Proceedings of the 2005 Annual Multicrop Aflatoxin/Fumonisin Elimination & Fungal Genomics Workshop

October 24 – 26, 2005
Raleigh, North Carolina USA

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

Cotton Incorporated

National Cottonseed Products Association

Arizona Cotton Research and Protection Council

The Cotton Foundation

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Introduction:

Aflatoxin and Fumonisin Elimination and Fungal Genomics Workshop – 2005
Raleigh, NC

If any of us were becoming smug in believing that aflatoxin would never be a real problem here in the US, the events starting at Christmas 2005 should have emphatically changed our minds. We may give thanks and relax somewhat that there was no human involvement with aflatoxin poisoning, but consumers did bring aflatoxin into their homes in contaminated food for their dogs. The latest statements from Cornell University College of Veterinary Medicine where the definitive diagnoses were made are that they believe that over 100 dogs have died in recent weeks. This follows cases of human ingestion of toxic amounts of aflatoxin and resulting morbidity and mortality from eating home grown corn in Kenya in 2004.

The presentations at the 2005 Aflatoxin Workshop which focused on preharvest aflatoxin control should give hope that aflatoxin can be eliminated as a serious problem during production of susceptible crops in the U.S. Innate crop resistance and good crop production practices need to be, at least, equal partners with statistically valid sampling; regular, sensitive and accurate product assays; proper commodity handling and processing practices and/or the use of absorbing clays in product formulations in assuring food product safety.

Highlights of the Presentations of the Workshop follow:

Fumonisins

Fusarium verticillioides is a fungal pathogen of maize throughout the world. The fungus can infect all stages of maize development and almost every tissue type of the maize plant, thus causing seedling blight, root rot, stem rot, and kernel rot. Each of these disease manifestations can result in severe economic losses. The fungus also can coexist relatively peacefully with maize as an endophyte, maintain a biotrophic growth habit throughout the entire growing season of the plant, and cause asymptomatic infections of kernels. During the colonization of maize kernels, *F. verticillioides* produces toxic secondary metabolites known as fumonisins. Fumonisins cause a range of species-specific health effects when ingested and are suspected to cause cancer and birth defects in humans. At the workshop session on Fumonisin Elimination, five presentations focused on three important research areas: breeding for resistance, understanding the basis for fumonisin production, and identifying fumonisin-contaminated grain along the marketing supply chain.

For many years, pathologists have known that the amount of fumonisin contamination in maize does not correlate to the severity of kernel damage caused by the pathogen. Maize breeders who have studied the inheritance of resistance have contended that disease severity, which is easy to score, and fumonisin contamination, which is expensive to analyze, are separable traits. Holland presented results from a study of two maize breeding populations, in which he examined disease severity (rot) and fumonisin content. Based on his results, Holland made the case that the most economical method to breed for resistance to fumonisin contamination would be to make selections based on disease severity.

One approach toward discovering potential targets for resistance is to study the basis of susceptibility. Three presentations in the session were in this category. First, Zimeri presented evidence that sphingolipid metabolism in maize roots is affected by fumonisin, suggesting that toxin production has a role in the pathogenesis of maize seedlings. She presented evidence that treatment of seedlings with fumonisin caused symptoms similar to pathogen-infected seedlings. Interestingly, the ancestors to maize, teosinte and *Tripsacum*, were more sensitive to fumonisin. Secondly, Kolomiets presented evidence that suggests the *LOX3* gene in maize, which encodes a 9-lipooxygenase, is associated with fumonisin production in diseased kernels. Kernels from a maize line with a mutated *lox3* supported growth of the pathogen but fumonisin production and conidiation were severely reduced. The result suggests that fatty acid hydroperoxides, which are the products of 9-lipooxygenase, have a role in regulating both development and secondary metabolism in the pathogen. Thirdly, Woloshuk presented evidence that fumonisin production in *F. verticillioides* is associated with the metabolism of endosperm starch. Kernels with reduced amylopectin due to immaturity or mutations in starch synthesis do not support fumonisin production by the pathogen. Key for the fungus is the expression of alpha-amylase, which degrades amylopectin to yield fumonisin-inducing alpha-1,6-glucosides.

To assure that the quality of grain is maintained along the supply chain from the producer to the end-user, new methodology for testing grains is needed, and technologies developed in near-infrared and reflectance (NIR) spectroscopy have become important tools. Wicklow described the application of NIR technology to identify and sort mold-damaged and mycotoxin-contaminated grain. His results showed that a NIR sorting device that uses two specific wavelengths to assess kernels was very effective in removing highly damaged and fumonisin-contaminated kernels.

Conventional Breeding: Resistance to Aflatoxin Production

Concerted cooperative effort and optimization of evaluation techniques are responsible for the significant progress now evident in the development of commercially acceptable varieties with low levels of aflatoxin in the commodity at harvest. Newly created resistant varieties of corn, peanut and almond are at the early stages of commercialization. Identification of plant genotypes with low levels of aflatoxin in seeds at harvest is relatively easy compared with the problem of moving resistance into a commercially acceptable variety that has the various quality traits and yield that are demanded by producers. Generally, the corn, peanut and almond varieties developed in this research also have the desired commercial traits and will likely be commercially used in the near future.

Several projects are continuing to identify new sources of resistance for corn where there is a wide variety of publicly available germplasm available for evaluation that may yield unique sources of resistance. Additional sources of resistance are of value especially when they have high levels of resistance controlled by relatively few genes. Perhaps the greatest challenge of conventional breeding is evaluating for resistance and/or susceptibility in the field.

Resistance in corn, peanuts, almonds and cotton can be successfully identified when environmental conditions are conducive for aflatoxin production. With peanuts, modification of the environment can create more favorable conditions for aflatoxin production. Corn scientists have joined together to evaluate hybrids created in the various research projects over a number of locations utilizing different inoculation techniques. This cooperative effort has been extremely valuable in characterizing the level of resistance as well as the agronomic characteristics of newly developed resistant hybrids. Conventional breeding can also identify crop characteristics than are strongly associated with low aflatoxin accumulation and select for those characteristics. In many cases, the associated traits are easier to select for and have higher heritability that aflatoxin accumulation per se. Perhaps the greatest success has been with almond where aflatoxin contamination is strongly associated with insect damaged kernels. Three different major components of insect resistance have been successfully used in breeding to reduce aflatoxin. Advanced selections of almond are demonstrating very low levels of insect damage and therefore low aflatoxin (Gradziel).

With peanut low levels of aflatoxin has been associated with drought tolerance and resistance to the peanut root knot nematode. Peanut varieties have been developed with drought resistance and have demonstrated lower aflatoxin contamination in multiple environments. It also is necessary to have resistance to tomato spotted wilt virus in a commercial peanut variety. Recently advanced breeding lines with resistance to both virus and nematode as well as acceptable yield and grade have been developed (Holbrook). Resistance to aflatoxin accumulation in corn has also been associated with resistance to drought stress and corn earworm. Varieties selected for resistance to drought and earworm resistance have lower aflatoxin contamination in the grain.

Conventional breeding is now incorporating the more recently developed molecular biology techniques to enhance chances of success. Molecular markers for corn are being used to transfer chromosome areas associated with genes conditioning resistance into commercially acceptable corn inbreds. This is highly advantageous because evaluation for aflatoxin accumulation in grain is not necessary at every cycle of breeding. Molecular markers are greatly enhancing the successful movement of genes from a source of resistance with poor agronomic characteristics into commercially used inbreds. Resistance has been incorporated into commercially used inbreds and hybrids that will be evaluated in precommercial trials over a number of locations in 2006.

Genomic and proteomic techniques are being utilized with corn and peanut to compare resistant and susceptible genotypes to attempt to identify characteristics of the resistant genotypes that are associated with low aflatoxin. Ultimately each potential mechanism associated with resistance will need to be crossed into common genetic backgrounds and evaluated in the field which will take several years and considerable effort. If successful, these techniques offer a possibility of identifying unique molecular markers to be used

in conventional breeding, and they may also identify actual genes for resistance. In the future it is highly likely that genes conditioning different mechanisms of resistance can be pyramided into commercially acceptable varieties resulting in extremely low levels of aflatoxin.

Control of aflatoxin contamination by genetic resistance has a major advantage in that it is cost-effective to the producer, environmentally friendly, and acceptable to the general public. It is highly likely that the techniques and procedures used in the development of varieties with low aflatoxin will be modified and used to develop varieties that control other mycotoxins.

Fungal Genomics

A number of inroads were made over the past year in improving our understanding of the genetic basis and functional genomics of fumonisin and aflatoxin production. We are seeing increased use of microarray analyses to get a better picture of the genetic processes involved in the infectivity and toxin production by these two agriculturally important fungi.

With regard to fumonisin, preliminary analysis has found a number of candidate fumonisin regulatory genes in *Fusarium verticillioides* (Brown). The gene *GBP1* was identified as being associated with the regulation of fumonisin in the *F. verticillioides* porobably independent of the FUM gene cluster (Sagram).

In *Aspergillus flavus*, it was found that oxidative stress and antioxidants affect aflatoxin biosynthesis, suggesting the antioxidative response systems of *A. flavus* are targets for control. Combined treatment of fungi with phenolics and inhibitors of the mitochondrial respiratory system effectively suppressed growth of *A. flavus*. Targeting genes in other antioxidative response, MAPK or vacuolar H(+)-ATPase (V-ATPase) systems should greatly improve methods for fungal control using combinations of compounds (Kim). The gene *veA* regulates both, formation of resistant structures (scletoria), and biosynthesis of aflatoxin, and other toxins, in *A. flavus*. In a *veA* mutant expression of *AflR* and aflatoxin was suppressed. Targeting *veA* could decrease *A. flavus* survivability by affecting sclerotial development. Homologues of *veA* were found across fungal genera indicating the possibility of targeting this gene for broad spectrum fungal control (Duran). A component of a signaling pathway that may modulate aflatoxin production in *A. flavus* was identified. This identified gene had the greatest similarity to *rdi,* a yeast gene that encodes a Rho-guanidine nucleotide dissociation inhibitor (Rho-GDI). Deletion of this gene caused a severe growth defects of *A. flavus* on minimal media, a moderate defect on complete media, and a temperature sensitive phenotype. This gene may play a central role in combining *A. flavus* proteins AflR and RasA for facilitating signaling control of aflatoxin production through a Rho-mediated pathway (Georgianna).

Microarray analyses were used as a means for establishing a framework for sorting gene expression for aflatoxin biosynthesis on conducive and nonconducive conditions based on temperature (Glassbrook). Other microarray experiments suggested that the gene *nadA* is upregulated by *AflR* to supply NAD$^+$ cofactors for the aflatoxin biosynthesis. However, knocking out this gene did not affect production of aflatoxin suggesting that there is compensation for NADH oxidase activity or that it is not required for aflatoxin production (Jacobus). Microarray anaysis of maize lines Va3, susceptible, and Mp313E, resistant, to *A. flavus* showed expression patterns for a number of maize genes when exposed to *A. flavus* (Kelly). Genes that are putatively involved in aflatoxin biosynthesis, regulation and signal transduction, fungal virulence or pathogenicity, stress response or antioxidation, and fungal development were identified from an *A. flavus* EST library. This was used to construct microarrays containing over ,000 unique gene amplicons. Microarray-based gene profiling has thus far identified hundreds of genes that are potentially involved in aflatoxin production. This research is expected to provide information for developing new strategies for control of aflatoxin contamination of agricultural commodities (Yu).

Genomic analysis research is underway to determine if gene clusters associated with secondary metabolism of *A. flavus* are within polymorphic subtelomeric domains as found in other closely related fungi (Nierman). A 5-X coverage of the genomic sequence of *A. flavus* was completed and preliminarily annotated as much as possible. A web browser has been set up at NC State allowing BLAST searches of genes, proteins and genomic sequences of *A. flavus* and other *Aspergillus* species including alignments of ESTs, and GO annotations (Payne).

Research was presented suggesting the potential for low-level recombination and gene flow between atoxigenic and toxigenic strains of *A. flavus*. How this may affect use of atoxigenic strains for biocontrol remains to be seen (Carbone).

Improvement of Aflatoxin Resistance *via* Genetic Engineering

All of the current research projects seek to either reduce aflatoxin contamination by retarding *Aspergillus* infection, or interfere with mechanisms involved in toxin synthesis. Strategies include transformation of crop species with either naturally occurring or synthetic antifungal genes, mutation breeding, and the development of reliable molecular markers to improve the efficiency of breeding programs to produce improved fungal resistance.

Three distinct projects attempt to reduce both infection and aflatoxin contamination in peanut (Ozias-Akins). First are transgenic peanuts that express an anti-apoptotic gene, Bcl-xl, the product of which is expected to reduce *Aspergillus* infection. Second is a mutation breeding program involving EMS-mutagenized peanut populations which are being studied by TILLING (Targeting Induced Local Lesions IN Genomes) to identify genes that might be manipulated to alter fungal infection and/or aflatoxin contamination. TILLING can identify mutants based on screening with gene sequence rather than for phenotype. The TILLING technique is being tested with an allergen gene, *ara h ,* for which there is sufficient genomic sequence. Gene-specific primer sets have been designed for TILLING so that mutations in each copy of *ara h 2* can be screened separately. Third, characterization of allergen gene sequence has also allowed the isolation of promoters that may be useful for antifungal gene expression, particularly when expression is to be targeted to the developing seed. This work is expected to yield, among other things, promoter sequences that will be useful in achieving tissue-specific expression of defensive transgenes in developing peanut seeds.

Since *Aspergillus* enters the maize plant through the silks, (Peethambaran) believes it might be possible to inhibit infection by identifying silk-protein markers associated with resistance. Thus, the proteome of silks from resistant plants is being compared with that from susceptible ones to attempt to identify proteins produced by native genes that are involved in resistance, and/or to identify protein markers that can be used in a marker-assisted breeding program. Proteins present in resistant lines have been identified and characterized, and have been successfully tested against *A. flavus in vitro*.

Resistance-associated proteins (RAPs) have been identified using proteomics to compare constitutive protein profiles between resistant and susceptible maize genotypes (Chen). Transformed corn plants were subsequently produced in which silencing of the target RAPs was observed in some lines, and a kernel screening assay demonstrated a significant increase in susceptibility to *A. flavus* colonization and aflatoxin production in these lines. These observations are consistent with the hypothesis that RAPs are directly involved in aflatoxin resistance in maize, and therefore genes encoding these proteins would be excellent candidates for use as molecular markers of resistant corn lines.

The production and testing of transgenic peanut lines expressing an active form of the maize ribosome inactivating protein, RIP 1 was reported (Weissinger). Peanuts expressing the active form of the RIP, Mod 1 were tested *in vitro* previously and have been shown to be resistant to *Aspergillus* infection All of the lines found to be resistant to *A. flavus* were subsequently tested for resistance against two leaf pathogens, *Sclerotinia minor* and *Sclerotium rolfsii*, using a detached-leaf test that permits quantification of fungal growth. Four transgenic lines derived from the runner type peanut, Georgia Green, and one line derived from the Virginia cultivar, NCV 1, were found to exhibit significant resistance compared with un-transformed peanut lines. These lines are now being tested against a range of other fungal pathogens, and will also be tested to determine the extent to which the enhanced resistance against *A. flavus* infection is reflected in reduction of aflatoxin contamination.

Testing continues of fertile, transgenic cotton plants expressing the synthetic antimicrobial peptide, D4E1 (Rajasekaran). Transgenic lines produced through *Agrobacterium*-mediated transformation (Rajasekaran et al. *Plant Biotechnology Journal* 3: 545-554. 2005) expressed the antifungal gene product, and *in vitro* assays of plant leaf extracts confirmed that D4E1 was expressed at sufficient levels to inhibit the growth of *Fusarium verticillioides* and *Verticillium dahliae*. Although *in vitro* assays did not show control of pre-germinated spores of *Aspergillus flavus*, bioassays with cotton seeds *in situ* or *in planta*, inoculated with a GFP-expressing *A. flavus*, indicated that the transgenic cotton seeds inhibited extensive colonization and spread by the fungus in cotyledons and seed coats. Transgenic T1 seedlings had significantly reduced disease symptoms and increased seedling fresh weight, and thus tolerance to the black root rot fungal pathogen of cotton, *Thielaviopsis basicola*. Field evaluation of T2 progeny for resistance against Fusarium wilt race 1 indicated that the transgenic entries had improved stand, up to 68%, compared with un-transformed controls at 43%.

