Proceedings of the 2007 Annual Multi-Crop Aflatoxin/Fumonsin Elimination & Fungal Genomics Workshop

October 22-24, 2007
Atlanta, Georgia

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Table of Contents

8TH ANNUAL FUMONISIN ELIMINATION WORKSHOP

Moderator: Scott Averhoff, Texas Corn Producers Board

PLATFORM PRESENTATIONS

POSTER PRESENTATIONS

20TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 1: Microbial Ecology

Moderator: Phillip Wakelyn, National Cotton Council

PLATFORM PRESENTATIONS

.

AGENDA

7th Annual Fungal Genomics
8th Annual Fumonisin,
20th Annual Aflatoxin Elimination Workshop

October 22-24, 2007

Marriott Perimeter Center Hotel – Atlanta, GA

SUNDAY, OCTOBER 21, 2007

4:00 – 6:00 REGISTRATION / POSTER ASSIGNMENTS

MONDAY, OCTOBER 22, 2007

7:00 – 8:00 CONTINENTAL BREAKFAST

7:00 – 5:00 REGISTRATION / POSTER ASSIGNMENTS

8:05 WELCOME: Howard Valentine, Executive Director, The Peanut Foundation

8:10 INTRODUCTORY REMARKS: Kay Simmons, ARS National Program Leader,
Plant Genetics and Grain Crops

7TH ANNUAL FUNGAL GENOMICS WORKSHOP

MODERATOR:

8:15 **Gene Expression Profiling of *Aspergillus flavus* in Comparison with *A. oryzae* for Identification of Genes Involved in Aflatoxin Biosynthesis and Pathogenesis.**
Jiujiang Yu[1], Gary A. Payne[2], William C. Nierman[3], Masayuki Machida[4], Bruce C. Campbell[5], Deepak Bhatnagar[1], and Thomas E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]North Carolina State University, Department of Plant Pathology, Raleigh, NC; [3]J. Craig Venter Institute, Rockville, MD; [4]National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan; [5]USDA-ARS, Western Regional Research Center, Albany, CA.

8:30 Computational and functional analysis of fungal secondary metabolite biosynthesis clusters. William C. Nierman[1], Nora Khaldi[2], Fayaz Seifuddin1, Jiujiang Yu[3], Gary Payne[4], Li-Jun Ma[5], Natalie Fedorova[1]. [1]J. Craig Venter Institute, Rockville, MD; [2]Trinity College, University of Dublin, Dublin, Ireland; [3]USDA Southern Regional Research Center, New Orleans, LA; [4]North Carolina State University, Raleigh, NC; [5]The Broad Institute of Harvard and MIT, Cambridge, MA.

8:45 Comparative Genome Hybridization Among Multiple Strains of *Aspergillus flavus* and *A. oryzae* Show Evidence of *A. oryzae* Domestication. Gary Payne[1], J. Yu[2], M. Machida[3], D. R. Georgianna[1], D. Bhatnagar[2], T. E. Cleveland[2], William Nierman[4], and R. A. Dean[1]. [1]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]USDA/ARS/SRRC, New Orleans, LA; [3]National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, Japan; [4]J. Craig Venter Institute, Rockville, MD.

9:00 Elucidation of the Functional Genomic Basis of Inhibition of Aflatoxin Biosynthesis by Antioxidants. Jong H. Kim[1], Jiujiang Yu[2], Noreen Mahoney[1], Kathleen L. Chan[1], Russell J. Molyneux[1], John Varga[3], Deepak Bhatnagar[2], Thomas E. Cleveland[2], William C. Nierman[3], and Bruce C. Campbell[1]. [1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]USDA-ARS Southern Regional Research Center, New Orleans, LA; [3]J. Craig Venter Institute, Rockville, MD.

9:15 – 9:35 PANEL DISCUSSION
Panel Chair: Bruce Campbell

9:35 – 10:00 BREAK AND POSTER VIEWING

8TH ANNUAL FUMONISIN ELIMINATION WORKSHOP

MODERATOR:

10:00 Genomic Analysis of *Fusarium verticillioides*. Daren W. Brown, Robert R. A. Butchko, and Robert H. Proctor. USDA-ARS, Mycotoxin Research Unit, NCAUR, Peoria, IL.

10:15 Disruption of Ceramide Biosynthesis and Accumulation of Sphingoid Bases and Sphingoid Base 1-Phosphates: A Mechanism for *Fusarium verticillioides* Effects on Root Development and Maize-Seedling Disease. L. D. Williams[1], A. E. Glenn[2], C. W. Bacon[2], M. A. Smith[3], and R. T. Riley[2,3]. 1Burdock Group Consultants, Vero Beach, FL; 2USDA-ARS, Toxicology and Mycotoxin Research Unit, Athens, GA; College of Agricultural and Environmental Sciences, University of Georgia, Athens, GA.

10:30 Characterization and Complementation of an Apparent *FUM* Gene Cluster Deletion in *Fusarium verticillioides*. Anthony E. Glenn[1], Nicholas C. Zitomer[1], Ronald T. Riley[1], and Robert H. Proctor[2]. [1]USDA-ARS, Toxicology and Mycotoxin Research Unit, Athens, GA; [2]USDA-ARS, Mycotoxin Research Unit, Peoria, IL.

10:45 Chitinase Activity in Fumonisin Resistant and Susceptible Corn Seedlings. Todd Naumann. USDA-ARS, Mycotoxin Research Unit, Peoria, IL.

11:00 – 11:15 PANEL DISCUSSION
Panel Chair: Tony Glenn

20TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 1: Microbial Ecology

MODERATOR:

11:15 Effects of Antagonistic Pseudomonas Strains on *Aspergillus flavus* and *fusarium verticillioides* in Soil. Jeffrey D. Palumbo[1], Teresa L. O'Keeffe[1], and Hamed K. Abbas[2]. [1]USDA-ARS, WRRC, Albany, CA; [2]USDA-ARS, Stoneville, MS.

11:30 Practical Application of *Pichia anomala* WRL-076 and Potential Use of Arbuscular Mycorrhizal Symbiosis in Almond Orchard. Sui Sheng T. Hua. USDA-ARS, Western Regional Research Center, Albany, CA.

11:45 Etiology: A Path to Improved Management of Aflatoxin Contamination. Peter J. Cotty[1], Claudia Probst[2], and Ramon Jaime-Garcia[2]. [1]USDA-ARS, Tuscon, AZ; [2]University of Arizona, Department of Plant Sciences, Tuscon, AZ.

12:00 – 1:00 LUNCH

1:15 The Experimental Use Program for Determining the Efficacy of Afl-Guard for Biocontrol of Aflatoxin in Corn. Joe W. Dorner. USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

1:30 Aflatoxin Control in Pistachios: Biocontrol Using the Atoxigenic Strain AF36, Survival of AF36, and EUP Status. Themis Michailides and Mark Doster. University of California, Davis/Kearney Agricultural Center, Davis, CA.

1:45 Aflatoxin Control in Figs and Almonds: Biocontrol Using the Atoxigenic Strain AF36. M. Doster, D. Felts, H. Eveillard, T. Charbaut, D. Morgan, H. Reu, and T. J. Michailides. University of California, Davis/Kearney Agricultural Center, Davis, CA.

2:00 Dynamics of Mycotoxin Concentrations in Aging Corn Residues in a No-till Mississippi Field. Hamed K. Abbas, C. Accinelli, R. M. Zablotowicz, C. A. Abel, A. H. Bruns, Y. Dong, and T. W. Shier. USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS.

2:15 Application Timing Influences Both Spore Yield of an Aflatoxin Biocontrol Product and Displacement of Aflatoxin-producing Fungi. Ramon Jaime-Garcia[1] and Peter J. Cotty[2]. [1]University of Arizona, Department of Plant Sciences, Tucson, AZ; [2]USDA-ARS, Tucson, AZ.

2:30 – 3:00 PANEL DISCUSSION
Panel Chair: Joe Dorner

3:00 – 3:30 BREAK AND POSTER VIEWING

Session 2: Detection, Extraction, and Analysis of Aflatoxin; Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

MODERATOR:

3:30 Phenolic Natural Products in Tree Nuts: Influence on Aflatoxin Levels in Oxidative- and Drought Stressed *Aspergillus flavus*. Noreen Mahoney, Jong H. Kim, Bruce C. Campbell, and Russell J. Molyneux. USDA-ARS, Western Regional Research Center, Plant Mycotoxin Research Unit, Albany, CA.

3:45 Maize Kernel Inhibitors of Aflatoxin Production in *Aspergillus flavus*. Robert Holmes[1], Rebecca Boston[2], and Gary A. Payne[1]. [1]Department of Plant Biology, North Carolina State University, Raleigh, NC; [2]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

4:00 The Role of the PAL/PAC Signaling Pathway During Transcription of Genes Involved in Differentiation and Sterigmatocystin Synthesis by *Aspergillus nidulans*. Francisco Delgado Virgen, José Ruiz-Herrera, and Doralinda Guzman-de-Peña. Depto. De Biotecnología y Bioquímica, Campus Guanajuato, Irapuato, Gto., Mexico.

4:15 – 4:45 PANEL DISCUSSION
Panel Chair: Russell Molyneux

6:00 – 7:00 RECEPTION

TUESDAY, OCTOBER 23, 2007

7:00 – 8:00 CONTINENTAL BREAKFAST

7:00 – 5:00 REGISTRATION / POSTER ASSIGNMENTS

8:00 Joe Spence
Deputy Administrator
Nutrition, Food Safety & Quality

Session 3. Crop Resistance – Conventional Breeding

MODERATOR:

8:45 **Use of Molecular Markers to Create Commercial Corn Hybrids with Low Aflatoxin.** Don White. University of Illinois, Department of Crop Sciences, Urbana-Champaign, IL.

9:00 **Field Techniques for Evaluation of Aflatoxin Contamination in Cotton.** M. W. Olsen[1] and P. J. Cotty[2]. [1]University of Arizona, Department of Plant Sciences, Tucson, AZ; [2]USDA-ARS, Tucson, AZ.

9:15 **Is Crop Resistance Through Conventional Breeding Relevant to Reducing Aflatoxin Contamination.** C. Corley Holbrook[1], David M. Wilson[2], Patricia Timper[3], Baozhu Guo[3], and Dana G. Sullivan[4]. [1]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [2]University of Georgia (Retired), Tifton, GA; [3]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [4]USDA-ARS, Southeast Watershed Research Laboratory, Tifton, GA.

9:30 **Developing Resistant Maize Inbreds: A Progress Review With Future Projections.** Robert L. Brown[1], Abebe Menkir[2], Ranajit Bandyopadhyay[2], Thomas E. Cleveland[1], and Zhi-Yuan Chen[3]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]International Institute of Tropical Agriculture; [3]Louisiana State University Agricultural Center, Baton Rouge, LA.

9:45 **Breeding Corn Germplasm for Reduced Aflatoxin Contamination.** Kerry Mayfield, Tom Isakeit, Gary Odvody, and William L. Rooney. Texas A&M University, College Station, TX.

10:00 Progress in Breeding Aflatoxin-resistant Corn. Wenwei Xu[1], Gary Odvody[2], and W. Paul Williams[3]. [1]Texas A&M University, Lubbock, TX; [2]Texas A&M University, Corpus Christi, TX.

10:15 – 10:45 BREAK AND POSTER VIEWING

10:45 Generation of Expressed Sequence Tags (ESTs) From Cultivated Peanuts for Gene Discovery and Marker Development. Baozhu Guo[1], Xiaoping Chen[2], Phat Dang[3], Corley Holbrook[4], Albert Culbreath[2], Brian Scully[1], Jiujiang Yu[5], William C. Nierman[6], and Thomas E. Cleveland[5]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]University of Georgia, Department of Plant Pathology, Tifton, GA; [3]USDA-ARS, National Peanut Research Laboratory, Dawson, GA; [4]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [5]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [6]J. Craig Venter Institute, Rockville, MD.

11:00 Development of Corn Inbred Lines with Reduced Preharvest Aflatoxin Contamination and Identification of Genes/Markers for Breeding and Germplasm Evaluation. Baozhu Guo[1], Anton Coy[2], Meng Luo[2], Matt Krakowsky[3], Hamed Abbas[4], and Dewey Lee[2]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]University of Georgia, Department of Crop and Soil Sciences, Tifton, GA; [3]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [4]USDA-ARS, Crop Genetics and Production Research Unit, Stoneville MS 38776.

11:15 Integrating Fungal Pathogen and Insect Vector Resistance for Comprehensive Preharvest Aflatoxin Control in Almond. Thomas M. Gradziel, Abhaya M. Dandekar, and Gale H. McGranahan. University of California, Department of Plant Sciences, Davis, CA.

11:30 Identification of Genes Associated with Resistance for Generation of Gene Specific Markers in Maize. Jeffery R. Wilkinson[1], Rowena Y. Kelly[1], Arun Ankala[1], and W. Paul Williams[2]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

11:45 Corn/Soybean Rotations & Aflatoxin. H. Arnold Bruns, Robert Zablotowicz, and Hamed K. Abbas. USDA-ARS, Stoneville, MS.

12:00 – 1:00 LUNCH

1:15 Impact of Lignin on Resistance to *Aspergillus flavus* Infection in Maize. Lindsay Spangler, Penn State University, University Park, PA.

1:30 – 1:45 PANEL DISCUSSION
Panel Chair: Don White

Session 4: Crop Resistance – Genetic Engineering

MODERATOR:

1:45 Current Status of the Atoxigenic Strain *Aspergillus flavus* AF36 for Controlling Aflatoxin in Arizona Cotton. Larry Antilla[1] and Peter Cotty[2]. [1]Arizona Cotton Research and Protection Council, Phoenix, AZ; [2]USDA-ARS, Tucson, AZ.

2:00 Improvement of Antifungal Peptide Technology for Control of Phytopathogens Including *Aspergillus flavus*. Kanniah Rajasekaran[1], Jeffrey W. Cary[1], Jesse M. Jaynes[2], and Thomas E. Cleveland[1]. [1]USDA-ARS-SRRC, New Orleans, LA; [2]Tuskegee University, Tuskegee, AL.

2:15 Maize Lipoxygenases Govern Production of Conidia and Mycotoxins by *Aspergillus flavus* and *Fusarium verticilliodes*. Michael Kolomiets[1], Xiquan Gao[1], Shawn Christensen[1], Yong-Soon Park[1], Tom Isakeit[1], Javier Betran[2], Kerry Mayfield[2], Won-Bo Shim[1], Jürgen Engelberth[3], Cornelia Göbel[4], Marion Brodhagen[5], Ivo Feussner[4], and Nancy Keller[6]. [1]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX; [2]Department of Soil and Crop Sciences, Corn Breeding and Genetics, Texas A&M University, College Station, TX; [3]Department of Biology, University of Texas, San Antonio, TX; [4]Albrecht-von-Haller-Institute for Plant Sciences, Georg-August University Göttingen, Göttingen, Germany. [5]Biology Department, Western Washington University, Bellingham, WA; [6]Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI.

2:30 Characterization of Stress-related Genes that Could Affect Aflatoxin Contamination. Ye Chu, Paola Faustinelli, Laura Ramos, and Peggy Ozias-Akins. University of Georgia, Tifton, GA.

2:45 Advancement and Testing of Transgenic Peanut with Enhanced Resistance to A. *flavus*. Arthur Weissinger, Xingfen Wang, Minsheng Wu, Thomas Isleib, and H. Thomas Stalker. Department of Crop Science, North Carolina State University, Raleigh, NC.

3:00 – 3:20 PANEL DISCUSSION
Panel Chair: Kanniah Rajasekaran

3:20 – 3:45 BREAK AND POSTER VIEWING

3:45 – 5:00 COMMODITY BREAKOUT SESSIONS

WEDNESDAY, OCTOBER 24, 2007

7:00 – 9:00 CONTINENTAL BREAKFAST

8:00 – 9:00 POSTER VIEWING

8:00 – 9:00 Commodity and Industry Leaders Meeting to discuss the Multi Crop Program

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

MODERATOR:

9:00 **Cost-Effectiveness of Aflatoxin Control in Multiple Crops.** Felicia Wu and Yan Liu. University of Pittsburgh, Graduate School of Public Health, Pittsburgh, PA.

9:15 **Investigations of Novel Control Tactics Against Navel Orangeworm for Management of Aspergillus in Tree Nuts.** Douglas Light, James Baker, John Beck, Gloria Merrill, Jeffrey Palumbo, Teresa O'Keeffe, Noreen Mahoney, and Bruce Campbell. USDA-ARS, Western Regional Research Center, Albany, CA.

9:30 **Getting to the Root of Nematode Involvement in Aflatoxin Contamination of Peanut.** Patricia Timper[1] and Corley Holbrook[2]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

9:45 **Volatile Emission of Mechanically Damaged Almonds: An Old and New Method of Investigation to Determine Potential NOW Attractants.** John J. Beck[1], Glory B. Merrill[1], Wai S. Gee[1], Douglas M. Light[1], James N. Roitman[1], Bradley S. Higbee[2], and James Bettiga[3]. [1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]Paramount Farming Company, Bakersfield, CA; [3]S&J Ranch, Madera, CA.

10:00 – 10:15 BREAK

10:15 Selectable Markers with Potential Activity against Insects; Plus Other Insect-oriented Strategies for Mycotoxin Reduction in Midwest Corn. Patrick F. Dowd[1], Eric T. Johnson[1], and T. Scott Pinkerton[1]. [1]USDA-ARS, NCAUR, Crop Bioprotection Research Unit, Peoria, IL.

10:30 NIR Spectroscopy as a Tool for Optimizing Sorting of White Corn Kernels Contaminated with Mycotoxins. Don Wicklow[1], Tom Pearson[2], and Dan Brabec[2]. [1]USDA-ARS-NCAUR, Peoria, IL; [2]USDA-ARS-ERU, Manhattan, KS.

10:45 Water Conservation and Preharvest Aflatoxin Contamination in Peanut and Corn. Russell C. Nuti[1], Joe Dorner[1], Ron Sorensen[1], Marshall Lamb[1], and Clint Truman[2]. 1USDA-ARS, National Peanut Research Laboratory, Dawson, GA; [2]USDA-ARS, Southeast Watershed Research Laboratory, Tifton, GA.

11:00 Contaminant Distribution in Tree- and Ground Nuts and Other Commodities. Natsuko Toyofuku and Thomas F. Schatzki. USDA-ARS, Western Regional Research Center, Albany, CA.

11:15 The Importance of Aflatoxin to the Ethanol Fuel Industry. Ashli E. Brown[1], W. Paul Williams[2], and Jeffery R. Wilkinson[1]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

11:30 – 11:50 PANEL DISCUSSION
Panel Chair: Patricia Timper

11:50 CLOSING REMARKS

12:00 – 1:00 LUNCH
Box lunch will be provided.

TOURS
CNN
Georgia Aquarium

CLOSING DINNER
Carter Center

POSTER PRESENTATIONS

A. Fungal Genomics, Regulation of Aflatoxin Biosynthesis

A-1 **Genes Differentially Expressed by Aspergillus flavus Strains after Loss of Aflatoxin Production by Serial Transfers.** Perng-Kuang Chang[1], Jeffrey R. Wilkinson[2], Bruce W. Horn[3], Jiujiang Yu[1], Deepak Bhatnagar[1], and Thomas E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [3]USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

A-2 **Evaluation of Aflatoxin Degradation by *Aspergillus flavus*.** Bharath Bolla[1], Ashli E. Brown[1], William E. Holmes[2], Arun Anakala[1], W. Paul Williams[3], and Jeffrey R. Wilkinson[1]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]Mississippi State University, Bio-Analytical Mass Spectrometry Laboratory, Mississippi State, MS; [3]USDA-ARS, Corn Host Plant Resistance Research Laboratory, Mississippi State, MS.

A-3 **SSR Fingerprinting VCG Groups of Aspergilli.** Biing-Ru Wu[1], Hamed K. Abbas[2], Bruce W. Horn[3], W. Paul Williams[4], and Jeffrey R. Wilkinson[1]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]USDA-ARS, Crop Genetic and Production Research Unit, Stoneville, MS; [3]USDA-ARS, National Peanut Research Laboratory, Dawson, GA; [4]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

B. Fumonisin Elimination

B-1 **A Single Extraction Method for the Analysis of Fumonisins, Sphingoid Bases, and Sphingoid Base 1-phosplates in Plant Tissue.** Nicholas C. Zitomer, Anthony E. Glenn, and Ronald T. Riley. USDA-ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens, GA.

B-2 **Native and Introduced Fusarium verticillioides Populations in Ears of Field-grown Plants.** Ida E. Yates[1], Darrell Sparks[2], and Anthony E. Glenn[1]. [1]USDA- ARS, Athens, GA; [2]University of Georgia, Athens, GA.

B-3 ***Fusarium verticillioides* Gene Expression Profiling by Microarray Analysis.** Robert A. E. Butchko, Daren W. Brown, Mark Busman, and Robert H. Proctor. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

B-4 **Incidence of Aflatoxins and Fumonisins in Corn Flour (Maseca), in Guadalajara Mexico.** Waldina P. Reyes Velasquez and Doralinda Guzman-de-Peña. Universidad de Guadalajara, Bioquímica y Biotecnología, Campus Guanajuato, Cinvestav, IPN, Mexico.

B-5 **Dissection of Naphthalene Acetic Acid Activity on Mycotoxicogenic Fungi.** Doralinda Guzman de Peña and Gloria Laura Anguiano Ruvalcava. Depto. Bioquímica y Biotecnología, Campus Guanajuato, Cinvestav, IPN, Mexico.

B-6 **Effect of Nixtamalization With and Without Corn Matrix on the Concentration and Toxicity of Fumonisins in Fusarium verticillioides Culture Material.** Tantiana D. Burns, Maurice E. Snook, Trevor R. Mitchell, Ronald T. Riley, and Kenneth A. Voss. USDA-ARS, Toxicology and Mycotoxin Research Unit, Athens, GA.

C. Aflatoxin – Crop Management and Handling, Insect Control, and Fungal Relationships

C-1 **Effect of *Aspergillus flavus* Inoculum Size, Maize Variety, and Cowpea Intercropping on Aflatoxin Production During Maize Storage.** Ekanao Tedihou[1], Kerstin Hell[1], Bernhard Hau[2], Rabiu Olatinwo[3], and Gerrit Hoogenboom[3]. [1]International Institute of Tropical Agriculture, Cotonou, Benin. [2]Institute of Plant Diseases and Plant Protection, Hannover University, Hannover, Germany; [3]University of Georgia, Biological and Agricultural Engineering, Griffin, GA.

C-2 **Peanut Contamination by *Aspergillus flavus* and Aflatoxin B1 in Granaries in Villages and Markets of Mali, West Africa.** Cecilia M. Tojo Soler[1], Gerrit Hoogenboom[1], Bamory Diarra[2], Farid Waliyar[3], Sibiry Traore[3], and Rabiu Olantiwo[1]. [1]University of Georgia, Department of Biological and Agricultural Engineering, Griffin, GA; [2]IER, Bamako, Mali; [3]ICRISAT, Bamako, Mali.

C-3 **Correlations Between Field Populations of the Root-knot Nematode and Aflatoxin Accumulation and Yield in Corn.** Matthew D. Krakowsky[1] and Richard F. Davis[2]. [1]USDA-ARS, Plant Science Research Unit, Raleigh, NC; [2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA.

C-4 **Spatial Distribution Patterns of Aflatoxin and Ear-Feeding Insect Damage in Pre-Harvest Corn.** Xinzhi Ni. USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

D. Aflatoxin – Crop Resistance – Conventional Breeding

D-1 **Genomic Profiling of Maize Response to FAW to Identify Resistance Markers.** Arun Anakala[1], Rowena Y. Kelley[1], W. Paul Williams[2], Dawn Luthe[3], and Jeffrey R. Wilkinson[1]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [3]Pennsylvania State University, Department of Crop and Soil Sciences, University Park, PA.

D-2 **Generation of Gene Specific Markers Associated with Aflatoxin Resistance in Maize.** J. Erik Mylroie[1], Rowena Y. Kelley[1], Tom D. Brooks[2], Gary L. Windham[2], W. Paul Williams[2], and Jeffrey R. Wilkinson[1]. [1]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

D-3 **SERAT: The Southeast Region Aflatoxin Trial.** Kerry Mayfield[1], Tom Isakeit[1], Wenwei Xu[2], Donald White[3], W. Brien Henry[4], Gary L. Windham[4], W. Paul Williams[4], Baozhu Guo[5], Hamed Abbas[6], Steven Moore[7], Quinton J. Raab[8], Daniel P. Gorman[9], James M. Perkins[10], R. Dewey Lee[11], and Matthew D. Krakowsky[12]. [1]Texas A&M University, College Station, TX; [2]Texas A&M University, Lubbock, TX; [3]University of Illinois, Urbana, IL; [4]USDA-ARS, Corn Plant Host Resistance Research Unit, Mississippi State University, MS; [5]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [6]Crop Genetics and Production Research Unit, Stoneville, MS; [7]Louisiana State University, Alexandria, LA; [8]BH Genetics, Moulton, TX; [9]Pioneer Hi-bred International, Cairo, GA; [10]Monsanto, Waterman, IL; [11]University of Georgia, Tifton, GA; [12]USDA-ARS, Plant Science Research Unit, Raleigh, NC.

D-4 **DNA Markers for Resistance to Post-Harvest Aflatoxin Accumulation in Virginia-type Peanuts (*Arachis hypogaea* L.).** C. E. Rowe, S. R. Milla-Lewis, and T. G. Isleib. North Carolina State University, Crop Science Department, Raleigh, NC.

D-5 **Screening Corn Accessions to Find New Genes with Resistance to Aflatoxin.** Steven H. Moore[1] and Hamed K. Abbas[2]. [1]Dean Lee Research Station, LSU AgCenter, Alexandria, LA; [2]USDA-ARS, Crop Growth and Production Unit, Stoneville, MS.

E. Aflatoxin – Microbial Ecology

E-1 **Effect of Stalk Infection Site on Kernel Infection of Maize by *Aspergillus parasiticus*.** Gary L. Windham and W. Paul Williams. USDA-ARS, Mississippi State, MS.

E-2 **Differential Expression of the Aflatoxin Biosynthesis Cluster Genes in Relation to the Quantity of Aflatoxin Production by Field Isolates of Aspergillus flavus.** Sui-Sheng T. Hua[1], Henry Shih[2], Siov Bouy Sarreal[2], and Perng-Kuang Chang[2]. [1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

E-3 **Control of Fungal Pathogens in an Organic Production System with a *Pichia anomala* Yeast.** D. E. Parfitt[1], S-S. T. Hua[2], W. Gee[2], S. B. Sarreal[2], A. A. Almehdi[1], H. Chan[1], M. Braga[3], T. Martin-Duval[4], and B. A. Holtz[4]. [1]University of California, Department of Plant Sciences, Davis, CA; [2]USDA-ARS, Western Regional Research Center, Albany, CA; [3]USDA-ARS, Madera, CA; [4]University of California Cooperative Extension, Madera, CA.

E-4 **Specificity of Eupenicillium and Penicillium species for the conidial heads of *Aspergillus* section *Flavi* and *Nigri*.** B. W. Horn[1] and S. W. Peterson[2]. [1]USDA- ARS, National Peanut Research Laboratory, Dawson, GA; [2]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

E-5 ***Aspergillus flavus*/*A. parasiticus* Levels in Almond Orchards and on Navel Orangeworm (A Major Pest of Almonds and Pistachios).** T. J. Michailides, J. Siegel, M. A. Doster, D. Felts, D. P. Morgan, H. Eveillard, T. Charbaut, L. Boeckler, and H. Reyes. University of California, Davis/Kearney Agricultural Center, Davis, CA.

