* prior to harvest. These characteristics make old corncobs a potential source of inoculum for *A. flavus* in corn and cotton fields. The objective of the current study was to determine the prevalence of *A. flavus* in corncobs and, consequentially, the potential of corncobs to serve as a source of *A. flavus* in cotton and corn crops.

Corncobs and soil samples were collected from 24 fields in South Texas during the planting seasons of 2001 and 2002. Corncobs were either from the previous season or from two seasons prior. In a second study, corncobs and soil were collected during the 2002 season from two fields on a biweekly basis in order to determine soil and corncob variations over the crop season. One of the two fields had corncobs from the corn season of 2001 and the other field had corncobs from the corn season of 2000. *A flavus* was isolated from both materials by a dilution-plate technique on selective media. Populations were quantified as colony forming units per gram (CFU/g), and at least 15 isolates from each sample were transferred to a V-8 juice agar to determine incidence of the highly aflatoxigenic S strain.

The results indicated that South Texas corncobs on average harbor 80 times more *A. flavus* propagules than the surrounding soil (110,000 CFU/g in corncobs versus 1,300 CFU/g in soil). In some individual fields, corncobs supported greater than 500 times the number of propagules than soil. Cobs known to have been in soil for two years, on average still contained 44 times as much *A. flavus* as the surrounding soil, and for some individual fields, the difference was greater than 70-fold. Greater than 50% of the corncobs tested had more than 25,000 CFU/g, and over 60% of the soil samples tested had less than 1,000 CFU/g. No significant difference in the incidence of the S strain of *A. flavus* was found in either corncobs or soil. The average percentage of S strain isolates was 11.1% for corncobs and 17.8% for soil. Preliminary data indicated that the presence of *A. flavus* in corncobs was variable over the season. There was less variation of *A. flavus* in soil. The percent of isolates belonging to the S strain varied little in both corncobs and soil. Corncobs left on soil surfaces remain for long periods and support large populations of *A. flavus.* Adopting harvest and cultivation practices that break up and incorporate corncobs below the soil surface should result in reduced residence of corncobs and reduce exposure of subsequent corn or cotton crops to aflatoxin producing fungi.

Insect Management For Reduction Of Mycotoxins In Midwest Corn. Fy-2002 Report

P.F. Dowd[1], J. Barnett[2] R.J. Bartelt[1], J.J. Beck[2], M.A. Berhow[1], J.P. Duvick[3], D.A Kendra[4], G.A. Molid[5], and D.G. White[6]. [1] Crop Bio-Protection Research Unit, USDA-ARS, NCAUR, Peoria, IL; [2] Syngenta Biotechnology, Research Triangle Park, NC; [3] Pioneer Hi-Bred, Johnston, IA; [4] Mycotoxin Research Unit, USDA-ARS, NCAUR, Peoria, IL; [5] Del Monte Foods, Manito, IL; [6] Dept. Crop Sciences, University of Illinois, Urbana, IL

Mycotoxin management studies: The computer program developed at USDA-ARS, NCAUR to predict mycotoxin levels in midwest corn has been well validated for fumonisin levels in 3 commercial fields in 2000 and 7 fields in 2001 (most fields were planted with two hybrids) using the generalized values (a version using customized values is under development). In 2000, rainfall was relatively common and insect damage was limited. A predicted level of ca. 1.5 ppm compared well to actual field means of 0.8, 1.3 and 1.6 ppm for previously studied hybrids. In 2001, the climate was drier during the summer, and insect damage varied according to planting date, with later planted corn being more heavily damaged by European corn borers. For "early" plantings, predicted fumonisin levels (ca. 0.5 ppm) were close to actual means in fields (0.2, 0.3, 0.4, 0.4, 0.6 ppm) except for one field, which ran well below the predicted value. We have a tentative explanation for the lower values of this one field, which was restudied in 2002. Later planted corn encountered more insect damage, but predicted values of near 2.0 ppm compared well with actual values of 2.2, 2.2 and 4.2 ppm. No aflatoxin was predicted for 2000, and none was found. In 2001, predicted aflatoxin values of circa 10 ppm were higher than mean values found in some fields, although some individual samples had 2 to 10 ppm. Initially, equations used for aflatoxin prediction in the program were based on published values from 1983 and 1987, but we knew this data was probably not detailed enough (no insect damage levels available) to predict values very well (although predictions of negative occurrences have all been correct for years from 1992 through 2000 in the area monitored). The new data has allowed for adjustment in the program which will be checked against data collected in 2002, another year that the program has predicted aflatoxin may be present. The program is currently being converted from DOS Basic to a format that will run in Windows, with the intent of producing a version that is commercial quality.

Seven commercial corn fields were intensively monitored for insects, mold inoculum, ear damage and mycotoxins in 2002; nearly all fields had two hybrids. Fields had considerable variation in distribution of *Fusarium subglutinans*, *F. proliferatum*, and *F. verticillioides*, as indicated by PCR analysis of material collected from leaf axils 7-10 days after pollination. However, all fields had at least some of each species present. Both corn earworms and European corn borers were common, but earlier planted fields escaped most of the corn borer damage in milk stage (which was above 80% in the late planted fields). Bt hybrids had less insect damage and symptomatic mold, and one Bt hybrid had significantly (10X) lower incidence of corn earworms than the corresponding

nonBt hybrid. Symptomatic *Fusarium* mold was mostly associated with insect damage, except for one hybrid. Mycotoxin analyses are pending. Commercially available traps were again found to be suitable for monitoring sap beetles when tested in sweet corn. Commercially available dusky sap beetle pheromone (based on the patent of Bartelt and Dowd) was available this year for the first time, and was found to be essentially equivalent in attractancy to dusky sap beetles monitored in sweet corn as the pheromone synthesized in house at NCAUR.

Plant resistance: Continued studies of functionally effective combinations of resistance mechanisms have produced significant increases in caterpillar mortality. For example, mortality to fall armyworms was increased from 0 to 55% when a digestive enzyme inhibiting protein was combined with a protein toxin. Compounds involved in Tex6 silk resistance to caterpillars were purified to near homogeneity, but insufficient quantities were available for NMR. Greater quantities of Tex6 silk were produced in 2002 for further chemical analysis. Constructs made from peroxidase genes cloned from corn have been tentatively matched with peroxidase isozymes (Dowd et al. 1995) associated with disease resistance and wounding using transient expression studies. A food plant-derived gene tentatively (confirmatory sequencing is needed) producing a novel protein previously identified as toxic to caterpillars at naturally relevant levels (Dowd et al. 1999) was cloned from a tropical food plant by PCR.

Publications

Behle, R.W., Dowd, P.F., Tamez-Guerra, P. and Lagrimini, L.M. 2002. Effect of transgenic plants expressing high levels of a tobacco anionic peroxidase on the toxicity of *Anagrapha falcifera* nucleopolyhedrosis virus to *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. 95: 81-88.

Dowd, P.F. 2002. Antiinsectan compounds derived from microorganisms. In: O.Koul and G.S. Dhaliwal, eds. Microbial Biopesticides. Taylor and Francis, New York, pp. 13-115.

Dowd, P.F. 2001. Biotic and abiotic factors limiting efficacy of Bt corn in indirectly reducing mycotoxin levels in commercial fields. J. Econ. Entomol. 95: 628-634.

Dowd, P.F., and White, D.G. 2002. corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) and other insect associated resistance in the maize inbred Tex6. J. Econ. Entomol. 95: 628-634.

Simmons, C.R. Grant, S., Altier, D.J., Dowd, P.F., Crasta, O., Folkerts, O. and Yalponi, N. 2001. Maize *rhm*1 resistance to *Bipolaris maidis* is associated with few differences in pathogenesis-related proteins and global mRNA profiles. Mol. Plant Microbe Inter. 14: 947-954.

Tree Nut Leaf Volatiles Using Rapid Znose Dynamic Headspace Analysis

James N. Roitman and Gloria B. Merrill. USDA ARS Western Regional Research Center, Albany, CA

Contamination of tree nuts with aflatoxin arises from infections of susceptible host nuts with spores of aflatoxigenic strains of *Aspergillus flavus* or *A. parasiticus* while the fruits are maturing or during harvest when the nuts are lying on the ground or being swept up and transported to processing facilities. Attacks by insects, especially codling moth and navel orangeworm, afford openings through which microbial spores, including those of aflatoxigenic and other fungi, can reach nut kernels. Reduction of insect damage should greatly reduce the levels of aflatoxin encountered in the final product. Because insects rely primarily on odors to locate mates and find hosts for laying their eggs, we have analyzed, pistachio and walnut leaves for emitted volatile substances. It has been our goal to determine which volatile substances emitted by the fruit and leaves of nut trees influence the behavior of insect pests either as attractants or repellents. Because the amounts of volatiles omitted from leaves are rather low it has been necessary to use polymer absorbent traps and pass purified air over fresh leaves for 16 to 24 hours in order to have sufficient material for GCMS analyses. A company in Southern California (Electronic Sensor Technology) has recently developed an instrument (marketed as the zNose™) capable of performing rapid GC analyses in the laboratory or in the field. We have recently purchased a zNose instrument and performed some preliminary experiments to evaluate its usefulness.

THE INSTRUMENT. The zNose comprises a portable case containing a small, refillable helium tank, gas regulator, gauges, and a port for attaching a laptop computer that functions as the instrument's controller. A six-foot long electronic cable and flexible carrier gas tube are bundled and attached to the handheld GC instrument. Volatile samples in air are sucked into the GC *via* a steel needle protruding from the face of the GC and pass through a miniature polymer absorbent trap. The trap is connected to the GC column *via* a rotary valve and heated rapidly to desorb the volatiles onto the column which is then temperature programmed. The entire sampling and GC analysis process take about 30 seconds.

RESULTS AND DISCUSSION. Fresh two gram samples of whole almond, pistachio or walnut leaves in 40 mL septum-capped vials were placed in a heater block at 35° for five minutes, attached to zNose chromatograph *via* the needle, sampled for three seconds and analyzed. The three leaf types produced very different traces. The zNose traces of mission almond, Kerman pistachio and Payne walnut leaves showed 9, 10 and 14 distinct peaks respectively. Corresponding chromatograms of trapped fresh leaf volatiles by GCMS on a 60m DB-1 column showed many more peaks (mainly monoterpenes and sesquiterpenes with a few esters, ketones and alcohols). The great advantages of the zNose instrument are the minimal sample size and preparation time (a few minutes vs. more than one day), the extremely rapid analysis time (30 seconds vs. 105 minutes) and portability. Disadvantages are the low resolving power of the less than one meter GC column, the necessity of using metal columns (heating is achieved by

passing current directly through the column) and reproducibility problems, where temperature and time of sampling greatly affect the results. We have only begun to explore the potential of this interesting instrument.