Crop Management

Insect control is a major focus of crop management. Past studies in multiple locations have indicated that significant reductions in fumonisins can occur on many corn hybrids expressing the Bt gene compared to nonBt hybrids when the target insect (European corn borer) is controlled at levels approaching 100%. However, this is only one of many different insect species that can promote mycotoxins in the various susceptible crops. Fall armyworm leaf feeding and root knot nematode damage have been investigated for their importance in contributing to the aflatoxin problem in corn in the southeast (Krakowsky et al.). Root knot nematodes appear to also promote aflatoxin in peanuts by enhancing the stress due to drought, but may also contribute through physical damage to pods (Timper et al.). Codling moths, which promote aflatoxin formation in walnuts, have been effectively controlled through use of a pear-derived attractant that can be applied in a microencapsulated form in combination with reduced rates of insecticides (Light). The activity of plant derived anthocyanins (Johnson), chitinase like enzymes, peroxidases, and corn RIP enzyme (Dowd) studied in dietary assays and transgenically in plants, indicate they have the potential to be variously combined into stable, broad spectrum insect resistance through introductions of compatible genes.

Management tactics in addition to insect control, especially when conditions favorable for mycotoxin formation can be identified, are also of potential use. Because aflatoxin can be degraded by ammonia, and because ammonia production is enhanced in plants treated with Liberty herbicide, there is potential that either Liberty-Link plants (which degrade the ammonia) or normal plants treated with relatively nontoxic levels of the herbicide, may have reduced levels of aflatoxin. One study (Bruns and Abbas) has reported no significant reduction of aflatoxin in corn in Mississippi, but another study indicated that low levels of Liberty could significantly reduce aflatoxin levels when applied sooner (*v.* later) after mid silk by ground to non Liberty-Link hybrids (Moore). Further validation of the results under the same timing and application rates used in the Louisiana study and additional optimization may lead to an additional control strategy for aflatoxin in corn.

Predicting when conditions favorable for mycotoxin formation occur is key to developing and implementing management tactics. Remote sensing of peanut canopy indicated that it was able to give more specific and timely estimates of genotype response to drought than visual observations (Sullivan). This technique could be used to enhance breeding progress of drought and aflatoxin resistant peanut varieties. A predictive computer program for mycotoxin occurrence in Midwest corn initially predicted that *Aspergillus flavus* inoculum was likely to be present at problem levels at corn silking, and subsequent predictions indicated that low levels of aflatoxin were likely to be present in corn at harvest in central Illinois in 2005 (Dowd et al.). Grain elevators in the area did reject loads of aflatoxin contaminated corn sporadically through October 2005. Wider distribution of this program, which has also given reliable predictions of fumonisin levels over the past 5 years, is likely to promote better management of mycotoxins in corn.

Microbial Ecology

Considerable progress has been made in the use of competitive nonaflatoxigenic strains of *A. flavus* for reducing aflatoxin contamination in peanuts in the southeastern United States and cottonseed in Arizona and Texas. Biocontrol agents in the form of spore-coated barley (peanuts) or colonized wheat (cotton) recently have been commercially applied to large areas of crops. Afla-guard®, the biocontrol product for peanuts, changed soil populations from 71% to 4% toxigenic strains of *A. flavus* and this ultimately resulted in an overall aflatoxin reduction of 85% in peanuts (Dorner). Biocontrol application in peanut fields provides an additional benefit by controlling aflatoxin contamination during peanut storage under suboptimal temperature and moisture conditions. Biological control in cotton may also have a carryover effect in reducing aflatoxin contamination of corn, a common rotation crop for cotton (Cotty). The primary focus now is to optimize the cost of producing inoculum and to identify environmental factors in the field most conducive to reduction of aflatoxins. Both soil texture and canopy shading are critical for sporulation of nonaflatoxigenic *A. flavus* on wheat (Antilla and Cotty). Questionnaires are being provided to cotton farmers using biological control to better correlate specific cultivation practices with effective reduction of aflatoxins.

Biocontrol technology using nonaflatoxigenic *A. flavus* strains also is being tested with pistachios and figs in California (Doster et al.). Biocontrol *A. flavus* strains applied to pistachio orchards persisted in

soil for at least two years and continued to displace native toxigenic strains (80–97% of isolates). Equally important, application of *A. flavus* AF36 does not appear to increase fungal decay of early split nuts. *A. flavus* AF36 was most prevalent in soils under drip lines in drip-irrigated fig orchards. As with pistachios, biological control did not increase the incidence of fig decay. Other biocontrol strategies that are being pursued include the use of yeasts and bacteria for controlling invasion of tree nuts by aflatoxigenic fungi. The yeast *Pichia anomala* WRL-076 reduced the frequency of *A. flavus* colonization of wounded pistachio nuts by up to 10-fold (Hua). Furthermore, *P. anomala* will grow at low water activities that are conducive to invasion by *A. flavus*. Bacterial populations associated with almond reproductive parts are being examined for strains that are antagonistic to *A. flavus* (Palumbo et al.).

Research in biological control technology has provided a wealth of knowledge concerning the genetics and dynamics of natural populations of aflatoxigenic fungi and how these populations interact with applied biocontrol strains. Several lines of basic research are being conducted to better understand the mechanisms underlying the inhibition and exclusion of toxigenic fungi from crops by biocontrol agents. A laboratory assay in which wounded viable peanut seeds are inoculated with soil from the field indicates that section *Flavi* species preferentially invade peanuts at 22–37 °C and 0.92–0.96 seed water activity (Horn). It also appears that competition within section *Flavi* in natural populations accounts for considerable reduction in aflatoxin contamination. Other research suggests that chitinase production is one mechanism underlying the parasitism of *Aspergillus* and *Fusarium* species by *Gliocladium catenulatum* (Kendra et al.).

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

Considerable advances have been made in the identification of naturally-occurring compounds in both corn and tree nuts that suppress the formation of aflatoxins. Two compounds (ABI-1 and ABI-2) have been identified in kernels of the resistant maize, Tex6, that suppress both fungal growth and aflatoxin biosynthesis (Payne). ABI-2 was a less potent inhibitor of fungal growth than ABI-1 but had more effect on aflatoxin biosynthesis. Both compounds appear to suppress transcription of pathway genes, but act differently on other regulatory genes. Initial structural characterization showed these compounds to be non-proteinaceous, heat labile, small molecules, while the mass spectrometric molecular weight suggested the presence of a nitrogen atom. The inhibitors appear to belong to the inositol polyphosphate class, related to phytic acid, although the latter compound showed no inhibitory activity.

Previous work on polyphenolic constituents of walnut that inhibit aflatoxin biosynthesis has been extended to other phenolic antioxidants present in almonds and pistachios (Molyneux). These included hydrolysable tannins; the flavonoid, catechin; and a series of phenolic acids common to tree nuts and other plant species. The commercial antioxidant, lauryl gallate, was also tested as a model for the anacardic acids present in pistachio hulls. The most potent compounds were pentagalloyl glucose, caffeic acid and lauryl gallate, each of which inhibited aflatoxin production by >99%. Testing in the presence and absence of peroxide showed that phenolic compounds were able to overcome aflatoxin production induced by oxidative stress and this was confirmed using singular gene deletion mutants of *Saccharomyces cerevisiae* as a model fungal system to examine functional genomics of oxidative stress responses. The results indicate that aflatoxin production is stimulated by oxidative stress and that phenolic compounds present in tree nuts are capable of suppressing aflatoxin biosynthesis, implying that breeding to enhance levels of natural phenolics should reduce the potential for aflatoxin contamination.

Dr. Jane F. Robens
National Program Leader
Food Safety and Health
Beltsville Agricultural Research Center
Agricultural Research Service, USDA
Beltsville, MD

AGENDA

**5ᵗʰ Annual Fungal Genomics,
6ᵗʰ Annual Fumonisin,
18ᵗʰ Annual Aflatoxin Elimination Workshop**

October 24-26, 2005

Marriott Crabtree Valley – Raleigh, NC

SUNDAY, OCTOBER 23, 2005

3:00 - 6:00   REGISTRATION / POSTER ASSIGNMENTS

MONDAY, OCTOBER 24, 2005

7:15   Load buses for travel to Cotton Incorporated World Headquarters (Cary, NC)

8:00   Doughnuts, Coffee, and Presentations and Tour of Cotton Incorporated

11:30   Depart for Bayer CropScience (Research Triangle Park, NC)

12:00   Lunch and Tour of Bayer CropScience (Gustafson) Research Labs

2:30   Depart for NC State University (Raleigh, NC)

3:30   Tour NC State University Centennial Campus Research Facilities

5:00   Return to Marriott Crabtree Valley

Dinner on your own

4:00 – 7:00   REGISTRATION / POSTER ASSIGNMENTS

TUESDAY, OCTOBER 25, 2005

7:00 – 8:00   CONTINENTAL BREAKFAST

7:00 – 5:00   REGISTRATION / POSTER ASSIGNMENTS

8:00   Welcome
Roy Cantrell, Cotton Incorporated

8:05   Introductory Remarks
Jane F. Robens, USDA-ARS, National Program Leader, Beltsville, MD

5TH ANNUAL FUNGAL GENOMICS WORKSHOP

MODERATOR: Roy Cantrell, Cotton Incorporated

8:10   **Finding Target Genes for Better Control of *Aspergillus*.** Jong H. Kim[1], Bruce C. Campbell[1], Jiujiang Yu[2], Gregory S. May[3], Kathleen L. Chan[1], Gary A. Payne[4], Deepak Bhatnagar[2], and Thomas E. Cleveland[2]. [1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [3]MD Anderson Cancer Center, University of Texas, Houston, TX; [4]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

8:25   **Comparative Genomic Analysis of Secondary Metabolite Gene Clusters of Closely Related Aspergilli.** William C. Nierman[1], Natalie D. Fedorova[1], Catherine M. Ronning[1], Jennifer Wortman[1], Masayuki Mashida[2], Jiujiang Yu[3], Thomas E. Cleveland[3], Deepak Bhatnagar[3], and Gary A. Payne[4]. [1]The Institute for Genomic Research, Rockville, MD; [2]Institute for Biological Resources and Functions, Nat. Inst. Of Advanced Ind. Sci. and Technol., Tsukuba, Japan; [3]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [4]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

8:40   ***Aspergillus flavus* Genomics in Discovering Genes Involved in Aflatoxin Biosynthesis.** Jiujiang Yu[1], Jeffery R. Wilkinson[1], William C. Nierman[2], H. Stanley Kim[2], Gary A. Payne[3], Bruce C. Campbell[4], Deepak Bhatnagar[1], and Thomas E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]The Institute for Genomic Research, Rockville, MD; [3]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [4]USDA-ARS, Western Regional Research Center, Albany, CA.

8:55   **Mining Expressed Sequence Tags (ESTs) Leads to Identification of Putative FUM Cluster Transcription Factor.** Daren W. Brown, Robert A. E. Butchko, Mark Busman, and Robert H. Proctor. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

9:10   **Release of the *Aspergillus flavus* Genome Sequence.** Gary A. Payne[1], B. Pritchard[1], Jiujiang Yu[2], William C. Nierman[3], Ralph Dean[1], Deepak Bhatnagar[2], and Thomas E. Cleveland[2]. [1]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [3]The Institute for Genomic Research, Rockville, MD.

9:25   **Production of Cyclopiazonic Acid, Aflatrem, and Aflatoxin is Regulated by veA, a Gene Necessary for Sclerotial Formation in *Aspergillus flavus*.** Rocio M. Duran[1], Jeffrey W. Cary[2], and Ana M. Calvo[1]. [1]Department of Biological Sciences, Northern Illinois University, Dekalb, IL; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

9:40 – 10:00   PANEL DISCUSSION
               Panel Chair: Gary A. Payne, North Carolina State University, Raleigh, NC

10:00 –10:20   BREAK AND POSTER VIEWING

6TH ANNUAL FUMONISIN ELIMINATION WORKSHOP

MODERATOR: Larry Antilla, Arizona Cotton Research and Protection Council

10:20   **Kernel Constituents Induce Fumonisin Production during Colonization by *Fusarium verticillioides*.** Charles Woloshuk, Purdue University, West Lafayette, IN.

10:35   **Genetics and Breeding of Host Resistance to *Fusarium* Ear Rot and Fumonisin Contamination.** James Holland. Department of Crop Science, North Carolina State University, Raleigh, NC.

10:50   **NIR Spectroscopy as a Tool for Optimizing Sorting of White Corn Kernels Contaminated with Fumonisin.** Tom C. Pearson[1] and Donald T. Wicklow[2]. [1]USDA-ARS, Grain Marketing and Production Research Center, Manhattan, KS; [2]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:05   **Maize *LOX3* Gene is Required for Fumonisin Biosynthesis and Conidiation of *Fusarium verticillioides*.** Xiquan Gao[1], Won-Bo Shim[1], Ivo Feussner[2], and Mike Kolomiets[1]. [1]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX; [2]Georg-August University of Göettingen, Göettingen, Germany.

11:20   **Toxicity Responses of Corn to the Mycotoxin Fumonisin B$_1$ in the Absence of *Fusarium verticillioides* Infection.** Anne Marie Zimeri, Lonnie D. Williams, Ronald T. Riley, and Anthony E. Glenn. USDA-ARS, Richard B. Russell Research Center, Athens, GA.

11:35 – 11:55   PANEL DISCUSSION
                      Panel Chair: Charles Woloshuk, Purdue University, West Lafayette, IN

12:00 – 1:00   LUNCH

18TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 1: Crop Resistance – Conventional Breeding

MODERATOR: Don Jones, Cotton Incorporated

1:00   **Progress on the Creation of Usable Commercial Inbreds and Hybrids with Low Aflatoxin in Grain Using Molecular Markers.** Don White and Torbert Rocheford, University of Illinois, Urbana, IL.

1:15   **Breeding Corn Germplasm for Agronomic Performance and Reduced Aflatoxin Contamination.** Javier Betrán[1], Tom Isakeit[2], Gary Odvody[3], and Kerry Mayfield[1]. [1]Soil & Crop Sciences Department, Texas A&M University, College Station, TX; [2]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX; [3]Texas A&M Research & Extension Center, Corpus Christi, TX.

1:30   **Interaction Between *Aspergillus flavus* Strains and Host Plant Genotypes Across Environments and Years.** Kerry Mayfield[1], Tom Isakeit[2], Gary Odvody[3], and Javier Betrán[1]. [1]Soil & Crop Sciences Department, Texas A&M University, College Station, TX; [2]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX; [3]Texas A&M Research & Extension Center, Corpus Christi, TX.

1:45   **Application of HACCP to Control Mycotoxins in Maize Breeding Programs.** David F. Kendra. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

2:00   **Suppression of Insect Mediated Aflatoxin Contamination of Almond.** T. M. Gradziel and A. H. Dandekar. Department of Plant Sciences, University of California at Davis, Davis, CA.

2:15   **Genetic and Genomic Approaches to Improve Host Resistance to Preharvest Aflatoxin Contamination in Corn and Peanut.** Baozhu Guo[1], M. Luo[2], H. Chen[3], A. E. Coy[2], Matthew D. Krakowsky[4], C. Corley Holbrook[4], X. Liang[5], R. Dewey Lee[2], and Craig K. Kvien[3]. [1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]Department of Crop and Soil Sciences, University of Georgia, Tifton, GA; [3]NESPAL, University of Georgia, Tifton, GA; [4]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [5]Guangdong Academy of Agricultural Sciences, Guangzhou, China.

2:30 – 2:50   BREAK AND POSTER VIEWING

2:50   Progress Toward Identifying New Sources of Genetic Variation Associated with Reduced Levels of Aflatoxin Accumulation in Maize. Thomas Brooks[1], Matthew Krakowsky[2], W. Paul Williams[1], and Gary Windham[1]. [1]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [2]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

3:05   Proteomic Identification of Maize Cob Proteins that Potentially Confer Resistance to Aflatoxin. Dawn S. Luthe[1], Olga Pechanova[1], Bele Peethambaran[1], Leigh Hawkins[2], Tibor Pechan[3], Gary Windham[4], Susan Bridges[5], and W. Paul Williams[4]. [1]Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [3]Life Sciences and Biotechnology Institute, Mississippi State University, Mississippi State, MS; [4]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [5]Department of Computer Science, Mississippi State University, Mississippi State, MS.

3:20   A Field Technique for Varietal Assessment of Second Phase Aflatoxin Contamination in Cotton. Mary W. Olsen[1] and Peter J. Cotty[2]. [1]Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ; [2]USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ.

3:35   Corn Hybrids with Exotic Germplasm and Low Aflatoxin. Wenwei Xu[1], Gary Odvody[2], and W. Paul Williams[3]. [1]Agricultural Research and Extension Center, Texas A&M University, Lubbock, TX; [2]Agricultural Research and Extension Center, Texas A&M University, Corpus Christi, TX; [3]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

3:50   Computational Tools for Protein Identification and Gene Ontology Annotation of the Maize Proteome. Susan M. Bridges[1], Julia E. Hodges[1], Gregory Bryce Magee[1], Nan Wang[1], Dawn S. Luthe[2], and W. Paul Williams[3]. [1]Department of Computer Science and Engineering, Mississippi State University, Mississippi State, MS; [2]Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [3]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

4:05   Progress in Breeding Peanut for Resistance to Preharvest Aflatoxin Contamination and Drought. C. Corley Holbrook[1], Baozhu Guo[2], David M. Wilson[3], Dana G. Sullivan[4], Emily Cantonwine[3], and Craig K. Kvien[5]. [1]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [3]Department of Plant Pathology, University of Georgia, Tifton, GA; [4]USDA-ARS, Southeast Watershed Research Laboratory, Tifton, GA; [5]NESPAL, University of Georgia, Tifton, GA.