E-6 ***Aspergillus flavus* Aflatoxin Occurrence and Expression of Aflatoxin Biosynthesis Genes in Soil.** Cesare Accinelli[1], Hamed K. Abbas[2], Robert M. Zablotowicz[3], and Jeffrey R. Wilkinson[4]. [1]Department of Agro-Environmental Science and Technology, University of Bologna, Bologna, Italy; [2]USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS; [3]USDA-ARS, Southern Weed Science Research Unit; [4]Mississippi State University, Department of Biochemistry and Molecular Biology, Mississippi State, MS.

E-7 **Characterization of Species of the *Aspergillus* Section Nigri Isolated from Field Corn Co-infected with *Aspergillus flavus*/*parasiticus* and the Potential for Ochratoxin A Production.** Edwin R. Palencia[1], Maran Klich[2], and Charles W. Bacon[3]. [1]The University of Georgia, Athens, GA; 2USDA-ARS, Southern Regional Research Center, New Orleans, LA; [3]USDA-ARS, Russell Research Center, Athens, GA.

F. Aflatoxin – Detection, Extraction, and Analysis of Aflatoxins; Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

F-1 **Versicolorin A, a Valuable Predictor of Aflatoxins Pollution.** Liu Da-Ling, Yao Dong-sheng, and Wong Hang Heng. Institute of Microbial Biotechnology, Ji-Nan University, China.

F-2 **Enzyme Biosensor to Detect Aflatoxin B1 and Sterigmytocystin.** Liu Da-Ling, Yao Dong-sheng, and Wong Hang Heng. Institute of Microbial Biotechnology, Ji-Nan University, China.

F-3 **An Analysis of the Relationship Between Aflatoxin Concentration and Hyperspectral Fluorescence Response in Maize.** Zuzana Hruska and Haibo Yao. Stennis Space Center, Mississippi.

F-4 **Determination of Aflatoxins in Raw Grain and Seeds at PPT Levels.** Victor Sobolev. USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

G. Crop Resistance – Genetic Engineering

G-1 **RNAi Silencing of the 14 kDa Trypsin Inhibitor Protein in Maize and Its Effect on Host Resistance to *Aspergillus flavus* Infection and Aflatoxin Production.** Zhi-Yuan Chen[1], Robert L. Brown[2], Thomas E. Cleveland[2], and Kennith E. Damann[1]. [1]Louisiana State University Agricultural Center, Department of Plant Pathology and Crop Physiology, Baton Rouge, LA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

G-2 **Aflatoxin and Insect Response of Conventional Non-Bt, MON 810 (Cry1Ab), and MON 89034 (Cry1A.105 & Cry2Ab2) Corn Hybrids in South Texas.** Gary N. Odvody and Charles F. Chilcutt. Texas Agricultural Experiment Station, Texas A&M University System, Corpus Christi, TX.

7th ANNUAL GENOMICS WORKSHOP

Moderator: Donald Chase, Georgia Peanut Commission

Gene Expression Profiling of *Aspergillus flavus* in Comparison with *A. oryzae* for Identification of Genes Involved in Aflatoxin Biosynthesis and Pathogenesis

Jiujiang Yu[1], Gary A. Payne[2], William C. Nierman[3,4], Masayuki Machida[5], Bruce C. Campbell[6], Deepak Bhatnagar[1] and Thomas E. Cleveland[1]

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Aflatoxins, the toxic natural compounds and the most potent carcinogens, are produced by *Aspergillus flavus*. The fungus is also an opportunistic pathogen that infects plants, animals and humans. The primary objectives of our *A. flavus* genomics program are to reduce and eliminate aflatoxin contamination in food and feed and to control fungal infection in preharvest crops such as corn, cotton, peanut and tree nuts. *A. flavus* is a saprobe having the ability to survive in the natural environment by extracting nutrition from plant debris and dead insects. The nature of its survivability could be related to the mechanisms of fungal pathogenicity. The *A. flavus* EST project and whole genome sequencing projects for this fungus have been completed. Three different types of *A. flavus* microarrays have been constructed and used in gene profiling and functional genomics studies. Genes that are potentially involved in aflatoxin formation and fungal infection have been identified. Data mining of the *A. flavus* genome demonstrated that *A. flavus* possesses a whole array of genes encoding enzymes that can breakdown organic materials. Gene expression profiling using microarray in comparison with the non-toxigenic and non-pathogenic *A. oryzae* strain demonstrated that genes for the degrading enzymes and for spore surface proteins are preferentially expressed in *A. flavus* but not in *A. oryzae*. These fungal degrading enzymes and spore surface proteins could play important roles for its pathogenicity. Better understanding the functions of these enzymes can help devise strategies in control fungal infection of agricultural crops. These properties can also be explored to create a highly efficient bio-degrader for biofuel production.

Computational and Functional Analysis of Fungal Secondary Metabolite Biosynthesis Clusters

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Fungi produce an impressive array of secondary metabolites including mycotoxins, pigments, antibiotics and other biologically active small molecules of great medical, industrial and agricultural importance. Genes responsible for secondary metabolite (SM) biosynthesis, export, and transcriptional regulation are often found in well-defined, contiguous regions or clusters. Up to now most fungal SM genes are misannotated or simply unannotated in public databases. We have developed a web-based SMURF software (Secondary Metabolite Unknown Regions Finder) that allow for easy identification of SM genes and pathways in fungal genomes. The predictions are based on PFAM and TIGRFAM domain content as well as on a gene's chromosomal position with respect to NRPSs, PKSs or DMATs genes, which often encode the first step in these pathways. We have applied SMURF to catalogue putative SM clusters in 25 publicly available fungal genomes. The analysis has revealed the striking variability of fungal SM pathways at both species and strain levels. It also confirmed the correlation between unicellularity and the absence of SMs and the prominent role of duplication in the creation of new pathways. With over 200 more fungal genomes projects currently underway, the SMURF software can be used for functional annotation and as a research tool to study the evolution and dynamics of fungal SM pathways.

Comparative Genome Hybridization among Multiple Strains of *Aspergillus flavus* and *A. oryzae* Shows Evidence of *A. oryzae* Domestication.

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Whole genome sequences are now available for two closely related fungi that have very different ecological niches. *Aspergillus flavus* is opportunistic plant and animal pathogen that produces the potent carcinogen, aflatoxin. In contrast, *A. oryzae* is used extensively in the food industry and thus is regarded as safe. Controversy has surrounded the ecological relationship between these two species for several years, but the prevailing hypothesis is that *A. oryzae* is actually a domesticated *A. flavus*. The new genomic data on these species along with an available DNA Affymetrix GeneChip microarray for *A. flavus* allowed us to more closely examine this relationship. DNA sequence comparison between the genomes of the two sequenced strains showed 265 and 244 unique genes to *A. oryzae* and *A. flavus*, respectively. To determine if these differences were reflected within populations of *A. flavus* and *A. oryzae*, DNA from three strains of each species was hybridized to the *A. flavus* microarray, which also contains elements representing the 265 unique *A. oryzae* genes. This comparative genome analysis of the six strains revealed only 129 unique *A. oryzae* genes and 43 unique *A. flavus* genes. An analysis of gene polymorphism between the six strains of *A. flavus* and *A. oryzae* revealed 1076 probes representing potential species-specific polymorphic sites. Verification of these predicted polymorphisms by genome comparison confirmed that 1022 sites are polymorphic between the two sequenced strains. Our data show that while these two fungal species are highly similar, they can be distinguished from each other by species-specific features. Further, these data support the contention that *A. oryzae* is a domesticated form or *A. flavus*. When we elucidate the specific mechanisms for the domestication of *A. oryzae* we may be able to use biotechnological tools to convert toxigenic *A. flavus* strains to non-producers.

Elucidation of the Functional Genomic Basis of Inhibition of Aflatoxin Biosynthesis by Antioxidants

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Chemogenomic analyses reveal how targeting cellular stress-response systems can chemosensitize fungi to prevent mycotoxin production or breakdown resistance to antifungal agents. Thus, chemosensitization can be useful for controlling fungi of agricultural and medical importance. Cultures of *Aspergillus flavus* treated with certain phenolic antioxidants grow normally but produce no aflatoxin. Alternatively, when treated with an oxidative stress agent, such as the organic peroxide *tert*-butylhydroperoxide, *A. flavus* produces twice the aflatoxin levels compared to untreated cultures. Hence, it appears that pathways involving responses to oxidative stress play a role in signaling mechanisms that modulate aflatoxin biosynthesis.

Microarray analyses of *A. flavus* treated with an antioxidant, caffeic acid, showed that all genes in the aflatoxin biosynthetic gene cluster were down-regulated. This down-regulation corresponded with a lack of production, or only very small amounts, of aflatoxin, by caffeic acid-treated cultures compared to untreated cultures. Alternatively, those genes involved in normal metabolic activities, such as amino acid biosynthesis, were expressed at normal levels, or were slightly up-regulated. The responses of these metabolic genes were concordant with the fact that the caffeic acid-treated fungi grew at normal levels. In view that all genes in the aflatoxin gene-cluster were down-regulated, in the caffeic acid treated fungi, genes in the signal transduction pathway regulating aflatoxin biosynthesis, upstream of this cluster, were precluded from signaling aflatoxin biosynthesis.

As a chemogenomic agent, caffeic acid was able to "surgically" confine the functional genomic response of *A. flavus* to just a few, readily definable genes in the microarray analysis. The most noteworthy of the up-regulated genes in this analysis were three genes corresponding to orthologs of the alkyl hydroperoxide reductase gene, *AHP1*, of *Saccharomyces cerevisiae*. The *AHP1* gene is involved in the detoxification of peroxides. Hence, regulation of aflatoxin biosynthesis appears to be connected to the oxidative stress-response system of the fungus.

Moreover, we found that targeting genes involved in oxidative stress responses in fungi can enhance the activities of conventional antifungal agents. Many of these antifungal agents, themselves, target either oxidative or osmotic stress/cell wall-membrane integrity response systems. For example, fungal resistance to fludioxonil by strains of *A. fumigatus*, the primary etiological agent of aspergillosis, was completely reversed by 2,3-dihydroxybenzaldehyde or 2,3-dihydroxybenzoic acid. These compounds targeted mitochondrial superoxide dismutase (*SOD2*) or glutathione reductase (*GLR1*) gene (or gene products), respectively. Moreover, resistant strains of pathogenic yeasts, *Candida* species and *Cryptococcus neoformans*, were rendered susceptible to antibiotics, such as amphotericin B, fluconazole or itraconazole, by phenolic compounds. These compounds, such as thymol, targeted cell wall integrity pathways. Both the oxidative stress and cell wall integrity responses share regulation through the HOG signaling pathway; perhaps an "Achilles' heel" for fungal control.

PANEL DISCUSSION: 7TH ANNUAL FUNGAL GENOMICS WORKSHOP

Panel Chair: Bruce Campbell

There were four presentations made in this session. Despite what might appear as a low number, these presentations exemplified tremendous progress in our understanding of the functional genomics of aflatoxin biosynthesis. This progress has provided fundamental tools that can be used to prevent aflatoxin biosynthesis and, thus, contamination of agricultural products. The scientists who have worked in this area are keenly aware of the needs of the industry for the development of methods to stop aflatoxin contamination. At times, molecular biological and genomic approaches may be perceived as too "adventurous" in nature, and not able to provide "practical" solutions to the aflatoxin problem. The four presentations in this session profoundly showed that the investment that has been made in the functional genomics approach has provided really practical tools that can be used to reduce or eliminate aflatoxin contamination. Aflatoxin contamination of crop plants has been a problem for a long time. If elimination of aflatoxin contamination were easy, the problem would have been solved before the idea of using genomic approaches came along. Well, the problem was not solved, and still is not solved. But, armed with the latest knowledge from the genomic researchers, we are a lot closer to solving this pernicious problem

The first presentation by Jiujiang Yu, USDA-ARS, Southern Regional Research Center, provided a strong overview of where we now stand with regard to the genome of *Aspergillus flavus* and the genomic analytical tools that are now available. These tools, mainly microarrays, provide the opportunity to gain insights in to how and why aflatoxins are produced. Three different types of *A. flavus* microarrays are now available. By using comparative genomics between toxigenic and non-toxigenic species of *Aspergillus* a number of genes required for fungal infection and aflatoxin biosynthesis have been identified. Using this approach, a number of genes encoding cell wall degrading enzymes in *A. flavus* were identified. Such enzymes are needed for the fungus to become infective. In fact, the genes for these enzymes show a higher capacity for expression in *A. flavus* over that of a non-pathogenic *A. oryzae*. A better understanding of how these genes function could play a role in preventing fungal infection. Moreover, they may have some promise for use in biodegradation, and production of bio-fuels.

The second presentation, by Bill Nierman of the J. Craig Venter Institute (JCVI), provided an overview of computer-based data bases that are now available to researchers in the functional genomics of mycotoxin production. Fungi not only synthesize toxins. They are also well known sources of many other types of secondary metabolites of industrial or medical importance, such as drugs (*e.g.*, the statins), antibiotics (*e.g.*, streptomycin, echinocandins, *etc.*) and a variety of pigments. This talk showed how the genes which manufacture these substances, along with the genes that regulate and transport these products, are found in clusters. Until recently, these genes were not annotated or were misidentified in publicly available databases. JCVI has developed web-based software dubbed "Secondary Metabolite Unknown Regions Finder" (SMURF) that can be used to ferret-out the genes and pathways involved in secondary metabolite production in fungal genomes. SMURF has been used to identify these genes and pathways in 25 different fungal genomes, to date. SMURF software is a very important tool that is now available to better understand the functional genomics of secondary metabolite production in fungi.

The third presentation was made by Gary Payne, North Carolina State University. Gary has been one of more, if not the most, instrumental person in fostering the genomic sequencing of *A. flavus*. In his presentation, he showed how the use of currently available genomic sequences of two closely related fungi, having differing ecological characteristics, can be used to understand fungal evolution. He compared the genomes of the toxin-producing, pathogenic *A. flavus* with that of *A. oryzae*, used in the food industry. Based on available sequence and microarray data, he showed these fungi have over 200 genes, each, unique to their respective genomes. However, upon hybridization of DNAs from various strains of each fungal species, he later found far fewer genes were unique to *A. oryzae* and *A. flavus*, respectively. He was also able to identify over 1000 polymorphic sites between the two taxa that were species-specific. In conclusion, it appears that the domesticated, non-toxigenic *A. oryzae* is a descendent of *A. flavus*. Thus, it may be possible to develop atoxigenesis in *A. flavus* along the same lines as occurred during the domestication of *A. oryzae*.

The final talk of the session was made by Bruce Campbell, USDA-ARS, Western Regional Research Center. This presentation provided an overview of the discovery of natural compounds in plants that prevent aflatoxin biosynthesis. These compounds, are known to be antioxidants. Contrastingly, treating *A. flavus* with an oxidative stressor, such as peroxide, drastically increases the biosynthesis of aflatoxin. Based on these observations, it appears that pathways involving oxidative stress response modulate aflatoxin biosynthesis. Caffeic acid, a natural antioxidant, was used as a "chemogenomic" probe to identify genes that may control aflatoxin biosynthesis. This was done using microarray analysis. It was found that all genes in the aflatoxin biosynthetic gene cluster were down-regulated in caffeic acid treated *A. flavus*. Based on this, it was concluded that genes in a yet unknown signal transduction pathway for aflatoxin biosynthesis, were precluded from signaling aflatoxin biosynthesis. The most noteworthy of the up-regulated genes in this analysis were orthologs peroxiredoxin genes of *Saccharomyces cerevisiae*. These peroxiredoxins are involved in the detoxification of peroxides but may also play a role in the signal transduction pathway for aflatoxin biosynthesis. In any case, either by expressing peroxiredoxins or eliminating organic peroxides with antioxidants, in either the fungus or in the host plant, should prevent aflatoxin biosynthesis, and thus contamination.

There were only a few questions asked of the presenters, during the panel discussion. One question concerned the presence of rodlet or hydrophobin proteins in spore surfaces that may be required for infectivity. It is believed that such proteins in the human pathogen, *A. fumigatus*, may be involved in infectivity. Thus, these proteins may also be involved in plant pathogenesis, as well, such has been reported for *Magnopartha*. There was another question on what role secondary metabolites of fungi may play in environmental competitiveness. The response was that, to date, there is no clear evidence that these compounds provide a direct competitive advantage against other competing organisms, or can be used for direct pathogenic purposes. For example, many insects that have a long evolutionary history of direct contact (ingestion) with fungi have the ability to detoxify or excrete mycotoxins, in most cases. The possibility that some of these compounds may be used as "sinks" for reactive oxygen species (ROS), produced as a host protective response to fungal infection, or during drought, was offered. This was in view that some secondary metabolites, such as aflatoxin, are metabolized under conditions of low oxidative stress.

Genes Differentially Expressed by *Aspergillus flavus* Strains after Loss of Aflatoxin Production by Serial Transfers

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Aflatoxins are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species. To better understand the molecular events that are associated with aflatoxin production, three separate nonaflatoxigenic *A. flavus* strains were produced through serial transfers of aflatoxigenic parental strains. The three independent aflatoxigenic/nonaflatoxigenic pairs were compared via transcription profiling by DNA microarray analyses. Cross comparisons identified twenty-two features in common between the aflatoxigenic/nonaflatoxigenic pairs. Physical mapping of the twenty-two features using the *A. oryzae* whole genome sequence for reference identified sixteen unique genes. Aflatoxin biosynthetic and regulatory gene expression levels were not significantly different between the aflatoxigenic/nonaflatoxigenic pairs, which suggests that the inability to produce aflatoxins is not due to decreased expression of known aflatoxin biosynthetic or regulatory genes. Of the sixteen in common genes, only one gene (*hcc*), homologous to glutathione S-transferase genes, showed higher expression in the nonaflatoxigenic progeny relative to the parental strains. The *hcc* gene was overexpressed in an aflatoxigenic *A. flavus* strain. No discernible changes in culture morphology or aflatoxin production were observed between the control and the *hcc* overexpression transformants. Possible roles of *hcc* and other identified genes are discussed in relation to regulation of aflatoxin biosynthesis.

Evaluation of Aflatoxin Degradation by *Aspergillus flavus*

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Aflatoxins are highly toxic and hepatocarcinogenic compounds produced by *Aspergillus flavus* and *A. parasiticus* during infection of corn (maize), peanuts, cotton seed, and tree nuts (figure 1).To minimize exposure to aflatoxins the U.S. Food and Drug Administration enforces a 20 ppb limit of aflatoxin content in foodstuffs. Aflatoxin contamination becomes increasingly problematic when corn is used as a feedstock for ethanol production. Although aflatoxin has not been reported in ethanol produced from contaminated products, the distiller's grains produced during fermentation show a marked increase in aflatoxin levels, typically 3-4 times the initial value. Distiller's grains are key components in many animal feeds and generate a large portion of revenue for distilleries. Though ethanol plants can utilize aflatoxin-contaminated corn to generate ethanol, the FDA limitations prevent utilizing the distiller's grains as animal feed products thus making the whole process uneconomic. Several chemical methods have been shown to be effective in removing aflatoxin; however, they are not approved by the FDA and are too expensive to be feasible on an industrial level. Thus, we are in the initial stages of characterizing aflatoxin degradation by both toxic and nontoxic *Aspergillus* species. The degradation of exogenously introduced aflatoxin has been confirmed by Thin Layer Chromatography (TLC) and LC-MS/MS and will serve as the basis for identification of genes and enzymes involved in this unique phenomenon. Identifying the factors responsible for this process will allow for the removal of aflatoxins from contaminated substrates by co-inoculation or expression of these factors in either cell free extracts and/or modified yeast for use in kernel and cob ethanol production; therefore, increasing the economic feasibility of ethanol as an alternative fuel as well as providing an additional market for a previously unutilized resource.

SSR Fingerprinting VCG Groups of Aspergilli

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The common method for grouping fungi species is utilizing selected probes and DNA hybridization. The fingerprinting analysis is based on the presence and absence of fragment at specific positions. Banding patterns of diverse strains were compared to each other and determine the similarity and dissimilarity. This technique is accurate but time consuming, in particular for a lot of samples. The new method is based on the same concept but analysis by the Tm of banding patterns. Monitoring of the Tm is held throughout PCR reaction, it is not necessary to complete a gel or DNA hybridization. If different fungi produce same banding patterns, they have same Tm values. Roche LightCycler® 480 Real-time PCR can hold samples up to 96 samples in a single real-time performance. These new method based on the common DNA fingerprinting is excellent and good for analysis of vast fungi samples.

8th ANNUAL FUMONISIN ELIMINATION WORKSHOP

Moderator: Scott Averhoff, Texas Corn Producers Board

Fusarium Genomics: Polyketide Synthases and mRNA Alternative Splicing

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Genomic technologies have greatly expanded the pool of fungal-related data in recent years. A significant challenge to researchers examining plant pathogenesis and mycotoxin biosynthesis is to efficiently mine this data to identify structural and regulatory genes and general regulatory mechanisms involved in these processes. We reasoned that a comparison of all of the polyketide synthases (PKSs) present in four *Fusarium* genomes (*F. verticillioides*, *F. graminearum*, *F. oxysporum* sequenced by the Broad Institute and *F. solani* sequenced by the Joint Genome Institute) may identify specific PKSs required for pathogen growth and/or for primary metabolism. BLAST analysis led to the identification of 58 PKSs, many of which have been examined previously to some degree. Phylogenetic analysis of the PKSs and analysis of predicted domains identified will be discussed. A number of years ago, we examined over 720 *F. verticillioides* fumonisin gene expressed sequence tags (ESTs) and found that eight of 17 *FUM* genes had mRNA species that differed in the pattern of processed introns. Based on the number of alternative splice events and their accumulation late in culture, we suggested that the AS forms play a role in fumonisin synthesis. In order to better understand alternative splicing (AS) in *F. verticillioides*, we searched the entire EST data base (87,000 ESTs) and identified AS events in 354 genes which correspond to approximately 4% of the genes in the database. Of the AS events in coding regions, 261 (76%) consist of retained intron, 22% have alternative spliced borders, and 2% have an exon(s) excised. This is significantly different than observed in humans or plants where intron retention events are around 10% and 56% respectively while exon skip events are around 58% and 8% respectively. A significant portion of the *F. verticillioides* AS transcripts could generate proteins with an altered amino acid sequence leading to multiple possible functional proteins from the same gene.

Disruption of Ceramide Biosynthesis and Accumulation of Sphingoid Bases and Sphingoid Base 1-Phosphates: A Mechanism for *Fusarium verticillioides* Effects in Maize-Seedling Disease

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In sweet corn at the seedling and seed maturation stages, *Fusarium* can be a serious field problem. The fungus *Fusarium verticillioides* infects maize and produces fumonisins, inhibitors of ceramide synthase. To determine the role of fumonisins in maize seedling disease, seeds were inoculated with fumonisin producing or non-producing strains of *F. verticillioide*s. Silver Queen (SQ) seedlings grown from seeds inoculated with the producing strains had detectable fumonisins in roots and soils, and sphingoid bases and sphingoid base 1-phosphates were elevated in roots. Leaf lesions and abnormal leaf developmental were only observed with producing strains and while non-producing strains of *F. verticillioides* caused reduced root and stalk growth the effect on growth was greatest with producing strains. Leaf lesion incidence and severity of effects on root and stalk growth were significantly correlated with fumonisin in roots and the extent of disruption of sphingolipid metabolism in roots. In a subsequent study with the fumonisin producing strain MRC826, seedlings grown from inoculated seeds were harvested on days 7, 14 and 21. Reduced growth of aerial plant parts and roots were seen in seedling as early as day 7, however, leaf lesions were not apparent until day 14. Fumonisins were detected in the roots of seedlings and was maximal on day 14. There were significant increases in free sphingoid bases on day 7 which were maximal on day 14. There were also significant increases in free sphingoid base 1-phosphates on day 7. However, the level of free sphinganine 1-phosphate (Sa 1-P) decreased significantly after day 7. These results suggest a time-dependent adaptive response to disrupted sphingolipid metabolism after prolonged fumonisin exposure. FB1 was also detected in leaf tissues grown from inoculated seeds. Surprisingly, FB1 was preferentially accumulated in leaves of SQ and W23 over FB2 and FB3. The levels of accumulation of FB1 in the 1st leaf paralleled the known susceptibility of the two lines to *F. verticillioides* induced leaf lesions and the levels of FB1 in all leafs of the susceptible sweet maize SQ variety were significantly ($p<0.001$) greater than in the resistant dent maize variety W23. Disruption of sphingolipid metabolism as measured by accumulation of phytosphingosine (Pso), sphinganine (Sa), Pso 1-phosphate (Pso 1-P), and Sa 1-P, was easily demonstrated in the SQ line. For all leaves, the MRC826 treated plants had significantly elevated sphingoid bases and their 1-phosphates, and the levels were not significantly different between leaves. This was not the case in the W23 line where significant increases occurred only in the 2nd leaf and 3rd leaf. In all cases the elevations in W23 were significantly less than the elevations observed in SQ. The results support the hypothesis that fumonisin is necessary and sufficient to produce the full spectrum of symptoms indicative of *F. verticillioides*-induced maize seedling disease in SQ and that the mechanism of action involves both the ability to accumulate FB1 and its disruption of sphingolipid metabolism.

Characterization and Complementation of an Apparent *FUM* Gene Cluster Deletion in *Fusarium verticillioides*

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The filamentous ascomycete *Fusarium verticillioides* is a worldwide pathogen of maize and produces the fumonisin mycotoxins. Contamination of maize kernels with fumonisin B_1 (FB1) is of significant concern because of its causal role in equine leukoencephalomalacia, porcine pulmonary edema, liver and renal carcinogenicity in laboratory rodents, and possible human carcinogenicity and neural tube birth defects. Fumonisins B_2, B_3, and B_4 (FB2, FB3, and FB4) also occur in maize but usually at concentrations lower than FB1. Most maize isolates of *F. verticillioides* produce the full complement of fumonisins. However a distinct population of *F. verticillioides* is pathogenic on banana and does not produce fumonisins. Fumonisin-producing strains from maize cause leaf lesions, developmental abnormalities, stunting, and sometimes death of maize seedlings (e.g., 'Silver Queen' hybrid), whereas fumonisin-nonproducing banana strains do not. Seedlings of Silver Queen inoculated with banana strains of *F. verticillioides* generally appear equivalent to uninoculated control seedlings. Production of fumonisins by *F. verticillioides* is dependent on a biosynthetic gene (*FUM*) cluster of 16 contiguous and co-expressed genes encompassing ~45.7 kb on chromosome 1. A Southern analysis of banana strains did not detect genes in the *FUM* cluster but did detect genes flanking the cluster. Nucleotide sequence analysis of the genomic region carrying the flanking genes revealed that the *FUM* cluster was absent in banana strains except for portions of *FUM21* and *FUM19*, which are the terminal genes at each end of the cluster. The remnants of these two genes were contiguous, suggesting that 43.9 kb of the *FUM* cluster was presumably deleted. PCR analysis of the *FUM21-FUM19* junction confirmed absence of the cluster in all banana strains examined. Cotransformation of banana strain NRRL 25059 with two overlapping cosmids (Cos6B and Cos4-5), which together contain the entire *FUM* cluster, yielded fumonisin-producing transformants that were pathogenic on maize seedlings. Conversely, maize strains that possess the *FUM* cluster but do not produce fumonisins because of mutations in *FUM1*, a polyketide synthase gene, were not pathogenic on maize seedlings. Together, the data indicate that fumonisin production may have been lost by deletion of the *FUM* cluster in the banana population of *F. verticillioides* but that fumonisin production could be restored by molecular genetic complementation. The results also indicate that fumonisin production by *F. verticillioides* is required for development of foliar disease symptoms on maize seedlings. However, the overall incidence and severity of seedling disease development are likely dependent on both the maize genotype and the amount of fumonisin produced by *F. verticillioides* strains.