Impact Of Phytoalexins And Lesser Cornstalk Borer Damage On Resistance To Aflatoxin Formation

B. Z. Guo[1], Victor Sobolev[2], C. C. Holbrook[3], and R. E. Lynch[1]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]USDA-ARS National Peanut Research Laboratory, Dawson, GA; [3]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA

In peanut, the mechanism of resistance to *Aspergillus flavus* has been reported as the capacity to synthesize phytoalexins, the antibiotic secondary metabolites. The lesser cornstalk borer (LCB) is one of the most destructive insects in peanut production area. Penetration of peanut pods by insects enhances infection of pods by *A. flavus/parasiticus* and aflatoxin contamination in peanut. Water activity is a measurement of the energy status of the water in a system, indicating how tightly water is bound. We use water activity in a pod to explain the drought stress placed on the plants and drought tolerance. Field experiments were carried out in the rainout shelters to study the influence of phytoalexins on resistance to aflatoxin formation in peanut lines and determine if damage to the peanut by lesser cornstalk borer compromises the resistance. We compared two peanut cultivars, Georgia Green (popular commercial cultivar) with a small root system and Tifton 8 (drought tolerance) with a large root system. Rainout shelter was moved over 60 days after planting. We measured water activity, phytoalexins, and aflatoxin concentrations in all samples. The preliminary analyses of 2001 samples indicate that peanut cultivar Tifton 8 has higher water activity under drought stressed condition than Georgia green. Three phytoalexins have been measured, *trans*-resveratrol, *trans*-arachidin-3, and *trans*-3-isopentadienyl-4,3', 5'-trihydroxystilbene. Damaged pods of Tifton 8 had higher concentrations of all three phytoalexins than Georgia Green. The total aflatoxins levels were lower in Tifton 8 than in Georgia Green. LCB damage significantly increased aflatoxin concentrations in all samples, but Tifton 8 had lower total aflatoxin contamination, which might be correlated with the concentrations of phytoalexins in the damaged pods.

Effect Of Foliar Application Of Particle Films On Drought Stress And Aflatoxin Contamination Of Peanut

Wilson, J. P.[1], C. C. Holbrook, [1] L. Wells, [2] B. Mandal,[2] D. Rowland, [3] and D. M. Wilson[2]. [1] USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [2] Univ. of Georgia, Dept. of Plant Pathology, Tifton, GA; [3] USDA-ARS, National Peanut Research Lab., Dawson, GA

Foliar application of Kaolin-based particle films has been shown to suppress insect pests and diseases in tree fruit, and to improve transpiration efficiency. Drought tolerant peanut lines have been shown to have greatly reduced aflatoxin contamination. The objective of this study was to evaluate the potential of SurroundTM particle film to reduce drought stress and preharvest aflatoxin contamination in peanut. Four genotypes were planted in a split plot design with five replications. Half of the plots received two sprays with surround, while the other plots were untreated. All plots were inoculated with a mixture of Aspergillus flavus and A. parasiticus about 60 days after planting and subjected to drought and heat stress for the 40 days immediately preceding harvest. Surround spray treatments had no effect on visual stress, and no effect on aflatoxin contamination. Plots receiving surround spray treatment had slightly less incidence of tomato spotted wilt virus (TSWV) in comparison to untreated plots. However, this did not result in a significant increase in yield, and no effect on TSWV was observed in two other field studies. Although foliar applications of particle films may be useful for producing certain vegetable and fruits, its use appears to have little value in peanut production systems.

Development Of Kairomone-Based Mass-Trapping Control Of Codling Moths And Reduction Of Aspergillus In Walnuts

D. M. Light, K. M. Reynolds, M. Brewer and B. C. Campbell. USDA-ARS, Western Regional Research Center, Plant Mycotoxin Research Unit, Albany, CA

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

We are investigating mass-trapping control methods using the pear-ester kairomone to directly manage female and male codling moths in walnut. The goals of the mass-trapping studies are to: 1) to simulate the potential of other kairomone-based controls (*e.g.*, attract and kill), 2) demonstrate a control strategy for small acreage organically-managed orchards, and 3) demonstrate a control strategy for orchard borders to eliminate immigration of mated – gravid CM females. Field studies were conducted to optimize the composition, delivery rate, and formulation of kairomone lures and combinational lures of kairomone and pheromone to create bisexual and gender-specific attractants for mass-trapping codling moth populations. Number -placement of traps in orchards was optimized for mass-trapping efficacy. Occurrence - degree of nut damage was evaluated by nut drop, canopy infestation, and harvest injury/damage.

Some particular kairomone-pheromone blends showed promise in the attractiveness of lure-baited traps. These kairomone-pheromone blend lures captured greater numbers of males than pheromone-alone baited traps, while female trap capture was the same for kairomone-alone and the blends. Seven replicated three acre plots of walnuts (three organic, two conventional, and two mating disruption) were set-up to evaluate the use of mass-trapping as a population control measure over the entire season. Plots were three acres with 64 total traps (ca. 15 m apart, 21 traps/acre). Traps were baited with combination lures. Moth capture rates, efficacy, and walnut damage rates were compared for the different orchards and management practices. For all mass-trapping plots, far greater numbers of moths were captured in the traps positioned at the block borders than those in the central – interior block region. With the combination lure significantly more males than females were caught, ranging from 1.3 to 4 times more males captured in walnuts. Late August and harvest damage rates were significantly lower (one half) in the mass-trapping blocks (1.4% damage) than control blocks (3.2% damage). Also, replicated studies were conducted to determine the optimal number of traps per acre to use in the mass-trapping tactic. We found a trap density of one trap every 15+ meters, *e.g.* every third tree in walnuts, evokes the best CM capture rate, while

attracting more of the targeted female population.

These studies show promise that the kairomone can attract and eliminate CM and thereby limit damage. Next season, these mass-trapping studies will expand in block size, replicated organic orchards, and as a border treatment in mating disruption orchards to diminish CM immigration from neighboring orchards.

AFLATOXIN/FUMONISIN ELIMINATION WORKSHOP

15th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 6: CROP RESISTANCE - CONVENTIONAL BREEDING

Moderator: *Jeff Nunley, South Texas Corn and Grain Association*

Molecular Marker Assisted Selection For Resistance To Aspergillus Ear Rot And Aflatoxin Production In Maize

Torbert Rocheford, Chandra Paul, and Don White. Department of Crop Sciences, University of Illinois, Urbana

Various research groups have mapped Quantitative Trait Loci (QTL) for resistance to Aspergillus Ear Rot and low levels of aflatoxin production in diverse genetic backgrounds. We are performing molecular marker assisted selection of QTL for resistance to Ear Rot and low aflatoxin production into a commercially elite line. We are using the inbred Mp313E chromosome arm 4L region with one or more QTL for low levels of aflatoxin, identified in studies performed in Mississippi. This demonstrates the power of marker assisted selection, as favorable QTL alleles in very diverse and unadapted germplasm sources can be utilized and introgressed into adapted, elite germplasm. The Mp313E chromosome arm 4L region, particularly bins 4.06-4.08 were selected with markers in (Mp313xFr1064)Fr1064 backcross1 and backcross2 progenies. These lines were selfed to develop BC_2S_1 progenies. These lines show good recovery of the Fr1064 agronomic plant phenotype. These lines are also being selfed, marker genotyped, and selected to develop advanced breeding lines homozygous for the Mp313E 4L segment. Early generation evaluation of a series of BC_2S_1 lines, which were randomly segregating for dosage and recombinants of the Mp313E/FR1064 chomosome arm 4L region, was performed on two replicates in 2001. The environment was conducive to aflatoxin production with average concentrations ranging from 120 to 1049 ppb. In the same field, the Fr1064 and FrB73 checks ranged from 650 to 700 ppb. Notably one of the best lines for low levels of aflatoxin, at 166 ppb, was homozygous for all markers for Mp313E on chromosome arm 4L. This shows a dramatic improvement in aflatoxin levels over the Fr1064 inbred. Some of the other lines with the lowest levels of aflatoxin have various number of markers homozygous for the Mp313E 4L chromosome region. These preliminary results suggest that molecular marker assisted selection is working for lower aflatoxin levels. We will analyze the 2002 data this winter. We have developed advanced generation near-isogenic lines for the Mp313E/Fr1064 chromosome 4L region. These are being testcrossed in Hawaii and will be evaluated in multiple locations next year. This collective set of analyses will provide much more definitive results on the relative success of this marker assisted program.

Marker assisted selection is also being performed to move desirable chromosome segments from Tex6 into the B73 background. We are selecting Tex6 segments with QTL for lower aflatoxins in bins 5.01 and 10.05-.07 in BC_2S_2 families. We are also pyramiding chromosome segments for resistance to ear rot and lower aflatoxin from Tex6 and Mp313E. A (Tex6xB73)B73 BC_1S_2 family with favorable QTL segments in bins 5.01 and 10.05-.07 was crossed with a (Mp313ExFr1064)Fr1064 BC_2S_1 family. The F1 was selfed and F_2 plants genotyped and plants homozygous or heterozygous for desirable QTL were selected and selfed. The $F_{2:3}$ seed will be greenhouse grown and genotyped to fix the desirable QTL. We are also following desirable QTL for ear rot on chromosome bin regions, notably bin 2.08 from Tex6. The pyramiding of favorable QTL from diverse genetic backgrounds, Tex6 and Mp313E, into a commercially elite background, is

apparently the first time this has been done for aflatoxins. Pyramiding appears to be the approach necessary to achieve useful, stable levels of resistance.

Use Of MI82 And Oh516 As Sources Of Resistance To Aspergillus Ear Rot And Aflatoxin Production And The Utilization Of BGYF As A Selection Tool

Don White, Laura Maupin, Kevin Busboom, Mike Clements and Torbert Rocheford. University of Illinois, Urbana, IL

Our objectives was to determine if the corn inbreds MI82 and Oh516 have alleles for resistance to Aspergillus ear rot and aflatoxin production that can be transferred to commercially used corn inbreds. We also want to determine the types and magnitudes of gene action, heritabilities, and the gains from selection for low levels of bright green-yellow fluoresence (BGYF), aflatoxin, and ear rot.

MI82 is an inbred line that was selfed from a corn hybrid commercially grown in India by D.G. White that has not been officially released by the University of Illinois. It has been shown to be a source of high levels of resistance in F_1 crosses and in several laboratory studies. In a generation mean analysis, dominance was the most important gene action associated with low levels of BFYF and aflatoxin. Heritabilities for both BGYF and aflatoxin were relatively high and the characters were high correlated. We believe that is possible to use BGYF in a program designed to select resistance to Aspergillus ear rot and aflatoxin production. In our studies using 447 backcross susceptible self families of the cross between MI82 and the susceptible inbred B73, if 30% of the families with the lowest BGYF were selected then 50% with the lowest aflatoxin levels were selected, the selected families grown in the next year were more resistant than if 15% of the 447 families with just the lower levels of toxin had been selected. Therefore, selection for both BGYF and aflatoxin was slightly more efficient. In addition, only half the number of aflatoxin analysees would need to be made which will result in a big cost savings.

With the resistant inbred line Oh516 we evaluated crosses of OH516 and B73 in multiple generations in a generation mean analysis study. That study is being repeated in 2002. Within that study 265 backcross susceptible selfed families are being evaluated as families per se and in test crosses with LH185 with inoculation in Urbana. The backcross families in test crosses also are being evaluated in two locations, in Texas, with inoculation using *A. flavus* spores coated onto autoclaved corn kernels. Preliminary indications are that OH516 is a very good source of resistance and likely will be as good as the inbred line Tex6.