4:20   **Searching for New Resistance and Control Measures of Aflatoxin in Corn.** Steven Moore[1], Hamed Abbas[2], and Mark Millard[3]. [1]Louisiana State University Agricultural Center, Alexandria, LA; [2]USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS. [3]North Central Regional Plant Introduction Station, Ames, IA.

4:35   **Development of Aflatoxin-resistant Maize Inbreds and Identification of Potential Resistance Markers through USA-Africa Collaborative Research.** Robert L. Brown[1], Zhi-Yuan Chen[2], Abebe Menkir[3], Ranajit Bandyopadhyay[3], and Thomas E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA; [3]International Institute of Tropical Agriculture, Ibadan, Nigeria.

4:50 – 5:10   PANEL DISCUSSION
Panel Chair: Don White, University of Illinois, Urbana, IL

6:00 – 7:30   POSTER VIEWING WITH HORS D'OUVRES AND BEVERAGES

WEDNESDAY, OCTOBER 26, 2005

7:00 – 8:00   CONTINENTAL BREAKFAST

7:00 – 5:00   REGISTRATION / POSTER ASSIGNMENTS

Session 2: Microbial Ecology

MODERATOR: Phil Wakelyn, National Cotton Council

8:00   **Effect of Fungal Competition on the Colonization of Wounded Peanut Seeds by *Aspergillus* Section *Flavi* from Natural Soil Populations.** Bruce W. Horn, USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

8:15   **Transfer of Aflatoxin Biocontrol Technology: Results of First Commercial Use in Peanuts.** Joe W. Dorner. USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

8:30   **Atoxigenic Strain Technology for Aflatoxin Control in Cotton.** Larry Antilla, Arizona Cotton Research and Protection Council, Phoenix, AZ.

8:45   **Managing Aflatoxins in Cotton-Corn Rotations.** Peter J. Cotty, USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ.

9:00 **Aflatoxin Control in Pistachios: Biocontrol Using Atoxigenic Strains.** <u>Themis Michailides</u> and Mark Doster. University of California, Davis/Kearney Agricultural Center, Parlier, CA.

9:15 **Aflatoxin Control in Figs: Biocontrol and New Resistant Cultivars.** <u>Mark Doster</u> and Themis Michailides. University of California, Davis/Kearney Agricultural Center, Parlier, CA.

9:30 **Identification of Bacterial Antagonists of *Aspergillus flavus* from California Almond Orchards.** <u>Jeffrey D. Palumbo</u>, James L. Baker, and Noreen Mahoney. USDA-ARS, Western Regional Research Center, Albany, CA.

9:45 **Biological Control of *Aspergillus flavus* by a Saprophytic Yeast Strain in Tree-nut Orchards: Progress in 2005.** <u>Sui Sheng Hua</u>. USDA-ARS, Western Regional Research Center, Albany, CA.

10:00 – 10:20 BREAK AND POSTER VIEWING

10:20 – 10:40 PANEL DISCUSSION
Panel Chair: Bruce W. Horn, USDA-ARS, National Peanut Research Laboratory, Dawson, GA

Session 3: Crop Resistance – Genetic Engineering

MODERATOR: Keerti Rathore, Texas A&M University

10:40 **Genetic Engineering of Peanut with Putative Antifungal Genes.** Y. Chu[1], P. Faustinelli[1], L. Ramos[1], K. Rajasekaran[2], J. Cary[2], and <u>P. Ozias-Akins</u>[1]. [1]Department of Horticulture and NESPAL, University of Georgia Tifton Campus, Tifton, GA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

10:55 **Transgenic Peanuts with Enhanced Resistance to *Aspergillus flavus*.** <u>Arthur K. Weissinger</u>, Department of Crop Science, North Carolina State University, Raleigh, NC.

11:10 **Identification, Characterization and Antifungal Activities of Silk Proteins in *Aspergillus flavus* Resistant and Susceptible Corn Inbreds.** <u>Bela Peethambaran</u>[1], Gary L. Windham[2], Leigh Hawkins[2], W. Paul Williams[2], and Dawn S. Luthe[1]. [1]Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

11:25   **Silencing the Expression of RAP Genes in Maize and the Effect on Host Resistance against *Aspergillus flavus* Infection and Aflatoxin Production.** <u>Zhi-Yuan Chen</u>[1], Robert L. Brown[2], Thomas E. Cleveland[2], and Kenneth E. Damann[1]. [1]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

11:40   **Genetic Engineering of Cotton for Phytopathogens Including *Aspergillus flavus*.** <u>Kanniah Rajasekaran</u>[1], Jeffrey W. Cary[1], and Mauricio Ulloa[2]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [2]USDA-ARS, Western Integrated Cropping Systems Research Unit, Shafter, CA.

12:00 – 1:00   LUNCH

1:00 – 1:20   PANEL DISCUSSION
                 Panel Chair: Arthur K. Weissinger, North Carolina State University, Raleigh, NC

Session 4: Crop Management and Handling, Insect Control, and Fungal Relationships

MODERATOR: Pat O'Leary, Cotton Incorporated

1:20   **Update on Validation and Distribution of a Computer Program for Predicting Mycotoxins in Midwest Corn.** <u>Patrick F. Dowd</u>. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

1:35   **Mechanism of Preharvest Aflatoxin Contamination in Peanut Infected by Root-Knot Nematodes.** <u>Patricia Timper</u>[1], C. Corley Holbrook[2], and David M. Wilson[3]. [1]USDA-ARS, Crop Protection and Management Research, Tifton, GA; [2]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [3]Department of Plant Pathology, University of Georgia, Tifton, GA.

1:50   **Experimental Use of the Pear Ester Kairomone to Improve Codling Moth Control in Walnuts.** <u>Douglas M. Light</u>, Paula I. Bouyssounouse, and Bruce C. Campbell. USDA-ARS, Western Regional Research Center, Albany, CA.

2:05   **Cultural Conditions Promoting Chitinase Production by *Gliocladium catenulatum*.** <u>David F. Kendra</u>[1], Michael J. Muhitch[2], Amber Anderson[1], and Cesaria E. McAlpin[1]. [1]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]Rochester College, Rochester Hills, MI.

2:20   **Liberty Link and Urea on Aflatoxin and Fumonisin Levels in Corn.** <u>H. Arnold Bruns</u> and H. K. Abbas. USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS.

2:35 – 2:55   BREAK AND POSTER VIEWING

2:55 – 3:15   PANEL DISCUSSION
Panel Chair: David F. Kendra, USDA-ARS, National Center for
Agricultural Utilization Research, Peoria, IL

Session 5: Detection, Extraction, and Analysis of Aflatoxins; Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

MODERATOR: Tom Wedegaertner, Cotton Incorporated

3:15   **Distribution of Aflatoxin in Non-irrigated Peanuts.** Thomas F. Schatzki and M. S. Ong. USDA-ARS, Western Regional Research Center, Albany, CA.

3:30   **Inhibition of *Aspergillus flavus* Aflatoxin Biosynthesis by Antioxidant Phytochemicals Occurring in Tree Nuts.** Russell J. Molyneux, Noreen Mahoney, Bruce C. Campbell, and Jong H. Kim. USDA-ARS, Western Regional Research Center, Albany, CA.

3:45   **Biochemical and Genetic Analysis of Gallic Acid in Walnuts in Relation to Aflatoxin Accumulation.** Ryann M. Muir[1], Elizabeth Ingham[1], Sandra Uratsu[1], Gale McGranahan[1], Charles Leslie[1], Noreen Mahoney[2], and Abhaya Dandekar[1]. [1]Department of Pomology, University of California, Davis, CA; [2] USDA-ARS, Western Regional Research Center, Albany, CA.

4:00   **Inhibition of Aflatoxin Production by Compounds in Corn Seeds.** Gary A. Payne[1], Robert A. Holmes[2], and Rebecca S. Boston[2]. [1]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Department of Botany, North Carolina State University, Raleigh, NC.

4:15 – 4:35   PANEL DISCUSSION
Panel Chair: Russell J. Molyneux, USDA-ARS, Western Regional Research Center, Albany, CA.

4:35 – 5:15   COMMODITY BREAKOUT SESSIONS

6:00 – 7:00   RECEPTION

7:00   BANQUET

POSTER PRESENTATIONS

A. Fungal Genomics, Regulation of Aflatoxin Biosynthesis

A-1   **Evolutionary processes in the aflatoxin gene cluster in *Aspergillus*.** I. Carbone[1], J. L. Jakobek[1], E. H. Moussa[1,2], J. E. Cox[1], and B. W. Horn[3]. [1]Center for Integrated Fungal Research, Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Present address: Faculty of Sciences II, Department of Biological Sciences, Lebanese University, Beirut, Lebanon; 3USDA-ARS, National Peanut Research Laboratory, Dawson, GA.

A-2   **Differential Gene Expression Levels for *Aspergillus flavus* Resistance in Two Inbred Maize Lines.** Rowena Y. Kelley[1], Deborah L. Boykin[2], Leigh K. Hawkins[3], and W. Paul Williams[3]. [1]Department of Plant and Soil Sciences, Mississippi State University, Mississippi State, MS; [2]USDA-ARS, Mid South Area Statistics Office, Stoneville, MS; [3]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

A-3   **Enhanced Activity of Fungicides by Positive Interaction with Natural Phenolic Agents; Target-gene Based Bioassays for Control of *Aspergilli*.** Jong H. Kim[1], Bruce C. Campbell[1], Jiujiang Yu[2], Noreen Mahoney[1], Kathleen L. Chan[1], Russell J. Molyneux[1], Deepak Bhatnagar[2], Thomas E. Cleveland[2], Gregory S. May[3], and Gary A. Payne[4]. [1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA; [3]MD Anderson Cancer Center, University of Texas, Houston, TX; [4]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

A-4   **Deletion of GBP1, a Gene Encoding a Monomeric G Protein, De-represses Fumonisin Biosynthesis in *Fusarium verticillioides*.** Uma Sagaram and Won-Bo Shim. Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX.

A-5   **A Link between Rho-Signaling and Aflatoxin Biosynthesis in *Aspergillus flavus*.** D. Ryan Georgianna[1,2], Michael S. Price[1,3], and Gary A. Payne[1,2]. [1]Center for Integrated Fungal Research and Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Genomic Sciences Graduate Program, North Carolina State University, Raleigh, NC; [3]Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC.

A-6   **nadA, a Gene Regulated by AflR, Does Not Appear to Affect Aflatoxin Production.** Carrie Jacobus[1], Gary A. Payne[2], and Niki Robertson[1,3]. [1]Department of Genetics, North Carolina State University, Raleigh, NC; [2]Department of Plant Pathology, North Carolina State University, Raleigh, NC;

[3]Department of Botany, North Carolina State University, Raleigh, NC.

A-7 **Metabolic Profiling of *Aspergillus flavus* during Aflatoxin Biosynthesis.** N. J. Glassbrook and G. A. Payne. Department of Plant Pathology, North Carolina State University, Raleigh, NC.

B. Fumonisin Elimination

B-1 **Mapping of QTL for *Fusarium* Ear Rot and Fumonisin Accumulation in Maize.** Leilani A. Robertson[1], Michael P. Jines[2], Peter Balint-Kurti[3], Craig E. Kleinschmidt[4], Don G. White[4], Gary A. Payne[5], Chris M. Maragos[6], and James B. Holland[3]. [1]Deptartments of Plant Pathology and Crop Science, North Carolina State University, Raleigh, NC; [2]Department of Crop Science, North Carolina State University, Raleigh, NC; [3]USDA-ARS, Plant Science Research Unit, Department of Crop Science, North Carolina State University, Raleigh, NC; [4]Department of Crop Sciences, University of Illinois, Urbana-Champaign, IL; [5]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [6]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

B-2 **Polyketide Synthase in *Fusarium verticillioides*: Can They be Exploited to Control Fumonisin Contamination in Corn?** Robert H. Proctor, Robert A. E. Butchko, Mark Busman, Anne E. Desjardins, Daren W. Brown, and Ronald D. Plattner. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

B-3 **Computational Studies on the Influence of Solvent on the Conformational Preferences and Selective Recognition of Fumonisins.** M. Appell, C. M. Maragos, and D. F. Kendra. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

B-4 **Characterization of Potential Fumonisin Regulatory Genes Identified by EST Analysis.** Robert A. E. Butchko, Robert H. Proctor, Daren Brown, and Murk Busman. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

B-5 **Fumonisins in Maize in Guatemala, Exposure Estimates, and Policies and Recommendations to Minimize Exposure.** Ronald T. Riley[1], Olga A. Torres[2], Rubin Grajeda[2], Edwin Palencia[2], L. Lopez de Pratdesaba[2], Anthony E. Glenn[1], Kerry O'Donnell[3], Mario Fuentes[4], and Marcy Speer[5]. [1]USDA-ARS, Richard B. Russell Research Center, Athens, GA; [2]Instituto de Nutricion de Centro America Y Panama, Calzada Roosevelt, Zone 11, Guatemala; [3]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL; [4]Institute of Agricultural Science and Technology, Guatemala; [5]Duke Center for Human Genetics, Duke University, Durham, NC.

B-6   **Fusaric Acid, a *Fusarium verticillioides* Miasma to *Bacillus mojavensis*, a Biological Control Bacterial Endophyte.** Charles W. Bacon and D. M. Hinton. USDA-ARS, Richard B. Russell Research Center, Athens, GA.

B-7   **Developmental Toxicity of *Fusarium verticillioides* and Fumonisin B[1] in LMBc and CD1 Mice: Comparing the *in vivo* Models.** Kenneth A. Voss[1], Ronald T. Riley[1], Tantiana D. Burns[1,2] and Janee B. Gelineau-van Waes[3]. [1]USDA-ARS, Richard B. Russell Research Center, Athens, GA; [2]Interdisciplinary Toxicology Program, University of Georgia, Athens, GA; [3]Department of Genetics, Cell Biology, and Anatomy, Nebraska Medical Center, Omaha, NE.

C. <u>Aflatoxin - Crop Management and Handling, Insect Control, and Fungal Relationships</u>

C-1   **Anthocyanins from Petunia Floral Structures that Inhibit Corn Earworm Development.** Eric T. Johnson and Patrick F. Dowd. USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

C-2   **Using a Remotely Sensed Crop Index to Enhance Selection for Drought Tolerant Peanuts.** Dana G. Sullivan[1] and C. Corley Holbrook[2]. [1]USDA-ARS, Southeast Watershed Research Laboratory, Tifton, GA; [2]USDA-ARS, Crop Genetics Breeding and Research Unit, Tifton, GA.

C-3   **Correlations between Biotic Stresses and Aflatoxin Contamination in Maize.** Matthew Krakowsky[1], Xinzhi Ni, Richard Davis, and Kedong Da. [1]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [3]Department of Entomology, Coastal Plain Experiment Station, University of Georgia, Tifton, GA.

D. <u>Aflatoxin - Crop Resistance – Conventional Breeding</u>

D-1   **Multilocation Evaluation of Aflatoxin Accumulation in Yellow Maize Hybrids.** Cody McKee[1], Tom Isakeit[2], Gary Odvody[3], Kerry Mayfield[1], and Javier Betrán[1]. [1]Soil & Crop Sciences Department, Texas A&M University, College Station, TX; [2]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX; [3]Texas A&M Research & Extension Center, Corpus Christi, TX.

D-2   **Southeastern Regional Aflatoxin Test (SERAT).** Michael Clements[1], Paul Williams[1], Steve Moore[2], Matthew Krakowky[3], Baozhu Guo[3], Don White[4], Wenwei Xu[5], Tom Isakeit[6], Tom Brooks[1], Gary Windham[1], Hamed Abbas[7], James Perkins[8], Daniel Gorman[9], Quinton Raab[10], Keith Arnold[10], David Smith[11], and Javier Betrán[6]. [1]USDA-ARS, Mississippi State, MS; [2]Louisiana

State University Agricultural Center, Alexandria, LA; [3]USDA-ARS, Tifton, GA; [4]University of Illinois, Urbana, IL; [5]Texas A&M University Agricultural Research and Extension Center, Lubbock, TX; [6]Texas A&M University, College Station, TX; [7]USDA-ARS, Stoneville, MS; [8]Monsanto Company Crop Protection, Waterman, IL; [9]Pioneer – Dupont, Cairo, GA; [10]B-H Genetics, Moulton, TX; [11]Zea Sage, Sycamore, Il.