Chitinase Isoenzyme Profiles in Seedlings of Fusarium Resistant and Susceptible Corn

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Plant chitinases have been implicated in both antagonistic and beneficial interactions with microorganisms. In an effort to better understand communication between Zea mays and its fungal environment we are developing a knowledge base of corn chitinase isoenzymes. Of specific interest is the identification of proteins whose activity levels are effected by the presence of Fusarium. We have established isoenzyme profiles of imbibed seeds and seedlings for five lines of corn; the Fusarium resistant hybrid Enerfeast and its inbred parents, the genetic model B73, and the Fusarium sensitive sweet corn Jubilee. Twelve unique enzymes were identified that are able to degrade chitin after electrophoresis. The enzyme profiles were further characterized in Enerfeast by dissection of young seedlings into six separate tissues prior to protein extraction. Four chitinases have been purified and their pI and substrate profiles have been determined.

PANEL DISCUSSION: Fumonisin Elimination

Panel Chair: Anthony Glenn

Summary of Presentations: The session was moderated by Scott Averhoff of the Texas Corn Producers Board. Ron Riley and Anthony Glenn from the USDA-ARS Toxicology & Mycotoxin Research Unit in Athens, GA presented a set of reports on the effects of fumonisin on corn seedling development and disease. Fumonisins were shown to accumulate and disrupt ceramide biosynthesis in susceptible corn seedlings, resulting in elevated concentrations of sphingoid bases and their 1-phosphates in plant tissues. The accumulation of these sphingoid bases may represent the basic cellular mechanism of action resulting in inhibited seedling development and disease. Resistant corn genotypes accumulated much less fumonisin in their tissues and had significantly less disruption of ceramide biosynthesis. Studies of seedling disease associated with fumonisin production were facilitated by a population of *Fusarium verticillioides* from banana which did not produce fumonisins due to a unique deletion of the fumonisin biosynthetic gene cluster. Through transformation-mediated complementation of the gene cluster deletion, molecular genetic evidence was presented indicating that fumonisin-nonproducing strains are not pathogenic on susceptible corn seedlings while fumonisin-producing strains cause necrotic leaf lesions, stunting, and even death. Daren Brown, representing the USDA-ARS Mycotoxin Research Unit in Peoria, IL, then presented phylogenetic data examining the polyketide synthase (PKS) genes from *F. verticillioides*, *F. graminearum*, *F. oxysporum*, and *F. solani* and specifically addressed the question of whether the distribution of conserved PKS genes might reflect pathogen specialization versus general housekeeping functions. He also presented data indicating that *F. verticillioides* exhibits a significant degree of alternative splice forms (ASFs) among its mRNA transcripts (~4% of genes). The significance of these ASFs is the subject of additional studies since they could possibly result in multiple functional proteins from the same gene.

Summary of Panel Discussion: A main theme within some of the questions concerned the possible selective advantages of fumonisin production and ASFs. What advantage would *F. verticillioides* gain from maintaining the fumonisin gene cluster and biosynthetic pathway? Dr. Glenn responded that since fumonisin-nonproducing strains are rarely isolated from field samples of corn, this would suggest a selective advantage for fumonisin production, which could include effects on host plant tissues that facilitate infection or nutrient acquisition or even microbial antibiosis to limit niche competition. A contrast was made to *Aspergillus flavus* since aflatoxin-nonproducing strains are readily isolated from field samples, thus suggesting that aflatoxin may not provide a significant advantage to the life cycle of *A. flavus*. A similar question of selective advantage was posed to Dr. Brown regarding ASFs. He responded that since ASFs are not specific to any particular class or functional group of genes, their function or advantage wasn't clearly evident. They may provide a level of temporal or developmental specificity or functionality for the expression of particular genes and therefore may represent another mechanism of gene regulation. Other general questions related to disease pathology, including what is the nominal dose of fumonisin B1 (FB1) for observable effects on corn seedlings? Dr. Riley responded that dose response experiments indicated that 1-5 ppm of FB1 resulted in measurable effects on plant development and increases in phytosphingosine, sphinganine, and their respective 1-phosphates due to disruption of ceramide biosynthesis. He stressed the importance of elevated concentrations of sphingoid bases and sphingoid base 1-phosphates in cells due to their role in numerous cell signaling pathways. The 1-phosphates in particular are emerging as important mediators of cell signaling in eukaryotic cells, including the signal-transduction pathway controlling guard cell turgor regulation and closure of stomatal apertures. Thus, perturbation of ceramide biosynthesis could effect the growth and development of corn plants even if disease symptoms are not evident.

A Single Extraction Method for the Analysis by Liquid Chromatography/Tandem Mass Spectrometry of Fumonisins, Sphingoid Bases, and Sphingoid Base 1-phosphates in Maize Leaf Tissue

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The fungus *Fusarium verticillioides* is a pathogen of many plants and is known to produce fumonisins. These toxins have been shown to contribute to the development of maize seedling disease. Fumonisin disruption of sphingolipid biosynthesis has been demonstrated to occur during such pathogenesis. A liquid chromatographic/mass spectrometric method was developed for the analysis of fumonisin content (FB_1, FB_2, and FB_3) in maize leaf tissue, as well as the elevation of biomarkers of sphingoid base disruption in those tissues (phytosphingosine, sphinganine, phytosphingosine 1-phosphate, and sphinganine 1-phosphate). This method involved a quick extraction (3 hours) using 1:1 acetonitrile:water + 5% formic acid (1 ml/10 mg tissue) and subsequent analysis on a linear ion trap mass spectrometer. Percent recoveries ranged from approximately 50 to 100 percent, and limits of detection ranged from 1fg/µl to 224fg/µl. To test the efficacy of the method, seed of susceptible and resistant maize lines were inoculated with a pathogenic, fumonisin-producing strain of *F. verticillioides*. The maize seedlings were then harvested, and fumonisin content, as well as sphingoid bases and their 1-phosphates, were measured in the leaf tissues. Fumonisin accumulation was evident in the leaves of inoculated plants and was significantly greater in the leaves of the susceptible maize variety than the resistant variety. The elevation of sphingoid bases and sphingoid base 1-phosphates was also significantly greater in the leaves of the susceptible maize line as compared to the resistant line. Unexpectedly, FB_1 was preferentially accumulated in the leaf tissues over FB_2 and FB_3. The method developed was effective, fast, and sensitive for use in determining these indicators of disease induced by infection and toxin production.

Frequency of Introduced and Native *Fusarium verticillioides* Populations in Ears of Field-grown Corn Plants

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The purpose of the current research was to compare the frequency of corn kernel colonization by an introduced F. verticillioides in comparison to kernels colonized by endemic *Fusarium* species. The introduced fungus was *F. verticillioides* PATg, a transformant with a selection gene, hph, for hygromycin resistance (hygr) and a reporter gene, gusA, coding for ß-glucuronidase (GUS). This isolate can be identified by growth on a selection medium, MMH, and confirmed by histochemical staining for GUS. Components of the treatments included seed incubation medium (BOA versus MMH), ear inoculation (yes versus no), ear inoculum (*F. verticillioides* versus water), silk development at inoculation (green versus brown) and site inoculated (shuck versus silk). Over 75% of the kernels contained endemic *Fusarium* species as indicated by mycelia proliferation on BOA medium following incubation of non-inoculated kernels. The mycelial proliferation on MMH medium occurred for 90% of the kernels originating from *F. verticillioides* PATg-inoculated ears. Ninety six percent of the mycelia originating on MMH medium were confirmed to be the introduced population by staining. Thus, approximately 4% of the population was not identified leaving the possibility that these mycelia could have arisen from a hygromycin-resistant endemic *F. verticillioides* genotype or genomic alterations of the introduced genes in the experimental strain PATg. Consequently, control of the problems caused by this fungus should be focused, not on eliminating endemic *F. verticillioides* populations, but on identifying the biological and ecological interactions leading to the deleterious aspects in the corn/*F. verticillioides* relationship.

Fusarium verticillioides Gene Expression Profiling by Microarray Analysis

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Fusarium verticillioides is a pathogen of maize and it can produce the toxic polyketide derived secondary metabolites called fumonisins. Fumonisins have been shown to cause animal diseases and are epidemiologically correlated to esophageal cancer and neural tube defects in humans. The genes necessary for fumonisin production are clustered and co-regulated. Fumonisins are but one type of polyketide secondary metabolite produced by *F. verticillioides* and the role or function of fumonisins has not been clearly defined. A number of genomic resources have become available over the past few years including the Fusarium verticillioides Gene Index (FvGI), the whole genome sequence from The Broad Institute and DNA microarrays. Through the efforts of whole genome sequencing projects, multiple polyketide synthase (PKS) genes have been identified in *F. verticillioides*. Only a few of the polyketide derived products of these PKS genes have been identified and characterized.

DNA microarrays specific to different filamentous fungi have been used to compare expression of multitudes of genes between different conditions. These conditions can include time courses, nutrient availability and the presence of host material in the case of plant pathogenic fungi. The *F. verticillioides* DNA microarrays have been used to compare the expression of the sixteen PKS genes between a fumonisin non-producing strain and the wild-type strain. A number of different relationships emerge from this analysis, the most prevalent being a decrease in expression of the PKS gene in the fumonisin non-producing background. However, one example stands out. Expression of one of the PKS genes appears to be higher in the fumonisin non-producing strain than in the wild-type. This might provide an avenue for the identification and characterization of a new polyketide from *F. verticillioides*. Furthermore, this type of analysis, screening for differential gene expressing in mutant strains of *F. verticillioides*, could lead to the identification of novel genes involved in secondary metabolism and potentially the plant-pathogen interaction as well.

Incidence of Aflatoxin and Fumonisins in Corn Flour (Maseca), in Guadalajara México

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The traditional process to make tortillas in México is the nixtamalization and it has been analyzed by different authors to evaluate the deteriorative activity on aflatoxin contamination in corn used for human consumption. It has been reported that the traditional process can decrease, up to 96% the aflatoxin content in corn. Furthermore, this process also decreases the fumonisin contamination in corn. However the process had to be modified by industries to fulfill population demand and to overcome technical problems such as alkalinization of soils by wastewater. Each industry has their own technique and it is not of public domain, therefore it is not possible to analyze the deteriorative activity on aflatoxin of the industrial processes.

The objective of this study was to determine the incidence of aflatoxin(AFB_1) and fumonisins (FB_1 and FB_2) as well as the level of contamination in corn flour (harina de nixtamal) prepared by industrial nixtamalization (Maseca) that was sold in supermarkets during May –July in Guadalajara city, México.

Fifty samples were bought at stores, such as Walmart, OXXO, Gigante, Soriana, and Comercial Mexicana at different regions of the city. Each sample was 5 kg and it was mixed thoroughly to take 15 subsamples of 60g, then 10g of every 5 subsamples were taken to form one composite. In this way we had 3 composites for each sample. When the aflatoxin concentration in the composites was below 20 $\mu gAFB_1$ /kg the subsamples were not analyzed; if the composite had 21 $\mu gAFB_1$/kg, the parental subsamples of composite should be analyzed; if the composite is 22-80 $\mu gAFB_1$/kg the 15 subsamples had to be analyzed. In the case of fumonisins only the composites were analyzed. For aflatoxin extraction, the modification of the method 1 AOAC (CB-method) was used (Guzmán –de-Peña et al, 1992). The quantification was done by HPLC at 364 nm, without derivatizaton. For fumonisin determination the Shephard method modified by Doko et al (1995) was used. Derivation of extracted fumonisins with OPA and mercaptoethanol was performed and quantification was done with an Agilent 1000 HPLC with fluorescence detector. Incidence of aflatoxin was 12%; for aflatoxicol 12% and 100% fumonisins. The level of AFB_1 contamination was below 20µg/kg, only one sample had 16.9 and the value was the mean of 18 analyses. The highest concentration of aflatoxicol was 1.8 µg/kg; it was not determine which type of aflatoxicol was present in this corn flour. It should be mention that aflatoxicol has been found in corn in Mexico (Anguiano Ruvalcaba et al., 2005). The level of fumonisin B1 contamination was higher than FB2 in all composites and the total concentration was from 147 to 816µg/kg. The concentration of fumonisins found was below that recommended by the Food and Drug Administration –USA. These results show that the samples of corn flour analyzed had low aflatoxin and fumonisins contamination. Therefore studies of incidence of these mycotoxins should be periodically performed in Mexico.

Dissection of Naphthalene Acetic Acid Potassium Salt Activity on Mycotoxicogenic Fungi

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Enormous effort has been invested by the scientific community in finding substances that inhibit growth and synthesis of mycotoxins by fungi, mainly because the negative effects in human health and agroeconomics. Among these substances the plant growth regulators 2, 4-dichlorophenoxy acetic (2, 4-D), jasmonic, gibberellic and naphthalene acetic acid were evaluated *in vitro* to determine their effect on growth, differentiation and mycotoxin synthesis in *Aspergillus nidulans* and *A. parasiticus.* Jasmonic acid 1mM had no effect on both fungal species. Giberellic acid had a stimulatory effect on mycotoxin synthesis, while 2, 4-D had no effect in any of the evaluated parameters. Interestingly Naphtalene Acetic acid K-salt (NAA K-salt) 50mM inhibited fungal growth by 75% and mycotoxin synthesis by 25%, and NAA K-salt, 100mM arrested spore germination of *A. parasiticus* and inhibited transcription of flbA and aflR genes, in both fungi (Bucio-Villalobos et al., 2005).

Further studies to pursue technological applications led us to dissect the activity of NAA on mycotoxicogenic fungi cultured in synthetic media and in corn grain, as well as the toxic activity in seed corn and eight days old chicks.

The inhibitory effect of 1M NAA K-salt on germination of *A. parasiticus* ATCC 16992*, A. flavus, A. nidulans, Fusarium and Penicillium spp* was evaluated. The substance inhibited germination of all fungi tested, spores only reached the swollen stage and no further growth was observed. When NAA K-salt was added at different stages of the germination process of *A. parasiticus* it was observed that the compound could not arrest the swollen stage but it inhibited protuberance of germling and growth no matter at what time the compound was added.

Different concentrations (1, 6, and 12 mg/50 ml PDB) added to *A. parasiticus* cultures ($1X10^5$ spores/50ml) diminished aflatoxin synthesis. Similar results were observed when 2M solution was added to 50g of corn naturally contaminated, however inhibition of 84% on aflatoxin synthesis was achieved when 1g of NAA K-salt was added to corn. To confirm this effect sterile corn was inoculated with heavy spore suspension of *A. parasiticus* and 1 g of NAA K-salt was added, the reduction on aflatoxin synthesis was 75%. Also, it was observed that the substance induced morphological and physiological changes in the fungus, after six months of exposure in dry conditions on corn and then recovered in PDA, *A. parasiticus lost* the green color, and the aspect was fluffy and decreased its toxicogenic capabilities.

When corn with NAA K-salt (1g/50g of corn) was fed during 6 days to 8 days old chicks, they did not present any symptom of toxicity. However, germination of seed corn is affected by high concentration of NAA K-salt solution.

Naphthalene acetic acid K-salt is a promising compound to inhibit growth of mycotoxicogenic fungi and aflatoxin synthesis. Citotoxic and genotoxic studies should be performed before technological applications can be pursued.

Effect of Nixtamalization with and without Corn Matrix on the Concentration and Toxicity of Fumonisins in *Fusarium verticillioides* Culture Material

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Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium verticillioides and F. proliferatum*. It is common in corn and is found in corn-based foods. FB_1 causes equine leukoencephalomalacia in horses, pulmonary edema in swine, kidney and liver toxicity and cancer in laboratory rodents, and neural tube defects (NTD, a type of serious birth defect) in mice. Its impact on human health is not certain, however, there is evidence suggesting that FB_1 and other fumonisins are risk factors for neural tube defects and cancer in populations that are dependent on corn as a diet staple. Minimizing exposure is therefore desirable.

Nixtamalization is the cooking method for making masa and tortillas. It involves cooking and steeping in alkaline water and has been shown to reduce FB_1 concentrations in cooked products, by a combination of extraction into the cooking liquid and conversion of FB_1 to hydrolyzed FB_1 (HFB_1). There is evidence that FB_1 can also bind to food matrix components so that it is not detectable by routine analytical methods. If this occurs during nixtamalization, the amount of reduction achieved could be overestimated and potential toxicity of the cooked products underestimated.

To address this possibility, *F. verticillioides* culture material (CM) was nixtamalized as is (NCM) or after being mixed with ground corn (NCMC). Additional portions of the CM were sham nixtamalized under non-alkaline conditions either alone (SCM) or with the corn (SCMC). The materials were then compared in a bioassay using kidney toxicity as the critical endpoint. Toxicity was assessed by microscopic examinations and by quantification of tissue sphinganine (Sa) and Sa 1-phosphate (biomarkers of fumonisin exposure) from rats that had been fed CM equivalent weights of the CM, NCM, NCMC, SCM or SCMC for up to three weeks. Control groups were fed diets amended with unprocessed corn (UC) or nixtamalized corn (NUC).

Both nixtamalization and the sham nixtamalization procedure reduced FB_1. As a result, FB_1 concentration was reduced from 9.1 ppm in the CM diet to 2.1 ppm in the NCM diet and 1.2 ppm in the SCM diet. Improved reduction was achieved when corn was present during nixtamalization (NCMC diet = 0.5 ppm FB_1). In contrast, the corn had no effect on reductions achieved during the sham process (SCMC diet = 1.0 ppm). Moderate lesions and widespread apoptosis were found in the kidneys from rats fed the CM. Less severe, subtle lesions with fewer apoptotic cells were found in the group given the NCM. The presence of corn during nixtamalization further reduced toxicity as lesions indicative of fumonisin exposure were not found in the rats fed NCMC or in rats fed SCM, SCMC, UC or NUC. Sa and Sa 1-phosphate findings showed a similar pattern. The concentration of Sa plus Sa 1-phosphate decreased in the groups as follows: CM (600 – 800 nmol/g) > NCM (400-600 nmol/g) > SCM and SCMC (30-90 nmol/g). Lowest concentrations (< 8 nmol/g) were found in the NCM, UC and NUC groups. Together, these results provide evidence that interactions between FB_1 and matrix constituents of corn take place during nixtamalization and that these interactions are beneficial, leading to reduced toxicity of the cooked product.

20th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 1: MICROBIAL ECOLOGY

Moderator: Phillip Wakelyn, National Cotton Council

Effects of Antagonistic Pseudomonas Strains on *Aspergillus flavus* and *Fusarium verticillioides* in Soil

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In previous work, we isolated *Pseudomonas chlororaphis* strain JP1015 and *P. fluorescens* strain JP2175 from cornfield soil and rhizosphere samples, and characterized these strains for antagonistic phenotypes against *Aspergillus flavus* and *Fusarium verticillioides* in culture media. Both bacterial strains produce at least one diffusible antifungal compound as well as chitinase activity, and strain JP1015 also produces β-1,3-glucanase activity. The current study involved determining whether these bacteria were antagonistic to *A. flavus* and *F. verticillioides* in the soil environment. Autoclaved soils from two different Mississippi corn fields were coinoculated with approximately 10^4 fungal spores per gram and varying concentrations of bacterial cells, and fungal and bacterial populations were enumerated over 16 days. Bacterial populations of both strains reached 10^9 cfu/g after 3 days, regardless of the starting inoculum concentration. Strain JP1015 reduced recovered *A. flavus* soil populations by 100-fold after 3 days, and by more than 10-fold after 16 days. Inhibition of *A. flavus* by strain JP2175 was dependent on the starting inoculum level, with high inoculum levels inhibiting *A. flavus* to a similar extent as strain JP1015. *F. verticillioides* was inhibited by both bacteria, but to a lesser extent than was *A. flavus.* Growth and antagonistic phenotypes of both bacterial strains was similar in both soil types. Microscopic evaluation of fungal growth during coculture with bacteria in soil filtrate media showed that *A. flavus* spore germination was inhibited when bacteria were added to spores, and vegetative growth was inhibited when bacteria were added following spore germination. Inhibition of *F. verticillioides* was observed to be predominantly during vegetative growth. These results suggest that soil amendment using these bacterial strains may be effective in reducing populations of mycotoxigenic fungi in the corn environment.

Application of *Pichia anomala* WRL-076 to Control *Aspergillus flavus* in Tree-Nut Orchard: Progress in 2006 and 2007

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The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins, of which the worst one is aflatoxin. Contamination of aflatoxin in tree nuts, peanuts, corn and cottonseed has been recognized as a serious food safety hazard to both human and animal. Aflatoxin levels of 2-4 ppb have been declared mandatory by importing European Countries. The aflatoxin issue causes a trade barrier for exporting almond and pistachioin. The major aflatoxin-producing fungus, *Aspergillus flavus* has a broad ecological niche and reproduces copiously. Even very low levels of infection of the crops by *A. flavus* can result in aflatoxin levels above these mandatory standards. Managing aflatoxin contamination via biological control using yeast as an antagonist to fungal pathogen may be a promising and environmentally-friendly approach.

A visual bioassay has been developed to screen several hundred yeast isolates for identifying effective yeasts inhibiting "both the growth of the *Aspergillus flavus* and aflatoxin production". One yeast strain, *Pichia anomala* WRL-076 has been demonstrated to effectively reduce colonization and sporulation of *Aspergillus flavus* on wounded nut-fruits of pistachio and almond. Field tests to control *A. flavus* in commercial pistachio orchard were carried out in collaboration with Dan E. Parfitt and Brent A. Holtz (University of California, Davis). Similar results were observed in almond on a small scale experiment conducted at Nickels Soil Laboratory, Arbuckle, CA.

The population of *P. anomala* was shown to increase on pistachio nut-fruits before harvest in the year of 2006 and 2007. Fungal population including *A. flavus* on early split and insect damaged pistachio decreased when the trees were sprayed with yeast suspension during growing season. An unexpected result was observed in 2006 to show that *P. anomala* reduced the population of *Burkholderia cepacia* (a human pathogen from irrigation water) on pistachio nut-fruits.

In conclusion the yeast strain, *P. anomala* WRL-076 has the potential to control a wide range of undesirable microorganisms in tree-nut orchard. Registration and commercialization of *P. anomal*a WRL-076 is warranted for providing the growers alternative option in orchard management.

Etiology: A Path to Improved Management of Aflatoxin Contamination

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Establishing the causal agent of a plant disease problem is an initial step in developing effective management strategies. Management procedures for prevention of aflatoxin-contamination frequently are directed at either controlling the environment or reducing host susceptibility. The process of identifying the most important aflatoxin producers can be complex. Members of the species that produce aflatoxins vary widely in their aflatoxin producing ability thus, the aflatoxin-producers that most frequently infect crops do not necessarily produce the most aflatoxin-contamination.

Aspergillus flavus and *A. parasiticus* are the most commonly implicated causal agents of aflatoxin contamination, with *A. flavus* by far the most important. Furthermore, *Aspergillus flavus* may be divided into two distinct morphotypes, the S and L strains. Each morphotype is composed of many clonal lineages (called vegetative compatibility groups or VCGs) defined by a vegetative compatibility system that limits gene flow between dissimilar individuals. Both morphotypes and VCGs differ in many characteristics; the most frequently studied of which is aflatoxin-producing ability. The S strain, on average, produces much higher concentrations of aflatoxins than the L strain. Consequently, if the S strain commonly infects crops, it is a primary target for management of aflatoxin contamination. Members of different L-strain VCGs vary widely in ability to produce aflatoxins, with some L-strain VCGs producing very large amounts of aflatoxins, while others produce very little or no aflatoxins.

Studies on the S strain of *A. flavus* illustrate both the potential importance of etiological knowledge and how minor components of infecting communities can be the most important etiological agents. Work in Arizona, Texas, California, and Kenya indicate that S strain members can be important etiological agents in both North America and Africa. Impacts of S strain isolates can be underestimated by either overlooking the S strain due to morphological differences or by preferentially selecting L strain isolates during primary isolations. In Arizona, S strain isolates infect a relatively low proportion of the cottonseed that fluoresces bright green-yellow and yet cause the vast majority of the aflatoxin contamination. Thus, the *A. flavus* strains most commonly infecting cottonseed were not the most important in the aflatoxin contamination process. S strain members also are the most important etiological agents of aflatoxin contamination in South Texas, even though S strain isolates are but a minor component of the *Aspergillus* section Flavi propagules present on the harvested crops. This morphotype also was the primary cause of the maize aflatoxin contamination events that resulted in hundreds of deaths in Kenya in 2004. The S strain is also a frequent infector of highly contaminated maize in North Central Texas. Although, it composes a small percent of maize infections in the coastal bend of Texas. Thus, the relative importance of specific agents to the contamination process may vary with crop, season, and region.

Strains of *Aspergillus flavus* vary in adaptation, seasonality, and sensitivity to various anti-fungal agents. Identification of the most important etiological agents allows direction of management practices towards the most important targets, ensures breeding efforts are directed appropriately, and allows timing of agronomic practices to minimize exposure of crops to agents representing the greatest risk. Because the fungi causing the most contamination may vary with region, field tests should utilize aflatoxin producers endemic to test areas and care should be exercised to prevent introduction of aflatoxin-producers from other regions.

The Experimental Use Program for Determining the Efficacy of Afla-guard® for Biocontrol of Aflatoxin in Corn

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Research conducted over several years has resulted in the development of a product for biological control of aflatoxin contamination. The biopesticide is now produced commercially under the trade name, afla-guard®, and it is approved for use in commercial peanut production. Afla-guard is used to establish a dominant population of a nontoxigenic strain of *Aspergillus flavus* (NRRL 21882) in soil where the applied strain competitively excludes toxigenic strains in the colonization of peanuts during periods of late-season drought. Results of studies conducted in 2005 and 2006 showed that afla-guard also has potential for controlling aflatoxin contamination in corn. Therefore, an experimental use permit (EUP) was sought from the US Environmental Protection Agency to allow large-scale testing to determine the efficacy of afla-guard for biocontrol of aflatoxin in corn. The permit was granted in May 2007 allowing treatment of up to 3000 acres of corn in Texas.

Southern Texas was chosen for the study because the potential for aflatoxin contamination there is historically high. To increase the likelihood of obtaining useful efficacy data, two different areas were included in the study. The first included about 1000 treated acres in Victoria, Calhoun, and Refugio counties along the southeastern Texas gulf coast, subsequently referred to as the Victoria area. The other included about 2000 treated acres in Uvalde and Medina counties located west of San Antonio, subsequently referred to as the Uvalde area. Approximately half the acreage was treated with the peanut-approved rate of 20 lb/ac and the other half was treated at 10 lb/ac. Afla-guard was applied aerially between May 7 and May 18 when the majority of corn in the Victoria area was in early silk and the majority in the Uvalde area was beginning to tassel. Non-treated control fields were identified in relatively close proximity to treated fields. As corn was harvested and delivered to buying points, approximate 10-12 pound samples were collected from both treated and untreated fields. Samples were shipped to the National Peanut Research Laboratory where they were ground in a Romer mill, and the entire ground sample was then combined with an equal weight of water and ground in a vertical cutter mixer to homogenize aflatoxins and *A. flavus* propagules. Separate subsamples were taken for aflatoxin analysis by HPLC and for dilution plating to determine the degree of *A. flavus* colonization of corn (results not complete at this time).