We believe that MI82 and Oh516 can be used as sources of resistance to improve commercially used hybrids. It also appears at this time that at least nine highly resistant inbreds have been identified that can improve commercially used hybrids. Our goal now must be to move genes from the resistant inbreds into commercially acceptable genotypes.

Aflatoxin And Fumonisin Distribution In Maize Populations

Steven H. Moore[1] and Hamed K. Abbas[2]. [1]LSU Agricultural Center, Baton Rouge, LA; [2]USDA-ARS, Stoneville, MS

New genes are needed to develop corn hybrids with resistance to *Aspergillus flavus* and subsequent aflatoxin biosynthesis. There is no commercial hybrid presently marketed with advertized resistance to this malady and the resistance that is available would likely not keep levels in grain below that needed for commercial sale in a high-incidence year such as 1998 in the southeastern U.S. Recently, good resistance was found in 'OH516', indicating that many resistant genes may reside in existing germplasm collections that have simply not been adequately screened. The ultimate goal of this research effort is to screen corn germplasm from North and South America, Mexico, and Africa until the genes needed to ensure safe grain production are identified and introduced into breeding programs. Before large-scale screening can proceed, several field-research questions must be resolved, including; (1) How many ears of each accession should be tested to make a confident assessment of resistance? (2) Will self-pollination be necessary to assess resistance? (3) How does artificial inoculation with *Aspergillus flavus* spores affect assessment? (4) Can ears be bulked to assess resistance? To answer these questions, a field trial was conducted in central Louisiana to determine the frequency distribution of aflatoxin and fumonisin resulting from factorial treatments of inoculated/non-inoculated (*Aspergillus flavus)* and self-pollinated/open-pollinated populations of susceptible 'B73' and resistant 'Tex6' inbreds. A split-plot design with 20 replications was used with inbred as main plot and pollination/inoculation treatment used as sub-plot. Harvested ears were rated for quality (1 – 10, *discarded if rating was 2 or less*), fungal growth (1 – 10), bright greenish-yellow fluorescence (1 – 10), aflatoxin (ppb), and fumonisin (ppm). Inoculating with *Aspergillus flavus* greatly increased fungal growth, BGYF, and aflatoxin. Fungal growth and aflatoxin were greater in the susceptible line. Aflatoxin increased while fumonisin decreased when plants were self-pollinated. There were no significant interactions between inbreds and inoculation or pollination methods, indicating that the ranking of inbreds did not change due to these treatments. The correlation coefficients of aflatoxin with BGYF and fungal growth were 0.93 and 0.87, respectively. Fumonisin was not affected by any variable other than pollination method. More analysis is needed to define distribution patterns so that the required number of ears needed to effectively assess aflatoxin and fumonisin can be determined.

Aflatoxin Accumulation In Maize Inbreds And Hybrids

Javier Betrán[1], Tom Isakeit[2], Gary Odvody[3], and Kerry Mayfield[1]. Texas A&M University: [1]Soil & Crop Sciences Department, College Station, TX; [2]Pathology Department, College Station, TX; [3]Pathology Dept., Corpus Christi, TX.

Preharvest aflatoxin (AF), a carcinogenic toxin produced by *Aspergillus flavus*, causes enormous economic losses to Texas and U.S. growers. Development of stress-tolerant corn and resistance to AF would add $50 million to Texas corn value annually, allowing profitable production and marketing. We are searching new sources of AF resistance by screening diverse maize germplasm under field conditions in Texas. Subtropical and tropical, temperate, Quality Protein Maize, and high and low oleic maize inbreds were evaluated for response to field AF accumulation under *A. flavus* inoculation in Texas. Most promising white and yellow inbreds in previous evaluations were further characterized *per se* and in several hybrid combinations. Inbred lines and their hybrids were evaluated under inoculation in three locations in South and Central Texas: College Station (CS), Weslaco (WE), Corpus Christi (CC) during 2002. Alpha-lattice design with 4 reps was used. Drought and heat stress was induced by late planting and limited irrigation. Inoculation was done by placing *A. flavus* colonized corn kernels on the soil surface between treatment rows around mid-silk stage. Quantification of AF was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest^{TM}). Different fatty acid composition in the seed can affect the degree of fungi sporulation. Inbreds derived from GEM (Germplasm Enhancement Maize) germplasm with different levels of fatty acids: high linoleic and low oleic (DKB830:S11a17-35-B, DKXL380:S08A12-24-B, BR52060:S0212-25-B, FS8B(T):N1802-35-1-B), low linoleic and high oleic (CUBA164:S1511b-15-B, DKXL380:S08a12-12-B, DKB844:N11b17-21-B, AR16026:S1704-32-B), together with resistant (CML176 and Tx601) and susceptible (Tx732 and Tx110) inbreds were evaluated for aflatoxin accumulation at WE and CS. AF average was 90.3 ng g^{-1} at CS and 630.8 ng g^{-1} at WE. CML176, Tx601, and CUBA164:S1511b-15-B were the most resistant inbreds across locations. Inbreds DKXL380:S08A12-24-B and BR52060:S0212-25-B had low AF in WE but high in CS. No significant differences were observed between the two groups with different fatty acid composition. Quality Protein Maize (QPM), o2 maize which has been selected for vitreous endosperm, grain quality and good husk coverage, can provide resistant factors to AF. Fifty QPM temperate adapted inbreds and their testcrosses with Tx804 were evaluated at CS, WE and CC. Commercial hybrids P31B13, P32R25, RX897, and DK668 were included as checks. AF average was 326.57 ng g^{-1} at WE and 105.7 ng g^{-1} at CS. The least susceptible hybrids with AF < 50 ng g^{-1} at WE were from QPM inbreds developed at Texas A&M from CIMMYT population 69. The most susceptible hybrids and inbreds were from high oil QPM inbreds. AF in commercial hybrids was 551.8 ng g^{-1} for P31B13, 108.8 ng g^{-1} for P32R25, 307.5 ng g^{-1} for Rx897, and 691.9 ng g^{-1} for DK668. The correlations between inbred *per se* and hybrid AF was 0.56** at WE. Hybrids among subtropical and tropical inbreds (CML323, CML285, CML288, NC300) and temperate inbreds (Tx732, B104, Tx770, Tx772, LH235 x LH236, LH252 x LH262) were evaluated at CS, WE and CC. AF

average was 220.1 ng g^{-1} at WE and 20.2 ng g^{-1} at CS. The hybrids with the lowest AF at WE were CML323 x NC300 (9.8 ng g^{-1}) and CML323 x CML288 (11.5 ng g^{-1}). AF in checks was higher with 1200.0 ng g^{-1} for P31B13, 413.7 ng g^{-1} for P32R25, 100.7 ng g^{-1} for Rx897, and 634.4 ng g^{-1} for DK687. Less susceptible hybrids involved exotic inbreds. Yellow inbreds TxX69's, CML323, Tx772, CML288, and NC300 can have resistant factors to AF.

Selection Of Peanut Breeding Lines With Resistance To Preharvest Aflatoxin Contamination And Improved Drought Tolerance

C. Corley Holbrook[1], Baozhu Guo[1], David M. Wilson[2], C. K. Kvien[2], Weibo Dong[2], Diane L. Rowland[3], and Meng Luo[2]. [1] USDA-ARS, Tifton, GA; [2] University of Georgia, Tifton, GA; [3]USDA-ARS, Dawson, GA

This is a long-term research project designed to result in the development of peanut cultivars with resistance to preharvest aflatoxin contamination (PAC). The initial objectives were to develop large-scale screening techniques to identify sources of resistance to PAC in peanut germplasm. Screening techniques were developed, and fifteen sources of resistance were identified. These sources of resistance were then used in a hybridization program to combine this resistance with acceptable agronomic performance. Several hundred $F_{4:5}$ breeding lines were created and tested for resistance. Nine breeding lines were identified that had relatively low aflatoxin contamination and relatively high yield when subjected to late season drought stress. We have also conducted cooperative research with other research groups to attempt to develop indirect selection techniques for resistance to PAC. Promising results were observed in the characterization of gene expression of phospholipase D (PLD) that is associated with drought tolerance and possibly aflatoxin contamination in peanut. Promising results were also observed with research on the effect of resistance to the peanut root-knot nematode and reduced aflatoxin contamination. In 2002 we added five drought shelters, continued the hybridization and selection program, continued research on the development of an indirect selection tool for reduced aflatoxin contamination, and began late generation yield testing and evaluation of the PAC resistant breeding lines.

Identification And Characterization Of Resistance Mechanisms To Preharvest Aflatoxin Contamination And EST/Microarray Programs In Corn And Peanut

B. Z. Guo[1], M. Luo[2], P. Dang[3], A. E. Coy[2], W. Dong[2], C. C. Holbrook[4], R. D. Lee[2], N. W. Widstrom[4], R. P. Niedz[3], and R. E. Lynch[1]. [1]USDA-ARS, Crop Protection and Management Research Unit; [2] University of Georgia Coastal Plain Experiment Station, Tifton, GA.; [3] USDA-ARS, U.S. Horticultural Research Laboratory, Ft. Pierce, FL; [4] USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

Corn and peanut become contaminated with aflatoxins when subjected to prolonged periods of heat and drought stress. To meet the challenge of prevention of preharvest aflatoxin contamination, it will be necessary to have a more detailed understanding of the organization and function of the genetic material of corn and peanut in response to environmental stresses. The genes that control functions leading to plant reaction to the environmental stress and fungal infection must be identified. Our research studies are focusing on identification of resistance genetic sources and characterization of the resistance mechanisms. Drought tolerance, for example, may be determined by many genetic factors. The "one-gene-at-a-time" approach for analyzing gene function is inadequate. It is now possible to locate multiple genes of plants in responding to environmental stresses. There are several major new molecular tools used for gene functional analysis, such as Expressed Sequence Tag (EST) and Micro-Array technology. Research progress has been made in our laboratories in genetic evaluation and selection for drought tolerant germplasm and gene identification and characterization. Over 600 single crosses of corn made in 2001 have been evaluated for drought tolerance, aflatoxin contamination and yield performance in 2002. Several F1s have been selected for large scale field test in 2003. We have concluded that LCB insect damage significantly increased aflatoxin production in peanut. The drought tolerant peanut lines have higher phytoalexin concentration and lower aflatoxin contamination than the drought sensitive lines. Water activity was higher in Tifton 8, the drought tolerant line, than Georgia Green, the drought sensitive line. Gene expression of *PLD* have be characterized in relationship with drought tolerance/sensitivity. We have initiated a new EST program in Tifton to acquire expressed gene sequence information in order to rapidly identify genes and understand their functions in whole genome level in response to drought stress and fungal infection. Exciting information has been acquired, such as some plant defense genes identified *via* EST as gene of small cysteine-rich antifungal protein, Ca^{2+}/H^{+}-exchanging protein, peroxidase, 14-3-3-like protein, glutathione S-transferase, and trypsin inhibitor.