D-3   Response of Aflatoxin of CIMMYT Germplasm in Southern USA. Dan Jeffers[1], Matthew Krakowsky[2], Paul Williams[3], and Javier Betrán[4]. [1]CIMMYT, Mexico D. F., [2]USDA-ARS, Tifton, GA; [3]USDA-ARS, Mississippi State, MS; [4]Texas A&M University, College Station, TX.

D-4   Phenotypic and Genotypic Characterization of a RIL Maize Mapping Population for Aflatoxin and Secondary Traits. Melanie Edwards, Monica Menz, Tom Isakeit, and Javier Betrán. Texas A&M University, College Station, TX.

D-5   Expression of LOX Pathway Genes in Corn Embryos Associated with *Aspergillus flavus* Resistance. A. Camas[1], L. Lopez[1], G. Windham[2], P. Williams[2], and D. S. Luthe[1]. [1]Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [2]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

D-6   Breeding for Increased Resistance to *Fusarium verticillioides* in Maize. Magen Starr[1], Leilani Robertson[1], James Holland[2], and Gary Payne[3]. [1]Department of Crop Science, North Carolina State University, Raleigh, NC; [2]USDA-ARS, Department of Crop Science, North Carolina State University, Raleigh, NC; [3]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

D-7   Quantitative Expression Analysis of Adversity Resistance Genes in Corn Germplasm with Resistance to Preharvest Aflatoxin Contamination. M. Luo[1], R. D. Lee[2], and B. Z. Guo[2]. [1]Department of Crop and Soil Sciences, University of Georgia, Tifton, GA; [2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA.

D-8   Peanut PR Protein, ß-1,3-glucanase, Induction by *Aspergillus flavus* and Copurification with a Conglutin-like Protein. X. Liang[1], B. Z. Guo[2], and C. C. Holbrook[3]. [1]Guangdong Academy of Agricultural Sciences, Guangzhou, China; [2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [3]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

D-9   Corn Husk Characteristics Potentially Associated with Resistance to Aflatoxin Contamination of Grain: A Preliminary Study. M. J. Clements and W. P. Williams. USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

D-10 **Chalcone Synthase, a Gene that Influences Both Drought Response and Aflatoxin Accumulation in Maize.** M. Gerau, D. Bush, D. Davis, C. Morriss, and G. Davis. Division of Plant Sciences, University of Missouri-Columbia, Columbia, MO.

E. Aflatoxin - Microbial Ecology

E-1 **Influences of Crops and Geographic Features on Communities of Aflatoxin-producing Fungi.** Ramon Jaime[1] and Peter J. Cotty[2]. [1]Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ; [2]USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ.

E-2 **Aflatoxin Contamination of Maize in Africa.** Claudia Probst[1], Henry Njapau[2], and Peter J. Cotty[3]. [1]Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ; [2]Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD; [3]USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ.

E-3 **Influences of Herbicides on Release of Atoxigenic Strains.** Nicholas P. Garber[1] and Peter J. Cotty[2]. [1]Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ; [2]USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ.

E-4 **Screening of Atoxigenic *Aspergillus flavus* Isolates for Ability to Inhibit Aflatoxin B$_1$ Production by Toxigenic *Aspergillus flavus*.** A. Jha, R. Sweany, and K. E. Damann. Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA.

F. Aflatoxin - Potential Use of Natural Products for Prevention of Fungal Invasion and/ or Aflatoxin Biosynthesis in Crops

F-1 **Identification of Two Maize Seed Compounds that Influence Aflatoxin Biosynthesis.** Robert A. Holmes[1], Rebecca S. Boston[1], and Gary A. Payne[2]. [1]Department of Botany, North Carolina State University, Raleigh, NC; [2]Department of Plant Pathology, North Carolina State University, Raleigh, NC.

F-2 **A New Peanut Phytoalexin with Stilbene and Tetronic Acid Moieties.** V. S. Sobolev[1], S. T. Deyrup[2], and J. B. Gloer[2]. [1]USDA-ARS, National Peanut Research Laboratory, Dawson, GA; [2]Department of Chemistry, University of Iowa, Iowa City, IA.

G. Aflatoxin - Detection, Extraction, and Analysis of Aflatoxins

G-1   **Examination of Error Components Associated with Quantification of Aflatoxin in Ground Corn Grain with In house CD ELISA.** M. J. Clements[1], G. L. Windham[1], C. M. Maragos[2], W. P. Williams[1], T. D. Brooks[1], L. K. Hawkins[1], and H. M. Gardner[1]. [1]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [2]USDA ARS, National Center for Agricultural Utilization Research, Peoria, IL.

G-2   **Using Hyperspectral Technology to Measure Fungal Growth and Assess Mycotoxin Contamination of Corn.** Z. Hruska[1], H. Yao[1], K. DiCrispino[1], K. Bradham[1], D. Lewis[1], J. Beach[1], R. L. Brown[2], and T. E. Cleveland[2]. [1]Institute for Technology Development, Stennis Space Center, MS; [2]USDA-ARS, Southern Regional Research Center, New Orleans, LA.

5ᵀᴴ ANNUAL FUNGAL GENOMICS WORKSHOP

Moderator: Roy Cantrell, Cotton Incorporated

Finding Target Genes for Better Control of *Aspergillus*

Jong H. Kim[1], Bruce C. Campbell[1], Jiujiang Yu[2], Gregory S. May[3], Kathleen L. Chan[1], Gary A. Payne[4], Deepak Bhatnagar[2] and Thomas E. Cleveland[2]
[1]*USDA-ARS, Western Regional Research Center, Albany, CA;* [2]*USDA-ARS, Southern Regional Research Center, New Orleans, LA;* [3]*MD Anderson Cancer Center, University of Texas, Houston, TX;* [4]*Department of Plant Pathology, North Carolina State University, Raleigh, NC*

Gallic acid, from hydrolysable tannins in the pellicle of walnut kernels, dramatically inhibits biosynthesis of aflatoxin by *Aspergillus flavus.* The genetic basis for this inhibition was found to take place "upstream" from the aflatoxin gene cluster, including its regulatory gene, *aflR*. Other antioxidant phenolics showed similar antiaflatoxigenic activity as gallic acid. *tert*-Butyl peroxide treatment of *A. flavus* resulted in an approximate doubling of aflatoxin biosynthesis compared to untreated fungi. The fact that oxidative stress and antioxidants affect aflatoxin biosynthesis suggests that the antioxidative response systems of *A. flavus* are targets for control. High throughput screening, using yeast, *Saccharomyces cerevisiae*, as a model fungus, quickly identified a number of fungal genes vulnerable to treatment by phenolic compounds. The assay also provided a means to assess the bioactivity of combinations of phenolics and certain fungicides affecting mitochondrial respiration. For example, the *sod2*Δ mutant was very sensitive to treatment by certain phenolics and commercial fungicides or drugs, strobilurins/antimycin A, both of which inhibit complex III of the mitochondrial respiratory chain. This effectiveness was verified by stressing this system in the target fungus, *A. flavus*, and using complementation analysis, wherein the mitochondrial superoxide dismutase (Mn-SOD) gene (*sodA*) of *A. flavus* in the ortholog mutant, *sod2*Δ, of *S. cerevisiae*, relieved phenolic induced stress. Mitochondrial antioxidative stress systems are important in fungal response to antifungals. Combined treatment of fungi with phenolics and inhibitors of this respiratory system effectively suppresses growth of *A. flavus,* synergistically. Identifying the genes in other antioxidative response systems in other pathogens should greatly improve methods for fungal control using combinations of compounds that target those genes.

Comparative Genomic Analysis of Secondary Metabolite Gene Clusters of Closely Related Aspergilli

William C. Nierman[1], Natalie D. Fedorova[1], Catherine M. Ronning[1], Jennifer Wortman[1], Masayuki Mashida[2], Jiujiang Yu[3], Thomas E. Cleveland[3], Deepak Bhatnagar[3] and Gary Payne[4]

[1]The Institute for Genomic Research, Rockville, MD; [2]Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan; [3]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [4]Department of Plant Pathology, North Carolina State University, Raleigh, NC

We have performed preliminary comparative analysis of the genome sequence of three closely related Aspergilli, *Aspergillus fumigatus* Afu293, *A. fumigatus* CEA10, and *Neosartorya fischerii* NRRL181. Among our observations, we noted that species and strain specific genes are often found in clusters within polymorphic subtelomeric domains. Some of these clusters contain secondary metabolism biosynthetic genes, which are one of the most variable groups of genes in these genomes. Frequent exchanges between nonhomologous chromosomes observed in subtelomeric regions may facilitate amplification and diversification of secondary metabolism and other niche adaptation genes in these fungi. With these observations in hand we are beginning to explore the genomes of two other closely related species, *A. flavus* and *A. oryzae*. We have undertaken to identify all of the secondary metabolite clusters in both of these organisms and to compare the identities of the clusters, their completeness, and the syntenic order of genes within these clusters. Our findings will be reported and contrasted to those observed in the two *A. fumigatus* strains and in *N. fischeri*.

Aspergillus flavus Genomics in Discovering Genes Involved in Aflatoxin Biosynthesis

J. Yu[1], J. R. Wilkinson[1], W. C. Nierman[2,3], H. S. Kim[2], G. A. Payne[4], B. C. Campbell[5], D. Bhatnagar[1], and T. E. Cleveland[1]

[1]*USDA/ARS, Southern Regional Research Center, New Orleans, LA;* [2]*The Institute for Genomic Research, Rockville, MD;* [3]*The George Washington University School of Medicine, Department of Biochemistry and Molecular Biology, Washington DC;* [4]*North Carolina State University, Raleigh, NC;* [5]*USDA/ARS, Western Regional Research Center, Albany, CA*

Aflatoxins are the most carcinogenic natural compounds among the known mycotoxins. *Aspergillus flavus* produces aflatoxins B1 and B2 and causes aflatoxin contamination of preharvest crops such as corn, cotton, peanuts and treenuts. Due to the significant health and economic impacts of aflatoxin contamination, the chemistry, enzymology, and genetics of aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* are being actively studied. In *A. flavus* there are eight chromosomes with an estimated genome size of about 33-36 Mbp that harbor approximately 12,000 functional genes. Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* led to the cloning of 25 clustered genes within a 70kb DNA region responsible for the enzymatic conversions in the aflatoxin pathway. Identification and elucidation of genes involved in aflatoxin biosynthesis through genomics is the key strategy in order to better understand the molecular mechanisms that control or regulate aflatoxin production. The widely used wild type strain *A. flavus* NRRL 3357 (ATCC# 20026) was used in the EST project. Sequencing and annotation of *A. flavus* ESTs from a normalized *A. flavus* cDNA library identified 7,218 unique EST sequences. Genes that are putatively involved in aflatoxin biosynthesis, regulation and signal transduction, fungal virulence or pathogenicity, stress response or antioxidation, and fungal development were identified from these ESTs. Microarrays containing over 5,000 unique *A. flavus* gene amplicons were constructed at The Institute for Genomic Research (TIGR). For gene profiling experiments using microarrays, the four basic culture media were used: yeast extract (YE, non-aflatoxin-producing), yeast extract sucrose (YES, aflatoxin-producing), peptone minimal salt (PMS, non-aflatoxin-producing), and glucose minimal salts (GMS, aflatoxin-producing). The mycelium was harvested at 48 and 96 hours respectively after inoculation. Gene profiling under aflatoxin-producing and non-producing conditions has thus far identified hundreds of genes that are potentially involved in aflatoxin production. The 3DNA Array 900MPXTM protocol of Genisphere gave the best results on detection sensitivity. There are 7802 scorable spots detected including the aflatoxin pathway genes (over 90% of the total spots on the array). Among them, 434 were at significant levels and 285 ESTs were found to be up-regulated. Further investigations on the functions of these genes by gene knockout experiments are underway. This research is expected to provide information for developing new strategies for control of aflatoxin contamination of agricultural commodities.

Mining Expressed Sequence Tags (ESTs) Leads to Identification of Putative FUM Cluster Transcription Factor

Daren W. Brown, Robert A.E. Butchko, Mark Busman and Robert H. Proctor
Mycotoxin Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL

Fumonisins are a family of mycotoxins produced by *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) and can be found contaminating maize throughout the world. *F. verticillioides* generally grows in maize tissue without causing disease symptoms, but under some conditions, can cause seedling blight and ear, root and stalk rots of maize as well as synthesize fumonisins. Fumonisins cause several different toxicoses in animals and, in humans, are epidemiologically associated with esophageal cancer and birth defects in some regions of the world. Fumonisins are synthesized via enzymes encoded by genes localized within a 42.5 kb portion of the *F. verticillioides* genome. Understanding fumonisin biosynthesis and its genetic regulation may lead to the development of novel methods to prevent contamination of maize with fumonisins and thereby eliminate the toxins from the animal and human food chain.

Over four years ago, we initiated a project with The Institute for Genomic Research (TIGR) to sequence thousands of *F. verticillioides* cDNAs in order to generate an expressed sequence tag (ESTs) database. The primary goal of this project was to identify genes that regulate fumonisin biosynthesis. We generated eight cDNA libraries, each from a different culture condition, from which TIGR sequenced over 111,000 clones. After analysis, over 87,000 high quality sequences (e.g. ESTs) were generated that correspond to 11,119 unique sequences. Transcripts corresponding to genes within the fumonisin gene cluster were found primarily in four of the eight libraries. Three of these libraries were constructed from RNA harvested from *F. verticillioides* grown in GYAM, a liquid medium that supports fumonisin production, for 24, 48/72, or 96 hours. The fourth library was constructed from growth on maize meal, which also supports fumonisin biosynthesis. We reasoned that a comparison of the ESTs from different libraries would identify differentially expressed genes important to fumonisin biosynthesis. Preliminary analysis has found a number of candidate fumonisin regulatory genes and functional analysis is in progress.

This report describes analysis of a set of ESTs that match genomic sequence adjacent to the fumonisin gene cluster. BLAST analysis of the genomic sequence corresponding to the ESTs indicated that it did not share similarity with any previously characterized sequence in GeneBank. In contrast, BLAST analysis of the ESTs indicated that they share significant similarity with transcriptional factors of the $Zn(II)_2Cys_6$ family. Analysis of the gene structure identified eight introns of which two are located within the sequence encoding the $Zn(II)_2Cys_6$ motif. A majority of the ESTs are alternative splice forms (ASFs) where an intron was either not excised or utilized an alternative 3′ splice sequence. All of the ASFs are unable to encode the predicted full length *FUM21* protein. The distribution of ASFs in the GYAM libraries is consistent with the pattern of ASF ESTs of four fumonisin biosynthetic genes and further support our hypothesis that ASFs serve a biological function. Gene deletion studies indicate that *FUM21* plays an important but not absolute role in fumonisin biosynthesis as deletion of *FUM21* reduced fumonisin production to 30% of wild-type. Studies are in progress to examine if the *FUM21* ASFs serve a biological function.

Release of the *Aspergillus flavus* Genome Sequence

Gary A. Payne[1], B. Pritchard[1], Jiujiang Yu[2], William C. Nierman[3], Ralph Dean[1], Deepak Bhatnagar[2], and Thomas E. Cleveland[2]

[1]*Department of Plant Pathology, North Carolina State University, Raleigh, NC;* [2]*USDA–ARS, Southern Regional Research Center, New Orleans, LA;* [3]*The Institute for Genomic Research, Rockville, MD*

Aspergillus flavus is a pathogen of maize, peanuts, cottonseed, and tree nuts, and contaminates them with the carcinogen, aflatoxin. There are no effective control procedures for the fungus. To gain a greater understanding of the factors responsible for pathogenicity and aflatoxin production, a whole genome sequencing project for *Aspergillus flavus* was initiated in 2003. This project, which is directed by Gary Payne, is funded primarily from the USDA/NRI Competitive Grants Program (USDA-CSREES Award Number: USDA-2003-05428) with supplemental funding from USDA/ARS/SRRC in New Orleans. The sequencing project is near completion and we are now in the manual annotation and comparative genomics phase.

Sequencing to 5-X coverage was done at The Institute of Genomic Research (TIGR) under the supervision of Dr. William Nierman. A multiple library strategy with different insert sizes was used to attain maximal genome coverage and maximal linkage of the assembled contigs. A combination of 3–4 kb and 10 kb insert size libraries and a 50 kb linking library were used. The sequence reads can be obtained from NCBI.

Automated annotation was done under the supervision of Dr. Jennifer Wortman at TIGR using annotation tools trained on genomic sequence of *A. oryzae* as well as *A. flavus* and *A. oryzae* ESTs. Dr. Jiujiang Yu at the USDA/ARS/SRRC directed the sequencing of the *A. flavus* ESTs, which have been critical to gene annotation. Fine finishing of the sequence, which includes closing the small gaps is near completion.