Environmental conditions were characterized by abnormally high rainfall amounts and unusually cool temperatures that were not conducive for aflatoxin contamination. As a result, aflatoxin concentrations in corn were generally quite low, and often not detectable. Nevertheless, treatment with afla-guard produced significant reductions in aflatoxin contamination. In the Victoria area, the mean aflatoxin concentration in 93 samples from untreated fields was 5.4 ppb compared with means of 2.3 ($n = 43$) and 0.5 ($n = 50$) ppb from fields treated with 20 and 10 lb/ac, respectively. Kruskal-Wallis analysis of variance on ranks revealed significant ($P < 0.05$) reductions in aflatoxin were associated with both treatment rates. Both treatment rates also significantly reduced aflatoxin in the Uvalde area where means for control, 10 and 20 lb/ac treatments were 0.9, 0.3, and 0.1 ppb, respectively. There were no significant differences between the two treatment rates in either area. Combining data from the two treatment rates and from both areas, aflatoxin in control peanuts averaged 3.4 ppb ($n = 167$) compared with 0.5 ppb ($n = 283$) in all treated peanuts, an 85% reduction. Chi-square analysis of the data showed that treatment with afla-guard also produced a significant increase in the percentage of samples not contaminated with aflatoxin. Of samples from untreated, control fields, 33.5% did not contain any detectable aflatoxin compared with 78.1% negative samples from treated fields. Although aflatoxin concentrations in corn were generally low, the data showed that treatment with afla-guard had a significant impact by reducing aflatoxin levels in corn.

Aflatoxin Control in Pistachios: Biocontrol Using the Atoxigenic Strain AF36, Survival of AF36, and EUP Status

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For the past several years, we have investigated the use of atoxigenic strains (strains not able to produce aflatoxins) of *Aspergillus flavus* as biocontrol agents to reduce aflatoxin contamination of pistachios in California. This approach has been very successful in commercial cotton fields in Arizona where the atoxigenic strain AF36 has substantially reduced aflatoxin contamination of cottonseed. Three promising atoxigenic strains, AF36, A564, and A815, occur naturally in commercial pistachio orchards in California (4.3, 0.8, and 0.7% of the 864 isolates of *A. flavus* from commercial pistachio orchards belonged to AF36, A564, and A815, respectively). In 2001 and 2002, these three promising atoxigenic strains were applied (as infected wheat seeds at the rate equivalent to 10 lbs/acre) in a flood-irrigated research pistachio orchard. In order to determine the survival and spread of the atoxigenic strains, soil samples were collected on 21 August, 2006. The density of *A. flavus*/*A. parasiticus* in the soil did not significantly differ among treatments and was approximately the same level as in 2005. The percentages of *A. flavus* isolates that were AF36 (4.9% of *A. flavus* isolates), A815 (10.8%), and A564 (2.4%) in the areas that were treated with each of those specific strains decreased substantially from the levels in 2005.

Starting in 2003, the atoxigenic strain AF36 was applied in a different research pistachio orchard, which was irrigated by microsprinklers. The strain AF36 has not been applied in this orchard since 2005. On 25 September, 2006 (during the normal harvest period), samples of soil, early split nuts, and normal nuts were collected. The density of *A. flavus*/*A. parasiticus* in soil was not significantly different between treated areas and untreated areas. The density of *Aspergillus* sect. *Nigri* in the soil in treated areas was approximately 22 times that of the density of *A. flavus*/*A. parasiticus*. The incidence of AF36 among *A. flavus* isolates in the soil increased slightly from 2005 to 2006 in the areas treated in 2004 and 2005 from 82.0% to 88.4%, but decreased in the areas treated in 2003 and 2004 from 84.8% and 46.5%. In addition, the treatments did not differ significantly in the density of *A. flavus*/*A. parasiticus* on the surface of the hulls of freshly harvested nuts. The density of *Aspergillus* sect. *Nigri* on the surface of nuts in treated areas was approximately 140 times that of the density of *A. flavus*/*A. parasiticus*. No kernel decay by *A. flavus* was found in the 2,270 early split nuts examined, suggesting that applying AF36 does not significantly increase decay of the nuts. Soil and nut samples were collected during the harvest period in 2007 in order to complete the study on the survival and spread of the AF36 previously applied. These samples are currently being evaluated.

We are also determining the spatial patterns of aflatoxin contamination throughout the pistachio-growing regions in California using processor library samples (each sample consists of 20 pounds of nuts taken at the processing plant as nuts are being unloaded from the orchard). A total of 2,232 library samples from the 2001 to 2005 harvests have been analyzed for aflatoxins. Furthermore, library samples from the 2006 harvest have been prepared for aflatoxin analysis. Currently we are completing the determination of the exact location for all the orchards involved in the study.

Preparations have been made for applying the atoxigenic strain AF36 in commercial pistachio orchards. On 3 May, 2007, the United States Environmental Protection Agency approved an Experimental Use Permit for applying AF36 in commercial pistachio orchards in California with the request that we quantify the spores of AF36 in the air in treated and nontreated orchards However, we are still negotiating with the California Department of Pesticide Registration concerning the details associated with the Experimental Use Permit, since they requested a protocol for the spore sampling

that differed significantly from the sampling requested by the EPA. In summer of 2007, we quantified the propagules of *Aspergillus* sect. *Flavi* and other *Aspergillus* spp. in the air using Burkard traps in four commercial pistachio orchards. We expect to be able to apply AF36 in commercial orchards in 2008 and then compare spores in the air of treated vs. nontreated orchards.

Aflatoxin Control in Figs and Almonds: Biocontrol Using the Atoxigenic Strain AF36

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For several years, we have investigated the use of atoxigenic strains (strains not able to produce aflatoxins) of *Aspergillus flavus* as biocontrol agents to reduce aflatoxin contamination of figs in California. This approach has been very successful in commercial cotton fields in Arizona where the atoxigenic strain AF36 has substantially reduced aflatoxin contamination of cottonseed. In 2003 and 2004, we applied the atoxigenic strain AF36 (as infected wheat at the rate of 41.2 g wheat/tree (equivalent to 10 lbs/acre)) once each year during early summer in a drip-irrigated Calimyrna fig orchard. No AF36 has been applied in this orchard since 2004. On 4 October, 2006 and on 19 September, 2007, soil and fruit samples were collected. The soil collected in 2006 had a higher density (59.7 cfu/g soil) of *A. flavus*/*A. parasiticus* in the areas under the drip lines where infected wheat had been placed (in 2003 and 2004) than in the middles (3.9 cfu/g) or under the drip lines in the untreated areas (2.3 cfu/g). Almost all of the *A. flavus* isolates (95.5%) obtained from the soil collected in 2006 under the drip lines in the areas previously treated with AF36 belonged to the strain AF36 compared to 36.6% in the middles and to 61.8% of the isolates from the untreated areas, suggesting that AF36 survived for at least two years at high levels and that there was movement of AF36 from the applied areas under the drip lines to untreated areas. No decay by *A. flavus* was found in dried figs in 2006 and in 2007 (900 figs were examined each year), suggesting that applying AF36 does not significantly increase decay of the figs. Our results suggest that the use of AF36 in fig orchards should result in the atoxigenic strain becoming the dominant *A. flavus* strain where applied without significantly increasing fig decay. The soil samples collected in 2007 are currently being evaluated.

The incidence of atoxigenic strains among *A. flavus* isolates occurring naturally in commercial fig orchards in California was determined. Out of 349 isolates of *A. flavus* from commercial fig orchards, 6.9% belonged to AF36. In another study, we determined the natural occurrence in commercial fig orchards of AF36 and 15 additional atoxigenic *A. flavus* strains (obtained from California orchards). The strain AF36 (5.0% of the *A. flavus* isolates) was the most common strain, whereas only three of the 15 atoxigenic strains were detected: CAM (2.5%), CAP (2.5%), and CAD (1.3%). Based on these findings, it seems likely that AF36 is the most common atoxigenic strain occurring naturally in commercial fig orchards in California. Also, the greater occurrence of AF36 among the strains examined suggests that using AF36 for the biocontrol of aflatoxin contamination in figs was a better choice than using any other strain.

In 2007 we initiated a project with almonds investigating the use of biocontrol to reduce aflatoxin contamination. The first objective was to determine the incidence of the atoxigenic strain AF36 naturally occurring in commercial almond orchards. Soil samples were collected in April and May, 2007 from 28 almond orchards spread throughout the almond-growing regions. AF36 occurred naturally in almond orchards throughout California, making up 7.8% of the *A. flavus* isolates (637 out of 900 isolates evaluated). However, AF36 was more common in the Southern region (12.6% of the *A. flavus* isolates) compared to the Central region (6.4%) and the Northern region (3.5%). Nut samples were also collected during the normal harvest period and are currently being evaluated.

The second objective for the almond project was to initiate biocontrol studies by applying the atoxigenic strain AF36 in a research almond orchard. On 28 June, 2007, wheat infected with AF36 was applied in a drip-irrigated almond orchard at the Nickels Soil Laboratory in Arbuckle, CA. Soil

samples were collected prior to applying the wheat in order to determine the density and strains of *Aspergillus* sect. *Flavi* present. The main species present in the soil was *A. parasiticus* (74.2% of the isolates) with *A. flavus* strain L (15.0%) and *A. flavus* strain S (10.8%) being much less common. Nut samples and additional soil samples were collected during the normal harvest period in August. These samples are currently being evaluated.

Dynamics of Mycotoxin Concentrations in Aging Corn Residues under Mississippi No-till Conditions

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Mycotoxins, including aflatoxins, fumonisins, cyclopiazonic acid (CPA), and zearalenone, produced by *Aspergillus* and *Fusarium* species when present in grain can cause serious health problems in livestock and humans. Little is known about the occurrence of these toxins in corn plant debris post-harvest. The objective of this study was to determine the overwintering mycotoxin levels in corn stover, cobs, and cobs containing grain on the soil surface three to six months after harvest. Isolines of the hybrid Pioneer brands 34B24 (Bt) or 34B23 (non-Bt) were planted in Elizabeth, MS in 2006. Samples were dried, ground and analyzed for aflatoxin, fumonisin, zearalenone, and CPA. At maturity, grain from Bt hybrid contained significantly less total aflatoxin than the conventional hybrid (109 vs. 200 ng g^{-1}). In stover residues less than 4 ng g^{-1} aflatoxin was observed in both hybrids. Higher aflatoxin levels were found in cobs (17 to 111 ng g^{-1}) and cobs containing grain (541 to 774 ng g^{-1}) with significantly greater levels in the non-BT hybrid compared to the BT hybrid ($Pr > 0.01$). Pooled over hybrids, fumonisin levels averaged 3 μg g^{-1} in stover, vs. 12 μg g^{-1} in cobs, and 120 μg g^{-1} in cobs with grain, with no difference between hybrids. The pattern of partitioning of zearalenone in corn residues was different than other mycotoxins in that the lowest concentration was observed in cobs with grain (mean = 0.08 μg g^{-1}) compared to cobs (mean = 0.50 μg g^{-1}) or stover (0.67 μg g^{-1}) with no DON or its derivatives (3A-DON, 15ADON, nivalenol) detected in any samples. The presence of high levels of these mycotoxins in corn residues could be detrimental to grazing livestock or wildlife.

Application Timing Influences Both Spore Yield of an Aflatoxin Biocontrol Product and Displacement of Aflatoxin-producing Fungi.

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Aspergillus flavus is the main cause of aflatoxin contamination of many crops, including cottonseed. Atoxigenic *A. flavus* strains are used as biocontrols to reduce aflatoxin contamination. The atoxigenic strain AF36 of *A. flavus* was initially developed for use in Western Arizona where current recommendations for use were also developed. Biocontrol treatments have expanded to central Arizona where agronomic practices and field topologies differ from Western Arizona. Yet, there are no location specific recommendations for atoxigenic strain use in central Arizona. Treatments are considered successful when a single application (10 lb/acre) results in displacement of 80% or more of the aflatoxin producers. For the applications to be effective, the atoxigenic strains must reproduce during crop development when environmental conditions are conducive to aflatoxin contamination. This requires applying the biocontrol agent at the appropriate time. The objective of the present study was to determine cotton phenology stages in central Arizona when atoxigenic strain applications will result in optimal displacement of aflatoxin producers.

Five commercial cotton fields near Casa Grande, AZ treated with *A. flavus* AF36 on four different dates (July 14 and 21, and August 1 and 14) in the summer of 2006. The product was applied in 12 rows of 50 m long at the recommended commercial rate of 10 lb of colonized wheat seed per acre. Plots were separated by at least 50 m. The number seeds remaining from each application date was monitored twice a week for three weeks. Lint from the two central rows of each plot was picked at harvest time and dried in a forced air drying oven at 45C for 48 hours. *A. flavus* was isolated from lint by dilution plate technique on modify rose Bengal medium. The percentage of the S strain was determined by colony morphology. Incidences of *A. flavus* AF36 were determined by testing 12 to 18 isolates of the L strain from each plot by vegetative compatibility. ANOVA was used to determine significances in displacement among application dates. Correlation analysis was used to determine the relationship between the number of remaining seeds (total and sporulating) and the percentage of displacement. Linear regression analysis was used to model displacement as a function of the number of sporulating product seeds. In the summer of 2007 applications were made according to crop phenology in three commercial fields in different areas (Yuma, Gila Bend and Eloy). The applications were made 1) at initiation of boll formation; 2) when the first bolls were 1.25 inches in diameter; 3) at first boll opening; 4) when 25 % of bolls were open; and 5) when 50 % of bolls were open. An untreated control was also included. Preliminary results indicate significant differences among application dates for displacement. The highest displacement percentages were obtained in the second (July 21) and the last (August 14) date of application with 83.3 % and 73.2%, respectively, while the non-treated control had a significantly lower percentage of displacement (14.5%). On the third date (August 1), displacement was reduced to (46.3 %). The dates with highest displacement also had the lowest S strain incidence (below 3 %), but were not significant different from the control (19.5 %). Correlation analyses demonstrated a significant positive correlation between the total number of seeds in the field two weeks after treatment and displacement. Displacement increased as the number of sporulating seeds present 10 to 14 days after application increased. The regression model indicates that the desirable displacement of over 80% is achieved when the number of sporulating seeds at 10 to 14 days after application is over 6 per m^2. Samples for the 2007 season are being analyzed. Since in 2006 there was a good positive relationship between sporulating seeds 14 days after application and displacement, this parameter was monitored in 2007. The earliest application (initiation of boll

formation) had the fewest sporulating seeds 2 weeks after application, while the application at first boll opening had the most (8.3 seeds/m^2).

Microbial Ecology Panel Discussion

Panel Chair: Joe Dorner

The discussion began with the topic of the toxicity of cyclopiazonic acid (another mycotoxin produced by *Aspergillus flavus*) and whether or not we should be concerned about it since it is not regulated by FDA. Dorner stated that although it is not as acutely toxic as aflatoxin B_1, it is still a potent toxin that has been implicated in natural intoxications, including the original Turkey "X" disease, which led to the discovery of the aflatoxins. He speculated that part of the reason there appears to be a lack of concern about the toxin is the difficulty associated with its analysis, which has precluded the conduct of surveys necessary to establish the scope of its natural occurrence and involvement in other mycotoxicoses. Don Wicklow asked whether or not the non-aflatoxigenic strains used for biocontrol of aflatoxin produced CPA. Dorner stated that the strain used in afla-guard does not; Hamed Abbas stated that his K49 strain does not; Themis Michailides stated that AF36 does produce it, and EPA has requested that they refine their procedures for measuring it.

Pat Dowd asked about the timing of application of afla-guard in corn, noting that Dorner's results from the Texas EUP showed as good an effect where it was applied later (during silking) as where it was applied earlier (prior to tasseling). The timing of application for the EUP was dictated by the timing of the granting of the EUP by the EPA. In the Uvalde area, most of the corn was not yet tasseling at the time of application, whereas in the Victoria area most of the corn was already silking. Dorner stated that the timing of application in corn has not been fully studied, but based on work in peanuts and prior studies in corn it was decided to try to time the application during maximum whorl opening just prior to tasseling. Apparently, however, a slightly later application was just as effective.

Jeff Palumbo was questioned about the temperature used for testing antagonistic *Pseudomonas* strains and he stated that they used 28° C in their experiments. Asked if he thought a different effect might be seen at a temperature such as 37° C, he said he would think so because the optimal temperature for the pseudomonads was 28-30° and heat stress at the higher temperatures would probably have a negative impact on the bacteria. He was also asked about potential adverse environmental effects associated with application of the antagonistic pseudomonads, and he replied that as far as he knew, the particular species he has been testing are not pathogenic. He did not know how they interacted with other microorganisms in the soil, but in other systems they have been shown to be normal residents of rhizopheric microbial populations.

Mark Doster received a question concerning the taking of almond samples from the ground as opposed to the tree. He stated that in this study they sampled from the ground because that is the way almonds are normally harvested. The trees are shaken and nuts fall to the ground, and they are swept up later. This is different from pistachio harvesting in which the pistachios are collected on a tarp and never touch the ground. He also said they may sample from both the ground and the trees in the future. In answer to a question concerning the mode action of AF36 in figs, he stated that what is occurring in research orchards does not seem to be typical of commercial fig production in California. However, in his opinion the competition between toxigenic and atoxigenic strains took place primarily on the orchard floor.

Peter Cotty was asked about aflatoxin contamination when both S (high aflatoxin producing) and L (lower or non-aflatoxin producing) strains of *Aspergillus flavus* were found in a crop. He commented that when the S strain is found alone, very high concentrations of aflatoxin are normally found. However, when the S strain is combined with the L strain, aflatoxin concentrations are usually much lower. The natural co-occurrence of L with S strains has a modulating effect on the concentration of aflatoxin found. Inoculation with atoxigenic L strains serves to increase the frequency of that natural modulation.

Hamed Abbas was asked about his work showing zearalenone in corn plant debris, but not deoxynivalenol (DON), which, historically, is usually found in corn along with zearalenone. He

reiterated that neither zearalenone nor DON were found in the corn, but he did find zearalenone in plant debris that had been exposed to the cooler conditions in winter that would favor its production by *Fusarium graminearum*. However, DON was not found.

Ken Damann commented that he agreed that etiology of aflatoxin contamination was important, particularly with regard to identification of subsets of *Aspergillus flavus* populations that are most infectious. He, therefore, believes it is incumbent on researchers who have large collections of VCG tester strains to release those to help facilitate those identifications.

The final question concerned the use of the L strain, AF36, for biocontrol as opposed to an atoxigenic S strain. Peter Cotty commented that they screened a large number of S strains and found some non-aflatoxigenic strains. However, they all accumulated aflatoxin precursors that also have some degree of toxicity making them less desirable candidates.

Effect of the Stalk Inoculation Site on Kernel Infection of Corn by *Aspergillus parasiticus*

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Aspergillus flavus and *A. parasiticus* may infect corn kernels on developing ears by several routes. Kernel infection is postulated to occur inside ears primarily by the colonization of silk tissues. Airborne conidia land on exposed silks, germinate, and then colonize the silks inside the ear. Once inside the ear, fungi colonize the glumes at the base of the kernels and the kernel surfaces. Another method of infection involves the vectoring of conidia by insects. Ear-feeding insects transport fungi inside the husks and predispose the kernels to infection by wounding kernels as they feed. Systemic movement through the stalk is another possible route of kernel infection by mycotoxigenic fungi. Recently, we demonstrated systemic movement of an *A. parasiticus* mutant through the corn stalk and into the ear shank tissues. Plants were inoculated by inserting infested toothpicks in the stalks between the 5th and 6th node below the lowest ear shoot. In as little as two weeks, the mutant moved up through the stalk into the ear shank tissues. Kernel infection levels in inoculated plants were found to be low ($\leq$ 1.5 %). It is possible that inoculating the stalk closer to the site of ear attachment may increase the level of kernel infection. This study was conducted to determine if the site of *A. parasiticus* inoculation on stalks would have an effect on kernel infection.

An *A. parasiticus* color mutant (NRRL 6111) was used in field studies conducted in 2006 and 2007. This isolate can be distinguished from wild-type *Aspergillus* spp. by its reddish-brown color and the production of norsolorinic acid (NOR) which is a visible orange intermediate of the aflatoxin biosynthetic pathway. The NOR mutant was cultured on V-8 agar with sterilized toothpicks placed on the surface. Three *A. flavus* susceptible corn hybrids (TV2100, RX938, Mp305 x Mp339) and a resistant hybrid (Mo18w x Mp313E) were inoculated at the VT stage by inserting *A. parasiticus* infested toothpicks into stalks 1, 2, 3, 4, or 5 nodes below the lowest ear shoot. At maturity, five ears from each inoculation treatment from each hybrid were harvested, dried, and individually hand shelled. Three hundred and ninety kernels from each ear were surface sterilized and plated on Czapek solution agar amended with NaCl (7.5%). After 7 days, the Petri dishes were examined for presence of the NOR mutant. Ears were also collected from non-inoculated plants interspersed among the inoculated plants, and processed as described above as a control treatment.

In 2006, the susceptible hybrids TV2100 and Mp305 x Mp339 had the highest levels of infection. Systemic infection of kernels in these hybrids was highest in plants inoculated 1, 2, or 3 nodes below the ear. The highest rate (1.5 %) of kernel infection for any of the inoculation treatments was in TV2100 plants inoculated 1 node below the ear. The resistant hybrid Mo18w x Mp313E had the lowest level of infection. The NOR mutant was only detected in two ears from plants inoculated 1 node below the site of ear attachment. The fungus was not detected in ears from plants inoculated at 2 to 5 nodes below the ear. The NOR mutant was not detected in any of the ears from the un-inoculated plants. In 2007, the fungus was not detected in ears of any of the hybrids regardless of the inoculation site.

Our studies have demonstrated that *A. parasiticus* can readily move up the stalk and into the ear/cob tissue. Kernel infection by the NOR mutant was highest in plants with inoculation sites closest to the ears. However, kernel infection rates remained relatively low. These findings are similar to those of other researchers working with *F. verticilliodes* on corn. Infection of kernels systemically via the stalk is possible, but it appears that the most likely route of infection by mycotoxigenic fungi is by colonization of silk tissues.

Differential Expression of Clustered Aflatoxin Biosynthesis Genes in Relation to the Quantity of Aflatoxin Produced by Field Isolates of *Aspergillus flavus*

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Aspergillus flavus Link is a fungus which produces carcinogenic compounds known as "aflatoxins" in agricultural crops such as corn, peanuts, cotton and tree nuts. The genes directly involved in aflatoxin (AF) production have been identified in an 82kb cluster. Genetic and molecular studies of AF biosynthesis led to the characterization of 29 genes. Two AF genes, *aflR* and *aflJ,* have been demonstrated to play important regulatory role in toxin production. A cAMP-dependant protein kinase, PKA has been reported to negatively regulate *aflR* .

Field isolates of *A. flavus* vary greatly in the amount of aflatoxin produced on potato dextrose agar. In order to define the molecular basis of toxin production and AF gene activation, we used real time RT-PCR assays to study the expression of the selected genes: *aflR*, *aflJ*, *pksA*, *omtB* and *pka.*

Real-Time PCR instrument (ABI 7300 Real Time PCR System) can automate a laborious process of quantifying gene expression. It covers a broad 10^7-folrd dynamic range. Data analysis, including standard curve generation and copy number calculation, is performed automatically. The chemistry of SYBR® Green (Molecular Probes) provides the simplest and most economic method allowing detection of PCR products via the generation of fluorescence signal when the dye binds to double stranded DNA.

Fungal cultures inoculated with *A. flavus* isolates at a final concentration of 10^5 spores/ml, were grown at 28°C in potato dextrose broth in a flask with shaking. After 20 h of incubation, total RNA was extracted and used for first-strand cDNAs synthesis with a Gold RNA PCR Reagent Kit (Applied Biosystems). RT-PCR primers were designed with ABI Primer Express™ 3.0 software, only the intron portions of the DNA sequence were selected from the gene sequences.. The primer pair with the best PCR profile was selected for each gene. RT-PCR analyses were performed to relatively quantify the amount of cDNA of each gene for each sample. Amplification of *A. flavus* 18S ribosomal RNA was used as the endogenous control due to its relatively stable expression level. SDS Software 1.3.1 was used to determine the C_T , the threshold cycle, for each reaction. However, due to the large amount of comparisons tests that require multiple calibrator samples, we used Microsoft Excel to carry out the expression level calculation using the same comparative C_T method calculation as in the SDS Software

The gene expression levels of atoxigenic strain CA16 were designated as calibrators. Both *aflR* and *aflJ* were expressed in low, intermediate and high AF-producing strains. Because these are regulatory genes, their levels of expression were low comparing to the structural genes, *pks*A and *omt*B. CA42, CA60 and CA 90 had the highest level of *omt*B gene expression. CA18 is a low AF producer, even though the expression of *omt*B is relatively high but the *psk*A is very low compared to intermediate and high AF-producers. The global regulatory gene *pka* was down regulated in all the high aflatoxin producers.

Real-time RT-PCR provides an excellent tool for understanding the fine tuning on how genes of AF biosynthesis and signaling interaction in individual strain.

Control of Fungal Pathogens in an Organic Production System with a *Pichia anomala* Yeast

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Pichia anomala strain WRL-076, developed as a biocontrol agent against *Aspergillus flavus*, was tested in an organic production field environment to determine it's efficacy for control of nut and cluster loss caused by fungal pathogens of pistachio. *Alternaria alternata* and *Botryosphaeria dothidea* can cause significant yield loss in pistachio orchards as a result of fruit cluster destruction. This problem is especially severe in organic production systems where conventional fungicides cannot be used. A preliminary field test of WRL-076 was conducted in 2005 and was followed by a larger scale yield trial in 2006. Application of the yeast resulted in significant reductions in cluster loss in 2005-2006 and significant increases in harvestable yield in 2006. Yield for the yeast treatment was 12% greater than for the control in 2006. Little damage from disease was observed in 2007. Consequently, the yield from treated trees was only about 6% greater than the control and was not significantly different. The yeast effectively controlled a waterborne bacterial contaminant that was found in the spray water midseason in 2006, and may be useful as a control measure against human pathogenic bacterial contaminants in the field.

Specificity of *Eupenicillium* and *Penicillium* species for the conidial heads of *Aspergillus* sections *Flavi* and *Nigri*

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The genus *Penicillium* comprises species that mostly colonize plant matter. However, early reports suggest that several species are capable of parasitizing *Aspergillus*. More recently *Eupenicillium ochrosalmoneum* and *E. cinnamopurpureum*, both with *Penicillium* anamorphs, have been observed sporulating on the heads of *Aspergillus* species belonging to section *Flavi* during the colonization of peanut seeds. Little is known about the host specificity underlying these *Aspergillus–Penicillium* associations. In this study, *Aspergillus* species representing nine taxonomic sections were paired in culture with *E. ochrosalmoneum*, *E. cinnamopurpureum*, and two new *Penicillium* species based on morphological and molecular characters, *P. parvulum* and *P. georgiense*. Phylogenic analysis using three loci shows that *P. parvulum* is a sister species of *E. cinnamopurpureum* and that *P. georgiense* is not closely related to *P. parvulum* or either *Eupenicillium* species, though its precise phylogenetic placement within the genus *Penicillium* is unresolved. *Eupenicillium ochrosalmoneum*, *E. cinnamopurpureum*, and *P. parvulum* sporulated predominantly on the heads of section *Flavi* species, including aflatoxigenic *A. flavus* and *A. parasiticus*. In contrast, *P. georgiense* was restricted to the heads of section *Nigri* species. *Eupenicillium* and *Penicillium* species were observed spreading from one *Aspergillus* head to another by means of aerial stolon-like hyphae. Internal hyphae within *Aspergillus* stipes were uncommonly observed (0-7%). Additional studies are required to clarify whether the *Eupenicillium* and *Penicillium* species are parasitic or simply epibiotic on their hosts.