Integrating Seed And Endocarp Based Resistance To Preharvest Aflatoxin Contamination In Almond

Thomas Gradziel and Abhaya Dandekar. Department of Pomology, University of California, Davis, CA

Resistance to preharvest aflatoxin contamination of almond (*Prunus dulcis*) is being pursued through the integration of both seed and endocarp-based resistance to insect infestation and, when *Aspergillus* infection is present, suppression toxin formation. Since previous research has shown that *Aspergillus* infection in almond follows insect damage to the seed, primarily by the almond pest *Amyeloise transitella*, or naval orangeworm (NOW), the control of this pest is being pursued through antibiosis of the seed and through improved shell seal. Seed based antibiosis has been identified in several almond breeding lines. Although the mechanisms appear complex, advanced selections with improved NOW resistance and good horticultural quality have been developed and are now being prepared a regional grower testing. Current NOW research is also examining the role of amygdalin, benzaldehyde, and cyanide biosynthesis to insect antibiosis in this cyanogenic species.

The control of NOW is also being pursued through the breeding of almond varieties with good kernel size, high levels of kernel-to-nut crack out, and high shell-seal integrity. Recent research has shown that the majority of fractures in the developing endocarp or shell occurred at the site of the aborted ovule. Furthermore, the structural failure of this tissue now appears to be the consequence of a modified tissue development rather than a structural failure at nut maturity. Interspecies integration has successfully transferred the high shell-seal/high crack-out characteristic from *Prunus webbii* to cultivated almond types.

The integrity of the almond shell seal under field conditions has been evaluated through visual including microscopic inspection, and through gas-flow measurements. In addition, a survey is now underway examining the occurrence, symptoms and signs of natural contamination of almond pellicle tissue by native fungal spores such as *Aspergillus niger*.

Several breeding lines derived from interspecies introgression have been identified as having a low toxin production following controlled inoculations. The underlying mechanisms for toxin suppression are being evaluated through biochemical and genomic analysis. Major candidates evaluated in 2002 include gallic acid and related compounds, with analysis still ongoing. Controlled crosses between parents selected for toxin suppression are now mature kernel-producing trees, and these genotypes are now being evaluated to determine the heritability for this trait.

Large-scale 2002 field testing of advanced selections expressing varying levels of NOW and *Aspergillus* resistance is now taking place at multiple test sites throughout the Central Valley almond production area. Results to date have supported lab findings and have demonstrated the feasibility for aflatoxin control using this integrated strategy.

Identification And Characterization Of Potential Resistance Markers Through Proteome Analysis

Zhi-Yuan Chen[1], Robert L. Brown[2], Thomas E. Cleveland[2], and Kenneth E. Damann[1]. [1]Deptartment of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA; [2]Southern Regional Research Center, USDA-ARS, New Orleans, LA

Resistant and susceptible maize germplasm has been identified from various sources through field and/or laboratory screening. Comparisons of protein profiles between resistant and susceptible genotypes have led to the identification of over a dozen resistance-associated (either unique or upregulated in resistant lines) proteins (RAPs). Some of these proteins have been identified as storage proteins (such as globulin 1, and 2, late embryogenesis abundant proteins), stress-related proteins (such as small heat-shock proteins, cold-regulated proteins, peroxidoredoxin antioxidant, aldose reductase, and glyoxalase I), and antifungal or potentially antifungal proteins (such as 14 kDa trypsin inhibitor, pathogenesis-related protein 10).

Further physiological and biochemical studies of several markers have been initiated to verify whether the proteins are involved in conferring host resistance to *Aspergillus flavus* infection/aflatoxin production. Resistant genotypes were found to generally contain higher levels of aldose reductase and glyoxalase I activities in mature kernels than susceptible ones. However, upon fungal infection, the aldose reductase activity decreases in resistant genotypes and increases in susceptible ones, whereas glyoxalase I activity increases or at least remains the same in resistant genotypes, but decreases in susceptible ones. Further *in vitro* studies found that maize glyoxalase I activity was inhibited by addition of aflatoxins. In addition, the substrate of glyoxalase I, methylglyoxal, was found to increase aflatoxin production in *A. flavus* cultures. Therefore, high levels of glyoxalase I activity, which may effectively maintain the methylglyoxal inside cells to a low level under stress conditions, can reduce the level of aflatoxin production when kernels are infected. The gene encoding PR10 protein has also been recently cloned from maize, and been introduced into E. coli and tobacco for further characterization of this protein. In vitro studies using the overexpressed PR10 protein from *E. coli* have demonstrated its RNase activity. Transgenic tobacco plants have been generated, tested for positive integration of PR 10 gene, and are currently being evaluated for possible antifungal activities.

Maize lines generated from crosses between domestic lines, and crosses between domestic and African lines recently became available. Variation in resistance has been observed among lines sharing the same parental background. Proteomic analysis of iosgenic or near isogenic lines these germplasm pools may yield identification of factors playing a major role in resistance to *A. flavus* infection/aflatoxin production.

Panel Discussion - Crop Resistance - Conventional Breeding

Panel Chair: Thomas Gradziel
Panel Members: Torbert Rocheford, Don White, Steve Moore, Javier Betran, C. Corley Holbrook, B. Z. Guo, and Zhi-Yuan Chen

Sui-Sheng Hua asked Tom Gradziel how almond pellicle ink-staining by *Aspergillus niger* could be used as a model system for fungal contamination of drying nuts in the field. Gradziel replied that since *A. niger* spores were ubiquitous in the field at this time and the signs of their colonization of vulnerable almond pellicles were unique, that the relative presence of pellicle ink-staining for different locations and years could prove useful as a general indicator of kernel vulnerability (i.e. incomplete shell-seal, excessive kernel moisture, etc.) to fungal spore contamination. Themis Michailides then asked whether the signs of *A. niger* contamination were consistent over the years of observation. Gradziel responded that this was one of the objectives of continuing study; preliminary results from 2001-2002 indicated a unique pellicle-staining pattern for *A. niger* with other types of pellicle injury appeared associated with different field-borne fungi as well.

Peter Cotty commented on the danger of over-interpreting BGYF results as presented by several of the presentations for maize breeding, since BGYF and aflatoxin contamination were not always highly correlated in his studies of cotton. Steve Moore acknowledged the inconsistencies between BGYF and actual aflatoxin contamination but defended its continued use as a valuable and convenient prescreen for aflatoxin contamination in maize.

Gene Expression Profiling In Kernel Developing Stages As Influenced By Drought Stress In Corn And Peanut

M. Luo[1], P. Dang[2], B. Z. Guo[3], R. D. Lee[1], C. C. Holbrook[4], R. P. Niedz[2], and R. E. Lynch[3]. [1]University of Georgia-Tifton Campus, Tifton, GA; [2]USDA-ARS, U.S. Horticultural Research Laboratory, Ft. Pierce, FL; [3] USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [4]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA

Preharvest aflatoxin contamination in corn and peanut is a severe problem in the southern U.S. The previous researches showed genetic differences in drought tolerance and the positive correlation between drought tolerance and reduced preharvest aflatoxin contamination in corn and peanut. The overall goals of this project are to better understand global gene expression profiling of drought tolerance at certain stages of seed development under stress of water deficiency, and to identify the biochemical pathways and genes controlling the resistance to *Aspergillus flavus* infection and aflatoxin contamination. The peanut variety A13 and inbred lines of GT-MAS:gk will be used in this project. Two cDNA libraries have been constructed for EST/microarray analysis. Some ESTs from peanut libraries have been sequenced, and there are 215 of 361 high quality ESTs that functions can be identified by BLASTx analysis. The preliminary ESTs show that some plant defense genes have been identified, such as heat-shock protein, Ca^{2+}/H^{+}-exchanging protein, peroxidase, small cysteine-rich antifungal protein, and ribosomal protein. We have full-length sequence of ribosomal protein gene identified by DD-RT-PCR and cloned by RACE.

Identification And Characterization Of Phospholipase D Gene (PLD) In Peanut And *PLD* Expression Associated With Drought Stress

B. Z. Guo[1], Y. Cao[2], G. Xu[2], C. C. Holbrook[3], and R. E. Lynch[1]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]Department of Entomology, University of Georgia, Tifton, GA; [3]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA

Preharvest aflatoxin contamination has been identified by the peanut industry as the most serious challenge. Drought stress is the most important environmental factor exacerbating *Aspergillus* infection and aflatoxin contamination in peanut. Development of resistant peanut cultivars would represent a major advance for the U.S. peanut industry. In this study, we identified a novel *PLD* gene, encoding a putative phospholipase D, a main enzyme responsible for the drought-induced degradation of membrane phospholipids in plants. The completed cDNA sequence was achieved by using the consensus-degenerate hybrid oligonucleotide primer strategy. We have used the sequence information encoded by the cloned fragments to amplify both the 5' and 3' ends of this gene to obtain a full length sequence. The deduced amino acid sequence shows high identity with known *PLD* genes, having similar conserved features. The *PLD* gene expression under drought stress in greenhouse has been studied using four peanut cultivars, Tift 8 and A13 (drought tolerant) and Georgia Green and 419A (drought sensitive). Northern analyses had showed that PLD gene expression was induced faster by drought stress in Georgia Green than in Tift 8. More peanut lines will be studied to characterize the PLD gene expression as marker for screening germplasm for drought tolerance and aflatoxin formation.

A Comparison Of Inoculation Techniques For *Aspergillus Flavus* On Corn.

G. L. Windham[1], W. P. Williams[1], P. M. Buckley[1], H. K. Abbas[2] and L. K. Hawkins[1]. [1]USDA, ARS, Mississippi State, MS; [2]USDA, ARS, Stoneville, MS

Field studies were conducted at Mississippi State University and Stoneville, MS, to compare conventional *Aspergillus flavus* inoculation techniques used to evaluate corn genotypes with granular application techniques used to establish *A. flavus* isolates in cotton fields in the Southwest (Bock and Cotty, 1999). Conventional inoculation techniques included injecting spores under husks using the side-needle technique or spraying spores on ears weekly for 5 weeks with a Solo backpack sprayer. The granular applications consisted of broadcasting *A. flavus* infected wheat in field plots. Granular applications were made 2 weeks prior to midsilk, at midsilk, or 2 weeks after midsilk. The *A. flavus* isolate 3357 was used for all inoculations in all studies. Six commercial corn hybrids (Asgrow RX938, DeKalb DK668, Pioneer Brand 33Y09, Pioneer Brand 3310, Pioneer Brand 33K81, and Terral TV2100) were included in the tests. Inoculation treatments and hybrids were arranged as a split plot. Inoculation treatments were assigned to main plots and hybrids were assigned to subplots. Ears were harvested 60 days after midsilk, dried, shelled, and ground. Aflatoxin contamination in 50-gram subsamples from each plot was determined using the Vicam AflaTest. *A. flavus* kernel infection was determined by surface sterilizing the seed and plating the seed in petri dishes containing Czapek's agar with salt. Corn plots inoculated with the side-needle technique had the highest level of aflatoxin contamination (731 ppb). Corn from plots inoculated with wheat prior to midsilk had 451 ppb of aflatoxin, while corn from sprayed plots had 236 ppb of aflatoxin. Plots inoculated with wheat at midsilk or 2 weeks after midsilk had the lowest levels of aflatoxin contamination for all inoculated plots. Highest levels of kernel infection were recorded in plots inoculated using the side-needle technique. The commercial hybrid Terral TV2100 had the highest levels of aflatoxin contamination and *A. flavus* kernel infection in all tests. The commercial hybrid Asgrow RX938 had the lowest levels of aflatoxin contamination and *A. flavus* kernel infection in all tests. The side-needle technique appeared to be superior to all other inoculation techniques used in these studies. However, in large scale tests where inoculation of individual ears by hand is impractical, the spray technique and application of *A. flavus* infected wheat prior to midsilk may be useful tools in evaluating corn for aflatoxin resistance. Further studies are planned to improve the efficacy of granular applications of *A. flavus*.