A web browser was developed at North Carolina State University under the direction of Beth Pritchard that allows Blast matches to genes, proteins and genomic sequence of other *Aspergillus* species, alignments of ESTs, and GO annotations. New annotations will be updated on the site. Links to the web browser and to other information on the sequencing project can be found at: www.aspergillusflavus.org.

The genome has been assembled into 79 scaffolds ranging in size from 4.5 Mb to 1.0 Kb. Over 75% of the genome is represented in the 10 largest scaffolds. The estimated genome size of 36.3 Mb is similar to that for *A. oryzae* (36.8 Mb), a closely related species. These two fungi are enriched in genes for secondary metabolism. *A. flavus*, for example, is predicted to have 34 polyketide synthases, 22 non-ribosome peptide synthases, 77 ABC transporters and 122 cytochrome p450 enzymes.

Aspergillus flavus and *A. oryzae* (the predominate fungus used in food fermentation) are closely related and are likely ecotypes. A comparison of the genomes of these two fungi will likely reveal information on changes that have occurred during the domestication of *A. oryzae*, and help identify pathogenicity factors in *A. flavus*.

Production of Cyclopiazonic Acid, Aflatrem and Aflatoxin is Regulated by *veA*, a Gene Necessary for Sclerotial Formation in *Aspergillus flavus*

R.M. Duran[1], J.W. Cary[2], and A.M. Calvo[1]

[1]*Department of Biological Sciences, Northern Illinois University. DeKalb, IL;* [2]*Food and Feed Safety Research Unit, USDA, ARS, Southern Regional Research Center. New Orleans, LA*

Targeting genes involved in Aspergillus mycotoxin biosynthesis and/or fungal development would contribute to reducing the detrimental effects of these mycotoxins. Contamination of crops such as cotton, corn, sorghum, peanuts, walnuts and other oil seeds by aflatoxin and other Aspergillus mycotoxins leads to significant economic losses in the U.S. and a health threat in developing countries. It is well established that the biosynthesis of natural products, including mycotoxins, is associated with fungal development. We have found that the *Aspergillus flavus veA* gene regulates both, formation of resistant structures (scletoria), and mycotoxin biosynthesis. Our results demonstrated that the *A. flavus veA* mutant is unable to produce aflatoxin, while aflatoxin B_1, B_2, G_1 and G_2 were detected in the *veA* wild type strain. The expression of *aflR* (encoding for a specific transcription factor that activates the aflatoxin gene cluster) and aflatoxin enzymatic genes was blocked in the *veA* mutant.

Our chemical analysis also indicated the absence of other metabolites in the *veA* mutant. For this reason we also examined whether *veA* is involved in regulating the production of other toxins in *A. flavus*. Specifically, we evaluated the production of cyclopiazonic acid and aflatrem. Cyclopiazonic acid is a specific inhibitor of calcium-dependent ATPase and induces alterations in ion transport across cell membranes. Interestingly, our results showed that the *A. flavus veA* mutant presents a decrease in CPA production with respect to the wild type strain. In the case of aflatrem, a potent tremorgenic mycotoxin, its production was completely blocked in the *veA* mutant, indicating that *veA* is also required for the synthesis of this mycotoxin. The aflatrem gene cluster (containing atm genes) has been recently identified and characterized. We found that the atm genes are not expressed in the *A. flavus veA* mutant. The fact that *veA* not only regulates aflatoxin production but also other mycotoxins adds to the value of *veA* as a possible control target. *veA* involvement in the production of *A. flavus* mycotoxins is consistant with our previous observations: deletion of *veA* in *Aspergillus parasiticus* and *Aspergillus nidulans veA* mutants results in elimination of aflatoxin and sterigmatocystin production respectively.

Furthermore, targeting *veA* could also aid in decreasing *A. flavus* survivability by affecting sclerotial development. Sclerotia are resistant structures that allow the fungus to survive under adverse conditions until the environment is favorable again for growth and plant infection. Molecular studies over the control of sclerotial development are limited. We have shown that the *A. flavus veA* mutant, as with *A. parasiticus*, is completely unable to produce sclerotia, indicating that the *veA* gene is essential for sclerotial morphogenesis.

The impact of this study on fungal disease resistance may well go beyond control of aflatoxin contamination of food and feed crops. Recently, we have found *veA* homologues across fungal genera, including species of agricultural and medical importance. Because *veA* has only been found in fungi, there is a great potential for using *veA* as a target for plant, animal and human disease control. Future studies might include the generation of transgenic plants resistant to fungal invasion and toxin production that are developed based on the production of inhibitors of *veA*. Additionally, this research might lead to anti-VeA fungal drugs that could be used to prevent human and animal disease.

PANEL DISCUSSION: Fungal Genomics Workshop

Panel Chair: Gary Payne, North Carolina State University

Panel Members: Bruce Campbell, William Nierman, Jiujiang Yu, Daren Brown & Ana Calvo

Questions for William Nierman
Q: Are there common telomere sequence repeats?
A: Yes, and we use that for genotyping these regions. Gene rearrangements mediated by these repeats may drive enrichment of unique clusters of genes in these regions. Of course, this is my speculation.
Q: Is it possible to compare tertiary sequence that is not usable at the nucleotide or amino acid level?
A: There has been a lot of discussion about this but probably not enough structures present to run the technology yet.

Questions for Bruce Campbell
Q: Does the addition of antioxidants affect fungal growth?
A: Not at levels we are testing.
Q: Will increasing antioxidant levels increase resistance in plants?
A: Yes
Q: Do you think that aflatoxin plays a role in oxidative stress?
A: Yes
Q: Have you compared the response of aflatoxin non-producing mutants with wild type strains for their response to oxidative stress?
A: No. There may be other ways the atoxigenic strains respond to oxidative stress other than aflatoxin biosynthesis. I'm sure even the toxigenic strains have other mechanisms, as well.

Questions for Jiujiang Yu
Q: Can you gain more information from the Affymetrix oligo arrays or from amplicon arrays?
A: There is a wider range of intensities and it is easier to spot and optimize amplicon arrays.
Comment from Nierman: Fungal arrays present new problems in that some oligos may not match message because we have a harder time with gene annotation of splice boundaries and mature message. We still lack some power on gene calling. Different gene calling programs give different 5′, 3′ boundaries and splice sites.

Questions for Ana Calvo
Q: Do you have the sequence for veA?
A: Yes
Q: What is the relationship between cleistothecia production and mycotoxin biosynthesis?
A: Morphogenesis is often associated with mycotoxin production, e.g. VeA is necessary for both mycotoxin production and cleistothecial production. Therefore, we expect genetic links. For example, the FadA pathway links asexual structures and mycotoxins. The link is not well defined for sexual structures at this time. Additionally, sclerotial development is not well characterized in general.
Q: Sclerotia production is linked with aflatoxin production but many strains lack sclerotia and still produce aflatoxin. Does the *veA* deletion strain produce aflatoxin? Does the mutant lose the ability to produce conidia?
A: Several processes are necessary for sclerotial production and *veA* contributes to these processes. The *veA* deletion mutant does produce conidia.
Q: What is the role of light in your fungus? Is it related to stress?
A: Our hypothesis is that in *A. nidulans* light is a signal to direct the formation of aerial spores if the fungus is near the surface and survival structures if the fungus is deeper in the soil. Several photoreceptors have been identified but we are not sure which ones affect veA.

Q: Do you think that mycotoxins are related to survival of these structures since their production is induced at the soil surface?
A: Maybe
Comment from Campbell: We have found that oxidative stress increases sclerotial development and melanization. Antioxidants suppress not only aflatoxin biosynthesis, but also melanization and sclerotial development…suggesting that sclerotial development and aflatoxin biosynthesis might have some common triggering mechanism.

Questions for Daren Brown
Q: Is Fum 21 in other fungi?
A: Yes, there are some fumonisin-like clusters in other fungi.

Evolutionary Processes in the Aflatoxin Gene Cluster in *Aspergillus*

I. Carbone[1], J.L. Jakobek[1], E.H. Moussa[1,2], J.E. Cox[1] and B.W. Horn[3]
[1]Center for Integrated Fungal Research, Department of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Present address: Faculty of Sciences II, Department of Biological Sciences, Lebanese University, Beirut, Lebanon; [3]National Peanut Research Laboratory, USDA, ARS, Dawson, GA

Aflatoxins are potent natural carcinogenic secondary metabolites produced by several species in the genus *Aspergillus.* Recently nonaflatoxigenic *A. flavus* strains were approved as biocontrol agents for use on cotton and peanuts. Although these biocontrol strains have proven to be effective, we have no knowledge of the long-term effects that introduced strains have on the evolution of aflatoxigenicity. Our hypothesis is that a low level of recombination and gene flow among *Aspergillus* species is significantly contributing to the persistence and further evolution of aflatoxigenic strains. We are currently testing this hypothesis by focusing on *A. flavus* and *A. parasiticus,* the two most abundant aflatoxigenic species. Our examination of nucleotide sequence variation in 21 intergenic regions across the entire aflatoxin gene cluster of *A. parasiticus* indicates the presence of recombination blocks and there is evidence that balancing selection has influenced genetic variation in these blocks. The same blocks appear to be conserved for putative orthologs of these genes in *A. nidulans, A. flavus,* and *A. fumigatus*, indicating the potential for an introduced biocontrol strain to acquire toxigenicity genes from indigenous strains via recombination or from sympatric species via horizontal transfer. We are currently examining the timing and frequency of these events in nature.

Differential Gene Expression Levels for *Aspergillus flavus* Resistance in Two Inbred Maize Lines

R. Y. Kelley[1], D. L. Boykin[2], L. K. Hawkins[3] and W. P. Williams[3]

[1]*Dept of Plant and Soil Sciences, Mississippi State University, Mississippi State, MS2; *[2]*USDA–ARS Mid South Area Statistics Office, Stoneville, MS; *[3]*USDA–ARS Corn Host Plant Resistance Research Unit, Mississippi State, MS*

cDNA microarray is a powerful gene expression tool which allows investigators to examine changes in the expression levels of thousands of genes simultaneously. cDNA microarrays and other genomic-scale hybridization-based technologies have improved throughput, sensitivity, and versatility for identifying differentially expressed genes. For example, microarray analysis has been used for profiling RNA levels in *Arabidopsis*, bacteria, yeast, and mammals. In maize, microarray technology has been applied to examine gene expression in response to disease, growth, water-deficient conditions and stress sensitivity. Recently, genes differentially expressed in *Aspergillus flavus* during aflatoxin biosynthesis were identified using microarray, but no work has been done using microarray to look at gene expression related to *A. flavus* infection and aflatoxin production in maize. Therefore, the objective of this experiment was to identify differentially expressed genes for *A. flavus* resistance in the Va35 (susceptible) and Mp313E (resistant) maize (*Zea mays* L.) lines using cDNA microarray analysis. Primary ears from plants in treated plots were inoculated with isolate NRRL 3357 of *A. flavus* 14 days after pollination and were harvested two days after inoculation. Uninoculated ears were harvested 16 days after pollination and used as a control. cDNA from the inoculated and uninoculated ears was labeled with Cy3 and Cy5 fluorescent dyes. Both samples were hybridized to the unigene 1.01.05 maize chip containing 5,065 EST contigs from EST libraries derived from immature leaf, endosperm, immature ear and the root.

Out of the 5065 ESTs analyzed, 123 or 2.4% of the total ESTs analyzed were significantly up-regulated for the susceptible inbred line Va35 and 95 or 1.8% of the total ESTs analyzed were significantly up-regulated for the resistant inbred line Mp313E, and 16 or 0.3% of the total ESTs were up-regulated for both the susceptible and resistant inbred lines. The expressed ESTs included genes with known functions such as stress response, metabolism, protein synthesis, cellular communication and signal transduction, transcription and RNA processing and photosynthesis-associated genes. Up-regulated ESTs also included genes with unknown functions, demonstrating the usefulness of microarray as a gene discovery tool.

This study provides characterization of the maize ear expression patterns for a number of maize genes when exposed to *A. flavus*. For a substantial number of the genes studied, previously published data are available on their expression patterns in other tissues but not when exposed to *A. flavus*; therefore, the current data provide initial information useful toward the characterization of genes expressed in maize exposed to *A. flavus* at two days after inoculation.

Enhanced Activity of Fungicides by Positive Interaction with Natural Phenolic Agents: Target-gene Based Bioassays for Control of Aspergilli

Jong H. Kim[1], Bruce C. Campbell[1], Jiujiang Yu[2], Noreen Mahoney[1], Kathleen L. Chan[1], Russell J. Molyneux[1], Deepak Bhatnagar[2], Thomas E. Cleveland[2], Gregory S. May[3] and Gary A. Payne[4]

[1]*USDA-ARS, Western Regional Research Center, Albany, CA;* [2]*USDA-ARS, Southern Regional Research Center, New Orleans, LA;* [3]*MD Anderson Cancer Center, University of Texas, Houston, TX;* [4]*Department of Plant Pathology, North Carolina State University, Raleigh, NC*

Signal transduction and stress-response genes of fungal pathogens play important roles for exerting pathogenesis and, in some cases, biosynthesis of mycotoxins. As such, they may serve as potentially viable targets for antifungal compounds. Results of our research, as presented in this poster, show that targeting genes in the mitochondrial respiratory chain pathways, MAPK or vacuolar H(+)-ATPase (V-ATPase) using safe, natural compounds can significantly elevate the sensitivity of fungi to commercial fungicides or drugs. The use of such compounds can result in lowering effective dosages, costs of treatment and potential for development of resistance.

Cellular targets of several conventional fungicides are already known. Examples include macro-molecular synthesis (*e.g.*, nucleic acids, amino acids, cell wall, *etc.*), cell division, signal transduction and respiration. Defects in any of these systems can lead to oxidative stress, with a resultant decrease in cell viability. Many defensive phenolic compounds are produced or released by plants during fungal infection. We theorize that disruption of cellular redox homeostasis using phenolics may inhibit fungal growth by disruption of cellular redox homeostasis.

The molecular target for strobilurin-related fungicides, such as azoxystrobin or kresoxim-methyl, is the mitochondrial respiratory *bc1* complex. Inhibition of this complex eventually leads to cellular oxidative stress caused by abnormal release of electrons from the respiratory chain. Using deletion mutants, we found at least five phenolic compounds that disrupt the normal function of mitochondrial respiration of yeast. Combined treatments of these phenolic agents and commercially available fungicides that are inhibitors of the mitochondrial respiratory chain have a 100 to 1000-fold synergistic fungicidal effect due to disruption of respiration and inhibiting the oxidative stress-response of the fungus. We also found that the *sakAΔ* (MAPK mutant) strain of *A. fumigatus* was much more sensitive to phenolics, indicating *sakA* likely is involved in regulation of the antioxidative stress response system in *A. fumigatus*, perhaps involving mitochondrial function/respiration. In addition, we found that the alkaloid berberine targets the activity of oxidative stress genes, and combined treatment of this alkaloid and certain phenolics resulted in >10,000 times greater fungicidal activity than either compound alone.

We conclude that natural compounds (*i.e.,* phenolics or alkaloids) can be developed as useful anti-fungal agents with the molecular target identified, leading to effective control of a broad spectrum of fungal pathogens.

Deletion of *GBP1*, a Gene Encoding a Monomeric G Protein, De-represses Fumonisin Biosynthesis in *Fusarium verticillioides*

U.S. Sagaram and W.B. Shim
Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX

Fumonisins are a group of mycotoxins produced by *Fusarium verticillioides* (perfect stage *Gibberella moniliformis*) that contaminate maize and other cereal crops. Among the B-series fumonisins, which occur under natural and laboratory conditions, fumonisin B_1 (FB_1) is the most abundantly produced and most toxic fumonisin. The occurrence of FB_1 has been linked to a number of human and animal disorders worldwide. To date, scientists have identified the fumonisin biosynthesis gene cluster that is responsible for synthesizing fumonisins in *F. verticillioides*. However, we still lack detailed understanding of how the fungus recognizes the external environmental/host signals to regulate FB_1 biosynthesis. In this study, we describe the molecular characterization of GBP1 and its role in FB_1 biosynthesis and conidiation in *F. verticillioides*. *GBP1* was identified as an expressed sequence tag (EST) up-regulated in *F. verticillioides fcc1* mutant that showed reduced conidiation and no FB_1 biosynthesis. The objective of this study was to test whether *GBP1* is involved in FB_1 biosynthesis or conidiation, or both. Sequence analysis showed that *GBP1* is a monomeric G-protein that encodes a putative 368-amino acid protein with similarity to DRG and Obg sub-classes of G-proteins that are involved in development and stress responses. Deletion mutant of *GBP1* (Δ*gbp1*) exhibited normal growth when grown on select defined media and corn kernels. Δ*gbp1* produced 58% greater FB_1 level than the wild type when grown on corn kernels. Complementation of Δ*gbp1* with wild-type *GBP1* restored FB_1 production to that of wild type. The data indicates that deletion of *GBP1* results in increased FB_1 production but does not affect conidiation. The deletion of *GBP1* did not affect the expression level of key FB_1 biosynthetic genes (*FUM1*, *FUM8*, and *FUM12*) and *ZFR1*, a positive regulator of fumonisin biosynthesis, suggesting that the increased FB_1 production in Δ*gbp1* is modulated via FUM gene cluster-independent mechanism. Further studies are necessary to clearly define the role of *GBP1* in FB_1 regulation.