Aspergillus flavus/A. parasiticus Levels in Almond Orchards and on Navel Orangeworm (A Major Pest of Almonds and Pistachios)

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Contamination of almonds with aflatoxins has become a huge issue relevant to food safety in the last few years. Rapid Alerts (http://ec.europa.eu/food/food/rapidalert/index_en.htm) for almonds issued by the European Union due to exceeding the regulated aflatoxin tolerance have reached to more than 45 up to date in 2007. Serious concerns and worries that bad publicity associated with the rejections of almond lots due to excessive levels of aflatoxins will partially negate recent positive reports of food nutrition researchers regarding the many dietary health benefits of almonds. In earlier research in the late 1970s, it was found that there is a close association of aflatoxin contamination and damage by the Lepidopteran pest navel orangeworm (NOW) (*Amyelois transitella*) considered as the number one pest of both almonds and pistachios. Not much research has been done on the role of NOW in spreading *Aspergillus* spp. The objectives of this study were to determine the levels 1) of *A. flavus/A. parasiticus* in almond orchards; and 2) of contamination of navel orangeworm by *A. flavus* and *A. parasiticus.*

All soils collected from 28 Nonpareil almond orchards in northern, central, and southern California had *A. flavus,* but only 21 orchards had *A. parasiticus*, and 26 orchards had *A. tamarii*. *A. flavus* was more common (45.4% of the isolates) in the southern orchards while *A. parasiticus* (46.5%) in the northern orchards, while *A. tamarii* was the least common (23.7%) in northern California almond orchards. Mean densities in soil for Sect. *Flavi* were 28.3, 25.0, and 36.5 cfu/g for the north, central, and south regions, respectively. Propagules ranged from a few in some orchards to more than 200 cfu/g in other orchards. Three distinct strains of *A. flavus*, S, M, and L, were recovered from almond orchard soils. Morphological characteristics of the M strain (medium size and number of sclerotia between the size and number of those produced by S and L strain isolates) resembled the features of the T strain reported by Cho, Zhang, & Cotty (Phytopathology 97:S22, 2007).

Navel orangeworm (NOW) adults that emerged in the spring from masses of almond mummies kept in buckets were heavily (up to 60% insects plated) contaminated with *A. flavus /A. parasiticus* and more than 90% with *A. niger*. NOW adults taken from sticky traps collected from an almond orchard in Madera had up to 8% infestation with *A. flavus/A. parasiticus*. Other *Aspergillus* spp. ranged from 4 to more than 80%. In general, there was an increase in the incidence of *Aspergilli* recovered from NOW adults over the season.

NOW adults removed from sticky traps set in two pistachio orchards were also contaminated (up to 15%) with *A. flavus /A. parasiticus* and other *Aspergillus* spp. NOW trapped on sticky traps set in one of the pistachio orchards later than earlier during the growing season were more contaminated with *A. flavus/A. parasiticus, A. niger,* and other *Aspergillus* spp. By late September, approximately 42% of the larvae collected from early-split nuts had *A. flavus/A. parasiticus,* after plating on media. Sclerotia of *A. ochraceus* and *A. niger* developed on the body of adult NOW moths after plating while sclerotia of *A. flavus* were not found. However, sclerotia of *A. flavus* were found on infected almond kernels. The high incidence of *A. flavus* and *A. parasiticus* on NOW (larvae and adults) suggests that this pest may play an active role (in addition to creating wounds) in vectoring and spreading the aflatoxigenic fungi in pistachios and almonds.

Aspergillus flavus / Aflatoxin Occurrence and Expression of Aflatoxin Biosynthesis Genes in Soil

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Aflatoxins (AF) are carcinogenic metabolites produced by several species of *Aspergillus,* including *A. flavus*. Although *A. flavus* is readily isolated from environmental samples, soil and plant material are considered the natural habitat of this fungus. Studies were conducted on a Dundee silt loam to ascertain the ecology of *A. flavus* in soil planted in corn the previous year and to estimate the occurrence and fate of AFB1 in soil. This soil was previously planted in Bt or non Bt corn and soil was sampled from five plots of each corn hybrid. Low levels of AF B1 (0.4 to 5.5 ng g^{-1}) were detected in soil, but recovery was variable among replicate samples. Native soil and autoclaved soil was amended with 10.0 ng g^{-1} AFB1 and incubated at 25°C. These soils were extracted with ethyl acetate at 0, 3, 6, 13, and 17 days after treatment and analyzed by HPLC. A rapid loss of extractable AF occurred in non-sterile soil (half life $\leq$ 5 days) which was not observed in autoclaved soil indicating biological detoxification. Plate counts using semi-selective media (MDRB agar) indicated this soil contained log (10) 3.1 to 4.5 propagules of *A. flavus* g^{-1} soil. Based on cultural methods, about 66% of these *A. flavus* were capable of AF synthesis. Using traditional PCR all five genes were detected in all soils confirming the plate count estimates of an abundant native *A. flavus* population. Reverse transcriptase (RT-PCR) analysis showed that the two regulatory genes, *aflR* and *aflS*, and structural genes, including *afllD, aflG* and *aflP*, of the AF biosynthesis pathway were expressed in surface soil. These data suggests that indigenous soil populations of *A. flavus* are physiologically active and have the potential to express several genes involved in AF biosynthesis in surface soil. Although AFB1 appears to be transient in soils, it is clear that AFB1 is produced in surface soil in the presence of corn residues as indicated by *A. flavus* CFU levels, AFB1 detection, and expression of aflatoxin biosynthetic genes.

Characterization of Species of the *Aspergillus* Section *Nigri* from Corn Field Isolates Co-infected with *Aspergillus flavus/parasiticus* Species and the Potential for Ochratoxin A Production

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The members of the *Aspergillus* section *Nigri*, known as black-spored aspergilli, can contaminate several substrates including maize. Although some species within the group can produce plant disease symptoms such as black mold in onions and maize ear rot, the main concern with *A. niger* aggregate contamination is the production of a chlorinated cyclic polyketide, ochratoxin A. This mycotoxin is nephrotoxic, teratogenic, and carcinogenic to animals. The correct identification of the black- spored aspergilli is relevant since it is not clear which strains within *A. niger* aggregate are capable of colonizing and producing ochratoxin A on maize and maize products. However this task has been difficult because the morphology of most of the members is very similar. In order to differentiate species within this aggregate we report the use of a repetitive-sequence-based DNA fingerprinting method, the DiversiLab fingerprinting system, a barcoding procedure that looks promising. The results indicated that the corn-isolated black-spored *Aspergillus* species are co-infecting sound corn kernels along with species of other *Aspergillus*, i.e., *A. flavus/parasiticus* and *A. ochraceus* as well as with *Fusarium verticillioides.* The black-spored aspergilli were present as both surface and internal infections as kernels surfaced disinfected also yielded black-spored aspergilli. Preliminary studies on the production of ochratoxin A by isolates from corn are presented. The barcoding system established that this procedure is a rapid and reasonably inexpensive procedure for identifying these fungi. Based on our results, some of the isolates belonged to the *A.* section *nigri* group, and some of the species might be able to produce ochratoxin A.

20th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 2: DETECTION, EXTRACTION, AND ANALYSIS OF AFLATOXIN; POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS

Moderator: Pat Donahue, Kraft Foods, Inc.

Phenolic Natural Products in Tree Nuts: Influence on Aflatoxin Levels in Oxidative – and Drought-Stressed Aspergillus flavus.

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California tree nuts (almonds, pistachios and walnuts) are an extremely valuable crop, with an aggregate value of $3.64 billion in 2005, of which $2.14 billion (59%) came from exports, primarily to the European Union (EU). These export markets are severely impacted by aflatoxin contamination of shipments. An increasing number of aflatoxin notifications by the Rapid Alert System for Food and Feed (RASFF) has led to special measures being imposed by the EU requiring that as of 1 September 2007 every consignment of almonds imported from the U.S. will be subjected to 100% surveillance (i.e. every consignment tested for aflatoxin). However, almonds tested under the Voluntary Aflatoxin Sampling Plan (VASP) before shipment will be subject to 5% surveillance.

These restrictions urgently require the identification of factors that can control aflatoxin production and accumulation in tree nuts. Our approach to the problem is based on the hypothesis that differential susceptibility of tree nut species and cultivars to contamination indicates presence of natural factors that inhibit aflatoxin formation. Thus, in spite of equal susceptibility to insect damage, only one RASFF aflatoxin notification for walnuts was issued in 2005-2006, whereas almonds and pistachios received 63 and 24 notifications, respectively. This data correlates well with our *in vitro* studies of aflatoxin susceptibility in California tree nuts, showing that walnut cultivars were extremely resistant to aflatoxin formation whereas almond cultivars were much more susceptible. Research has therefore been designed to identify endogenous constituents of almonds/pistachios/walnuts that suppress aflatoxin formation and the environmental factors responsible. The advantages of this approach are: no artificial compounds or organisms are required; resistance compounds are amenable to manipulation through breeding, selection and agronomic practices; and, anti-aflatoxigenic compounds are present throughout growth, harvest, processing, shipping and distribution. A corollary is that natural constituents provide probes to investigate chemogenetics of aflatoxin biosynthesis and catabolism

Experiments *in vitro* have shown that oxidative stress induced by addition of *tert*-butyl hydroperoxide significantly enhanced aflatoxin production by *Aspergillus flavus* strain 4212, a pistachio isolate, whereas fungal weight was only slightly reduced. These results have been corroborated with *A. flavus* strain 3357, a peanut isolate. Incorporation of natural antioxidants occurring in tree nuts into the media reduced the aflatoxin formation by 59.5-98.8%, while fungal weights were restored to the levels of control over a nine-day time course experiment with addition of the antioxidant tannic acid.

Time course experiments under high (96%) and low (15%) humidity conditions showed similar effects for both *A. flavus* 4212 and 3357, with aflatoxin levels being maintained at low humidity but declining by >120% under high humidity. Drought stress in plants produces reactive oxygen species and is therefore a form of oxidative stress. In the presence of tree nut antioxidants low humidity aflatoxin production was reduced by >82%. Sclerotia production was much greater under high humidity conditions but the presence of antioxidants did not significantly affect this parameter. HPLC analysis of aflatoxin production under high humidity conditions showed that although aflatoxin declined by 58% between day 6 and day 10, no new detectable compounds were produced, indicating complete disruption of the molecule, including the aromatic ring system. Avoidance of drought-stress conditions and the presence of sufficient levels of natural antioxidants in tree nut crops should therefore result in lower aflatoxin levels, without the formation of other potentially toxic precursor or catabolic metabolites.

Antifungal Activity in Tex6 Maize Seeds is Associated with a Chitinase and Zeamatin that Appear to Act Synergistically

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Production of pathogenesis-related (PR) proteins in response to pathogen infection is a hallmark of plant disease resistance. Through our efforts to identify antifungal and anti-aflatoxigenic activities present in kernels of the maize inbred line Tex6, we have purified two antifungal proteins, the major seed chitinase (Chitinase A) and the thaumatin-like protein zeamatin. These two proteins co-purify through liquid chromatography steps, presumably due to their almost identical pIs (8.16 and 8.18, respectively) predicted from the protein sequences. Removal of ChiA from an active fraction by chitin-affinity separation abolishes the antifungal activity observed when both proteins are present. Re-addition of ChiA protein restored the antifungal activity. Presumably, ChiA facilitates increased access of zeamatin to the fungal plasma membrane by modifying the fungal cell wall. ChiA and zeamatin are encoded by members of large gene-families, many of which are induced by *A. flavus* infection, as demonstrated by DNA microarray analysis of field-inoculated maize ears. We are testing if the interaction between ChiA and zeamatin is a general or specific phenomenon and evaluating their degree of synergism. We will also determine the abundance of these two proteins, and their degree of interaction in other maize lines.

The Role of the PAL/PAC Signaling Pathway during Transcription of Genes Involved in Differentiation and Sterigmatocystin Synthesis in *Aspergillus nidulans.*

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Over the years, numerous studies have contributed to establish the physiological determinants that lead to mycotoxin production in *Aspergilli*. Among the most important of these determinants are carbon and nitrogen sources and pH of the medium. It is clear now that simple carbohydrates such as glucose and sucrose induce Aflatoxin (AF) biosynthesis in *A. parasiticus* and Sterigmatocystin (ST) biosynthesis in *A. nidulans*. It has also been widely reported that ammonium supports AF biosynthesis in *A. parasiticus* while nitrate stimulates ST production by *A. nidulans*. However, studies focusing on the effect of pH on mycotoxin synthesis have often produced complex and at times contradictory results.

Regulation of fungal metabolism by pH is mostly effected through the PAL/PAC signaling pathway, described for the first time in *A. nidulans*. PacC is a transcription factor that is activated at alkaline pH by the products of 6 pal genes that sense external pH and transduce the activating signal, which leads to repression of genes expressed preferentially under acidic growth conditions and promotes the expression of genes expressed preferentially under alkaline growth conditions, including PacC itself.

The objective of this work is to elucidate the role of this signaling pathway in the regulation of genes involved in conidiation and ST synthesis in *A. nidulans*. To achieve that, we used three *A. nidulans* strains kindly donated by Miguel A. Peñalva (CSIC, Spain): MAD134 (biA1, palA1, wA3), which is an acidity-mimicking mutant; the alkalinity-mimicking mutant MAD135 [biA1, pacC^{c}14 (5-492)] and their parental strain MAD002 (biA1). They were grown on Kafer's minimal medium with or without 100 mM citrate buffer, supplemented with 5% sucrose and incubated at 37 °C for 72 h. We evaluated growth, conidiation, ST synthesis and the expression level of some of the genes from the G-Protein/cAMP/PKA signaling pathway that connects conidiation and ST/AF production in *Aspergilli* under these conditions.

Our results with the parental strain, MAD002, show that there is an effect of the initial pH of the medium on growth and activation of ST synthesis, which took place under alkaline growth conditions. Supporting this, the acidity-mimicking mutant, MAD134, presented reduced growth and did not produce ST under acidic, neutral or alkaline growth conditions; while the alkalinity mimicking mutant MAD135 showed the opposite phenotype.

The ST pathway-specific regulator gene aflR proved to be regulated by the Pal/Pac signaling pathway; however, we found that the genes fluG and flbA are not regulated by such regulatory circuit.

Interestingly, the observed expression levels of the main transcription factor involved in conidiation; brlA, suggest that it might be regulated by pacC at alkaline pH, but not at acidic pH; therefore, there must be at least an additional conidiation's regulatory mechanism active at low pH values.

Panel Discussion: Detection, Extraction and Analysis of Aflatoxin; Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

Panel Chair: Russell Molyneux

Questions and Answers (Gary Payne):

Q. Do you have a prediction as to why Tex6 is aflatoxin resistant?
A. Probably an earlier expression of the inhibitory compounds.

Q. What is the nature of these compounds?
A. Small, organic compounds (MW <500).

Q. Are all the inhibitors from Tex6? What is the percentage inhibition due to these compounds
A. Yes they are from Tex6. The inhibitors work in concert with each other, not sure how much activity is due to each compound alone.

Q. Are chitinase and zeamatin close in the corn genome?
A. Not known.

Q. Were non-inoculated, non-wounded controls included?
A. Yes, but not analyzed due to expense.

Questions and Answers (Russell Molyneux):

Q. Is there an antioxidant that will reduce aflatoxin below the EU level?
A. All the experiments were done *in vitro* so it is impossible to answer that question. However, the correlation with presence of phenolics is strong, with walnuts, which have the highest levels, showing virtually no aflatoxin contamination. It is important to note that the phenolic natural products are present throughout all stages of growth, harvesting and processing, so they always have potential to inhibit aflatoxin biosynthesis.

Q. What are the other minor peaks in the HPLC analysis of time-course of aflatoxin production and catabolism?
A. Not identified but possibly aflatoxin biosynthetic precursors.

Q. Is there any difference observed due to position of the hydroxy groups on the aromatic ring?
A. Antioxidant activity is known to be dependant on position and number of hydroxy groups but no structure-activity study was done. The focus was on compounds that occur naturally in tree nuts.

Questions and Answers (Francisco Delgado Virgen):

Q. How was RT-PCR quantified?
A. By brightness of the band, after reaction with ethidium bromide and UV irradiation.

Analysis of the Relationship between Aflatoxin Concentration and Hyperspectral Fluorescence Response in Maize

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The focus of the present experiment was to analyze the relationship between fluorescence emission and aflatoxin concentration in single corn kernels. Hyperspectral imaging technology offers a novel non-invasive approach toward screening for toxigenic fungi and the presence of toxins associated with them by identifying a given specimen based on its spectral signature. Our previous research has determined that additional spectral information about the fungus as well as the presence or absence of the toxin may be gained from combining hyperspectral imaging with UV excitation, where the resulting fluorescence image reveals more information than the reflected image. The objective of the present study was to take advantage of the UV elicited response of corn kernels contaminated with *A. flavus* expressed under 365 nm UV excitation and analyze the response measured with a hyperspectral VNIR sensor using statistical models.

Infected corn samples were harvested in 2006 from a cornfield in Tifton, GA. Kernels exhibiting fluorescence were identified under UV light source with excitation wavelength centered at 365 nm, and extracted. Several adjacent, non-glowing kernels from the same ear were also extracted and used as controls. Only whole, undamaged kernels were imaged using ITD's VNIR 100E hyperspectral sensor. The fluorescence image was acquired under the UV light source with 500 ms integration time due to the low light intensity. A dark current image was taken for calibration purposes. Two calibration methods were used for image calibration. A radiometric calibration process was used to convert the raw digital counts into percent reflectance using the dark current image, and the Internal Average Relative Reflectance (IARR) calibration method which normalizes images to a scene average spectrum was also applied. Regions of interest were created for each kernel to separate corn from background and each kernel was assigned a unique identifier. Spectral signatures and statistical information were extracted from each kernel.

After imaging, each corn kernel was crushed and weighed. Each sample was extracted with methanol/water (80/20%). Extracted samples were diluted and passed through AflaTest affinity columns. Samples were eluted with pure methanol and measured with a fluorometer from VICAM.

VNIR-fluorescence emission spectrum was correlated with aflatoxin content of each corn kernel across all wavelengths between 400 and 600 nm. A weak negative correlation of the two measures was noted between 400 and 450 nm with r = -0.67. Multiple linear regression model across all 74 fluorescence bands was used to predict aflatoxin content based on spectral information. When the non-positive estimates were assigned to zero by the model, the Coefficient of Determination $r^2 = 0.72$. Resubstitution and Cross-Validation, two-class discriminate analysis models, were used to separate two classes: contaminated (≥ppb level) and control (<ppb level). The ppb levels used were: 4 (European standard), 7 (ethanol standard), 20 (US food standard), and 100 (US feed standard). Resubstitution fits a single classifier to the data, and applies this classifier in turn to each data observation. Cross-validation (in leave-one-out form) removes each observation in turn, constructs the classifier, and then computes whether this leave-one-out classifier correctly classifies the deleted observation. Overall, the Resubstitution model showed higher classification accuracy compared to the Cross-Validation model. For example, the average classification accuracy for the 20 ppm level was 87% according to the Resubstitution model and 84% according to the Cross-Validation model. The two models differ in classifier error estimates.

Our results illustrate the potential of using fluorescence hyperspectral curves for estimating aflatoxin content in individual corn kernels.

Determination of Aflatoxins in Raw Grain and Seeds at PPT Levels

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A chemical cleanup procedure for low-level quantitative determination of aflatoxins in major economically important agricultural commodities using HPLC has been developed. Aflatoxins were extracted from a ground sample with MeOH-H_2O (80:20, v/v), and after a cleanup step on a minicolumn packed with Florisil, aflatoxins were quantified by HPLC equipped with a C_{18} column, a photochemical reactor, and a fluorescence detector. Water-MeOH (63:37, v/v) served as the mobile phase. Recoveries of aflatoxins B_1, B_2, G_1, and G_2 from peanuts spiked at 5, 1.7, 5, 1.7 ng/g were 89.5 ± 2.2, 94.7 ± 2.5, 90.4 ± 1.0, 98.2 ± 1.1, respectively (mean ± SD, %, n=3). Similar recoveries, precision, and accuracy were achieved for corn, brown and white rice, cottonseed, almonds, Brazil nuts, pistachios, walnuts, and hazelnuts. The quantitation limit for aflatoxins in peanuts was 15 pg/g for aflatoxin B_1, and 5 pg/g for aflatoxin B_2. The minimal cost of the minicolumn allows for substantial savings compared with available commercial aflatoxin cleanup devices.

20th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 3: CROP RESISTANCE – CONVENTIONAL BREEDING

Moderator: David Gibson, Texas Corn Producers Board

Use of Molecular Markers to Create Corn Hybrids with Low Aflatoxin

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This project is creating high yielding, commercially acceptable, corn hybrids with resistance to Aspergillus ear rot and low levels of aflatoxin using genes from known sources of resistance. This is being accomplished by using molecular marker assisted backcrossing to move chromosome regions associated with resistance from resistant inbreds Tex6 and Mp313E into the commercially elite, but susceptible, inbred lines FR1064, LH195RR, LH310, LH311 and LH312. With FR1064 we have crossed FR1064 with Mp313E and backcrossed three times to FR1064 while selecting for the chromosome four regions from MP313E that have been associated with resistance. Two inbreds have been selected for commercialization. I also am pyramiding the chromosome region from Mp313E with chromosome regions from Tex6 associated with resistance. These lines are much different than those with just the Mp313E chromosome four crossed into FR1064. For those lines I took a version of MP313E crossed with FR1064 and backcrossed twice to FR1064 with chromosome four from Mp313E and crossed it with a line that was developed from the cross of B73xTex6 then backcrossed to B73 and selfed that had resistance from Tex6 on chromosomes 8,10,2, and 5. The resulting lines are later in maturity than resistant versions of FR1064. Two versions of the pyramid lines have been selected for commercialization. In years and locations where differences are significant, testcross hybrids with genes from just Mp313E or the pyramid lines have less aflatoxin than crosses with the original FR1064 and similar or better yield.

In the summer of 2007, I had 11 locations in southern areas in cooperation with two seed companies where resistant hybrids were evaluated for yield and, in many cases, aflatoxin. Hybrids were created by crossing three different commercial male lines with selected resistant lines. B-H genetics had plots at five locations in Texas (two locations near Ganado, and one at Garwood, Granger, and Knippa) and Terral Seed had six plots in Mississippi, Alabama, Texas and Louisiana. This is in addition to SERAT evaluations and a test by Steve Moore from LSU. In general, the resistant hybrids compared favorably with respect to yield compared to commercially used hybrids when the same male parents were used. In many cases they had yields superior to or equal to commercial hybrids where parentage was unknown. They did not compete well with hybrids that had LH310 or LH311 as female parents when conditions were favorable for yields above 200 bushel.

In Urbana, I had 9 acres of seed production using three male parents to make even greater volumes of hybrid seed for testing next year. Also, at Urbana, the resistant inbreds were self pollinated in order to produce large volumes of seed. Producing commercial bag quantities of hybrid seed is taking considerable time especially considering I am not well equipped for such production.

I also am making good progress in crossing resistance into the widely used line, LH195RR. I currently am at backcross six self. This summer several hundred plants of the backcross six self generation were genotyped using 40 molecular markers in the chromosome 4 region by Monsanto. We identified 168 homozygous lines that are being crossed to LH210 in Hawaii nursery and will be evaluated next summer for yield and aflatoxin in two locations. From that, the most promising will be selfed. LH195 with resistance from Mp313E should be more resistant than the FR1064 lines because LH195 is better adapted to southern environments. I also am backcrossing chromosome 4 regions from Mp313E into LH310, LH311, and LH312. Backcross three will be done in the greenhouse this winter. These three inbreds have clearly demonstrated very high yield and are available with complete sets of transgenic traits.

I also am creating lines with various segments of chromosome four from the resistant line Mp313E in a LH195 background. With that project Monsanto is providing detailed molecular marking of the chromosome 4 region and we expect to identify several hundred selections with different parts of that

chromosome. Eventually lines can be created that will have different segments of the chromosome 4 region associated with resistance. Those lines will need to be phenotyped for resistance. Those lines will be very helpful in determining how much of the chromosome four is required for resistance.

Generally, I am making very good progress toward creating high yielding, agronomically acceptable hybrids with lower aflatoxin in grain than many commercially used hybrids. This research is creating germplasm and techniques that can be used by any commercial company to create resistant hybrids.

Field Based Assessment of Cotton Cultivars for Aflatoxin Contamination

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Aflatoxins produced by Aspergillus species are problematic in cottonseed produced in the irrigated agriculture of the desert southwestern United States. When used for animal feed, restrictions on the amount of allowable aflatoxin contamination in seed often limits or eliminates sales of cottonseed to dairies and feed lots. Even though wide scale planting of new "Bt varieties" of cotton have eliminated much of the damage to bolls that is a major entry point for Aspergillus flavus, aflatoxin contamination is still a major problem. At present there are no assays available to compare susceptibility of cotton cultivars to aflatoxin contamination under field conditions. Development of a reliable, flexible field assay and elucidation of the etiology of contamination of seed in uninjured bolls are the objectives of this project.

In 2006 trials, experiments were conducted at The University of Arizona Maricopa Agricultural Center (MAC) near Maricopa, Arizona using commercially available portable greenhouses to increase both temperature and humidity in the cotton canopy. Greenhouses covered a 2 row by 6 ft block of cotton. A variety trial was conducted to compare susceptibility to aflatoxin contamination in cultivars Hammer, DP449BR, ST4892 and ST 5599. Relative humidity and temperature were monitored using HOBO data loggers. All plots were inoculated, and there were four replications of each treatment. In another study at The University of Arizona Campus Agricultural Center (CAC) near Tucson, Arizona, individual bolls were inoculated when closed, fully open or partially open and covered with plastic bags for 6 days.

Results of the MAC greenhouse variety experiment indicate that cotton in greenhouses was exposed to sustained relative humidity of 90 to 100% and temperatures of 27 to 43^{o}C; cotton outside was exposed to relative humidity of 60 to 100% and temperatures of 21 to 32^{o}C. Results show that fuzzy seed from both partially open and fully open bolls inside greenhouses had significantly more aflatoxin contamination than similar bolls outside the greenhouses, but there was no significant difference in aflatoxin contamination among the varieties tested. Within greenhouse treated cottonseed, there was significantly more BGYF pigment, an indication of infection, in partially open bolls compared to fully open bolls. Results at CAC show that seed from bolls inoculated when closed were not contaminated, that there was very little contamination of fully open bolls, and that there was significantly higher contamination in partially open bolls in Hammer, a variety characterized by weak seed coats.

Results show that the portable greenhouses may provide a reliable and efficient way to evaluate the relative susceptibility of cotton cultivars to aflatoxin contamination. Advantages of these greenhouses include mobility, low cost and easy availability. The primary disadvantage is the labor involved in placing them securely in the field. Results from individually bagged bolls indicate that fungal entry and aflatoxin contamination may be taking place when bolls just begin to open. Further studies may show whether varietal differences in timing of boll opening correlates with aflatoxin contamination.

Is Crop Resistance Through Conventional Breeding Relevant to Reducing Aflatoxin Contamination?