Bock, C. H., and Cotty, P. J. 1999. Wheat seed colonized with atoxigenic *Aspergillus flavus*: characterization and production of a biopesticide for aflatoxin control. Biocontrol Sci. Technol. 9: 529-543.

Evaluation Of Inoculation Methods For Aspergillus Ear Rot And Aflatoxin Contamination Of Corn

L.M. Maupin[1], D.G. White[1], and J.M. Perkins[2]. [1] Department of Crop Sciences, University of Illinois, Urbana, IL; [2]Monsanto Company, Waterman, IL

The purpose of this experiment was to evaluate inoculation methods that could reduce time and expense associated with inoculating corn genotypes to evaluate relative resistance to aflatoxin production in corn grain by *Aspergillus flavus* Link:Fr. An inoculation technique that allows for identification of resistant susceptible genotypes must result in adequate amounts of aflatoxin in grain for separation of genotypes with limited time, expense, and worker exposure to airborne fungal spores. It was our desire to devise an inoculation technique that fit these criteria to be used in the southern United States for evaluating families of the backcross susceptible self generations of a marker population in test crosses at several locations. For inoculation techniques we chose to adapt methods of others who produced inoculum of nontoxigenic *A. flavus* isolates for biological control and techniques used by G.N. Odvody in Texas for classification of resistance. Two inoculation methods using corn kernels coated with *A. flavus* conidia placed either on the soil surface or down the whorl of the plant were evaluated. The ground and whorl inoculation methods require similar time and labor. However, we speculated that placing inoculum in the whorl of the plant may allow conidia of *A. flavus* to be more readily disseminated to the ears of the plants. For inoculum preparation, autoclaved corn was inoculated with conidia of *A. flavus* and incubated in micropore bags (Model: D2X-bag, Northwest Mycological Consultants, Corvallis, OR) for three weeks. Eight kg of the corn with sporulating *A. flavus* was then mixed with 600 ml of cottonseed oil (Archer Daniels Midland Co., Decatur, IL) and conidia suspended in the oil. Four kg of the oil and corn mixture was then added to 20 kg of autoclaved corn in a cement mixer. Then 350 g of diatomaceous earth was added to the mixture to adhere the oil and conidia onto the corn kernels. The final product, corn coated with *A. flavus* conidia, was stored in a cooler for two weeks and then shipped to Texas. Two inoculation methods (ground and whorl) and a control were evaluated in three replicates using 12 hybrids at four locations in Texas. For the ground inoculation, inoculum was placed on the soil surface in the row at a rate of 700g per 25m row. For the whorl inoculation, inoculum was placed down the whorl at a rate of 5g per plant. All inoculations were completed at the V9-V10 growth stage. The four locations in Texas were Moody (central), Ganado (upper gulf), Tynan (lower gulf), and Harlingen (deep south). All locations were furrow irrigated except the upper gulf location that was not irrigated. Over all locations and hybrids aflatoxin levels with whorl inoculation (395ng/g) were significantly higher ($P = 0.05$) than soil surface inoculation (249ng/g) or noninoculated (94ng/g). The whorl inoculation also was the most consistent in identifying more resistant and susceptible hybrids over the four locations. This inoculation is being used in 2002 to evaluate backcross susceptible self families of the cross between the resistant Oh516 and the susceptible B73 in test crosses with LH185 in Ganado and Batesville, TX.

Aflatoxin Concentration In Commercial Corn Hybrids Across Five Louisiana Locations

Steve Moore[1], Hamed Abbas[2], Ken Damann[1], Rick Mascagni[1], Jim Rabb[1], Lester Brown[1], Warner Hall[1], Bobbie Johnson[2], Millie Deloach[1], Brad Guillory[1] and Jose Liscano[1]. [1]LSU Agricultural Center, Baton Rouge, LA; [2]USDA-ARS, Stoneville, MS

Aflatoxin contaminated grain is a major economic concern for Louisiana corn producers and has been widespread at high or moderate levels in two of the past five years. Aflatoxin is produced by the fungus *Aspergillus flavus* and is enhanced when hot and dry conditions exist. The objective of this research was to identify possible resistance in commercial corn hybrids. Fifty-three hybrids in the 2001 Corn Hybrid Performance Trial were tested for aflatoxin in two replications at five locations. Ten ears from each entry at four locations were inoculated with *Aspergillus flavus* spores using a pin-bar cushion after mid-silk. Inoculated ears plus ten additional non-inoculated ears were harvested at maturity at four locations, bulked, shelled, and ground to a fine meal, then sent to the USDA-ARS laboratory at Stoneville, Mississippi where they were analyzed for aflatoxin and fumonisin concentrations. Highest aflatoxin occurred at the Red River Research Station in Bossier City where hot and dry conditions prevailed for much of the growing season. The second highest aflatoxin concentration occurred at Alexandria. Both of these locations appear to be good screening sites for resistance. No location had fumonisin levels of much concern, which is good news for Louisiana producers, since many of the test locations had moderate temperature and moisture conditions. Aflatoxin and fumonisin concentrations were determined for each hybrid. 'Dekalb DKC 68-70' had the lowest overall aflatoxin concentration and ranking among inoculated hybrids and also ranked lowest in a test conducted at Baton Rouge where corn grain was analyzed in the Department of Plant Pathology. Although some hybrids appeared to have superior resistance, another year of testing is needed to verify results from 2001. Sixty-six hybrids were inoculated at five locations in 2002 and analyses are now being conducted to determine aflatoxin and fumonisin concentrations. Following measurement of these toxins, a complete analyses will be made to determine genotype times environmental interactions and resistance levels of individual hybrids.

Comparison Of Aflatoxin Production In Normal- And High-Oleic Backcross Derived Peanut Lines

H.Q. Xue[1], T.G. Isleib[1], G.A. Payne[2], R.F. Wilson[3], W.F. Novitzky[3], and Greg O'Brian[2]. [1]Department of Crop Science, N.C.State Univ., Raleigh, NC; [2]Department of Plant Pathology, N.C. State Univ., Raleigh, NC; [3]USDA-ARS, N.C. State Univ., Raleigh, NC

Linoleic acid has been reported to alter aflatoxin production by *Aspergillus flavus* grown *in vitro*. The effect of the high-oleate (low-linoleate) trait of peanut was tested by comparing normal oleic lines with high-oleic backcross-derived lines. Seeds were blanched, quartered and inoculated with *A. flavus* conidia, placed on moistened filter paper in petri dishes, and incubated for 8 days. In one experiment, dishes were stacked in plastic bags in a Latin square design with bags and positions in stacks as blocking variables. Position effects were pronounced. Background genotype had no significant effect on aflatoxin content. High-oleic lines averaged nearly twice as much aflatoxin as normal lines. Interaction between background genotype and oleate level was not detected. In a second experiment, dishes were arranged on plastic trays enclosed in plastic bags and stacked with PVC spacers between trays. Fungal growth and aflatoxin production were greater than in the first experiment. Background genotype, oleate level, and their interaction were significant. The mean of high-oleic lines was almost twice that of normal lines, but the magnitude of the difference varied with background genotype. Special care should be taken with high-oleic lines to prevent growth of *Aspergillus* spp. and concomitant development of aflatoxin contamination.

Response Of White Food Corn To Aflatoxin Contamination

Javier Betrán, Tom Isakeit, Gary Odvody, Kerry Mayfield, Dennis Transue, Sandeep Bhatnagar, Dan Makumbi, Rosan Ganunga. Texas A&M University

Preharvest aflatoxin (AF) contamination is one of the main limitations for corn production in Texas causing enormous health and economic losses. In the USA, grain with more than 20 ng g^{-1} of aflatoxin B1 is banned from interstate commerce and with more than 300 ng g^{-1}, cannot be used as livestock feed. At present, there are no elite inbreds resistant to AF that can be used directly in commercial food corn hybrids. We have estimated the response to aflatoxin accumulation of white food corn inbreds and hybrids under inoculation. Both white food inbred lines and their hybrids were evaluated under inoculation in three locations in South and Central Texas: College Station (CS), Weslaco (WE), Corpus Christi (CC) during year 2001 and 2002. The material included subtropical, tropical and temperate inbreds. Commercial white food corn hybrids were included as checks. Alpha-lattice design with number of reps from 4 to 9 was used. Drought and heat stress was induced by late planting and limited irrigation. Inoculation was with a conidial suspension of *A. flavus* injected 6-10 days after midsilk by using the nonwounding silk channel inoculation technique at CS and WE during 2001. At CS and WE during 2002 and at CC both years inoculation was done by placing *A. flavus* colonized corn kernels on the soil surface between treatment rows around mid-silk stage. Quantification of AF was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest™). Significant differences for AF among inbreds *per se* were detected in all locations. The average AF was 1341.2 ng g^{-1} (range: 115.3 to 2675.0 ng g^{-1}) at WE during year 2001. The inbreds with the lowest AF contamination were CML176, CML78, and Tx130. At CS during 2002 the average AF accumulation was 79.8 ng g^{-1} (range: 0.0 to 462.7 ng g^{-1}). The inbreds with the lowest AF were CML269, Tx130, and Tx807. At WE during 2002 the average AF was 157.1 ng g^{-1} (range: 0.0 to 1125.0 ng g^{-1}). The inbreds with the lowest AF were Y21, CML373, and CML78. The inbreds with the most consistent lowest AF across evaluations were CML78, Tx130 and CML269. Significant differences among the hybrids were also detected in all locations. Average AF for experiment 2212A involving hybrids among subtropical (CML311, CML78, CML322) and temperate (Tx114, Tx110, Tx130) lines was 101.2 ng g^{-1} at CS in 2001, 659.2 ng g^{-1} at WE in 2001 and 170.9 ng g^{-1} at WE in 2002. The hybrids with the lowest AF across evaluations were Tx110 x CML78, CML269 x Tx110, and CML78 x CML311. Average AF for experiment 21107 was 126.9 ng g^{-1} at CS, 872.7 ng g^{-1} at CC, and 487.9 ng g^{-1} at WE. Hybrid CML269 x CML176 was the least susceptible and most consistent hybrid across evaluations. Commercial checks and Tx114 and Tx110 hybrids were among the most susceptible hybrids. Average AF for experiment 22107 was 192.2 ng g^{-1} at WE and 9.8 ng g^{-1} CS. At WE the hybrids with the lowest AF were CML269 x CML176 and CML78 x CML269. The most promising white food corn inbreds for reducing the risk of AF in Texas growing conditions are CML269, CML176, CML78, Tx130, and Tx807. Most of these inbreds have subtropical or tropical origin and hard endosperm. Their hybrids were more resistant to AF than commercial white food hybrids.