A Link between Rho-Signaling and Aflatoxin Biosynthesis in *Aspergillus flavus*

D. Ryan Georgianna[1,2], Michael S. Price[1,3], and Gary A. Payne[1,2]
[1]North Carolina State University, Center for Integrated Fungal Research and Department of Plant Pathology, Raleigh, NC; [2]North Carolina State University, Genomic Sciences Graduate Program, Raleigh, NC; [3]Duke University Medical Center, Department of Molecular Genetics and Microbiology, Durham, NC

The transition between growth, development, and secondary metabolism in filamentous fungi is mediated by complex signaling pathways. Recently, we identified a component of a signaling pathway that appears to modulate aflatoxin production. Transcriptional analysis of *Aspergillus flavus* grown on media conducive or non-conducive for aflatoxin biosynthesis revealed a gene whose expression paralleled that of the aflatoxin pathway regulatory gene *aflR*. This identified gene had the greatest similarity to *rdi1*, a yeast gene that encodes a Rho-guanidine nucleotide dissociation inhibitor (Rho-GDI). Deletion of this gene (designated *afrdiA*) in *A. flavus* resulted in a 97% reduction in aflatoxin production. The deletion also caused a severe growth defect on minimal media, a moderate defect on complete media, and a temperature sensitive phenotype. These observed growth phenotypes in the *A. flavus* Δ*rdiA* strain were not similar to the reported phenotype for the *Saccharomyces cerevisiae rdi1* null mutant. A similar mutant phenotype was identified in yeast resulting from deletion of a gene encoding a Rho-GTPase associated protein, Bem4p. A blast search of the *A. flavus* genome revealed no sequences with significant similarity to *bem4*, including *afRdiA*. Although believed not to be a Rho-GDI, the yeast Bem4p is thought to have interactions similar to those of the yeast Rdi1p, both known to be capable of associating with guanidine nucleotide bound Cdc42p and Rho1p. We propose a model in *A. flavus* using inferences from the yeast Rho-signaling pathway and yeast systemic deletion project where afRdiA plays a central role in combining known interactions of the *A. flavus* proteins AflR and RasA with homologs of the yeast proteins Rho1, Cdc42, and Bbc1 for facilitating signaling control of aflatoxin production through a Rho-mediated pathway.

The NADH oxidase, NadA, and its Role in Aflatoxin Biosynthesis

Carrie Jacobus[1], Gary Payne[2], Niki Robertson[1,3]
[1]Department of Genetics, North Carolina State University, Raleigh, NC; [2]Department of Plant Pathology, North Carolina State University, Raleigh, NC; [3]Department of Botany, North Carolina State University, Raleigh, NC

The *nadA* gene is part of a gene cluster for sugar metabolism that lies adjacent to the aflatoxin gene cluster in *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. The enzyme encoded by *nadA* converts NADH to NAD^+, cofactors needed for certain reactions in the aflatoxin biochemical pathway. Microarray experiments comparing gene expression between a wild type strain of *A. parasiticus* and an *aflR* deletion mutant showed that *nadA* expression was significantly reduced in the mutant background. These results were confirmed by quantitative RT-PCR and are consistent with the presence of a putative AflR binding site upstream of the coding region of *nadA*. We hypothesized that NadA may be needed to supply NAD^+ cofactors for the aflatoxin biosynthetic pathway and hence is upregulated by AflR. In order to investigate a connection between *nadA* expression and aflatoxin production, a gene replacement construct was used to knock out *nadA* expression in *A. flavus*. Aflatoxin production in the presence of sucrose, fructose, or glucose was investigated in three independent *nadA* mutants. In each case, aflatoxin levels in the mutant strains were similar to those produced by the wild type strain. This suggests that NADH oxidase activity is somehow compensated for in the mutants or that the NADH oxidase is not required for aflatoxin production. Investigations are currently underway to characterize additional phenotypes of these mutants to better understand the role of NAD^+ in aflatoxin biosynthesis.

Metabolic Profiling of *Aspergillus flavus* during Aflatoxin Biosynthesis

Norm Glassbrook and Gary A. Payne
North Carolina State University, Raleigh, NC

Metabolic profiling techniques were applied to characterizing *Aspergillus flavus* during under growth conditions conducive to aflatoxin production (28 ˚C) and those not conducive to production (37 ˚C).

Samples analyzed by gas chromatography coupled with mass spectrometry (GC/MS) or liquid chromatography coupled with mass spectrometry (LC/MS).

Differences were observed in the small molecule composition of *Aspergillus* grown under conditions conducive to aflatoxin production (28 ˚C) and that grown under conditions not conducive to production (37 ˚C).

Observed changes in metabolite levels were mapped onto biochemical pathways along with results of microarray analyses of comparable experiments. This type of pathway mapping provides a framework for sorting large quantities of metabolic profiling data and for formulating testable hypotheses.

6TH ANNUAL FUMONISIN ELIMINATION WORKSHOP

Moderator: Larry Antilla, Arizona Cotton Research and Protection Council

Kernel Constituents Induce Fumonisin Production during Colonization by *Fusarium verticillioides*

Charles Woloshuk and Burt Bluhm
Purdue University, West Lafayette, IN

Questions remain unanswered regarding the interactions between the maize kernels and *Fusarium verticillioides* that lead to the accumulation of fumonisins. We have evaluated the role of kernel endosperm composition in regulating fumonisin B_1 (FB_1) biosynthesis. We found that kernels lacking starch due to physiological immaturity did not accumulate FB_1. Quantitative PCR analysis indicated that kernel development also affected the expression of fungal genes involved in FB_1 biosynthesis, starch metabolism, and nitrogen regulation. A mutant strain of *F. verticillioides* with a disrupted alpha-amylase gene was impaired in its ability to produce FB_1 on starchy kernels, and both the wild-type and mutant strains produced significantly less FB_1 on a high-amylose kernel mutant of maize. When grown on a defined medium with amylose as the sole carbon source, the wild-type strain produced only trace amounts of FB_1, whereas it produced large amounts of FB_1 when grown on amylopectin. Furthermore, the addition of dextrin to amylose induced FB_1 production. We conclude that enzymatic hydrolysis of amylopectin induces FB_1 production in *F. verticillioides*.

Genetics and Breeding of Host Resistance to Fusarium Ear Rot and Fumonisin Contamination

J.B. Holland
USDA-ARS, Plant Science Research Unit, Department of Crop Science, North Carolina State University, NC

To investigate the inheritance of resistance to Fusarium ear rot and fumonisin contamination in maize, we estimated heritabilities and genetic correlations of these two aspects of disease in two maize populations. One population of 215 BC1S1 lines derived from the first backcross of GE440 to FR1064 (GEFR population) was developed by Dr. Don White), and was tested in replicated, double-inoculated field trials in four environments. The second population was 143 recombinant inbred lines derived from the cross of NC300 to B104 (NCB population), developed by Dr. Goodman, and evaluated in three environments. Heritabilities of ear rot and fumonisin content in the GEFR population were 0.75 and 0.47, respectively, and their genetic correlation was 0.96. In the NCB population, ear rot and fumonisin content heritabilities were 0.86 and 0.80, respectively, and their genetic correlation was 0.87. These results suggest that direct selection for reduced fumonisin content is theoretically optimal for reducing susceptibility to fumonisin content, but that indirect selection against ear rot may be economically most efficient at reducing fumonisin content because ear rot is much easier and faster to score than fumonisin content. Both populations were also fingerprinted with at least 105 SSR markers. QTL for both traits were mapped in both populations, and many QTL for fumonisin were also detected in the same regions as QTL for ear rot. However, some QTL appeared to have effects on ear rot but not fumonisin, and vice versa. Therefore, indirect selection on ear rot may not be effective at selecting for all of the fumonisin-reducing alleles. QTL had relatively small effects (maximum of 18% of phenotypic variation) and were largely different between populations, so marker-assisted selection will be hindered by the genetic complexity of resistance to both traits.

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

T.C. Pearson[1] and D.T. Wicklow[2]

[1]*USDA-ARS, Grain Marketing Research and Production Research Center, Manhattan, KS;* [2]*USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL*

Near infrared and reflectance spectra (500–1700 nm) were analyzed to determine if they could be used to identify single whole white corn kernels contaminated with fumonisin. Kernels used for the study were obtained from processors in Illinois, Indiana, Kentucky, and Nebraska. Kernels were visually examined and grouped into six symptom categories: asymptomatic, chalky tip end, yellow-tan tip end, red streaks, 50% discolored, and 100% discolored. 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 endosperms side of each kernel. After spectra acquisition, kernels were weighed 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. Total fumonisin (B_1, B_2, and B_3) was measured with a fluorometer after extracts were purified with immunoaffinity columns (Fumonitest, Vicam, Watertown, MA) using the procedure recommended for corn, sorghum, and 17% protein poultry feed. The 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. Mycological evaluations, performed on grain sub-sampled from each symptom category and state, revealed that the five kernel groupings risk producing false positives.

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 fumonisin. It was found that using the wavelength pair of 500nm and 1200nm, approximately 77% of the kernels having high levels of fumonisin (>40ppm) were correctly classified. Additionally, approximately 96% of the kernels having low levels of fumonisin (<2 ppm) were correctly classified. In contrast, if only a single band is selected for distinguishing contaminated kernels, the accuracy for kernels having low fumonisin levels (<2 ppm) drops to approximately 83%. Thus, use of a dual band sorting machine for removal of white corn contaminated with fumonisin would result in 13% less good product being removed than with a monochromatic sorter.

Previous work with yellow corn showed that approximately 85% of the aflatoxin and fumonisin could be removed by high speed sorters using the spectral bands of 750nm and 1200nm. It was hypothesized that the 750nm band was detecting some color changes in fungal infested kernels while the 1200nm band was responding to increased porosity of the degraded endosperm. Insect damaged kernels have low absorbance at 1200nm, due to feeding and fungal infestation, and would all be rejected. In the case of white corn, 500 nm was found to be more accurate than 750nm for the visible spectral band. This may be due to the white corn germ and endosperm being of more uniform color than yellow corn kernels with a white germ. Because yellow corn absorbs more light at 500nm, asymptomatic yellow corn kernels can be distinguished from white corn kernels.

Maize *LOX3* Gene is Required for Fumonisin Biosynthesis and Conidiation of *Fusarium verticillioides*

Xiquan Gao[1], Won-Bo Shim[1], Ivo Feussner[2], and Mike Kolomiets[1]
[1]*Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas;* [2]*Georg–August–University Goettingen, Germany*

In this study we tested the hypothesis that 9-lipoxygenases (9-LOXs) and their metabolites are mycotoxin susceptibility factors in corn that are induced and utilized by *Fusarium verticillioides* and other phytopathogenic fungi to increase fungal sporulation and mycotoxin production in seeds. This hypothesis is based on three key observations: (1) oxylipins produced from linoleic and other free fatty acids in *Aspergillus* spp., so called psi-factors, are potent regulators of sporogenesis and mycotoxins synthesis; (2) the primary products of plant 9-LOX reactions, fatty acid hydroperoxides $9S$-HPOT(D)E, which are structurally similar to psi-factors, strongly induce both *Aspergillus* conidiation and mycotoxin production *in vitro*; (3) transcript levels of a maize 9-LOX gene, *ZmLOX3,* are induced in corn lines that are susceptible but not resistant to aflatoxin contamination. To test our hypothesis, we generated maize near-isogenic lines (NILs) that are either *Mutator*-insertional mutant or wild type at the *ZmLOX3* locus. Currently, mutants and wild type NILs are at the BC_4F_4 stage in B73 genetic background which is susceptible to fumonisin contamination. Oxylipin profiling suggested that germinating *lox3* mutants are devoid of most 9-LOX derived fatty acid hydroperoxides. Fumonisin B_1 production and conidiation of *F. verticillioides* were drastically reduced when the fungus was grown on mutant *lox3* kernels providing a strong support of our hypothesis. Moreover, conidiation of a distantly related fungal species, *Colletotrichum graminicola*, a causal agent of anthracnose leaf blight, was significantly reduced on *lox3* mutant leaves as compared to wild type leaves. Importantly, fungal biomass of both pathogens was not affected by the *lox3* mutation. These data strongly support our hypothesis that 9-LOX-derived metabolites positively regulate both fungal conidiation and mycotoxin production and are susceptibility factors in maize.

Toxicity Responses of Corn to the Mycotoxin Fumonisin B_1 in the Absence of *Fusarium verticillioides* Infection

A.M. Zimeri, L.D. Williams, R.T. Riley, and A.E. Glenn
USDA-ARS, R.B. Russell Research Center, Toxicology & Mycotoxin Research Unit, Athens, GA

Fusarium verticilliodes, the causative agent of ear rot in corn, produces the mycotoxin fumonisin B_1 (FB_1), which is a potent competitive inhibitor of ceramide synthase, a key enzyme necessary for sphingolipid metabolism. Consuming corn and corn products laden with FB_1 causes a range of species specific diseases in animals and also has been shown to cause cancer in laboratory rodents. FB_1 may also contribute to esophageal cancer and neural tube birth defects in humans. While FB_1 contamination is typically greater when ear rot damage is more severe, recent reports have indicated corn hybrids can be infected with *Fusarium* and contaminated with FB_1, yet do not always exhibit phenotypic signs of ear rot. We hypothesize that breeding programs may have inadvertently selected for endophytic fungal associations whereby *F. verticillioides* infects corn but does not cause severe ear rot, yet may still contaminate corn with significant levels of FB_1. We have begun to address the role that FB_1 may play in the overall biology of the fungal association with corn and have developed an experimental system based on observations that FB_1-producing strains of *F. verticillioides* are pathogenic against seedlings of susceptible corn hybrids while non-producing strains cause no disease symptoms. Numerous corn lines were screened for their sensitivity or insensitivity to FB_1 to evaluate which phenotype was most prevalent within corn. This study was enhanced by a new strategy for disinfesting seed both externally and internally using chlorine gas. The great advantage with this technique was that seed were not imbibed and could thus be sterilized more efficiently and stored until needed. Sterile seed of each corn line were place on agar supplemented with various concentrations of FB_1 (0, 1, 10, and 100 μM). After 7–10 days, root and shoot weight (wet and dry) and length were noted. Teosinte and *Tripsacum* were also evaluated for their sensitivity to FB_1 since teosinte is the likely progenitor of modern corn and *Tripsacum* is the sister taxon to *Zea*. Results indicated corn seedlings were in general sensitive to FB_1, with teosinte and *Tripsacum* also being very sensitive. Only one corn line, W23, was very insensitive to FB_1. In both sensitive and insensitive corn lines, low levels of FB_1 (1 μM) stimulated root and shoot growth. Sensitive corn lines had severely inhibited growth of roots and greatly reduced germination rates when exposed to higher concentrations of FB_1 ($\geq$10 μM). W23 germinated and grew well even on 100 μM FB_1. Thus, data supported FB_1-sensitivity as ancestral while insensitivity of corn to FB_1 toxicity may be recently derived. Though FB_1 alone caused stunting of aerial tissues and reduced root mass, it was not sufficient to cause the full suite of seedling blight disease symptoms caused by a *Fusarium* infection. To determine if other secondary metabolites work synergistically with FB_1 to cause disease, we germinated seedlings in the presence of fungal extracts with and without FB_1. Solvent extracts were made of FB_1-producing and non-producing strains grown on corn for 14 days. We found that seedlings exposed to extracts containing FB_1 exhibited seedling blight disease as seen in plants infected with wild-type *Fusarium*. Seedlings exposed to extracts that did not contain FB_1 grew similar to the control plants. In addition to mechanistic examinations of FB_1 toxicity in sensitive and insensitive seedlings, future work also will focus on whether FB_1 is absorbed and translocated throughout the plant. We will also investigate the impact of FB_1 and other molecules on systemic signaling within corn seedlings.