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During this 20th annual meeting of the Multicrop Aflatoxin Elimination Group we thought it would be appropriate to evaluate whether crop resistance through conventional breeding is relevant to reducing aflatoxin contamination. Our presentation revolved around this issue, with a primary focus on results from peanut. There are two requirements for successful plant breeding. First, there must be genetic variation for the trait of interest. Second, we must have accurate and reliable screening techniques that can be used to identify individuals with favorable genetic combinations. Results presented in this and previous workshops have clearly indicated that there is genetic variation for reduced aflatoxin contamination in corn and tree nuts. Conventional breeding has not be an important focus for the cotton group, however, preliminary data has been presented that indicated there is also genetic variation for this trait in cotton. For peanut, we screened the core collection and identified 11 accessions that showed at least a 50% reduction in aflatoxin over multiple environments. Drought tolerant material was also evaluated resulting in the identification of 10 additional accessions that showed at least a 50% reduction in aflatoxin. It is likely that work in the area of molecular genetic will also add to the genetic variability available for conventional breeding efforts. Accurate and reliable screening techniques are more problematic. Environmental effects on aflatoxin contamination for all crops are large, and data sets typically have large CV's. We have developed rainout shelters and a field inoculation technique to reduce CV's. We also use a data scaling system so that we can make comparisons across environments. We have used the tools we have to develop late generation breeding lines with relatively low aflatoxin and relative high yield, and are releasing C76-16 as peanut germplasm with improved resistance to drought and aflatoxin contamination. Additional releases of germplasm and/or cultivars are expected. Peanut root-knot nematodes have been shown to increase aflatoxin contamination of peanut. We have recently released Tifguard as a nematode resistant cultivar, and hope it can be used as a tool to reduce aflatoxin contamination throughout the Southeastern peanut production region. It seems likely that crop resistance through conventional breeding will play a critical role in reducing aflatoxin contamination in U.S. agriculture.

Developing Resistant Maize Inbreds: A Progress Review with Future Projections

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One goal of the research collaboration between Southern Regional Research Center (SRRC) and the International Institute of Tropical Agriculture (IITA) is to develop maize inbreds, through breeding and selection that have good resistance against aflatoxin contamination by *Aspergillus flavus* in useful agronomic backgrounds. U.S. parents for the initial crosses contributed by SRRC were CI2, MI82, T115, GT-MAS:gk, OH516, SD18, Mp420, B73xTex6 and MO17xTex6; 4001, 1368, Babangoyo, KU1414, 9071, and 9450 comprised the parents contributed by IITA. The former group had demonstrated resistance to aflatoxin contamination in numerous field trials while the latter group, selected in Central and West Africa for moderate to high resistance to fungal ear rots (by several fungi including *A. flavus* and *Fusarium verticillioides*), showed potential for aflatoxin-resistance in the Kernel Screening Assay (KSA) and potential for contributing new traits to the pool of resistance genes. Two populations were made from crosses: a temperate population consisting of 75% U.S. germplasm and a tropical population with a 50% contribution from each source. Lines were selfed and visually selected on foliar disease resistance and agronomic characteristics through the S_4 generation. After this generation, lines were selected on aflatoxin-resistance through KSA and field trials and on agronomic performance. Presently, 8 inbred lines in S_8 to S_{10} stages of inbreeding are being registered and released as sources of resistance. The selection of these lines for release was based not only on aflatoxin-resistance, but on good agronomic characteristics as well. These lines can be used in U.S. breeding programs for development of commercial cultivars with aflatoxin-resistance and in African national programs. They may also be useful in combating foliar diseases.

The second goal of the research collaboration is to identify markers in the resistant inbred lines coming out of the breeding program. Towards this goal, comparative proteomics is being employed to identify proteins associated with resistance (RAPs) in near-isogenic lines (varying in aflatoxin accumulation by the KSA), discovered among program breeding materials. Most RAPs identified are constitutively produced and fall into one of four protein categories: 1) antifungal; 2) storage; 3) stress-related; or 4) other (including putative regulatory proteins). Previous investigations have demonstrated that while inducible proteins are needed for kernels to resist aflatoxin accumulation, production of a high level of constitutive proteins in resistant kernels is a major factor differentiating them from susceptible kernels.

Several RAPs have been further characterized through physiological and biochemical investigations to determine their potential role in resistance and, therefore, fitness as markers for breeding programs employing the above-germplasm. Corresponding genes of several RAPs have been sent to ARS-Mississippi State for QTL analysis. Three, the 14 kDa trypsin inhibitor (TI), glyoxalase I (GLX I), and pathogenesis-related protein 10 (PR10) are being investigated using RNAi gene silencing as well. PR10-silenced transgenic kernels have shown significant, and in the case of some transgenic events, even drastic increases in susceptibility to *A. flavus* growth and aflatoxin production.

More aflatoxin-resistant inbred lines are expected to be released over the next several years through the IITA-SRRC collaborative project. These lines can serve not only as sources of resistance for U.S. breeding programs and African national programs, but can be used as parents to make new

crosses (possibly initiating new combinations of resistance genes) for continued breeding efforts to combat aflatoxin contamination of maize.

Breeding Corn Germplasm for Reduced Aflatoxin Contamination

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Our goal is to breed corn germplasm concurrently for reduced aflatoxin contamination and improved agronomic performance. Selection for reduced aflatoxin contamination is based phenotypic traits which include long tight husk cover, flint kernel texture, good anthesis silking interval and good kernel integrity. Our germplasm is coming from crosses between and among temperate, subtropical and tropical germplasm and selected for combinations of the previously mentioned traits. Yellow and white corn inbreds were evaluated as hybrids with either LH195 or LH210 as the tester. Yellow trials were evaluated for aflatoxin at College Station, Weslaco and Corpus Christi and white trials were evaluated for aflatoxin at College Station and Weslaco. The trials at College Station and Weslaco were inoculated utilizing the silk channel method 10-20 days post flowering, while the trials at Corpus Christi were inoculated by distributing colonized kernels between the rows at silking time.

Yellow inbreds derived from crosses containing CML288 (25 ng g^{-1}) and CML327 (24 ng g^{-1}) or derived from the LAMA (23 ng g^{-1}) populations tended to accumulate less aflatoxin than commercial checks (111, 633 and 120 ng g^{-1}). Environmental conditions at College Station were not favorable for aflatoxin accumulations due adequate rain and cool temperatures during flowering and grain fill. The Corpus Christi location of the yellow trial is currently being quantified. Several white inbreds derived from CML269 and Tx130 (31.9 and 56.2 ng g^{-1}) and lines derived from CML78 (32.9 ng g^{-1}) accumulated fewer aflatoxins than the checks (179.9 and 130.8 ng g^{-1}). Each of the previously mentioned white inbreds had hybrid combinations with grain yield superior to or equal to the commercial checks. A set of Recombinant Inbred Lines created from the cross of B73o2 and CML161 was evaluated for QTL for reducing aflatoxin accumulations. Transgressive segregation was observed in the RILs. Significant QTL were detected on chromosome 1, with other potential QTL detected on chromosomes 3, 5 and 9. We participated in the SERAT Trial, to further characterize and evaluate our material at multiple locations across the southern U.S. and Illinois. The College Station SERAT location had significant differences for grain yield ($p>.0001$) and aflatoxin ($p>.01$).

Several yellow and white sets of germplasm have shown reduced aflatoxin accumulations over multiple locations and agronomic performance which is equal to or better than commercially available hybrids. Further evaluations of the QTL are planned in hybrid combinations to determine stability. Multilocation evaluations allows for better estimation of aflatoxin accumulations. Making crosses between lines which have had a previous reduced response to aflatoxin accumulations could further reduce aflatoxin accumulations further improve agronomic performance.

Progress in Breeding Aflatoxin-resistant corn

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Aflatoxin contamination of corn by *A. flavus* is a chronic problem in the southern United States where a hot and dry environment stresses the plants and increases aflatoxin production. Producers need new hybrids that are adapted to the southern environments and resistant to aflatoxin contamination. Our strategy for breeding aflatoxin resistant corn is to improve drought and heat tolerance, improve corn earworm resistance, and stack the resistance genes from known aflatoxin resistant sources such as MP715 into the germplasm adapted to the southern environments. The objective of this study was to develop multiple-stress tolerant corn inbred lines and hybrids to reduce aflatoxin contamination.

This report presents the results from two experiments. Exp.-1 used 21experimental and four commercial hybrids (Pioneer hybrid 31B13, Garst 8285, Triumph 1416, and DKC66-80) and was conducted in 2005 and 2006. Exp. 2 used 26 experimental hybrids and four checks (P31B13, Gartst 8285, DKC66-80, and BH8913RRYCGB) and was conducted in 2007. Both experiments were carried out in Lubbock, Halfway, Dumas, Corpus Christi, and Beeville in Texas and Mississippi State, MS. The Lubbock and Halfway locations had optimum and limited irrigation treatments. The experimental hybrids in both studies were different and developed by the corn-breeding program of the Texas Agricultural Experiment Station (TAES) in Lubbock, TX. They were chosen for improved drought tolerance and earworm resistance. In Lubbock and Halfway, plants were inoculated one week after silking by injecting 3-ml *A. flavus* conidia (1.5×10^6 conidia/ml) into silk channels. In Corpus Christi, Beeville, and Mississippi State, corn kernels colonized by *A. flavus* strain NRRL3357 were distributed between all rows when the first hybrid was at the mid-silking stage. A limited late planting date was used in Corpus Christi, Beeville and Mississippi State to encourage severe drought stress at later stages of maturity. The tests used a randomized complete block design with nine replications at Corpus Christi and Beeville, and three replications in other locations. Ears from each plot were hand-harvested, rated for corn earworm feeding damages and ear traits, and then threshed grain yield. Grains were initially ground in a Romer mill at the coarse grind setting. After thoroughly mixing the ground kernels, a 200 g sub-sample was ground again at the finest grind setting on the mill. Aflatoxin B1 assay was done on 50 g sub-samples of the finely ground material for each composite replication using the Vicam immunoassay/ fluorometer system. Standard ANOVA was performed to test the difference among hybrids. Natural log transformation of aflatoxin data was made before data analysis.

Results of Experiment 1: In 2005, the average aflatoxin of 25 hybrids was 6268 ppb at Beeville, 2023 ppb at Corpus Christi, and 1038 ppb at Mississippi State. In 2006, the average aflatoxin of 25 hybrids was 1029 ppb at Corpus Christi and 612 ppb at Mississippi State. The aflatoxin was significantly different among hybrids. Combined over five environments, S2B73BC x NC300 and S1W x CML343 had 87% and 72% less aflatoxin than the average of four check hybrids (2194 ppb), while WA22W x S1W, Tx202 x CML343, and CUBA117:S15-1A-1 x Tx205 had 62%, 51%, and 51% less aflatoxin than the check means, respectively.

Results of Experiment 2: Results from Mississippi showed that S2B73BC x NC300 (37 ppb), S2B73 x NC300 (59 ppb), B110xCML343xS1)xB73)F5xMP715-1-4-7-2-1-1-B-1 x C2A554-4 (55 ppb), B110xCML343xS1)xB73)F5xMP715-1-4-7-2-1-1-B-1 x C2A554-4 (89 ppb), and B110xCML343xS1)XB73)F5xMP715-1-4-7-B-1-1-2-1 x C2A554-4 (101 ppb) had significantly lower aflatoxin than the checks (280-2400 ppb) and test mean (378 ppb). The lines derived from the breeding crosses of MP715 (aflatoxin resistant source) appear to have good levels of aflatoxin. In

both experiments, the hybrids with much lower aflatoxin than commercial checks also produced comparable grain yield.

Generation of Expressed Sequence Tags (ESTs) from Cultivated Peanuts for Gene Discovery and Marker Development

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Aflatoxin contamination caused by *Aspergillus* fungi is a great concern in peanut production worldwide. Pre-harvest *Aspergillii* infection and aflatoxin contamination are usually severe in peanuts that are grown under drought stressed conditions. Genomic research can provide new tools to study plant-microbio interaction and enhance crop genetic improvement. However, genome research in peanut is far behind other crops due to the shortage of essential genome infrastructure, tools, and resources. The objectives are to develop tools and resources and provide putative genes and EST-based markers for peanut researchers and provide microarray technology for peanut community to study peanut-Aspergillus interaction and to mitigate aflatoxin contamination in peanut. We have completed sequencing the 5' ends of a total of 44,064 clones from 10 cDNA libraries, resulting in over 13,824 UniESTs, which will be used for production of a peanut long-oligo microarray for gene expression profiling. These sequence data have been made available to the community in order to develop genomic tools and resources for deciphering the chromosomal location and biological function of genes in the peanut genome and mitigating peanut food safety issues (CD037499 to CD038843, ES702796-ES717096, ES717097-ES724546, ES751523-ES768453). Our research objectives are two folds: 1) construction of peanut 70-mer oligo microarray in collaboration with Dr. Nierman and Dr. Yu, and 2) development of markers/genes associated with the resistance. A total of 13,824 ESTs have been assembled. Oligo probes were designed by comparing peanut EST sequences against *A. flavus* sequences, resulting in 8,402 oligos designed from 8,402 ESTs and 5,422 ESTs that no oligos were designed. The design of the microarray will contain the 8,402 peanut genes and over 12,000 *A. flavus* genes. The availability of this peanut-Aspergillus array will give us an edge in studying peanut-Aspergillus interactions and help us identify genes involved in both fungal invasion and crop resistance. A panel of 16 diverse peanut genotypes has been screened for genetic diversity, and two mapping populations have been developed between Tifrunner and GT-C20 and SunOleic 97R and NC94022 for advancement to RILs (recombinant inbred lines).

Development of Corn Inbred Lines with Reduced Preharvest Aflatoxin Contamination and Identification of Genes/Markers for Breeding and Germplasm Evaluation

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Host plant resistance is a highly desirable tactic that can be used to manage aflatoxin contamination. Screening and identification of corn germplasm for resistant traits for crop improvement and molecular marker development will bring new genetic diversity into US corn germplasm. Using the combination of genetic and genomic approaches to elucidate crop defense pathways and understand the resistance mechanism and regulation will enhance genetic breeding for better crop with improved resistance. The field screening and evaluation of inbreds from different regions of the world in 2006 and 2007 have identified some lines with reduced aflatoxin contamination. Two inbred lines, GT601 (AM-1) and GT602 (AM-2), have been released. GT601 (AM-1) (PI 644026) and GT602 (AM-2) (PI 644027) are yellow dent maize (*Zea mays* L.) lines developed and released jointly by the USDA-ARS Crop Protection and Management Research Unit and the University of Georgia Coastal Plain Experiment Station in 2006. GT601 (AM-1) and GT602 (AM-2) were developed by seven generations of self-pollination from a maize population GT-MAS:gk (PI 561859). This population was derived from an older hybrid visibly segregating for fungal infection by *Aspergillus flavus,* and selected for resistance to the fungal infection and reduction of aflatoxin contamination.

Maize line Tex6 was reported to have reduced aflatoxin contamination. By using maize 70-mer oligo-array, our goal was to study gene expression profiles in the kernels during late developmental stages in order to understand gene expression patterns of storage components, especially defense related genes. We analyzed temporal patterns of gene expression profiles in developing kernels at 25, 30, 35, 40 and 45 days after pollination (DAP) under normal irrigation or drought stressed condition. In comparison with 25 DAP, expression of 211 genes was significantly different at 45 DAP ($P < 0.05$), and 134 genes was up-regulated at normal irrigation condition. In contrast, under drought stressed condition we identified 154 defense-related genes expressed significantly (two fold change) from 35 DAP to 45 DAP, and 73 genes identified as down regulation are classified as disease resistance, stress response, and antioxidants. Further analysis will be conducted to compare these defense-related genes identified under normal irrigation or drought stressed condition, and will be used to evaluate corn germplasm and inbred lines for association of these gene expressions and phenotypic traits such as drought tolerance and aflatoxin contamination.

Integrating Fungal Pathogen and Insect Vector Resistance for Comprehensive Preharvest and Postharvest Control of Aflatoxin and Almond.

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The record, 2.3 billion pound, 2007 California almond crop has necessitated a large proportion of harvested almonds to be field-stored under plastic tarps for weeks to months prior to processing. Environmental conditions within these temporary storage units have been shown to be conducive to continued *Aspergillus flavus* (AF) infection as well as the development of the navelorange worms (NOW) which acts as vectors to contaminate otherwise protected kernels. Under these conditions postharvest aflatoxin contamination can be as serious as preharvest contamination, particularly since standard control strategies of rapidly field-drying of kernel meatsand protecting drying nuts from worm feeding, are not applicable. Controlled laboratory studies have demonstrated that 1st-instar NOW larvae will initially feed on almond hull tissue for days to weeks until they've achieved suitable size to penetrate almond shell and seedcoat barriers to kernel feeding. Susceptible genotypes, such as the predominant variety *Nonpareil*, have been shown to be more susceptible to fungal infection of the hull tissue which, in turn, appears to promote NOW development and survival. Almond varieties and breeding lines have been shown to differ in both hull resistance to fungal development as well as seedcoat resistance to NOW infestation. We have released the new almond variety *Sweetheart*, which combines both hull and seedcoat -based resistance and has shown promising levels of pre-and post-harvest control of NOW infestation and AF infection in long-term regional field trials. The variety *Sweetheart* also possesses very high kernel quality and good yield potential making it immediately attractive to commercial growers.

Identification of Genes Associated with Resistance for Generation of Gene Specific Markers in Maize

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The opportunistic pathogen *Aspergillus flavus* infects important seed crops such as corn, cotton, peanuts, almonds and pistachios. In maize natural sources of resistance have been identified that exhibit significantly reduced aflatoxin accumulation; however, efforts to increase host resistance through traditional plant breeding have yielded little success. Using the maize microarrays we compared resistant (Mp313E) and susceptible (Va35) inbred maize lines 48 hours post-*A. flavus* infection. A total of 234 genes were identified as showing significant changes in expression during infection (P value ≤ 0.05), 123 of these genes were up-regulated in the susceptible line Va35, 95 up regulated in the resistant line Mp313E, 14 up regulated or unchanged during infection in both lines, and 1 single gene down regulated in both lines during infection. Of these genes 73 could be mapped within the maize genome with 28 within previously identified resistance QTL. Comparisons of the biological profile response, as characterized by GO annotation, of these genes revealed a striking difference in response to infection between the maize lines. Among two of the identified important genes are a tryptophan synthase in Mp313E and a transport inhibitor response protein in Va35. These two genes are critical components of the auxin pathway, thus possible playing a direct role in regulating pathogen response. When tryptophan is considered in light of *A. flavus* NRRL 3357 cultures grown in the presence of tryptophan accumulating less aflatoxin than cultures without tryptophan it is possible that these genes products directly affects aflatoxin production by *A. flavus*. Utilizing genes that can be linked to plant defense directly or through chromosome mapping gene-specific primers capable of discriminating between the resistant and susceptible genotypes are being developed. These gene-specific primers will be a valuable tool in the introgression of resistance into elite production lines.

Mycotoxin Levels and *Aspergillus flavus* Colonization of Corn and Soybean Under Different Cropping Sequences.

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A four year field experiment was initiated in 2005 to determine the effects of eight corn (*Zea mays* L.)-soybean (*Glycine max* L. Merr.) rotation schemes on aflatoxin and fumonisin contamination of the crops and colonization of the grain by *Aspergillus flavus*. Both the corn hybrid and soybean cultivar are glyphosate resistant and furrow irrigated. Aflatoxin levels in soybean averaged 2.3 and < 0.5 ppb in 2005 and 2006, respectively, while higher levels ($P \leq 0.05$) were found in corn 16.7 ppb and 37.1 ppb (2005 and 2006, respectively). *A. flavus* colonization was greater in corn (log 1.9 cfu g^{-1}) compared to soybean < 1.3 cfu g^{-1} in 2005. In 2006 similar colonization was observed among both crops (~log 3.3 cfu g-1). A higher frequency of aflatoxigenic *A. flavus* isolates were associated with corn compared to soybean in both years. Higher levels of fumonisin contamination were found in corn (2.6 ppm and 0.9 ppm in 2005 and 2006, respectively) compared to soybean <0.2 ppm in both years. The experiment has not completed a full sequence of rotation treatments and thus complete assessment of alternate cropping systems on mycotoxin contamination is not yet possible.

Impact of Lignin on Resistance to *Aspergillus flavus* Infection in Maize

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Aspergillus flavus is a pathogenic fungus that can cause the accumulation of carcinogenic aflatoxins in maize and other oil-rich seeds such as cotton and peanut. Caffeoyl-CoA-O-methyltransferase (CCoAOMT), an enzyme in the lignin biosynthetic pathway, is more abundant in maize inbreds resistant to *A. flavus* infection and aflatoxin accumulation than in susceptible inbreds. Evaluation of lignin in cobs resistant or susceptible to *A. flavus* was done by studying lignin composition, cob protein antifungal properties, and characterizing the CCoAOMT gene. Lignin was extracted from an *A. flavus* resistant inbred (Mp313E) and a susceptible inbred (SC212m) 21 days after silking (DAS). Acetyl bromide and thioacidolysis extractions determined that there were no differences in total lignin content between the two inbreds. Thioacidolysis monolignol tests showed that resistant inbreds had two times more guaiacyl subunits than SC212m. Syringyl lignin was more prevalent in SC212m than Mp313E. Although both inbreds have the same amount of total lignin, more syringyl lignin in SC212m may make it easier for *A. flavus* to infect the cob. *In vitro* antifungal activity assays were performed using resistant and susceptible cob proteins extracted 21 DAS and analyzed 25 days after inoculation. The assay found that Mp313E and SC212m cob protein extracts inhibited fungal growth more than a buffer control, but there was no significant difference in fungal inhibition between the two inbreds. More research is needed to determine the overall impact of lignin on *A. flavus* resistance in maize.

Panel Discussion: Crop Resistance - Conventional Breeding

Panel chair: Don White

Summary of Presentations : The presentations included in the conventional breeding session also included one on crop rotation. A presentation on cotton dealt primarily with an inoculation technique that could allow classification of varieties that are likely to have higher aflatoxin. The results indicate some genetic variation which may be of value. The conventional breeding program with peanuts has been very successful. A number of sources of resistance have been identified and, through breeding, varieties with lower aflatoxin and favorable yield are being developed. A variety that has root knot nematode resistance which will likely result in less aflatoxin is being released. In another project, expressed sequence tags are being generated from cultivated peanuts which should be very useful in studying resistance and eventually creating molecular markers. There were a number of talks on corn that included studying potential mechanisms of resistance, developing new sources of resistance and developing commercially usable hybrids with acceptable yield and lower aflatoxin production than currently used commercial hybrids. Several novels sources of resistance have been or are being created. Several projects have created corn hybrids with acceptable yield and lower aflatoxin.

Summary of Panel Discussion: There was a discussion on the differences between the crops and its relationship to breeding programs. With tree nuts and peanuts the germplasm is in the public sector and new varieties are developed by the public sector. Therefore breeders have unrestricted access to breeding materials. In contrast, with cotton and corn most of the contemporary germplasm is owned by a private companies and new varieties are developed by the private sector. Therefore, public researchers must execute intellectual property agreements with private companies in order to access better yielding genetic material. Also, with both crops, growers demand transgenic traits for insect resistance which also requires input from the private sector that hold the rights to these traits. It is generally thought by the panel that outstanding progress has been made in peanuts in corn. Varieties with good yield, disease resistance and other traits that have lower aflatoxin are being developed. It was pointed out that in order for varieties with low aflatoxin to be commercially used they must yield the same or more than currently used varieties. There were questions on details of some of the presentations especially with cotton where evaluation of resistance is just now being done. There also were questions on a relationship of low aflatoxin in corn and resistance to other ear rot pathogens. Generally, much of the germplasm with low aflatoxin in grain is not necessarily resistant to Fusarium ear rot. Thus the relationship between resistance to Aspergillus ear rot and Fusarium ear rot is not good.

Genomic Profiling of Maize Response to FAW to Identify Resistance Markers

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Corn is contaminated every year with aflatoxin in one or more regions of southern states of Georgia, Lousiana, Texas, North Carolina and Mississippi. In 1998, about 50% of the corn produced in Mississippi State was found to be contaminated with aflatoxin above legal limits. Aflatoxins are potential carcinogens produced by *Aspergillus* fungi which easily invade corn plants through physical damages done by the insects like Fall Army Worm (FAW) and South western corn borer. Hence production of corn genotypes which are resistant both to fungal and insect infection should be adopted. We are working with resistant genotypes of maize and trying to find genes common to both fungal and insect resistance. In this move, we are performing a genomic profile to identify candidate genes involved in maize defense response during insect and as well as in fungal infections through high-throughput micro array analysis. SNPs will then be identified to generate molecular markers to assist pilot breeding program which will eventually benefit both corn breeders and corn producers.

Generation of Gene Specific Markers Associated with Aflatoxin Resistance in Maize

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Aflatoxins are the most potent naturally occurring carcinogens known. These mycotoxins are produced by the fungi *Aspergillus flavus* and *A. parasiticus* during infections of maize (corn), peanuts, cotton, and tree nuts. In Mississippi and other southern states, high heat and drought produce the ideal conditions for fungal growth and aflatoxin production in maize**.** To prevent the devastating health effects of aflatoxins these contaminate crops are destroyed, resulting in a large economic burden for southern farmers. Thus, the prevention of aflatoxin contamination in corn is of vital economic and health importance. Generation of maize lines resistant to aflatoxin accumulation is one of the most promising avenues of research; however, lack of gene-specific markers for resistance has hampered these efforts. Microarray studies of resistant (Mp313E) and susceptible (Va35) maize infected with *A. flavus* NRRL 3357 identified 234 genes that showed differential expression. By mapping these 234 genes to aflatoxin resistance Quantitative Trait Loci (QTL) of Mp313E, a subset of 25 genes have been selected for further study. These genes are being analyzed in numerous maize lines using the Roche LightCycler 480 to identify single nucleotide polymorphisms (SNP). Identified SNP are being used to generate gene specific markers capable of discriminating between the resistant and susceptible maize genotypes. These gene-specific primers will be a valuable tool in the introgression of resistance into elite production lines.

SERAT: The Southeast Region Aflatoxin Trial

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Aflatoxin contamination is a chronic problem of corn producers in the southeast United States. For the last five years, research groups from Georgia, Mississippi, Louisiana, Texas and Illinois have been screening corn germplasm under specific local environments. Several sources of resistance to aflatoxin accumulation have been identified and released, however; currently none is used directly in commercial hybrids. Aflatoxin accumulation is severely affected by the environment. Genotype by environment interaction for aflatoxin, different relative genetic response across environments, is normally significant. A testing network of environments across major growing areas affected by aflatoxin has been established to identify the most consistent stable sources of resistance. SERAT is a multilocation and multistate regional test of the most promising germplasm from each breeding program. Participants provide seed of a few hybrids and a testing location. Evaluations are conducted under inoculation with *A. flavus* following the protocols commonly used by each research group. In addition to aflatoxin, grain yield and other agronomic traits such as maturity, lodging, plant height, grain moisture, test weights, etc. are recorded. In 2007, SERAT tests were conducted at 10 locations: Alexandria, LA; Starkville, MS; Urbana, IL; Halfway, Ganado and College Station, TX; Tifton, Camilla, Leesburg and Cairo, GA. Tests at Halfway, TX, and Camilla, Leesburg and Cairo, GA were not inoculated. The test at Leesburg, GA was lost due to drought. Average aflatoxin was 178 ng g^{-1} at College Station and 755 ng g^{-1} at Tifton. Grain yield was variable across environments. Average grain yield was 176 bu a^{-1} at College Station; 202 bu a^{-1} at Tifton; 203 bu a^{-1} at Cairo; 148 bu a^{-1} at Camilla; 164 bu a^{-1} atHalfway; and 132 bu a^{-1} at Starkville. Most of the experimental hybrids accumulated lower aflatoxin than commercial checks and several experimental hybrids had similar grain yields to commercial checks. SERAT has fostered collaboration between researchers working to lower susceptibility aflatoxin accumulations in corn. Germplasm from each program has been identified which has lowered susceptibility to aflatoxin accumulation and comparable grain yields to commercial checks. With this collaborative regional test, we expect to identify germplasm with the most stable responses for lowered aflatoxin accumulations, along with other beneficial agronomic traits, and increase collaboration among researcher groups from different states.