Relationship Between Pod Galling From Root-Knot Nematodes And Aflatoxin Contamination In Peanut

Timper, P.,[1] Wilson, D. M.,[2] and C. C. Holbrook[1]. [1]USDA ARS, Tifton, GA; [2]Department of Plant Pathology, University of Georgia, Tifton GA

A field study was conducted to determine whether aflatoxin levels in peanut, *Arachis hypogaea*, were correlated with pod and root galling caused by the peanut root-knot nematode. The experiment was conducted under a rain- out shelter containing 24 cement plots (each 2 x 2 m) with the following treatments: root-knot nematodes alone (RKN), Aspergillus flavus (Af) alone, RKN + Af, and no nematodes or fungus. Each treatment was replicated six times. Peanut seedlings, either infected with RKN or uninfected, were transplanted into half the plots. Inoculum of Af was sprinkled over the plant canopy at mid bloom. Drought was induced after pod set by covering plots during rain with a fiberglass shelter. Pod- and root-gall indices were determined for all plants in the plot and averaged. Pods from each plot were bulked, shelled, and a subsample of kernels was used to determine aflatoxin concentration and percentage colonized by Af. In plots treated with Af, aflatoxin concentrations were 850 to 1800 ppb and were unaffected by the presence or absence of nematodes. However, in plots without added fungus, aflatoxin concentrations were 2 ppb in plots without nematodes and 1800 ppb in plots with nematodes. There was a correlation between aflatoxin concentration and pod-gall index: with increasing pod galling, there was an increase in aflatoxin concentration ($P=0.001$, $r=0.82$). Colonization of kernels by Af also increased with increasing pod galling ($P=0.04$, $r=0.42$).

Evaluation Of Physiological Measures Of Drought Stress To Indirectly Select For Reduced Aflatoxin Contamination In Peanut.

Weibo Dong[1], C. Corley Holbrook[2,] Baozhu Guo[2], Meng Luo[3,] David M. Wilson[3,] Craig K. Kvien[3], and Diane L. Rowland[4]. [1]UGA; [2]USDA-ARS, Tifton, GA; [3]UGA, Tifton, GA; [4]USDA-ARS, Dawson, GA

Developing and utilizing resistant cultivars is a prospective way to reduce or eliminate preharvest aflatoxin contamination in peanut (Arachis hypogaea). Indirect selection tools would aid in the development of peanut cultivars with resistance to aflatoxin contamination. The objectives of this study were to examine the relationships between visual drought stress rating, SPAD chlorophyll meter reading (SCMR) and aflatoxin contamination in a range of peanut, and to evaluate the possibility of using physiological measures of drought stress to indirectly select for reduced aflatoxin contamination in peanut. Ten peanut genotypes were selected for the experiment conducted in Tifton, GA. Drought and heat stress conditions were imposed by covering the entire test plots with a mobile greenhouse. Significant differences of SCMR and visual drought stress ratings were observed among the genotypes. Five breeding lines showed lower drought stress ratings than the drought tolerant line Tifton8, and three of them had a very low aflatoxin contamination in 2001. Gk7HO/L, which had high aflatoxin contamination in 2001, showed the highest drought stress in this experiment. The results indicated that visual drought stress rating was a steady trait in peanut. No significant correlations between SCMRs and drought stresses were found. We are conducting more research to determine the correlation between SCMR and drought tolerance, and between SCMR and aflatoxin contamination in peanut.

Protection Of Kernels From Infection By *Aspegillus Flavus* Is Decreased In Opaque-2 Mutants Of Maize

Kirsten Nielsen[1], Mariana Kirst, Rebecca S. Boston[1], and Gary A. Payne[2]. Departments of [1]Botany and [2] Plant Pathology, NC State University, Raleigh, NC

The maize inbred W64A and its near isogenic mutants *opaque-2* (*o2)*, *o2-Agroceres (o2-Agro)*, *o2-Italian* (*o2-It)*, and *floury-2* (*fl2)* were screened for resistance to infection by an *Aspergillus flavus* transformant that expresses a green fluorescent protein reporter gene. Kernels homozygous for the *o2* mutant alleles had maize ribosome-inactivating protein 1 proenzyme (proRIP1) levels 50-100-fold lower than normal W64A kernels whereas W64A *fl2* had proRIP1 levels equivalent to those of normal kernels. Kernels from ears inoculated with GFP-*A. flavus* were cultured for evaluation of infection of the endosperm by GFP-*A. flavus*. Kernels with low levels of proRIP1 had an infection rate by GFP-*A. flavus* of almost 50%. In contrast, kernels with high proRIP1 levels had only a 2% infection rate. These results suggest that maize proRIP1 plays a role in protection of the kernel against invasion by *Aspergillus flavus.*

Maize Mapping Populations For Identifying Qtls For Fumonisin Accumulation And Ear Rot Resistance

L. A. Robertson, G. A. Payne, D. G. White, and J. B. Holland. North Carolina State University, Raleigh, NC

GE440 and NC300 were identified in previous studies as potential sources for resistance to fumonisin accumulation and *Fusarium* ear and kernel rot. Two mapping populations, GE440 x FR1064 and NC300 x B104, were created to identify the loci associated with disease resistance and resistance to fumonisin accumulation. Our research is focused on answering two key questions: **(1)** Do some QTLs for ear rot resistance also confer resistance to fumonisin accumulation?, and **(2)** Are QTLs consistent across populations? In 2002, the GE440 x FR1064 population was grown at Mt. Olive, NC and the NC300 x B104 population was grown at Clayton, NC. Populations were replicated twice at each location. Primary ears were inoculated with a mixture of three isolates each of *F. verticillioides* and *F. proliferatum*. Inoculated ears were rated for the percentage of kernels rotted. Replicate plots of parental lines and the five most and five least rotted lines from the GE440 x FR1064 population were selected for toxin analysis. The grain was ground, bulked by plot, and evaluated for fumonisin concentration using HPLC. Our results to date indicate that QTLs for both traits are segregating in both populations. GE440 and FR1064 differed significantly for ear rot, but not for fumonisin concentration. NC300 and B104 did not differ significantly for ear rot, but NC300 did have significantly less fumonisin. Our results indicate that genes controlling Fusarium ear rot and fumonisin accumulation resistance are segregating in both populations. Therefore, we expect to be able to map QTLs for resistance to both the disease and mycotoxin accumulation in these populations.

Identification Of Genes Differentially Expressed During Aflatoxin Biosynthesis In *Aspergillus Flavus* And *Aspergillus Parasiticus* .

G.R. OBrian, A.M. Fakhoury and G.A. Payne. Department of Plant Pathology, North Carolina State University, Raleigh NC

A complex regulatory network that involves both nutritional and developmental signals regulates the production of aflatoxins as well as that of many other fungal secondary metabolites. To gain a better understanding of the genes involved in the regulation and biosynthesis of aflatoxin, we employed DNA arrays to identify a set of genes that are differentially expressed during aflatoxin production and are thus potentially involved in aflatoxin biosynthesis. cDNA clones from a library enriched for transcripts induced during aflatoxin biosynthesis were arrayed on nylon filters. Hybridization of the filter with target cDNA made from transcripts expressed during conducive and non-conducive conditions for aflatoxin production resulted in the identification of over 10000 expressed clones. Quality sequence was obtained for 2200 clones. Further analysis resulted in the identification of 753 unique expressed sequence tags. Many of these ESTs showed sequence similarity to genes known to be involved in metabolic and regulatory pathways; however, no known function could be ascribed to over 50% of the ESTs. The 753 ESTs were arrayed on glass slides and used to identify differentially expressed genes during the temporal induction of aflatoxin biosynthesis. An analysis of three independent experiments identified 26 genes with relatively higher levels of expression during aflatoxin biosynthesis and 18 genes more expressed prior to aflatoxin biosynthesis. No predicted function could be ascribed to 17 of the 26 genes that were more expressed during aflatoxin biosynthesis.

Identification And Characterization Of The Lipoxygenase Gene Family Of Maize

Michael Kolomiets[1], Pedro Navarro[2], Jinglan Zhang[1], Nasser Yalpani[2], Carl Simmons[2], Robert Meeley[2], and Jon Duvick[2]. [1]Department of Plant Pathology and Microbiology, Texas A&M University; [2] Pioneer Hi-Bred Intl., Inc.

Lipoxygenases (LOX) are dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids such as linolenic and linoleic acids. Correlative evidence strongly implicates lipoxygenases and their metabolic products in the resistance mechanisms to diverse plant pathogens and in accumulation of mycotoxins. LOX activity and accumulation of the LOX-derived volatile compounds correlated with aflatoxin resistance (Wright *et al*., 2000). Additionally, LOXs and their products may be involved in the signaling cross talk between *Aspergillus* and the plant hosts. Dr. Keller's group has shown that 9-LOX-derived fatty acid hydroperoxides induce aflatoxin production by *Aspergillus flavus* while 13-fatty acid hydroperoxides had the opposite effect. This finding suggested that unsaturated fatty acids and/or their derivatives may act as signaling molecules in production of mycotoxins such as aflatoxin. In agreement with this hypothesis, it was found that expression of a 9-LOX gene was down-regulated in lines resistant to aflatoxin contamination but was not affected in susceptible lines (Wilson *et al*., 2001).

As the first step in our attempt to clearly define LOX function in interaction of corn plants with *Aspergillus flavus* and *Fusarium verticillioides*, we have undertaken a genomic scale analysis and identification of the entire LOX multigene family of maize by using the Pioneer and DuPont EST collection. Bioinformatics analysis of ESTs derived from more then 130 different cDNA libraries identified 471 LOX ESTs. These ESTs were grouped into 36 clusters, contigs and singletons. Full insert sequencing has been carried out for the longest cDNA clones that represented each of the groups. Because several clones were not full length, we used 5'RACE cloning technique to isolate the remaining sequence. Sequence analysis revealed that maize genome contains at least 11 different LOX genes. This number has been independently confirmed by Southern blotting analysis and by identification of chromosome location of each individual gene by using gene-specific PCR primers and genomic DNA from oat-maize addition lines. Homologs of two of the LOX genes have never been reported in any other plant species and can be grouped into two novel classes of plant LOX genes. RNA profiling and Northern blotting results suggested that many of the LOX genes are up- or down-regulated by pathogens, their elicitors or defense-associated signaling molecules such as JA, ethylene and SA suggesting their involvement in pathogen-induced defense responses. Reverse genetics strategy is underway to identify maize mutants in which function of is interrupted by insertions of *Mutator* transposable elements in their coding sequences. To date, *Mu*-element insertions have been identified in 8 of 11 LOX genes. These mutants will undergo thorough testing for disease resistance and mycotoxin accumulation levels to unambiguously define LOX function in defense responses and interaction between pathogens and the plant host.