PANEL DISCUSSION: Fumonisin Elimination

Panel Chair: Charles Woloshuk

Panel Members: James Holland, Don Wicklow, Mike Kolomiets, Anne Marie Zimeri

The panel discussion began with an announcement by Woloshuk that the proposal to sequence the genome of *Fusarium verticillioides* was funded. It is anticipated that sequencing and assembly will be completed by May 2006. Special appreciation was given to the USDA-ARS Mycotoxin Research Unit in Peoria and Syngenta. The Mycotoxin Research Unit made public all their EST sequences and Syngenta made public the genomic sequence data. Their actions provided significant support and leverage to the proposal. Acknowledgement was also given to those who supplied letters in support.

Woloshuk was asked whether the commercial hybrids or imbred lines were used in his study. His reply was commercial hybrids (Beck's Hybrids). Woloshuk was asked about pH changes in kernels overtime. His reply was that he did not present the results in his presentation, but pH was monitored during the study. The initial pH of uninoculated kernels was very similar for all kernel stages and did not change over the coarse of the experiment. Colonization by the wild type fungus resulted in increased pH in blister and milk stages, decreased pH in dent and mature stages, and little pH change in dough. Woloshuk was asked about shifts in the types of fatty acids that might occur during kernel development. Woloshuk could not answer this question but commented that most of the lipids was in the germ tissues. There was no comment from the other panel members or the audience. Woloshuk was asked about the correlation between results obtain from kernel assay and field assays. He replied that he believed there should be a correlation. He noted the field studies by Gary Payne's group, who observed naturally occurring kernel infections as early as the milk stage of development.

Holland was asked to clarify the discrepancy between the very high estimates of genetic correlations between Fusarium ear rot and fumonisin content and the identification of some QTL that affect only one of the two traits. His reply was that both methods involve estimation and may have different errors associated with them, so that the discrepancy is an artifact of the use of different statistical techniques. He proposed to directly test the hypothesis that the genes affecting Fusarium ear rot are largely the same as the genes affecting fumonisin content by selecting against ear rot in one population and evaluating the effect of selection on fumonisin content. This study is underway.

Zimeri was asked if fumonisin has been detected in the soil without plant material. She indicated that Ron Riley was the person who performed the experiment to answer this question, and that did not know if he separated the plant material from the soil.

Wicklow was asked about the cost of applying the NIR grain sorter. He replied that the commercial high-speed dual-wavelength sorter (ScanMasterII 2000 DE, Satake-USA, Houston, TX) they tested for removing white corn contaminated with fumonisin had a capacity of 7000 kg per hour. Potential use would be on high value grains or as a cost effective method for salvaging good quality kernels from grain lots rejected for damage and mycotoxin contamination.

Kolomiets was asked about the growth characteristics of his lox3 mutants. He indicated that while plant height was reduced up to 30% of the wild type, the grain weight and yield from the mutants was not affected when grown in the greenhouse. Further testing of lox3 mutants will be performed in the field during next growing season. Kolomiets was also asked about the specific content of the kernel oils. He replied that with limited seed only germinated kernels were tested for some complex lipids and no significant difference from wild type were detected. Future studies would answer this question.

QTL Mapping for Fusarium Ear Rot and Fumonisin Contamination Resistance in Two Populations of Maize (*Zea mays*)

Leilani A. Robertson[1,2], Michael P. Jines[2], Peter Balint-Kurti[1,4], Gary A. Payne[1], Donald G. White[3], and James B. Holland[2,4]

[1]*Department of Plant Pathology, North Carolina State University, Raleigh, NC;* [2]*Department of Crop Science, North Carolina State University, Raleigh, NC;* [3]*Department of Crop Sciences, University of Illinois, Urbana–Champaign, IL;* [4]*USDA–ARS, North Carolina State University, Raleigh, NC*

Fusarium verticillioides and *F. proliferatum* are fungal pathogens of maize that cause ear rot and contaminate grain with fumonisins, a family of mycotoxins that adversely affects animal and human health. The objective of this study was to identify QTL for resistance to Fusarium ear rot and fumonisin contamination in two maize populations, comprised of 215 $BC_1F_{1:2}$ families from the first backcross of GE440 to FR1064 (GEFR) and 143 recombinant inbred lines from the cross NC300×B104 (NCB). QTL mapping was used to investigate whether QTL were consistent across populations and the genetic relationships between resistances to ear rot and to fumonisin contamination. In the GEFR population, six QTL explained 43.6% of the phenotypic variation for mean ear rot resistance across environments and nine QTL with one epistatic interaction explained 66.6% of the variation for mean fumonisin concentration across environments. In the NCB population, five QTL explained 31.3% of the phenotypic variation for mean ear rot resistance across environments and six QTL and three epistatic interactions explained 50.1% of the phenotypic variation for mean fumonisin concentration across environments. Three QTL in the GEFR population and four QTL in the NCB population affected both ear rot and fumonisin concentration. Three ear rot and three fumonisin contamination resistance QTL mapped to similar positions in the two populations. One QTL localized to chromosome 4 appeared to be consistent for both traits across both populations.

Polyketide Synthases in *Fusarium verticillioides*: Potential Targets to Control Fumonisin Contamination in Corn

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

The fungus *Fusarium verticillioides* negatively impacts corn production in two ways; it produces the mycotoxins fumonisins and it causes diseases such as ear and stalk rot. Fumonisins can cause a number of animal diseases, including cancer and neural tube defects in laboratory rodents. In humans, consumption of fumonisin-contaminated corn has been correlated epidemiologically with esophageal cancer and neural tube defects. Sorting of individual kernels from *F. verticillioides*-inoculated corn revealed that fumonisin levels are substantially higher in symptomatic kernels compared to asymptomatic kernels (Desjardins & Plattner, Plant Dis. 1998; 82: 953–958). This finding indicates that a reduction in ear rot should reduce fumonisin contamination in corn. Thus, a goal of our research is to identify factors that contribute to the ability of *F. verticillioides* to cause ear rot because such factors are potential targets for disease control.

Production of some polyketide-derived metabolites contributes to the ability of a number of fungi (e.g. *Cercospora*, *Cochliobolus* and *Phylosticta*) to cause plant disease. Therefore, polyketides may contribute to the ability of *F. verticillioides* to cause corn ear rot. Polyketide synthases (PKSs) typically catalyze an early step in the biosynthesis of polyketides, and disruption of a PKS gene blocks production of the corresponding polyketide(s). Fifteen PKS genes have been identified in the *F. verticillioides* genome (Kroken et al., Proc. Nat. Acad. Sci. USA 2003; 100: 15670–15675). To determine the role of *F. verticillioides*-produced polyketides in pathogenesis, we are disrupting each PKS gene. To date, we have disrupted eight of the PKS genes and determined that one (*PKS10*) is required for production of the mycotoxins fusarins (also shown by Song et al., ChemBioChem 2004; 5: 1196–1203) and another (*PKS3*) is required for production of the dark pigment in the walls of the sexual fruiting bodies of *F. verticillioides*. Pathogenicity tests with all eight PKS mutant strains are in progress.

Previous Northern blot analysis indicate the fifteen genes in fumonisin biosynthetic (*FUM*) gene cluster, including the PKS gene *FUM1*, exhibit similar patterns of expression. This co-expression of *FUM* genes was also detected by microarray analysis of over 11,000 *F. verticillioides* EST sequences. Based on these results, we are using microarray analysis to examine the expression patterns of the *F. verticillioides* PKS genes and their flanking genes to identify polyketide biosynthetic gene clusters. Preliminary analysis suggests that two PKS genes, *PKS4* and *PKS10*, are part of polyketide biosynthetic gene clusters. The eight contiguous genes on one side of *PKS10* exhibit co-expression while the genes on the other side do not. Sequence comparisons indicate that six of the co-expressed genes encode enzymes (e.g. oxidoreductases and a carboxmethly transferase) consistent with the predicted fusarin biosynthetic pathway. The seven contiguous genes adjacent to *PKS4* also exhibit co-expression.

Computational Studies on the Influence of Solvent on the Conformational Preferences and Selective Recognition of Fumonisins

M. Appell, C.M. Maragos, and D.F. Kendra
USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL

Selective recognition is an important factor in the development of materials to bind and detect mycotoxins, including fumonisins. At the molecular level, selective recognition can be modeled as the interaction of the mycotoxin with the binding material. This type of molecular recognition is highly dependent on the conformation of the mycotoxin and the binding site. In addition, solvent has an effect on conformations of the interacting species and the binding interactions. In our computational studies, we identified several stable conformations for fumonisins A_1, A_2, B_1, B_2, and B_3 in explicit water and *in vacuo*. Calculations of the preferred conformations of fumonisins interacting with potential binding sites will provide useful information for the design of fumonisin selective binding materials.

Using Genomics Approaches to Characterize Potential Fumonisin Regulatory Genes

Robert A.E. Butchko[1], Robert H. Proctor[1], Daren W. Brown[1], Charles P. Woloshuk[2], Burton H. Bluhm[2] and Mark Busman[1]
[1]*Mycotoxin Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL;*
[2]*Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN*

Fusarium verticillioides can cause seedling disease, stalk rot and ear rot of maize and also produces the mycotoxins fumonisins. Fumonisins are polyketide derived secondary metabolites synthesized through a multi-step biosynthetic pathway by enzymes encoded by a co-regulated cluster of genes (*FUM* gene cluster). Fumonisins are toxic to both humans and animals and have most recently been described as teratogenic, causing neural tube defects in mice. In an effort to reduce or eliminate fumonisin contamination of maize we are employing genomic resources to elucidate the genetic regulation of fumonisin production. Three types of *F. verticillioides* genomic resources are available: expressed sequence tag (ESTs) libraries, microarrays and whole genome sequence.

We have developed an EST library containing over 87,000 sequences, in collaboration with The Institute for Genomic Research (TIGR), which represents 11,119 different sequences. The cDNA libraries were constructed from mRNAs isolated from eight different growth conditions. The publicly accessible TIGR *F. verticillioides* Gene Index (FvGI) incorporates all available sequence data into one database and at present, includes 11,126 total unique sequences. We have utilized the EST libraries to identify possible regulatory genes. Comparison of libraries from conditions where the *FUM* gene cluster is not transcribed and conditions where the *FUM* gene cluster is transcribed has allowed the identification of a number genes with similarity to regulatory type genes which may have a role in the transcriptional regulation of the *FUM* gene cluster. We have disrupted a number of these candidates, six of which have an effect on the transcription of the *FUM* genes.

We have also generated a NimbleGen oligonucleotide microarray, in collaboration with TIGR, based on the FvGI. The microarrays consist of approximately 180,000 24-base pair probes or features, with each sequence in the FvGI represented by a set of 12 probes. We have validated the microarrays using mRNA generated from wild-type *F. verticillioides* cultured on fumonisin-inducing media. mRNA was isolated at 6 time points over 5 days and was used to probe the microarrays. *FUM* genes exhibited patterns of expression expected based on previous Northern analysis. Analysis of this time course experiment will allow us to set baseline levels of expression across the set of genes represented on the chip for comparison in other experiments. We are currently investigating differential gene expression between wild-type *F. verticillioides* and a *FCK1* mutant cultured on whole maize kernels, as well as on dissected endosperm and germ tissue.

Recently, 4X coverage of the *Fusarium verticillioides* genome generated at Syngenta and assembled at the Broad Institute was made available to the public. The intersection of whole genome sequence, EST libraries and microarrays is allowing us to more comprehensively define genes and describe their expression at the transcription level.

Fumonisins in Maize in Guatemala, Preliminary Exposure Estimate, and Policies and Recommendations to Minimize Exposure

Ronald T. Riley[1,*], Olga A. Torres[2], Edwin Palencia[2], L. Lopez de Pratdesaba[2], Anthony. E. Glenn[1], Kerry O'Donnell[3] and Mario Fuentes[4]

[1]*Toxicology and Mycotoxin Research Unit, R. B. Russell Agricultural Research Center USDA-ARS, Athens, GA;* [2]*Instituto de Nutricion de Centro America Y Panama, Guatemala, Central America;* [3]*Microbial Genomics and Bioprocessing Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL;* [4]*Institute of Agricultural Science and Technology, Guatemala, Central America*

From 2000-2003, maize samples were collected from fields in the highlands (>1650 m) and lowlands (< 360 m) of Guatemala. The results showed that maize grown in the lowlands had significantly higher levels of fumonisins than the maize grown in highlands. Approximately 92% of the samples from the lowlands collected at harvest in 2002 contained detectable levels of FB_1, whereas, only 5% of the samples collected at harvest from the highlands contained detectable fumonisins. However, 27% of samples of the 2002 crop collected from storage in the highlands immediately before harvest of the 2003 crop contained ≥0.3 ppm FB_1 compared to only 2% of the samples collected at harvest in 2002. All (100%) of the *Fusarium* infected kernels (60/180) analyzed from nine random lowland samples (20 kernels/sample) were infected with *F. verticillioides* (60/60) and no other *Fusarium* species, whereas, in samples from the highlands (n = 9) only 5% (2/43) of the *Fusarium* positive kernels (43/180) were *F. verticillioides*. All the *F. verticillioides* isolates were able to produce fumonisin in culture. In FY 2005 maize samples (n = 236) from the 2004 crop were analyzed from highland and lowland markets in Guatemala. The results show that lowland maize, highly contaminated with fumonisin, is sold in highland markets in Departments where the incidence of neural tube defect is sometimes very high. Thus, fumonisin exposure in high risk areas will be greatest in groups that obtain their maize from the market place since we have shown that maize that is grown in the highlands contains very low levels of fumonisins. Based on a recall study in women conducted in the Central Highlands, a preliminary assessment of daily intake of total FBs was estimated. Consumption of nixtamalized maize products made from lowland maize could result in exposure exceeding the provisional maximal tolerable daily intake (2 μg total fumonisins/kg bw) with over 50% of the maize samples. Policies and recommendations to minimize fumonisin exposure in Guatemala have been discussed. These recommendations are intended to establish a prudent public health policy that will minimize risks to human health while also minimizing negative impacts on the maize industry. They can be achieved through the use of good agricultural and good processing/cooking practices and education of high risk populations and health providers in Guatemala.

Fusaric Acid, a *Fusarium verticillioides* Miasma to *Bacillus mojavensis*, a Biological Control Bacterial Endophyte

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

Antagonisms among microorganisms are strategies that maintain both inter- and intra- specific competition, which is particularly important among those microorganisms that are ecological homologues. *Fusarium verticillioides* is systemically localized in corn and is prevalent in the roots as opposed to the shoot axis of corn, and is best described as a root endophyte. During its early biotrophic phase of its association with corn, hyphae dwell within the intercellular spaces of corn. A biocontrol bacterium, *Bacillus mojavensis,* is patented as an endophytic biocontrol agent of plant diseases. The intention is to replace the fungus with this endophytic bacterium as a management strategy which operates under the broad mechanism of competitive exclusion. Under greenhouse conditions, corn infected with the bacterium shows increased growth and rooting, seedling vigor and disease resistance. Also under these conditions, fumonisin concentration in corn is reduced by 60% in the presence of the fungus, and fungus infection, expressed as CFU/gram of plant tissue, is also reduced. However, use of this bacterium under field conditions and contrary to greenhouse conditions, *F. verticillioides* is superior in colonizing corn plants pre-inoculated with the bacterium. Of the many toxins produced by *F. verticillioides*, fusaric acid might be involved in this effect. Fusaric acid (5-butylpicolinic acid), first discovered during the laboratory culture of *F. heterosporum*, was one of the first fungal metabolites implicated in the pathogenesis of wilt symptoms of plants. In addition to this role in plant pathogenesis, fusaric acid is mildly toxic to mice, and has several important pharmacological properties, and perhaps its major importance in animal toxicity may be synergistic interactions with other naturally co-occurring mycotoxins. It was determined that fusaric acid accounted for the reduction in bacterial growth and resulting decrease in biocontrol activity. Fusaric acid supplied to cultures of the bacterium, at a concentration as low as 22 μM, accounted for a 41% reduction in growth of the bacterium. It is also toxic to this bacterium. Fusaric acidless mutants of *F. verticillioides* were ineffective in colonizing *B. mojavensis*-infected maize, suggesting that fusaric acid is one important defense mechanism for the fungus. The results indicate that the biocontrol bacterium must be modified to resist fusaric acid before its use under field conditions. Two fusaric acid tolerant bacterial mutants have been developed that are endophytic and antagonistic to the fungus. These mutants will form the basis of subsequent field testing for the control of *F. verticillioides*.