DNA Markers for Resistance to Post-Harvest Aflatoxin Accumulation in Virginia-type Peanuts (*Arachis hypogaea* L.)

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Reduction of aflatoxin contamination is a long-standing objective of the U.S. peanut industry. Besides adopting certain cultural, harvest, and storage practices, resistant cultivars should be an effective and low-cost part of an integrated aflatoxin management program. However, aflatoxin contamination is expensive to measure and exhibits high environmental variation. Marker-assisted selection (MAS) can improve the efficiency and cost effectiveness of selection for traits of this sort. The objective of our research is to screen F_2 segregating populations derived from crosses between *A. cardenasii*-derived low-aflatoxin producing germplasm lines and high-aflatoxin producing cultivars, with candidate AFLP markers in order to identify markers closely linked to genetic factors controlling aflatoxin accumulation in these lines. The ultimate goal of our research is to utilize linked markers in a MAS program that could ultimately lead to peanut varieties with reduced potential for aflatoxin contamination.

A set of germplasm lines derived from an interspecific hybrid between *Arachis hypogaea* and a related diploid wild species, *A. cardenasii*, has been shown previously to have increased genetic potential to resist aflatoxin accumulation. We screened a collection of those lines for DNA polymorphism with particular attention paid to GP-NC WS 2 and other lines exhibiting reduced accumulation of aflatoxin. After screening 256 AFLP primer combinations, 835 polymorphic bands were identified among the interspecific lines. F-tests were performed at each of the 835 marker loci to test the null hypothesis that there were no significant differences in the mean aflatoxin value for genotypes with the marker versus genotypes without the marker. Results of the statistical analysis identified 57, 66, and 58 markers as being significantly associated with reduced accumulation of aflatoxin B1, aflatoxin B2, and total aflatoxin, respectively, in the interspecific lines (see Table 1 in the attachments section). During the summer of 2006, crosses were made between species-derived lines exhibiting reduced accumulation of aflatoxin with high aflatoxin producers in order to create populations for subsequent genetic work. F_1 hybrid seeds from these crosses were sent to our winter nursery in Puerto Rico, and F_2 progenies were harvested in the spring of 2007. Because the aflatoxin assay is a destructive one, embryos must be removed from cotyledons and cultured in regeneration media in order to regenerate full plants for DNA extraction, subsequent marker work, and generation advancement. In addition, given that F_2 seed was to be used immediately after harvest, potential problems with seed dormancy needed to be evaluated. Earlier this year, work in our laboratory aimed at determining the best protocols for tissue culture of peanut embryos, and evaluating dormancy problems.

Currently, we are in the process of regenerating plants from embryo culture. These populations will be evaluated for their ability to support aflatoxin accumulation using an *in vitro* assay, and also for presence or absence of candidate markers. Genotypic and phenotypic data produced in these tests will be analyzed in order to identify AFLP markers linked to reduced accumulation of aflatoxin. Linked markers can be used in the future to improve the efficiency of selection when transferring the low aflatoxin production of the interspecific lines into elite peanut breeding materials.

Screening Corn Accessions to find New Genes with Resistance to Aflatoxin.

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New genes are needed to develop corn hybrids with improved resistance to *Aspergillus flavus* and aflatoxin biosynthesis. In order to find new genes for resistance, corn lines must be screened. An original objective of this research was to screen new maize germplasm from the world collection until adequate resistance is found. The screening process requires two seasons. In season one, accessions are hybridized with 'B73' to enhance large ear size and uniform maturity. In season two, F1 seed are screened in replicated field trials. A progeny row of F1 seed is planted in each experimental unit and inoculated with *A. flavus* (90 million spores/ml) using a hand-held pin bar cushion. At the end of the season, corn from harvested ears are ground to a coarse meal and rated for bright greenish-yellow fluorescence (BGYF). Samples from accessions with low BGYF are sent for analyses at the USDA-ARS facility at Stoneville, Mississippi. Either 300 or 500 accessions have been planted each year since 2003. Over thirty accessions have been identified with potential resistance to *Aspergillus flavus* and/or aflatoxin biosynthesis. New F1 hybrids are being prepared this winter (2007-2008) in order to test these accessions alongside resistant checks in replicated field trials at multiple locations during the growing season in 2008. With replicated multi-location data, we hope to determine in 2008 if superior resistance has been found.

20th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 4: CROP RESISTANCE – GENETIC ENGINEERING

Moderator: Robert Curtis, Almond Board of California

Current Status of the Atoxigenic Strain Aspergillus flavus AF36 for Controlling Aflatoxin in Arizona Cotton

Larry Antilla[1] and Peter J. Cotty[2].
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The concept of competitive displacement provides the basis for the application of a unique biological control strategy which has been used over the past several years in Arizona to limit levels of aflatoxin expression on cottonseed. The inability of certain strains of the fungus *Aspergillus flavus* to produce aflatoxin provides the mechanism through which such control has been made possible. The discovery of this phenomenon led to a cotton industry plan for area wide control of aflatoxin and the development of a grower owned facility designed to produce commercial scale quantities of the most commonly occurring atoxigenic strain in Arizona known as AF36. Progressive improvements in production, distribution and application of AF36 have been made since the facilities inception in 1999. To date, this program has expanded to encompass more than 150,000 acres of cotton in Arizona, Texas and southern California with data verifying displacement of aflatoxin producing fungi. A collaborative effort involving USDA, ARS and the Arizona Cotton Research & Protection Council, a state agency funded solely by the Arizona cotton industry, enabled the ACRPC to develop and implement commercial scale production capabilities and to obtain a Section 3 registration for AF36 in Arizona, Texas and southern California.

Because of declining cotton acreage and extensive crop rotation practices, Arizona cotton producers are attempting to expand both the scope of opportunity and the window of treatment timing in order to enhance the area wide influence of atoxigenic strain applications. One way to address these issues is by making atoxigenic strain applications to non-cotton crops following harvest or termination based on the theory that displacement of toxin producing strains will occur on residual organic matter occupying fallow field conditions. Several cropping scenarios are being considered in order to provide such test data including winter and spring cole crops (i.e. lettuce, broccoli, cauliflower, etc.), melons (grown primarily spring and fall), and alfalfa hay stands, which are being terminated for future rotation to other crops. In all these scenarios, pre-treatment soil sampling and analysis for fungal community composition, followed by post-treatment sampling in increments of six months to one year, are being utilized to help determine treatment effects. Although the work is field directed, microbiological analyses are critical links to assess product quality and composition of fungal communities in treated fields. These analyses are performed in the Quality Control and Assessment Laboratories at the ACRPC manufacturing facility and by the ARS lab in Tucson. The ACRPC laboratories are in the process of assessing 3,000 to 5,000 isolates of *A. flavus* by vegetative compatibility analyses during 2007. This is in addition to product quality assessments which are an integral part of the manufacturing process.

On the manufacturing front, cooperative studies with the USDA ARS Lab in Tucson are being conducted relative to the current wheat seed substrate being used for AF36. Issues such as shelf life of the inoculated product, and field loss to bird, insect and rodent predation are issues to be considered relative to atoxigenic strain product stability and durability at the field level. Studies are underway to examine commercial scale quantities of alternative substrates such as sorghum as well as various additives designed to minimize product loss due to predation. ACRPC field and lab personnel will be utilized to establish replicated sampling and evaluation of efforts of the various treatments prescribed.

The durability of competitive displacement by AF36 against the highly toxigenic S strain continued to be demonstrated in the 2006 crop season. Despite highly diverse crop rotation patterns, producers in Yuma County have utilized AF36 over the past four years with consistent results.

Average percentages of AF36 versus S strain on cottonseed from treated crops have been 76%/3%; 80%/6%; 72%/3% and 76%/4% respectively for the years 2003-2006. This is most encouraging when compared to pre-treatment levels of 4% AF36 and 51% S strain taken in 2000 before program inception.

Improvement of Antifungal Peptide Technology for Control of Phytopathogens including *Aspergillus flavus*

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Following the demonstration of the antifungal effects of the synthetic peptide D4E1 both *in vitro* and *in planta* against phytopathogens (*Verticillium dahliae, Fusarium verticillioides*, *Aspergillus flavus* and *Thielaviopsis basicola*), we undertook the following steps to a) increase the antifungal effects of the synthetic peptide D4E1 *in planta* and b) facilitate its detection and quantitation *in planta*.
We synthesized a dimer form of the synthetic peptide D4E1 and peformed *in vitro* assays for its antifungal activity against fungal pathogens. Not only did we find out that the dimer form of the peptide is less toxic to fungal pathogens such as *Aspergillus flavus* but we also determined that it may be toxic to plant cells as well. We were unable to regenerate tobacco plants following transformation with the dimer gene by either *Agrobacterium* method or biolistic transformation of plastids. The reasons for the less toxicity of the dimer D4E1 towards pre-germinated fungal conidia may be related to aggregation problems; however, its possible toxic nature to plant cells is yet to be resolved. In summary, we have discontinued using the dimer gene which could have improved our chances of *in vitro* detection and quantitation using antibodies raised against the dimer, which is not possible with the less antigenic monomer due its small size (17 aa).

Our efforts with the transgenic cotton plants expressing the monomeric form of D4E1 is continuing. Our previous field experiments in 2005 to evaluate the antifungal traits of transgenic cottons expressing the D4E1 gene yielded inconclusive results primarily due to the presence of non-transformed escapes among the transgenic population. We have since avoided this problem by reinitiating seed increase in the greenhouse and testing each plant by PCR for the presence of the D4E1 gene. New field experiments are planned for 2008 possibly in three locations in CA, AL, and AZ.

Transplastomic tobacco plants expressing D4E1 have shown very encouraging results *in vitro* when assayed against *Aspergillus flavus*, *V. dahliae* and *F. verticillioides*. We have already shown that transplastomic tobacco plants expressing a magainin analog are extremely antifungal to plant pathogens including *A. flavus*. We have begun new efforts to transform cotton via plastid transformation by constructing suitable plastid transformation vectors.
We have also initiated several experiments to produce transgenic cotton and tobacco expressing heterologous antifungal genes from wheat (hordothionin) and maize (mod1, a synthetic version of maize ribosome inhibiting protein). We have shown that pure hordothionin is extremely effective against *A. flavus*, *V. dahliae* and *F. verticillioides* at very low concentrations (0.5 to 10 μM). Both transgenic tobacco and cotton plants expressing the hordothionin are yet to be analyzed for antifungal activity. Transgenic peanut expressing the mod1 gene have been shown to retard the growth of *A. flavus* and inhibit the growth of *Sclerotium* and *Sclerotinia* sp..

Maize Lipoxygenases Govern Production of Conidia and Mycotoxins by *Aspergillus flavus* and *Fusarium verticillioides*.

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Contamination of seed with aflatoxin and fumonisin is a serious food and feed safety hazard as these mycotoxins are the most potent natural carcinogens that adversely affect diverse farm animals, poultry and humans. They are produced upon infection of corn ears by the seed-infecting fungi *Aspergillus flavus* and *Fusarium verticillioides*. Oxylipins are a large class of oxygenated fatty acids most of which are products of the lipoxygenase (LOX) pathway. Plant oxylipins function as signals in defense and development. In fungi, oxylipins are potent regulators of mycotoxin biosynthesis and sporogenesis. Recent data suggest that oxylipins may act as signaling molecules in cross-kingdom communication that either induce or inhibit sporogenesis and production of mycotoxins by the fungi. To test this hypothesis, we have cloned and characterized 12 maize LOX genes and generated mutants in which function of majority of these genes is disrupted by *Mutator* transposon insertions in their coding sequences. Analysis of available *LOX* mutants showed that some of them are significantly more resistant to leaf blights, stalk rots and to contamination with fumonisins and/or aflatoxins suggesting that these genes are susceptibility factors in maize. The results will be presented showing that the host oxylipin metabolism governs the outcome of maize-fungal interactions and that production of plant oxylipins may be modulated by fungi for their own advantage to facilitate pathogenesis and production of spores and mycotoxins. Our data also indicates that the two mycotoxigenic fungi *A. flavus* and *F. verticillioides* may depend on different LOX genes to induce (or repress) spore and mycotoxin production.

Characterization of Stress-related Genes that Could Affect Aflatoxin Contamination

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Aflatoxin contamination has been a major food safety concern for the peanut industry. Production of aflatoxin by *Aspergillus flavus* is correlated with the level of stress a plant encounters. Previous studies have shown that peanut plants subject to stresses such as drought, heat, or insect damage accumulate higher levels of aflatoxin. In order to reduce aflatoxin in peanut, we have worked on four proteins or protein families that are involved in stress responses. Chloroperoxidase (CPO) was initially isolated from *Pseudomonas pyrrocinia*. Expression of CPO in tobacco demonstrates significant reduction in *A. flavus* growth (Jacks et al., 2000). CPO was transformed into peanut through microprojectile bombardment and transgenic CPO-expressing peanut progenies exhibited antifungal activity when tested with an *A. flavus* strain AF70-GFP in a seed inoculation assay. The second protein introduced into peanut through genetic transformation was Bcl-xL, a human anti-apoptotic gene. Bcl-xL transgenic plants of other species show less negative responses to a wide range biotic and abiotic stresses. Our Bcl-xL transgenic peanut also demonstrated resistance to paraquat, a chloroplast-targeted herbicide. In both CPO and Bcl-xL transgenic events, transgene silencing was observed among progenies. The demethylation agent azacytidine was tested for reactivation of CPO transgene expression; however, no reactivation was observed. In addition to employing transgenes from other species, we also have studied peanut endogeneous genes: *ara h 2* and lipoxygenase (*lox*). Ara h 2 is a major peanut allergen that can be recognized by IgE from >90% of peanut allergic individuals. Other than being an allergen and a seed storage protein, Ara h 2 also functions as a trypsin inhibitor. In maize silencing a 14 kDa trypsin inhibitor promotes the growth of *A. flavus* and increases production of aflatoxin (Chen et al., 2007). In an effort to produce hypoallergic peanut, we silenced *ara h 2* using RNA interference. Ara h 2 silenced transgenic lines show a significant reduction in Ara h 2 protein level. Our Ara h 2 silenced lines can be a useful tool to study the role of Ara h 2 on aflatoxin production in peanut. Lipoxygenases in plants catalyze the oxygenation of polyunsaturated fatty acids into a variety of hydroperoxide compounds. The role of LOXs in fungal-host interaction has been clearly demonstrated in maize studies. A ZmLOX 5 knock-out mutant line shows significant reduction in *A. flavus* growth and inhibition of aflatoxin accumulation. A ZmLOX 3 mutant has reduced fumonisin but increased aflatoxin production (Kolomiets et al., 2007). Peanut LOX genes are also implicated in *A. flavus* fungal infection. PnLOX 1, a mixed-function LOX, shows increased expression upon *A. flavus* infection (Burow et al., 2000), whereas PnLOX2-3 (13-LOX) expression was severely reduced (Tsitsigiannis et al., 2005). Currently there has been no thorough study of the LOX gene family in peanut. We used the catalytic region of the maize LOX gene family and three other conserved regions to screen a cDNA phage library generated from peanut seeds. Eighteen positive phage clones were sequenced. A new LOX isoform that demonstrates polymorphism to published peanut LOX genes was identified. Three clones appear to be chimeras of LOX and other unrelated genes. One clone retains an intron sequence. Another clone has a 63 nt deletion in the coding region. Further sequencing and 5' RACE are needed to complete the coding region analysis. Those LOX genes that are responsive to *A. flavus* challenge will be further characterized.

Advancement and Testing of Transgenic Peanuts With Enhanced Resistance to *A. flavus*

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RIP 1 is a ribosome inactivating protein from maize which has been shown to inhibit hyphal growth of *Aspergillus flavus in vitro*. 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. We have transferred this gene into peanut in an attempt to control aflatoxin contamination by retarding fungal growth.

Peanuts cv. 'Georgia Green' and 'NCV 11' were transformed by co-bombardment of mature embryos with two plasmids, one containing a gene encoding the active RIP, Mod 1, the other carrying a hygromycin phosphotransferase gene, a selectable marker. Numerous transgenic events were recovered that have been shown to express the Mod 1 protein. Efficacy tests of Mod 1 transgenic peanuts indicated that detached cotyledons from transgenic lines expressing Mod 1 retarded growth of *Aspergillus flavus* isolate "32-8". Some of these events also inhibited the growth of *Sclerotium rolfsii* and *Sclerotinia minor* in *in vitro* assays.

T3 plants from nine events selected for Mod 1 expression, resistance to A. flavus , reduction of aflatoxin and availability of seed were grown in a field trial at Oxford Tobacco Research Station, Oxford, NC during the summer of 2007. A randomized complete block design with four replicates was used. Each plot contained 10 plants. Samples were taken from all plants in the experiment and are currently being tested for the presence of Mod 1 by PCR. PCR positive plants will be assayed for aflatoxin contamination. Further, disease scores and observation of events for off-types were carried out. However, due to extreme drought and high temperatures, fungal disease was not observed in the nursery, and it is not known if any plants were infected with *A. flavus* at this time. These conditions also slowed maturation, and the plots will not be harvested until the first week of November.

A crossing program intended to move beneficial transgenes into elite peanut lines and other lines of interest was continued during this reporting period. Two events, screened by PCR for Mod 1 were used during the winter and summer of 2007 to carry out a broad array of crosses with conventional lines. In some cases, the conventional parent was chosen for inclusion in the crossing program because the line naturally exhibits lower aflatoxin contamination, and therefore is assumed to be carrying one or more genes affecting this trait. The goal in these crosses is to combine the transgenic and native sources of resistance to achieve more consistent and perhaps more significant resistance in progeny.

Other crosses are being made with conventional lines that are high in oleic acid. While these lines show improved keeping quality because of the high oleic acid content, they have also been shown to be especially susceptible to aflatoxin contamination. We hypothesize that by transferring the Mod 1 gene into the high oleic acid lines, it may be possible to retain their desirable qualities, while reducing their susceptibility to *Aspergillus* infection, and thus reducing their tendency to become contaminated with aflatoxins. In addition to crossing Mod 1 parents onto numerous lines with high O/L ratios, two sets of crosses were specifically chosen to test the hypothesis that Mod 1 expression can reduce aflatoxin contamination in these materials. NC 7, a conventional line, and cv. 'Brantley', a derivative of NC 7 with high O/L were used as females in crosses with the two Mod 1 transgenic parents. A similar pair consisting of the cv. 'Gregory' and a derivative line with high O/L were also included in the crossing program. Progeny of these crosses will be tested for the presence of Mod 1 and also tested for expression of the Mod 1 protein. Positive progeny will subsequently be

back-crossed to their respective untransformed parent repeatedly to recover the phenotype of the recurrent parent. Progeny will then be subjected to tests for O/l ratio, Mod 1 and resistance to aflatoxin contamination.

PANEL DISCUSSION: Crop Resistance - Genetic Engineering

Panel Chair: Kanniah Rajasekaran

Panel members: Ye Chu, Michael Kolomiets, and Arthur Weissinger

Four presentations were made in this session on genetic engineering of corn, peanut and cotton for resistance to *Aspergillus* spp. and Dr. K. Rajasekaran opened the panel for discussion. In response to Dr. Corley Holbrook's question on possible reasons for gene silencing in transgenic peanuts, Dr. Art Weissinger answered that several factors including transgene copy number and promoter homology are important. Rajasekaran answered another question regarding the safety of the antifungal peptide D4E1 and reiterated that D4E1 is denatured quickly and it is safe to animals and human beings. Dr. Zhiyuan Chen asked about the source of *lox* mutants that Dr. Mike Kolomiets talked about. Dr. Kolomiets answered that they are from Pioneer Seed Company and Cold Springs Harbor Laboratory. Dr. Victor Nwosu asked Dr. Julie Chu whether the transgenic peanuts showed increased amount of seed storage proteins and Dr. Chu responded saying that it has not been studied yet. She also answered Dr. Don White's question regarding any possible difference in allergenic peanut protein in transgenic peanuts by indicating that both transgenic and control peanuts did not differ in the content of allergenic Arah2 and Arah1. Discussions were also held on possible relationships between expression of an anti-apoptotic gene and virus resistance in response to a question from Dr. Patrick Dowd.

RNAi Silencing of the 14 kDa Trypsin Inhibitor Protein in Maize and its Effect on Host Resistance to *Aspergillus flavus* Infection and Aflatoxin Production

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Maize (*Zea mays* L.) is one of the major crops that are susceptible to *Aspergillus flavus* infection and subsequent contamination with aflatoxins, the potent carcinogenic secondary metabolites of the fungus. Maize genotypes resistant to *A. flavus* infection and /or aflatoxin contamination have been identified and compared with susceptible genotypes for differences in protein profiles. Several resistance-associated proteins have been identified through proteome analysis and sequenced. One of them is a 14 kDa trypsin inhibitor (TI) which shows antifungal activity against a broad spectrum of plant pathogens. To further investigate whether the high level expression of TI plays a direct role in host resistance of maize to *A. flavus* infection and/or aflatoxin production, an RNAi gene silencing vector was constructed using Gateway technology and transformed into the immature maize embryos. Sixty-six transgenic ears representing 18 independent transformation events were produced. Ten ears representing 6 independent transformation events were selected for further evaluation, and kernels from seven ears were confirmed positive for transformation. TI production was reduced by 10 to over 85% in the RNAi silenced transgenic kernels compared to control lines when analyzed through 2 D PAGE. The kernel screening assay (KSA) also revealed that transgenic kernels with reduced levels of TI production were more susceptible to *A. flavus* colonization and aflatoxin production. This study clearly demonstrates the importance of the 14 kDa trypsin inhibitor expression in maize resistance to *A. flavus* infection and/or aflatoxin production.

Aflatoxin and Insect Response of Conventional Non-Bt, MON 810 (Cry1Ab), and MON 89034 (Cry1A.105-Cry2Ab2) Corn Hybrids in South Texas

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Field research was conducted in 2005 and 2006 to determine contribution of the combined Bt proteins Cry1A.105-Cry2Ab2 to reduced levels of insect injury and aflatoxin content, and increased agronomic performance through comparative evaluations against similar conventional non-Bt hybrids and hybrids containing Cry1Ab (MON 810). In 2005, non-Bt, MON 810, and two events of Cry1A.105-Cry2Ab2 (MON 89034 and MON 89597) were utilized in two conventional hybrid backgrounds for a total of 8 hybrids. An additional hybrid background was added in 2006 but MON 89597 was dropped from experiments, for a total of 9 hybrids. In both years, sites at Corpus Christi and Beeville, TX were planted about 4 wk later than normal to increase potential for drought stress. Inoculum was produced by growing a high aflatoxin-producing isolate of *A. flavus* (NRRL3357) on autoclaved corn kernels. When the first hybrids reached mid-silk at any site, inoculum was distributed on the soil surface between treatment rows at the rate of 1 kg dry seed equivalent per 200 ft. Naturally occurring insect populations affecting plants at both locations were primarily corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*). Insect pest data included whorl injury (0-9 rating / 10 plants), ear injury (average length of injury from tip in cm / 10 ears). Ears were harvested when kernel moisture in all hybrids was below 15%, then yield per plot was recorded. All grain from each replicate (ca 30-40 ears) was ground in a Romer mill and a subsample was analyzed for aflatoxin content (ppb) using the Vi-Cam Aflatest P immunoassay system. Aflatoxin was statistically analyzed using log10 transformed values. In 2005, there were significant interactions between location and event for all variables of interest except yield, whereas in 2006 there were no interactions so event was analyzed with locations combined. In 2005, at both experiment locations, insect injury to whorls and ears of corn hybrids containing MON 89034, MON 89597, or MON 810 was significantly less than in non-Bt hybrids. Compared to MON 810 hybrids, those with MON 89034 and MON 89597 had significantly lower whorl insect injury only at Corpus Christi but ear injury was lower at both locations. Average aflatoxin content at Beeville was extremely high ranging from about 2066 to 8815 ppb. Though high, aflatoxin content of both the MON 89034 and MON 89597 hybrids were significantly lower than the MON 810 and non-Bt hybrids. Aflatoxin content in MON 810 and non-Bt hybrids was not significantly different at Beeville and there were no differences in aflatoxin content (294-427 ppb) among any hybrids at Corpus Christi. The effect of event on yield, evaluated across all hybrids and locations, showed that hybrids containing either MON 89034 or MON 89597 had significantly higher yields than MON 810 and non-Bt hybrids which were not different from each other. In 2006, insect injury to whorls and ears was significantly less in MON 89034 hybrids than in MON 810 and non-Bt hybrids. Yield was significantly higher and aflatoxin content significantly lower (646 ppb) in MON 89034 hybrids than in MON 810 (898 ppb) and non-Bt hybrids (1220 ppb). MON 810 hybrids had significantly lower insect injury to whorls and ears and higher yields than non-Bt hybrids but aflatoxin content was not different. The combined Cry1A.105-Cry2Ab2 Bt proteins may provide a valuable genetic Bt tool for use in corn hybrids to reduce plant and ear insect injury and lower both aflatoxin content and yield loss associated with that injury.

20th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

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

Moderator: Victor Nwosu, Mars Snackfood US, LLC.

Cost-Effectiveness of Aflatoxin Control Methods: Economic Incentives

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Multiple sectors in crop industries – growers, elevators, handlers/shellers, processors, distributors, and consumers – are affected by aflatoxin, and have the potential to control it. The research conducted by the Aflatoxin Elimination Workgroup and collaborators has identified causes of aflatoxin contamination and offered solutions in the form of aflatoxin control methods. Now it is important for industries to adopt these methods. What would increase adoption of aflatoxin control methods? There are three important points: providing economic incentives, proving or improving cost-effectiveness of the control methods, and education and outreach.

The problem is that there is often a mismatch in economic incentives, in that different sectors bear the brunt of aflatoxin costs for different crops. In corn and cottonseed, growers bear most of the cost; whereas in peanuts and tree nuts, shellers and handlers bear most of the cost. Hence, peanut and tree nut growers may have no economic incentive to apply preharvest aflatoxin control; yet this is where the best control can occur. Postharvest control options are limited and in many cases are not yet approved by EPA or FDA. The Kaldor-Hicks efficiency criterion may help to get around this economic dilemma. This is a kind of economic efficiency in which one sector can compensate another to achieve Pareto optimality: a state in which at least 1 sector is left better-off and no one is worse-off. Applied to aflatoxin control, growers could be compensated by shellers/handlers or cooperatives to adopt preharvest aflatoxin control methods. However, these methods must be cost-effective for this arrangement to work.

We presented 4 case studies of cost-effectiveness to reduce aflatoxin in different crops: AF36 in cottonseed, Bt corn, Afla-Guard in peanuts, and storage/shipment aflatoxin control in almonds. AF36 has already been approved and has shown effectiveness in aflatoxin reduction in Arizona cottonseed. We asked: would growers have incentives to apply it on a yearly basis? We calculated that the cost of AF36 application is $5.50-$15/A, while the expected benefit is $14.88-$39.67/A. Even in years in which aflatoxin would be naturally low, AF36 represents just 2.2-6% of the total value of cottonseed production. We advise growers to aim for the lowest application cost (~$0.50/A) and for industry to communicate AF36 as an "insurance policy" to growers.