Literature Cited

Wilson R.A., Gardner H.W., and Keller N.P. 2001. Cultivar-Dependent Expression of a maize

Lipoxygenase Responsive to Seed Infesting Fungi. Mol Plant-Microbe Interact **8**:980-987. **Wright M.S**., Greene D.M., Zeringue H.J., Bhatnagar D., and Cleveland T.E. 2000. Effects of volatile aldehydes from Aspergillus-resistant varieties of corn on Aspergillus parasiticus growth and aflatoxin accumulation. Toxicon **38**:1215-1223

Use Of GFP-Tagged *Aspergillus Flavus* To Monitor Fungal Growth In Developing Ears Of Resistant And Susceptible Corn Hybrids

Zenaida V. Magbanua[1], W. Paul Williams[2], Gary Windham[2] and Dawn S. Luthe[1]. [1] Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2] USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS

We used a GFP-transformed *Aspergillus flavus* strain to needle inoculate mid-maturation ears and follow the path of fungal invasion in resistant and susceptible hybrids of *Zea mays*. In all ear cross-sections examined, the level of fluorescence was higher in susceptible compared to resistant lines. Our data suggest an extensive penetration of the rachis tissues from 1 to 5 days post-inoculation in susceptible but not resistant lines. At 10 and 20 days post-inoculation, fluorescence levels were low (slightly over background) and did not differ significantly between resistant and susceptible lines. We observed differences in fluorescence expression in the endosperm and embryos of all lines. In susceptible lines, the fungus appeared to preferentially grow from the mid-ear inoculation site to the tip of the ear. There was less fluorescence in sections taken toward the base of the ear. This may indicate that the fungus uses nutrient flow inside the ear to achieve systemic infection. While data analysis is still ongoing, we present a model for *A. flavus* invasion of the ear. The fungus enters through wounding of the ear or by capture of fungal spores by the silk. Both of these cases potentially could lead to penetration of the rachis tissues and eventually to invasion of the rachilla, pith, pericarp, endosperm and embryos as early as 1 to 3 days post-inoculation in susceptible plants. Successful invasion must occur at mid-maturation ear, when all the necessary nutrients for optimum fungal growth are available.

Host Specific Differences In Preharvest Grain Infection By Toxigenic Fungi In Dryland Pearl Millet And Corn

J.P. Wilson,[1] W.W. Hanna,[1] D.M. Wilson,[2] and A.E. Coy.[3] [1]USDA-ARS Crop Genetics & Breeding Res. Unit; [2]Dept. Plant Pathology, [3]Dept. Crop & Soil Sciences, Univ. Georgia, Coastal Plain Expt. Stn., Tifton, GA

Pearl millet is a promising alternative feed grain for the Southern Coastal Plain. Preharvest grain infection by potentially toxigenic fungi in pearl millet and corn were compared in 2000 and 2001. Hybrids were grown in dryland conditions at several planting dates to allow variation in flowering time. Grain was harvested and fungi were isolated in the laboratory. Fungal isolation differed by host species in both years. Across years, mean isolation frequencies of *Aspergillus flavus*, *Fusarium verticillioides*, *F. semitectum*, and *F. chlamydosporum* from corn were 4.3, 62.3, 0.1, and 0.0%, respectively; those from pearl millet were 0.1, 0.3, 55.9, and 23.8%. Isolations of *F. verticillioides* and *F. semitectum* differed by planting date. In 2000, aflatoxins in corn and pearl millet averaged 135.6 and 0.1 ppb, and fumonisins averaged 6.1 and 0.0 ppm, respectively. Differences in preharvest mycotoxins are likely due to host specific differences in preharvest fungal infection of the grain.

Resistance To Aflatoxin Production From The MI82 Corn Line

Laura M. Maupin[1], Michael J. Clements[2], and Donald G. White[3]. [1]Sunburst Plant Disease Clinic, Turlock CA; [2]USDA-ARS CHRRU, Mississippi State, MS; [3]University of Illinois Department of Crop Sciences, Urbana, IL

Our objectives were to determine if the corn (*Zea mays* L) inbred MI82 has alleles for resistance to Aspergillus ear rot (caused by *Aspergillus flavus* Link:Fr) and aflatoxin production in grain that can be transferred to commercially used inbreds, and to determine the types and magnitudes of gene action, heritabilities, and gain from selection for low levels of bright greenish-yellow fluorescence (BGYF), aflatoxin, and ear rot with MI82. Also, we hoped to determine if selection against BGYF would substantially reduce the concentration of aflatoxin in grain. Primary ears and ground grain from inbred MI82 (P_1), the susceptible inbred B73 (P_2), and the F_1, F_2, F_3, BCP_1S_1, and BCP_2S_1 generations developed from these inbreds were evaluated for BGYF, concentration of aflatoxin in grain, and severity of Aspergillus ear rot in 2000 and 2001. Dominance was the most important gene action associated with low levels of BGYF and a low concentration of aflatoxin in grain. Heritabilities for low levels of BGYF (83.5%), aflatoxin (74.1%), and ear rot (62.8%) were high. Correlation coefficients between aflatoxin and BGYF were high in both years (r = 0.75 and 0.79). Unlike aflatoxin, BGYF was not affected by the year in which plants were grown. Selection for low levels of BGYF prior to selection based on the concentration of aflatoxin in grain was more effective than selection for either factor alone. MI82 has value in programs designed to improve the resistance of commercially used corn inbreds.

Evaluation Of The Contribution Of Intact Seed Coats Versus Internal Mechanisms Of Corn Kernel Resistance To Aspergillus Colonization And Aflatoxin Production

D.T. Wicklow[1], K.N. Busboom[2] and D.G. White[2]. [1]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]Dept. of Crop Sciences, University of Illinois, Urbana IL

The objective of this experiment was to evaluate maize resistance to *Aspergillus flavus* infection and aflatoxin contamination in undamaged grain from wound-inoculated ears. Several inbreds and F_1 crosses with demonstrated resistance or susceptibility to aflatoxin, as determined by the pinboard inoculation method, were further evaluated using the >punch-drill & pipe-cleaner= method of wound-inoculation. At harvest, 20 to 24 non-damaged kernels surrounding each wound-site are removed from the ear and examined for bright greenish yellow fluorescent (BGYF) kernels and aflatoxins. The method emphasizes kernel resistance to the parasitic abilities of the fungus and is based upon the occurrence of BGYF kernels and the extent to which these kernels become contaminated with aflatoxin. FR1064 x Mp313E, FR1064 x Oh516 and Oh516 had no BGYF kernels in the undamaged grain near points of wound-inoculation and recorded the lowest aflatoxin levels among all genotypes. However, BGYF kernels with substantial aflatoxin contamination were recovered in the other grain on these ears indicating that the individual kernels were not immune to *A.flavus* or aflatoxin production. The F_1 crosses FR1064 x Mp313E; FR4310 x Mp313E; FR4310 x Mp 420 were shown to have a source of internal grain resistance to *A flavus* and aflatoxin. The resistance to toxin production of these genotypes can be attributed to inbred parents that resist seed coat tearing or provide internal sources of kernel resistance. None of the F_1 crosses we examined combined these resistance phenotypes.

PARTICIPANTS

Last Name	First Name	Affiliation	Phone	Fax	E-mail
Abbas	Hamed	USDA,ARS,CG & PRU	662-686-5313	662-686-5218	habbas@ars.usda,gov
Alexander	Charles	NCGA	252-745-3097	252-745-7484	calexander@costalnet.com
Anguiano	Gloria Laura	CINVESTAV-IPN, Lab.de Micotoxinas	0152 462-623-9600	0152 462 624-5996	ganguian@ira.cinvestav.mx
Antilla	Larry	Arizona Cotton Research & Protection Council	602-438-0059	602-438-0407	lantilla@azcotton.com
Applegate	Cynthia	Office of the TX State Chemist	979-845-1121	979-845-1389	caa@otsc.tamu.edu
Averhoff	Scott	SARA Farms	972 937-8507	972 937-3587	scottaverhoff@hotmail.com
Bacon	Charles	USDA, ARS, Russell Research Center	706-546-3142	706-546-3116	cbacon@saa.ars.usda.gov
Balthrop	Jim	Office of the TX State Chemist	979-845-1121	979-845-1389	James-Balthrop@tamu.edu
Bean	Brent	TX Co-Op Extension	806-677-5600	806-358-9718	b-bean@tamu.edu
Betran	Javier	TX A & M Univ.	979-845-3469	979-862-1931	javier-betran@tamu.edu
Bhatnagar	Deepak	USDA, ARS, SRRC	504-286-4388	504-286-4419	dbhatnag@srrc.ars.usda.gov
Brooks	Tom	USDA, ARS,Corn Host Plant Resistance Research Unit	662-325-7996	662-325-8441	tbrooks@msa-msstate.ars.udsa.gov
Brown	Daren	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6230	309-681-6689	browndw@ncaur.usda.gov
Brown	Robert	USDA, ARS, SRRC	504-286-4359	504-286-4419	rbrown@srrc.ars.usda.gov
Bruns	Arnold	USDA, ARS	662-686-5279	662-686-5218	abruns@ars.usda.gov
Butchko	Robert	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6379	309-681-6689	butchkora@ncaur.usda.gov
Cary	Jeff	USDA, ARS, SRRC	504-286-4269	504-286-4419	jcary@srrc.ars.usda.gov
Casasent	David	Carnegie Mellon	412-268-2464	412-268-6345	casasent@ece.cmu.edu
Chen	Zhi-Yuan	Louisiana St. Univ.	504-286-4343	504-286-4419	zchen@srrc.usda.ars.gov
Chlan	Caryl	UL Lafayette	337-482-5916	337-482-5660	cchlan@louisana.edu
Clements	Michael	USDA,ARS,Corn Host Plant Resistance Research Unit	662-325-8023	662-325-8441	mclements@msa-msstate.ars.usda.gov
Cleveland	Ed	USDA. ARS, SRRC	504-286-4387	504-286-4269	eclevela@srrc.ars.usda.gov
Cotty	Peter	USDA, ARS, SRRC	504-286-4391	504-286-4496	pjcotty@srrc.udsa.gov
Croker	Betsy	NCGA	202-628-7001	202-628-1933	croker@dc.ncga.com
Damann	Bud	LSU Ag Center	225-578-1401	225-578-1415	kdamann@agetr.lsu.edu
Dandekar	Abhaya	Univ. of CA at Davis	530-752-7784	530-752-8502	amadanadekaar@ucdavis.edu
Dixon	Kenya	USDA, ARS	662-686-5313	662-686-5218	
Dombrink-Kurtzman	MaryAnn	USDA, ARS, NCAUR, Mycotoxin Research	309-681-6254	309-681-6689	dombrink@ncaur.usda.gov
Donaldson	Gerald	TX Corn Producers Board	979-282-9592	979-657-1147	donfarm@wcnet.net
Dong	Weibo	U. of GA, USDA, ARS	229-386-3176	229-391-3701	wdong@tifton.cpes.peachnet.edu
Dorner	Joe	Natl. Peanut Research lab	229-995-7408	229-995-7416	jdorner@nprl.usda.gov
Doster	Mark	Univ. of CA.	559-646-6585	559-646-6593	mark@uckac.edu