Commercially available Bt corn events have shown significantly lower fumonisin levels around the world compared with non-Bt isolines. A new variety in late development has shown significant aflatoxin control as well. We ask: how effective must it be to make it economically worthwhile? Even if the seed premium for this new Bt corn event were $20/A, the added cost is just $0.15/bu compared with potential losses of $1-$3/bu for aflatoxin-contaminated corn. Hence, Bt corn is cost-effective if it can reduce aflatoxin-related problems by just 5-15%, aside from its other grain quality improvements. In today's ethanol boom, the problem of mycotoxins in corn is more important than ever.

In the peanut industry, shellers bear the brunt of aflatoxin cost: ~$32.75/A on average, with high variability by year and region. Biocontrol method Afla-Guard's expected benefits are $22.92-$29.47/A, while costs of application are $19-$24/A. Shellers benefit at the margin if they pay growers to apply Afla-Guard; adoption would increase if material costs decrease.

Tree nut industries, especially almonds, suffer the bulk of aflatoxin-related loss through rejection of exported consignments to the EU. As of October, the EU had rejected 57 U.S. almond lots in 2007: nearly a $600,000 cost. Given extremely high aflatoxin levels in some U.S. lots upon arrival in EU ports, and the unlikelihood of such lots leaving the U.S. in such a state, the problem may lie in aflatoxin accumulation during transportation. Another problem is high concentration of aflatoxin in a small number of nuts. Aflatoxin control strategies should not cost more than ~$400,000-$600,000/yr;

otherwise it would make more sense to accept a certain number of rejections, although this may bear indirect costs of lowered reputation for high-quality nuts.

In summary, it is important to consider economic incentives for aflatoxin control: is the need preharvest, postharvest, or both? What about the method? If there is a mismatch, it is possible to realign markets so that sectors with economic incentives to reduce aflatoxin can do so. Then it becomes important to assess the cost-effectiveness of different methods. Our analysis suggests ways in which to think about, estimate, and communicate cost-effectiveness: as an insurance policy, where to cut costs if possible, how to compare expected benefits and costs, and how to convey these ideas to growers and handlers.

Investigations of Novel Control Tactics against Navel Orangeworm for Management of *Aspergillus* in Tree Nuts

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The boring of moth larvae into tree nuts provides the primarily invasion pathway for *Aspergillus*. Our goals are to, first better define insect – host plant – *Aspergillus* relationships and secondly, devise benign, reduced-risk, species-specific control systems to diminish insect-caused nut damage through the use of novel host-plant kairomones and/or microbial agent tactics. Specific insect – host plant interactions and dependences might present particular critical life-cycle vulnerabilities that then might be exploited and manipulated for management and control of the insect pest and thereby *Aspergillus* invasion and aflatoxin contamination of tree nuts. Olfaction is the primary sense used by insects, in particular Lepidopterous moth pests, to detect and assess host nuts for locating ovipositional sites by female moths and feeding sites by larval worms. The navel orangeworm, *Amyelois transitella,* is the chief moth pest that has historically been associated with the introduction of *Aspergillus* species and the occurrence of aflatoxin in all tree nuts, almonds, pistachios and walnuts, and also figs.

Currently, we lack critical management tools to better detect and control the navel orangeworm (NOW), including the lack of a season-long dependable monitoring lure and reduced-risk microbial insecticides. Presently, no pheromone-based lure is available due to its unstable nature and the lure for the "egg traps" is "almond presscake" that is only seasonally effective from winter through spring but is ineffective/unreliable at the crucial summer "hull-split" pre-harvest period of highest vulnerability of nuts to NOW attack. Critical life-cycle vulnerabilities of NOW are: 1) feeds only on kernels, can't feed or penetrate hulls and shells, thus must seek prior openings (hull-shell splits) or prior damage incurred by primary attacking pests (peach twig borer on almonds and codling moth on walnuts), 2) does not diapause and must find and overwinter in residual orchard nuts present on the ground or "stick-tights"- "mummies" that remain on the tree, and 3) multiple eggs are selectively laid on specific nuts, wherein multiple worm and pupal stages occur. Female moths discriminate and lay eggs selectively on susceptible nuts, including mummies, prior-damaged nuts, and hull – shell split nuts. We are headspace-collecting and GC-MS analyzing the odors emanating from these specific, vulnerable host resources and performing both laboratory (GC-EAD, flight tunnel) and field bioassays to define - identify the specific attractant volatiles present and formulate an optimal lure.

Laboratory experiments have clarified the association of NOW nut damage and "vectoring" with *Aspergillus* infection and levels of aflatoxin contamination. We have demonstrated that newly hatched neonate larvae can carry on their body setae *A. flavus* spores and readily transport spores to almond kernels, upon which the number of worm feeding holes or penetrations is correlated with the amount of aflatoxin accumulated (10 to 498,000 ppb B1 aflatoxin per kernel).

Further, we have discovered an insect virus that is infective to NOW larvae and we are presently pursuing a patent application through ARS OTT. Laboratory experiments have demonstrated that NOW infection by this virus expresses dose-dependent activity in both neonate mortality and the retardation of growth and instar development. Limited field trials on single replicate walnut trees have demonstrated that this viral agent reduced kernel damage significantly better than three, registered conventional synthetic insecticides, two organophosphates and an insect growth regulator.

These studies show that the NOW does vector *A. flavus* and its nut-penetrating damage does promote/facilitate aflatoxin accumulation. Further pursuit of a kairomone-based monitoring lure and a viral insecticide will both enhance the control and management of NOW and *Aspergillus.*

Getting to the Root of Nematode Involvement in Aflatoxin Contamination of Peanut

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Infection of peanut by root-knot nematodes (*Meloidogyne arenaria*) can lead to an increase in aflatoxin contamination of kernels when the plants are subjected to drought stress during pod maturation. The nematode can infect both the roots and peanut pods. We recently showed that root infection in the absence of pod infection by *M. arenaria* can lead to greater aflatoxin contamination in the kernels; however, it is not known whether wounding of pods by nematodes can also play a role in aflatoxin contamination by serving as entry points for toxigenic *Aspergillus* spp. Our objectives were: 1) to determine the contribution of pod galling caused by root-knot nematodes to the increase in aflatoxin contamination, and 2) to determine whether nematode-resistant peanut genotypes reduce the risk of preharvest aflatoxin contamination in soil infested with root-knot nematodes.

A greenhouse experiment was conducted in which pods and roots were physically separated. Pod set was restricted to soil-filled pans (41 cm dia. x 10 cm depth), while the roots grew underneath the pan into a pot. The experiment had a factorial arrangement of treatments: pod zone with and without nematodes, and root zone with and without nematodes. The four treatment combinations were replicated 10 to 13 times. Grain infested with *Aspergillus flavus/A. parasiticus* was added to the soil surface (pods zone) at mid bloom. Plants were subjected to drought stress 40 days before harvest. In both 2004 and 2005, there was very little pod galling from *M. arenaria*, and there was no difference in aflatoxin concentrations between treatments with and without nematodes in the pod zone. In 2006, there was heavy pod galling with galls present on 53% of the pods. We also found greater aflatoxin concentrations in the kernels when nematodes were added to the pod zone than when they were not (572 vs 19 ppb; $P = 0.03$). In the 2006 experiment, nematode infection of roots had no effect of aflatoxin concentrations in the peanut kernel. Based on these and previous results, it appears that nematode infection of either the roots or pods can lead to greater aflatoxin contamination of peanut kernels.

A field microplot study was conducted in 2006 to determine whether there was lower aflatoxin concentrations in nematode-resistant than in susceptible peanut when exposed to *M. arenaria*. Two sister lines with good resistance to Tomato Spotted Wilt Virus were used: C724-19-15 which is highly resistant to *M. arenaria* and C724-19-25 which is susceptible to the nematode. Half of the 24 plots were inoculated with nematodes at two different times (at plant and after pegging) and the other half were not inoculated with nematodes. There were six replicates of each treatment combination. All plots were inoculated with *A. flavus*/*A. parasiticus*. Drought was induced 5 to 6 weeks before digging. Root-galling from *M. arenaria* was so severe in the susceptible peanut that kernel yield averaged only 14 g compared to kernel yields of 218 to 234 g in the resistant peanut and the susceptible peanut without nematodes. Aflatoxin concentrations were also greater ($P = 0.04$) in the susceptible peanut (137 ppb) than in the resistant peanut (12 ppb). This experiment will need to be repeated to confirm that aflatoxin contamination of peanut in soil infested with *M. arenaria* is reduced when a nematode-resistant peanut is grown.

Volatile Emission of Mechanically Damaged Almonds: An Old and New Method of Investigation to Determine Potential NOW Attractants

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Volatile emission of plants is typically measured by removal of the plant-part, transportation to a laboratory, and subsequent volatile analyses via a number of accepted methodologies. Studies performed by our laboratory have shown the volatile emission of removed plant parts are essentially identical to the volatiles produced if the plants parts were injured. Using a new method of on-plant analysis, the volatile output of control and damaged almonds was performed during the 2007 growing season. After completion of data analyses, the results of this method will be compared to previous almond volatile collection method results obtained by this laboratory. The methodology development of this technique will be discussed.

Selectable Markers with Potential Activity Against Insects, Plus Other Insect-Oriented Strategies for Mycotoxin Reduction in Midwest Corn

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Reduction of insect damage has the potential to greatly reduce the levels of mycotoxins in corn, as have studies with Bt corn have shown. However, the large number of insect species involved necessitates the development of comprehensive insect control to most effectively utilize this strategy.

One strategy that we have explored is field based management, which relies on a predictive computer program that allows farmers to make more informed decisions for applying insect or mold control, including early harvest and drying. The program recently has been converted to a web-based version and is awaiting loading. Additional data has been collected for popcorn to allow expansion to that commodity. The field-corn version of the program continues to detect conditions where aflatoxin may result and has done well in predicting fumonisin levels.

Our recent progress towards comprehensive insect resistance in corn has included identification of a hexosaminidase which enhanced insect resistance in corn transformants, enhanced anthocyanin production that has increased insect resistance in nongrain tissues, molecularly evolved peptides with enhanced activity against insects, and greatly enhanced efficacy of functionally different multigene combinations in the model plant tobacco.

Selectable markers genes that are plant-derived and/or nonmicrobial antibiotic related have the potential to enhance acceptability of transformed plants. The protein product from the model anticell-death insect virus AcMNPV P35 gene significantly enhanced mortality of fall armyworms to the nonhost virus AgMNPV in dietary assays. Some transformed corn lines caused greatly enhanced mortality to corn earworms and fall armyworms; and overall feeding was significantly lower in lines that had the gene introduced compared to those that did not. Fall armyworms fed P35 lines had enhanced mortality to AgMNPV compared to those fed on non-P35 lines.

The blue fluorescent mutant *bf-1* line of maize putatively has an altered anthranilate synthase gene. We identified a single point mutation in a *bf-1* anthranilate synthase a subunit mRNA that caused a leucine to proline change at residue 531. This unique change is in the substrate-binding domain, as opposed to other mutations in other plants of this enzyme that have involved the tryptophan binding feedback region. Transgenic maize callus expressing the *bf-1* mutant anthranilate synthase was resistant to the inexpensive selecting agent 6-methyl anthranilate. Additionally feeding by corn earworms and fall armyworms was inversely correlated to the levels of the *bf-1* anthranilate synthase produced in regenerated corn plants.

NIR Spectroscopy as a Tool for Optimizing Sorting of White Corn Kernels Contaminated with Mycotoxins

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Near infrared reflectance spectra (500-1700 nm) were analyzed to determine if they could be used to identify single whole white corn kernels contaminated with aflatoxin and fumonisin. Kernels used for the study were obtained from grain lots harvested in 2006 from commercial fields in Southern Texas. Kernels were visually examined and grouped into four symptom categories: asymptomatic, showing 25% to 50% discoloration, and discolored BGYF kernels. Friable kernels and fragments were not included in this study as they are usually removed by existing cleaning equipment at grain elevators. Spectra were acquired on both the germ side and endosperm side of each kernel. After spectra acquisition, kernels were weighted individually and then placed in groups of five according to their classification based upon symptoms of fungal infection and numerical sequence within each pill box. Aflatoxin B1 or total fumonisins (B1, B2, and B3) were measured with a fluorometer after extracts were purified with immunoaffinity columns (Aflatest or Fumonitest, Vicam, Watertown, MA). The aflatoxin or fumonisin level of each five-kernel group then was assigned to each individual kernel from that group. Kernels were analyzed in groups instead of individually to reduce cost and analysis time.

For high speed sorting operations, whole spectra cannot be acquired at throughput rates that are economically feasible. Most commercial sorting machines are able to only measure one spectral band of light while some machines can measure two bands. Discriminate analysis was used to select the optimal pair of wavelengths to identify kernels containing mycotoxins. It was found that using the wavelength pair of 500nm and 1200nm, approximately 87% and 93% of kernels having high levels of aflatoxin (>100 ppb) and high levels of fumonisin (>40 ppb), respectively, were correctly classified. Additionally, approximately 96% of the kernels having low levels of aflatoxin (<10ppb) and fumonisin (<2ppm) were correctly classified as uncontaminated. Kernels having minor symptoms (24% to 50% discolorations) had lower classification accuracies (80%), than those with discolorations covering more than 75% of the kernel (88%), or discolored BGYF kernels (91%).

A commercial sorting machine (Satake DE) was set up to sort corn using light at 500 and 1200 nm and reject 4% to 9% of the incoming corn. 10 kg samples of corn were used in all sorting experiments. Incoming mycotoxin levels in three lots of corn used in these sorting experiments ranged from 23 to 150 ppb aflatoxin and 0.4 to 0.6 ppm fumonisin. In one pass through the sorter, aflatoxin was reduced 19-75% (average 45%) and fumonisin was reduced an average of 60%. Two passes through the sorter reduced aflatoxin by 88%, and below 20 ppb in each of the three lots of corn. A limited number of samples run through a monochromatic sorter that has higher spatial resolution using the 515 nm filter reduced aflatoxin by 61% in one pass and 82% in two passes. It was found that some kernels of white corn with minor symptoms of discoloration could have very high contamination levels of aflatoxin without exhibiting external BGYF. Thus, two passes through the sorter were required to reduce aflatoxin below the regulatory limit of 20 ppb.

In earlier sorting experiments with commercially harvested yellow corn that was contaminated with equivalent levels of aflatoxin (average 75ppb), we were able to reduce aflatoxin below 20 ppb with a single pass through the same Satake DE machine (Pearson et al. 2004). Differences in pericarp thickness, kernel vitreosity (hardness) and the presence or absence of carotenoids, could influence both kernel symptom expression and the ability of NIR light to detect fungal damaged endosperm.

Water Conservation and Preharvest Aflatoxin Contamination in Peanut and Corn

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Water remains a major limiting resource in consistent crop production in the Southeast US. Avoiding drought stress is the main factor for preventing preharvest aflatoxin contamination in peanut and corn. Furrow diking is a tillage operation that creates a series of basins and dams in the furrow to catch water delivered by either rainfall or irrigation. Improving water capture for field crops in the Southeast would improve irrigation efficiency while reducing input costs and has the potential to affect preharvest aflatoxin contamination by improving soil moisture. A series of field experiments was conducted in 2005-2007 near Dawson, Georgia using furrow dikes in irrigated and non-irrigated peanut and corn. The objectives included monitoring soil moisture levels to determine if water can be saved in irrigated systems with furrow dikes compared to those in conventionally tilled systems. Irrigator Pro, a decision based computer software program, was used for irrigation recommendations. In non-irrigated experiments, soil moisture and yield parameters were monitored to document impact of furrow dikes within those systems. Large samples were processed to quantify the presence of aflatoxin at harvest in peanut and corn.

The 2005 growing season had abundant rainfall. Peanut and cotton crops only required one irrigation. Furrow diked corn was irrigated 3 times and non-diked corn required 5 irrigations. Despite abundant rainfall, higher levels of soil moisture were maintained in plots with furrow dikes compared to conventional plots. Similar yields were attained in all crops regardless of furrow diking in 2005. Levels of aflatoxin in corn ranged from 7 to 191 ppb and none was found in peanuts in 2005. The 2006 and 2007 seasons required more frequent irrigation because of drought conditions. Aflatoxin in corn was significantly impacted by irrigation in these 2 drought years, but furrow diking did not reduce preharvest aflatoxin contamination. Non-irrigated corn yield was improved 17% by furrow diking in 2007. No aflatoxin was found in peanuts grown in 2006, while yield of like treatments were similar. At the time of this meeting, the 2007 peanuts had not been harvested. Trends for aflatoxin prevention in corn are evident in these studies. Strong results for soil and water conservation, yield improvement, and economic feasibility for furrow diking have been shown in associated studies. Furrow diking improves the efficiency of water capture, however, there must be water in the system to take advantage of. Further investigation is necessary to either strengthen/disprove the reliability of furrow diking tillage to affect preharvest aflatoxin contamination of crops in the Southeast US.

Contaminant Distribution in Tree- and Ground Nuts and Other Commodities

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The computation of sample distributions for a non-parametric lot distribution is described. The method makes use of a c-program, running on a desktop PC to evaluate the complete sample set of up to 10^11 configurations into 15 concentration bios by an exhaustive search. Use of this calculation allows the computation of a lot distribution with probabilities up to 20X larger than those allowed under an algorithm developed by Schatzki in 1995, i.e. up to lambda = 2.0, rather than 0.1. The method has been applied to sample distributions up to 4000 kernels (pistachios), 5000 kernels (almonds) and 200 kernels (peanuts). Programs are available from the authors.

The Importance of Aflatoxin to the Ethanol Fuel Industry

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Aflatoxins are highly toxic, hepatocarcinogenic compounds produced by *Aspergillus flavus* and *A. parasiticus* during infection of corn (maize), peanuts, cotton seed, and tree nuts. Aflatoxin becomes problematic when contaminated corn is used as a feedstock for ethanol production. Although, aflatoxin has not been reported in ethanol produced from contaminated products the distiller's grains produced during fermentation show a marked increase in aflatoxin levels, typically 3-4 times the initial value. Distiller's grains are key components in many animal feeds and generate significant revenue for distilleries. Therefore, ethanol manufactures should not use aflatoxin-contaminated corn to generate ethanol due to the 20 ppb FDA limitations of aflatoxin on animal feeds. Though, previously not enforced in DDG the FDA has informed the Mississippi State Chemical Lab that DDG testing is imminent, increasing the need for methods to decontaminate aflatoxin tainted corn. Although, several chemical methods have been shown to be effective in removing aflatoxin they are not approved by the FDA and are too expensive to be feasible on an industrial level. Thus, we have begun to characterize aflatoxin degradation by *Aspergillus* species. The observed degradation of exogenously introduced aflatoxin has been confirmed by TLC and LC-MS and is serving as the basis for identification of genes and enzymes involved in this unique phenomenon. Identification and characterization of the factors responsible for the degradation of aflatoxins will allow contaminated substrates to be used for ethanol production. This research has the potential to greatly increase ethanol's economical feasibility as an alternative fuel and provide an additional market for a previously unused resource.

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

Panel Chair: Patricia Timper
Panel Members: F. Wu, D. Light, J.J. Beck, P. Dowd, D. Wicklow, R. Nuti, T.F. Schatzki, and A.E. Brown

Ashli Brown was asked several questions related to her presentation on reducing alfatoxins in the byproducts of ethanol production. Q 1. How do you intend to apply the degradation compound to such a large volume of material? Degradation is expected to take place during the fermentation process. Q 2. Why did you choose LCMS instead of another aflatoxin test? The equipment was available to us and we feel it is more reliable than other methods such as ELISA. Q 3. Have you tried using binders to take up the aflatoxin like they do in the EU? No, but we are looking into it. Q 4. Do you have a robust sampling method for distiller's grain? No, that is a main concern with no answer yet.

Felicia Wu was asked questions related to her presentation on the economics of aflatoxin mitigation programs. Q 1. Did you include in your analysis the 'value-added' benefits of using pre-fractionation in ethanol production? No, we did not, since at the moment few, if any, ethanol plants are taking advantage of this. Fractionation involves removing the part of the kernel that contains the highest concentration of mycotoxin, so that it is not present in the ethanol co-products. If this practice were adopted by ethanol plants, it could significantly lower the risk of mycotoxins in ethanol co-products. Q 2. Are fumonisins found in ethanol or by-productions (Q was asked to Ashli, but answered by Felicia)? A study led by Bothast (1992) examined fumonisin in ethanol co-products. Since there are several different co-products, and they examined fumonisin in only one of them, we cannot say what the overall concentration factor was, but it doesn't contradict the rule of thumb of 3-times concentration. Q 3. What do you think of the economics of using ethanol co-products to fuel ethanol plants rather than being used for animal feed? This would be a good way to avoid having excess mycotoxins in animal feed, but ethanol plants would probably rather sell their co-products for animal feed because it fetches a higher price, even considering transportation costs.

Don Wicklow was asked a question related to his presentation on sorting white corn contaminated with mycotoxins using a high speed commercial sorting machine. Q. Is it worth it to go through this sorting process – how much will it cost to sort? Yes, it can be worth the cost, but it depends on the value of the product. One grain processor felt that they could lose up to 14% of their white corn kernels when removing (rejecting) the aflatoxin contaminated kernels and still make a profit because incoming grain contaminated with over 20 ppb aflatoxin can not be used in foods and is of considerably less value. One 8 channel machine can sort 10 tons of grain per hour and costs about $60K.

John Beck was asked a question related to his presentation on a method for determining potential insect attractants to damaged almonds. Q. Why didn't you use nonpareil almonds in your tests? Both nonpareil and 'Monterey' almonds were investigated in 2006; however, the results for the nonpareil were not consistent and therefore not included in the presentation.

Effect of *Aspergillus flavus* inoculum size, maize variety, and cowpea intercropping on aflatoxin production during maize storage.

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Aflatoxin contamination of maize (*Zea mays*) by *Aspergillus flavus* is a serious problem with significant health implications in many parts of West Africa. Post harvest aflatoxin contamination is usually influenced by prevailing conditions during maize growth in the field. The main goal was to identify factors that directly or indirectly enhance production of aflatoxin during maize storage. The objective of this study was to determine the effects of initial inoculum level, type of variety and impact of intercropping on the rate of aflatoxin production in maize during storage. The two maize varieties used in this study were *Gbogbe*, a local maize variety with a maturity period of 90 days, and *TZSR-W* (Tropical *Zea mays* Streak Resistant White), an improved variety with a maturity period of 120 days. A local variety of cowpea (*Vigna unguigulata* (L) Walp) was used as intercrop with the maize varieties. Experimental plots were inoculated with *Aspergillus* (*T-strain*). The field experiment was conducted between June and September 2006. The improved variety (TZSR-W) had significantly higher level of Colony Forming Unit (*CFU)* ($P = 0.0212$), aflatoxin B1 ($P = 0.0038$), and aflatoxin B2 ($P = 0.0014$) contamination compared to the local variety (*Gbogbe*). The *Aspergillus* (*T-strain*) inoculation level and variety selection had major impact on the production of aflatoxin in stored maize. Intercropping of maize with cowpea during the growing season did not show any significant impact on *CFU* level. The initial inoculum level and the water content of maize after harvest may have played significant role in *A. flavus* infection initiation and development during storage. These factors could serve as useful inputs in developing a model for predicting the rate of aflatoxin production and level of contamination during maize storage. Avoidance of environmental conditions that favors infection development in the field, utilization of management practices that minimizes primary inoculum or sources of inoculum, and harvesting when the water content is optimal may help prevent or reduce aflatoxin contamination in maize.

Peanut Contamination by *Aspergillus flavus* and Aflatoxin B1 in Granaries of Villages and Markets of Mali, West Africa

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Peanut (*Arachis hypogoea*) is an important crop in Mali, West Africa. However, one of the main problems related with its production and commercialization is the detection of high levels of Aflatoxin B1 in peanuts and derived products for consumption. Thus, research is needed in order to improve the quality of peanut grains during production and storage by reducing the contamination rate by *Aspergillus flavus* and Aflatoxin B1.

The objectives of this work were to determine 1) The rate of progress of *Aspergillus flavus* and Aflatoxin B1 in rustic granaries located in 26 villages and also in the granaries located in the corresponding 26 markets in Mali and 2) The association between *Aspergillus flavus* and Aflatoxin B1 with environmental variables outside the peanut granaries.

During the period 1999/2000, 2000/2001 and 2001/2002 peanut samples were collected every three months from 26 granaries located in villages and from 26 granaries located at the local markets for *Aspergillus flavus* and Aflatoxin B1 determination. A statistical analysis (T-Test) of the weather variables for the locations with the highest levels and the lowest levels of Aflatoxin B1 was conducted.

The preliminary results showed that the peanut stored and consumed in rural areas are highly contaminated by A*spergillus flavus* and the Aflatoxin B1, with average rates of Aflatoxin B1 significantly above the accepted international standards especially between the months of June and December. The analysis of the rate of Aflatoxin B1 increase reveled that the greatest increase in Aflatoxin B1 usually occurred between June and August. Thus, the statistical analysis (T-test) of daily weather data was focused between June and August for years 1999, 2000 and 2001. The village Tiele had one of the highest values of Aflatoxin B1 in year 2000 and relatively low values in years 1999 and 2001. For Tiele, the average maximum humidity for June 1999 was 79%, for June 2000 was 91%, while the average minimum humidity for June 1999 was 34.3% and for June 2000 was 47.3%. Statistical differences were found between June 1999 and June 2000 (T-test). The analysis of the aflatoxin B1 levels in the villages and its relation with the weather conditions outside the storage reveled that for one village (Tiele) high air humidity values were a factor that assisted in the development of *Aspergillus flavus* and consequently in the Aflatoxin B1 levels of the granary.

Natural Distribution Patterns of Aflatoxin Contamination and Ear-Feeding Insect Damage in Pre-Harvest Corn: Summary of Two-Year Data

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Natural distribution patterns of ear-feeding insect damage and aflatoxin levels were examined in 2005 and 2006. A pre-harvest corn field (1/6 hectare) was sampled according to an 8x8 m grid each year. The husk coverage and insect damage were assessed in the field. Insect damage assessment included the corn earworm, *Helicoverpa zea* (Boddie), the maize weevil, *Sitophilus zeamais* Motschulsky, as well as the brown, *Euschistus servus* (Say), and southern green stink bug, *Nezara virdula* (L.). The top ears from five plants were collected at each grid point in the field when the kernel moisture was at 12% for aflatoxin quantification. The aflatoxin levels in 2005 were positively correlated to the number of the maize weevils ($r = 0.3$, $P = 0.002$, $n = 92$), but not to either corn earworm or stink bug damage, or husk coverage (P-values > 0.05). In contrast, the data collected in 2006 showed that aflatoxin levels in corn samples were positively correlated to both maize weevil number ($r = 0.46$, $P = 0.0001$, $n = 76$) and percentage of stink bug-damaged kernels ($r = 0.36$, $P = 0.0012$, $n = 76$), but not to other indices (i.e., corn earworm damage or husk coverage) (P-values > 0.05). The mean aflatoxin levels were not different ($F = 0.74$, $df = 1, 166$, $P = 0.3917$) between 2005 (33.78 ppb, $n = 92$) and 2006 (25.51 ppb, $n = 76$). The roles of insect feeding and other environmental factors on aflatoxin and other mycotoxin accumulations in pre-harvest corn are also discussed.

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