Last Name	First Name	Affiliation	Phone	Fax	E-mail
Dowd	Patrick	USDA	309-681-6242	309-681-6686	dowdpf@mail.ncaur.usda.gov
Eastwood	DeLyle	USDA, ARS, WRRC	510-559-5955	510-559-5684	delyle@pw.usda.gov
Faga	Betsy	N. American Millers Association	202-484-2200	202-488-7416	bafga@namamillers.org
Galindo-Castro	Ivan	Empresas Polar, Caracas, Venezuela	(58212) 202-3062	(58212) 202-3065	ivan.galindo@empresas-polar.com
Gibson	David	TCPB	806-763-2676	806-762-2674	dgibson@texascorn.org
Glenn	Tony	USDA, ARS, Russell Research Center	706-546-3119	706-546-3116	aaglenn@saa.ars.usda.gov
Gradziel	Tom	Univ. of California	530-752-1575	530-752-8502	tmfradziel@ucdavis.edu
Gray	Gary	Blue Diamond Growers	916-446-8389	916-446-8697	ggray@bdgrowers.com
Guo	Baozhu	USDA	229-387-2334	229-387-2321	bguo@tifton.cpes.peachnet.edu
Guzman De Pena	Doralinda	CINVESTAV-IPN, Lab.de Micotoxinas	0152 462-623-9648	0152 462-624-5596	dguzman@ira.cinvestav.mx
Hajovsky	Bart	B-H Genetics	361-771-2255	361-771-2211	theb.hajovsky@ykc.com
Hammond	Bruce	Monsanto Co.	314-694-8482	314-694-8575	bruce.g.hammond@monsanto.com
Hartman	Ernest	2080 Leissner Rd., Seguin, TX	830-303-6071		
Hawkins	Leigh	USDA, ARS, Corn Host Plant Resistance Research Unit	662-325-2707	662-325-8441	lhawkins@msa-msstate.ars.usda.gov
Henry	Michael	Food & Drug Administration	301-827-0161		mhenry@czmfda.gov
Holbrook	Corley	USDA, ARS	229-386-3176	229-391-3701	holbrook@tifton.cpes.peachnet.edu
Hua	Sylvia	USDA, ARS, Western Regional Research Center	510-559-5905	510-559-5777	ssth@pw.msda.gov
Hurley	Mike	Dried Fruit Export Assn'.	559-233-0604	559-233-9819	mikehurley@dfaofca.com
Isakeit	Thomas	TX A & M Univ.	979-862-1340	979-845-6499	t-isakeit@tamu.edu
Jacobs	Merle	Almond Bd. Of California	209-343-3222	209-550-5494	mjacobs@almondboard.com
Jaime	Ramon	USDA. ARS. SRRC	504-286-4283	504-286-4496	rjaime@srrc.ars.usda.gov
Johnson	Bobbie	USDA, ARS, CG & PRU	662-686-5243	662-686-5218	bjjohnson@ars.usda.gov
Kallus	Teddy	B-H Genetics	361-771-2755		
Keller	Henry Lee	TCPB	830-931-2579	830-538-2368	hmkeller@stic.net
Kelley	Rowena	Miss. St. Univ.	662-325-2739	662-325-8441	rkelley@ars.usda.gov
Kendra	Dave	USDA, ARS, NCAUR, Mycotoxin Research	309-681-6579	309-681-6689	kendrad@ncaur.usda,gov
King	Edgar	ARS, USDA	662-686-5265	662-686-5459	eking@ars.usda.gov
Klein	Bob	CA Pistachio Comm.	559-221-8294		bobK@pistachios.org
Kolomiets	Mike	TX A & M Univ.	979-458-4624	979-845-6483	kolomeits@tamu.edu
Kondo	Tatsuhiko	University of Tokyo	81-3-5841-5133	81-3-5841-8022	aa07029@mail.ecc.u-tokyo.ac.jp
Lee	Dewey	Univ. of Georgia	229-386-3430	229-386-7308	deweylee@uga.edu
Light	Doug L	USDA, ARS, WRRC	510-559-5831	510-559-5777	dlight@pw.usda.gov
Linz	John	Michigan St. Univ.	517-355-8479X126	517-353-8963	jlinz@msu.edu
Luo	Meng	Univ. of GA, USDA	229-387-2377	229-387-2321	mluo@tifton.cccpes.peachnet,edu
Luthe	Dawn	Miss. St. Univ.	662-325-7733	662-325-8664	dsluthe@ra.msstate

Last Name	First Name	Affiliation	Phone	Fax	E-mail
McDaniel	Rusty	Douglass King Seed Co.	361-449-2959		rustymc@the-i.net
Maragos	Chris	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6266	309-681-6689	maragocm@ncaur.usda.gov
Mattila	Anna Maria	Purdue Univ.	765-496-2428	765-496-0363	amattila@purdue.edu
McAlpin	Cesaria	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6248	309-681-6689	mcalpice@ncaur.usda.gov
Michailides	Themis	Univ. of CA.	669-646-6546	559-646-6593	themis@uckac.edu
Mims	Will	SC Corn & Soybean Assn'.			grnfarms@ftc-l.net
Molyneux	Russ	Western Regional Research Center, ARS	510-559-5812	510-559-6129	molyneux@pw.usda.gov
Moore	Steven	LSU	318-473-6524	318-473-6535	smoore@abctr.lsu.edu
Morgan	David	Univ. of CA.	559-646-6562	559-646-6593	morgan@uckac.edu
Muir	Ryann	Univ. of CA, Dept. of Pomplogy	530-752-5325	530-752-8502	rmmuir@ucdavis.edu
Murphy	Emory	Georgia Peanut Commission	229-386-3470	229-386-3501	emory@gapeanuts.com
Nemchenko	Andriy	TX A & M Univ.	979-458-4624	979-845-6483	nemchenko@tamu.edu
Nunley	Jeff	S. TX Cotton & Grain Association	361-575-0631	361-572-0960	jnunley@stcga.org
Obsta	Bill	B-H Genetics	361-771-2755	361-771-2211	
Odvody	Gary	TX Ag. Exp. Station	361-265-9201	361-265-9434	g-odvody@TAMU.edu
Ozias-Akins	Peggy	Univ. of Georgia	229-386-3902	229-386-3356	ozias@tifton.uga.edu
Payne	Gary	NC St. Univ.	919-515-6994	919-513-0024	gary_payne@ncsu.edu
Perkins	Jim	Monsanto Co.	815-758-9524	815-758-3117	jim.perkins@monsanto.com
Plattner	Ron	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6241	309-681-6689	plattnrd@ncaur.usda.gov
Poole	Gavin	Institute for Techology Development	228-689-8176	228-689-8175	g[oole@pvtech.org
Proctor	Bob	USDA, ARS, NCAUR, Mycotoxon Research	309-681-6380	309-681-6689	proctorh@ncaur.usda.gov
Raab	Quinton	B-H Genetics	361-771-2755		
Rains	Glen C.	Univ. of Georgia	229-391-3735	229-386-3958	grains@tifton.uga.edu
Rajasekaran	Kanniah	USDA, ARS, SRRC	504-286-4482	504-286-4217	krajah@srrc.ars.usda.gov
Ramos	David	Walnut Marketing BD.	530-756-0531	530-756-2582	deramos@ucdavis.edu
Richard	John	Romer Labs, Inc.	636-583-8600	636-583-6553	jrichard@romerlabs.com
Riley	Ron	USDA, ARS, Russell Research Center	706-546-3377	706-546-3116	rriley@saa.ars.usda.gov
Robens	Jane	USDA, ARS	301-504-5381	301-504-5467	jfr@ars.usda.gov
Robertson	Leilani	NC State Univ.	919-749-7020		larobert@unity.ncsu.edu
Rocheford	Torbert	Univ. of Ill., Dept. of Crop Sciences	217-333-9643	217-333-9817	trochefo@uiuc.edu
Roitman	James	USDA Western Research Lab	510-559-5784	510-559-5777	jnr@pw.usda.gov
Sacher	Bob	Conagra Foods	949-437-2811	949-437-3074	bsacher@cagpc

Last Name	First Name	Affiliation	Phone	Fax	E-mail
Sakuda	Shohei	University of Tokyo	81-2-5841-5133	81-3-5841-8022	asakuda@mail.ecc.u-tokyo.ac.jp
Sampley	Dottie	USDA, Risk Management Agency, OK City RO	405-879-2735	405-879-2741	dottie.sampley@rm.fcic.usda.gov
Sanchez	Beatriz	Empresas Polar, Caracas, Venezuela	(58212) 202-3062	(58212) 202-3065	beatriz.sanchez@empresas-polar.com
Schatzki	Tom	USDA. ARS, WRRC	510-559-5672	510-559-5777	tom@pw.usda.gov
Schuster	Greta	West Texas A&M Univ.	806-651-2552	806-651-2938	
Simmons	Kay	USDA, ARS, NPS	301-504-5560	301-504-6191	kws@ars.usda.gov
Smyth	Doug	Kraft Foods	973-503-4877	973-503-2968	smythd@nabisco.com
Snyder	Jennifer	Corn Refiners Association	202-331-1634		jsnyder@corn.org
Stubbs	Robert	Office of the TX State Chemist	979-845-1121	979-845-1389	RTS@otsc.tamu.edu
Supak	James	Office of the TX State Chemist	979-845-1121	979-845-1389	j-supak@otsc.tamu.edu
Talley	Justin	West Texas A&M Univ.			
Tsitsigiannis	Dimitrios	Dept. of Plant Pathology, Univ. Wisconsin	608-265-2060	608-263-2626	dimtsi@plantpath.wisc.edu
Valentine	Howard	American Peanut Council	706-579-1755	706-579-1754	pnuttech@alltel.net
Verstuyft	Eugene	B-H Genetics	361-771-2755		
Walker	Scott	Monsanto Co.	812-768-5676	812-768-6586	scott.l.walker@stl.monsanto.com
Weissinger	Arthur	NC State Univ.	919-515-2704	919-515-7959	arthur@unity.ncsu.edu
White	Don	University of Illinois	271-333-1093	217-244-1230	d-white@uinc.edu
Wicklow	Don	NCAUR-ARS-USDA	309-681-6243	309-681-6686	wicklodt@ncaur.usda.gov
Williams	Dean	Douglass King Seed Co.	210-661-4191	210-661-8972	dwilliams@dkseeds.com
Williams	Paul	USDA, ARS, Corn Host Plant Resistance Research Unit	662-325-2735	662-325-8441	wpwilliams@msa-msstate.ars.usda.gov
Wilson	David	Univ. of Georgia	229-386-3368	229-386-7285	dwilson@tifton.uga.edu
Windham	Gary	USDA, ARS, Corn Host Plant Resistance Research Unit	662-325-2594	662-325-8441	glwindham@msa-msstate.ars.usda.gov
Woloshuk	Charles	Purdue Univ.	765-494-3450	765-494-0363	woloshuk@purdue.edu
Worley	Kit	Worley Grain Co.	512-446-3211	512-446-5794	worley@tex1.net
Xu	Wenwei	Texas A & M Univ.	806-746-4015	806-746-6528	wwe-zu@tamu.edu
Yates	Ida	USDA, ARS, Russell Research Center	706-546-3523	706-546-3116	iyates@saa.ars.usda.gov
Youngker	Chuck	AZ Cotton Research & Protection Council	602-438-0059	602-438-0407	
Yu	Juijiang	USDA, ARS, SRRC	504-286-4405	504-286-4419	juiyu@srrc,ars.usda.gov
Zhang	Jinglan	TX A & M	979-458-4624	979-845-6483	zhangjl@tamu.edu

Index of Authors