Developmental Toxicity of *Fusarium verticillioides* and Fumonisin B$_1$ in LM/Bc and CD1 Mice: Comparing the *in vivo* Models

Kenneth A. Voss[1], Ronald T. Riley[1], Tantiana D. Burns[1,2] & Janee B. Gelineau-van Waes[3]

[1]*Toxicology & Mycotoxin Research Unit, RB Russell Agricultural Research Center USDA-ARS, Athens, GA;* [2]*Interdisciplinary Toxicology Program, University of Georgia, Athens, GA;* [3]*Department of Genetics, Cell Biology & Anatomy, Omaha, NE*

The human health effects of *Fusarium verticillioides* and fumonisins are uncertain. There is evidence however suggesting that fumonisins disrupt folate utilization and increase the risk of neural tube defects (NTDs = birth defects cause by failure of the neural tube to close properly) in populations that depend heavily on fumonisin-contaminated corn as a food source. Fumonisin B$_1$ (FB$_1$) was not teratogenic when given orally (gavage) to pregnant CD1 mice on gestation days (GD) 7–15 whereas intraperitoneal (ip) injection of ≥5 mg/kg BW FB$_1$ on GD7 and GD8, the critical time for neural tube closure, to pregnant LM/Bc mice caused NTDs. Experiments were therefore done to compare the incidence of NTDs in litters of LM/Bc and CD1 dams given FB$_1$ by two different dosing protocols: (a) dietary exposure to fumonisins (provided by adding *F. verticillioides* culture material to the diet) beginning 5 weeks before mating and (b) ip administration of FB$_1$ on GD7 and GD8.

The results of the feeding studies were inconclusive. Diets containing 50 ppm FB$_1$ did not cause NTDs in either strain. At the maternally toxic dose of 150 ppm FB$_1$, one of five LM/Bc litters was NTD positive (1/10 fetuses affected) whereas fetal death rates were higher but no NTDs were found in the CD1 strain (n = 9 litters). In a second feeding trial using LM/Bc mice, NTDs were not found in the fetuses of females fed diets containing 150 or 300 ppm FB$_1$.

A dose-related increase in NTDs was found in the litters of CD1 dams (n = 8–10/dose level) given FB$_1$ by ip injection on GD7 and GD8: 0, 11, 0, and 40 percent of the litters were NTD positive at doses of 0, 15, 30 and 45 mg/kg BW FB$_1$, respectively. This result was confirmed in a second experiment. NTDs were found in 0, 8.3, 16.6, 36.4, 54.5 percent of the litters of CD1 dams (n = 8–12/dose level) given 0, 10, 23, 45 or 100 mg FB/kg BWt FB$_1$ ip on GD7 and GD8. In affected litters of dams given ≤45 ppm FB$_1$, 33 percent or less of the CD1 fetuses had NTDs. The number of NTD positive fetuses from affected litters of CD1 dams given 100 mg/kg BWt FB$_1$ tended to be higher: 15 to 100 percent exhibited NTD (average mean for the group = 42 percent). In contrast, 100 percent of the litters and ≥50 percent of the fetuses from LM/Bc dams given ≥15 mg/kg FB$_1$ by this ip dosing schedule were NTD positive.

These results indicate that (a) both mouse strain and dosing regimen affect NTD induction; (b) induction of NTDs by ip FB$_1$ exposure during the critical time for neural tube closure is not unique to the LM/Bc mouse strain; (c) LM/Bc mice are more sensitive to NTD induction than CD1 mice; and (d) unequivocal induction of NTDs by dietary exposure to fumonisins remains to be shown. Comparative studies using fumonisin-exposed LM/Bc and CD1 mice will be useful for elucidating the physiological and biochemical events involved in NTD formation *in vivo*.

18ᵀᴴ ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 1: CROP RESISTANCE — CONVENTIONAL BREEDING

Moderator: Don Jones, Cotton Incorporated

Creation of Commercial Hybrids with Low Aflatoxin in Grain using Markers

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

This project is creating high yielding, commercially acceptable, corn hybrids with high levels of resistance to Aspergillus ear rot and low levels of aflatoxin in grain. 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 and LH195RR.

With FR1064 we have crossed FR1064 with Mp313E and back crossed three or four times to FR1064 while selecting for the chromosome four region from MP313E that has been associated with resistance. We now have five inbred lines at the self seven level of inbreeding that have the chromosome region four from Mp313E. These lines, at various stages of backcrossing and selfing, have been evaluated for aflatoxin production and yield as test cross hybrids with several inbreds including FR4341 at locations in Texas, Mississippi, Illinois and the various locations provided by SERAT. In general, in experiments where differences occur, the back crossed resistant inbreds have 50 to 90% less aflatoxin in test cross hybrids than FR1064 in comparable test crosses. Although the resistant lines with chromosome four from Mp313E are approximately 94% similar to FR1064 they are later in maturity, have better husk coverage, and better yield in experiments done in Texas.

We also are 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 we took a version of MP313E crossed with FR1064 and back crossed 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. Therefore, the pyramid lines have both B73 and FR1064 which contribute yield and agronomic characters and both MP313E and Tex6 chromosome regions associated with resistance. The resulting lines are later in maturity than resistant versions of FR1064. They also have greater plant height and more drought resistance. When evaluated as test cross hybrids these lines usually have less aflatoxin in grain than comparable test cross hybrids with FR1064. In some experiments they have better resistance than the resistant lines with just chromosome four from Mp313E. However, in other experiments they are similar or higher in aflatoxin. They have demonstrated the highest level of resistance when conditions were extremely favorable for aflatoxin production.

We also are using molecular markers to backcross resistance from MP313E on chromosome four into LH195RR. We have backcross two or three versions selected for chromosome four. We need to make six backcrosses to LH195 in order to effectively recover the agronomic characters of LH195 which is very widely used in corn hybrids from southern Illinois to the deep South.

With guidance from Quinton Raab of B-H Genetics we have concentrated on identifying potential male parents to be used with the resistant female parents that we are developing. Inbred lines related to Stiff Stalk Synthetic such as FR1064, B73 and LH195RR are usually used as female parents of commercial hybrids because of good seed quality and rapid seedling emergence. The male parents that we have identified as contributing good yield in hybrid combinations with our resistant female parents include FR6942, LH256 and TR9352Bt. These inbreds are widely used as male parents in corn hybrids from Central Illinois to the deep South. This summer we produced commercial bag quantities of some of our resistant inbreds with LH256 and TR9352. B-H Genetics is producing bag quantities of crosses with FR6942 in Florida this winter. The seed will be processed and seed treated so that large yield trials can be conducted in 2006. We also are increasing seed of resistant lines in Hawaii this winter so that even greater amounts of commercial seed can be produced in 2006. We also will be evaluating additional male lines with resistance that have been developed by Javier Betrán. Crosses between two resistant inbreds may well result in hybrids with greater levels of resistance.

Breeding Corn Germplasm for Agronomic Performance and Reduced Aflatoxin Contamination

Javier Betrán, Tom Isakeit, Gary Odvody, and Kerry Mayfield
Texas A&M University, College Station, TX

Our program has evaluated, identified and developed corn inbreds with resistant factors that can reduce the risk of aflatoxin and have a good agronomic performance in hybrids. We have used three locations in South Central Texas and inoculation with *Aspergillus flavus* (isolate NRRL3357) using the nonwounding silk channel or colonized corn kernels on the soil surface. At harvest, infected ears were husked, rated for kernel integrity and visible fungi colonization, shelled, ground with a mill, and evaluated for aflatoxin. Quantification of aflatoxin was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest™). These experimental screening techniques and inoculation have facilitated the display of genetic differences among inbreds and hybrids, and increased heritability in aflatoxin evaluations. Multiyear and multi-location testing has permitted the estimation of how much is the genotype by environmental interaction in aflatoxin accumulation and the identification of white and yellow lines with the most consistent resistance. The replicated evaluation of lines and hybrids in several locations and years was instrumental to identify genotypes with the best response in different environments. Inbreds CML323, Tx772, CML288, NC300, FR2128, CML338, CML161 and experimental lines TxX69's and TxLAMA among the yellows, and inbreds CML176, CML269, CML78, and Tx807, and Tx experimental lines derived from crosses among CML269, Tx110, CML78, and CML270 among the whites are the most promising lines to contribute resistant factors to aflatoxin. Most of these lines have subtropical or tropical origin, an indication that exotic germplasm can harbor genes that can contribute to reduce the risk of aflatoxin. In addition, these exotic lines have shown good combining ability and agronomic performance in crosses with temperate adapted inbreds LH195 and LH210. Some Argentine commercial hybrids (e.g., AX889 and Condor) have also shown less aflatoxin concentrations than U.S. commercial hybrids. Low aflatoxin accumulation was associated with good husk coverage, flinty endosperm texture, and good kernel integrity. It seems plausible to select for associated traits having high heritabilities and strong correlation with aflatoxin to reduce the risk of aflatoxin contamination. Early maturing hybrids were more susceptible due to lack of adaptation, bad husk coverage and soft endosperm. The relationship between aflatoxin accumulation in inbreds and their hybrids has been variable. The correlation between inbred and hybrids have been of low predictive value in some experiments and high in others. The type of germplasm evaluated, the number of lines, and the genetic variation present has influenced this relationship. The use of genetic designs such as diallels, factorial designs and generation means analysis where lines are evaluated in several crosses have facilitated the identification of those lines that perform better across hybrid combinations. Recombinant inbred line (RIL) populations have been developed from selected lines (e.g., CML176 and CML161) to map potential genomic regions or QTLs associated with response to aflatoxin and other secondary traits. The combined evaluations for aflatoxin and agronomic performance has facilitated the selection for adaptation, yield potential, stability, and reduced aflatoxin risk. Ultimately, we aim to incorporate aflatoxin resistant factors into elite genetic backgrounds suitable to produce commercial hybrids.

Interaction Between *A. flavus* Strains and Host Plant Genotypes Across Environments and Years

Kerry Mayfield, Tom Isakeit, Gary Odvody, and Javier Betrán
Texas A&M University, College Station, TX

Do interactions occur between genetically different isolates of *Aspergillus flavus* and different genotypes of maize? Currently one isolate of *A. flavus* has been used for inoculation in our trials, although isolates of this species are known to exhibit a range of toxigenic capacity at different environments. Our objective was to determine if there is interaction between genetically-different isolates of *A. flavus* and several genotypes of maize. Two experiments were conducted in 2004 and 2005, one with hybrids and one with inbreds. The hybrid trial contained four commercial hybrids and four TAMU experimental hybrids. The inbred trial included two white inbreds, two high lysine inbreds, and one yellow inbred. Inbreds and hybrids were selected for maturity and previous response to aflatoxin (AF). The hybrid trial was planted at College Station, TX (CS), Weslaco, TX (WE) and Corpus Christi, TX (CC) in 2004 and at CS and WE in 2005. The inbred trial was planted at CS and WE both years. An alpha-lattice field experimental design was used in the hybrid trial, and a randomized complete block design in the inbred trial, both with four reps. Hybrids and inbreds were inoculated using the silk channel inoculation method using isolates L1, F1, I5 (isolated from soil in a maize field in San Patricio County, Texas) and NRRL3357 (AF3357). Isolates were inoculated with in the same row and kept separate by marking individual inoculated plants with colored tape. Plots were hand harvested, shelled and ground prior to quantification of AF using Vicam Aflatest™. Data analysis was conducted using SAS Proc GLM. Significant differences among genotypes were detected in both inbred and hybrid trials in 2004 and 2005 at both locations. Significant differences among the different isolates were also obtained for aflatoxin concentration. Aflatoxin concentrations across environments per isolate in inbreds were 202 ng g^{-1} for AF3357, 323 ng g^{-1} for F1, 206 ng g^{-1} for L1, and 506 ng g^{-1} for I5. In hybrids, aflatoxin averages were 129 ng g^{-1} for AF3357, 370 ng g^{-1} for F1, 138 ng g^{-1} for L1, and 197 ng g^{-1} for I5. Isolates F1 and I5 produced more aflatoxin than commonly used isolate AF3357. Graphs showed slight interaction between genotype and isolate; however, this interaction was non significant at any location or trial, and neither across locations and years. Significant isolate by environment interactions were detected in the hybrids both years and across environments. Isolate F1 produced more aflatoxin in CS in 2005, AF3357 in CC in 2004, L1 in CS both years, and I5 in CC in 2004. One isolate of *A. flavus* may be used in screening for resistance, however; results may be variable in years that environmental conditions are unfavorable for that isolate. A mixture of local isolates may ensured more consistent aflatoxin concentrations to differentiate among testing maize genotypes.

Application of HACCP to Control Mycotoxins in Maize Breeding Programs

David F. Kendra
Mycotoxin Research Unit, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL

Maize ear rot and associated mycotoxin contamination are serious problems for maize growers around the world. In the U.S. corn-belt severe ear rot and mycotoxin outbreaks occur sporadically while they are serious problems yearly in other regions such as the southeastern U.S. During hybrid selection, commercial maize breeders routinely discard genotypes that are visibly susceptible so commercial hybrids are generally somewhat resistant to ear rot; however, little information is available on mycotoxin resistance levels for commercial maize hybrids. Due to increased public concern over food safety and its role in trade policy development and negotiations, mycotoxins are now more closely monitored with at least 99 countries having official regulations for food and/or feed. During the last three decades, the Hazard Analysis Critical Control Point (HACCP) system has been gradually introduced and applied successfully by the food industry to introduce risk assessment based evaluations for potential contamination of food products with pathogenic micro-organisms and physical and chemical safety hazards, including mycotoxins. HACCP is a pro-active, highly structured, systematic quality management system that includes the identification, evaluation and control of hazards in the entire agricultural system. As a result of the increased importance of mycotoxins in global trade, this paper recommends that corn breeders implement a HACCP based approach to develop hybrids that meet or exceed international regulations for regulated mycotoxins in order to ensure competitiveness of U.S. farmers in the global market.

Characterizing Components of Insect-Based Resistance to Preharvest Aflatoxin Contamination in Almond

T.M. Gradziel and A.M. Dandekar
Plant Sciences Department, University of California, Davis, CA

Aflatoxin contamination in almond is strongly associated with insect damaged kernels. Preharvest damage is caused primarily by the naval orangeworm (*Amyelois transitella*) while postharvest infestations involve both naval orangeworm and the Indian meal moth. Incorporating resistance to these insects complements efforts to develop aflatoxin resistant varieties and allows selection strategies which typically demonstrate less environmental variance then plant disease screening methods, allowing higher final heritability. Three major components of insect resistance have been successfully utilized in breeding for aflatoxin resistance: high shell-seal integrity, nonpreference/antibiosis, and toxicity. The integrity of the almond shell-seal is determined primarily by the inner endocarp layer, particularly in the region adjacent to the vascular bundles feeding the ovules. In addition to a high response-to-selection, this approach has allowed the development of resistant genotypes having kernel/shell ratios exceeding 65%. In nonpreference/antibiosis, insects show reduced preference towards resistant genotypes and when infestation occurs, show longer larval development times when feeding on resistant genotypes. Advanced selections demonstrating very low levels of field infestations and almost total suppression of postharvest infestation have been developed. Insect toxicity is achieved through the selection of high amygdalin levels. Since almond is a cyanogenic species, insect feeding will result in the breakdown of amygdalin, forming benzaldehyde and the toxin cyanide. High amygdalin levels in the kernel and/or hull and seedcoat have proven effective in controlling insect damage of mature nuts in the field. Because amygdalin accumulation within the kernel and/or seedcoat occurs late during seed maturation, developing nuts may be susceptible to insect infestation during earlier maturation stages. Intermediate levels of amygdalin in the still developing fruit of high-amygdalin genotypes as well as fully mature seed of intermediate-amygdalin genotypes may show higher levels of insect damage. Benzaldehyde appears to be a powerful attractant to oviposition and feeding by these insects and it is hypothesized that higher infestation results from the ability of benzaldehyde to act as an attractant at levels too low for cyanide toxicity.

Genetic and Genomic Approaches to Improve Host Resistance to Preharvest Aflatoxin Contamination in Corn and Peanut

B.Z. Guo[1], M. Luo[1,2], H. Chen[2], P. Dang[3], A.E. Coy[2], M.D. Krakowsky[4], D. Davis[5], W. Xu[6], X. Liang[7], C. Holbrook[4], R.D. Lee[2], M. Bausher[3], A. Culbreath[2], P. Ozias-Akins[2], and Craig K. Kvien[2]

[1]*USDA–ARS, Crop Protection and Management Research Unit, Tifton, GA;* [2]*University of Georgia, Tifton, GA;* [3]*USDA–ARS, Ft. Pierce, FL;* [4]*USDA–ARS, Crop Genetics and Breeding Research Unit, Tifton, GA;* [5]*University of Columbia, MO;* [6]*Texas A&M University, Lubbock, TX;* [7]*Guangdong Academy of Agricultural Sciences, Guangzhou, China*

Plant-host resistance is a highly desirable tactic that can be used to manage pest problems. Screening and identification of crop plant germplasm for resistant traits for crop improvement and molecular marker development will bring new genetic diversity into U.S. corn/peanut gemplasm. 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 cultivars and improved disease resistance. Corn inbred lines, GT-A1-1, GT-P2 and GT-P56 selected from GT-MAS:gk population, are in late stage of testing and will be released soon. Corn inbred lines, A638 (early, 100 days) and CY1 (115 days), are selected from germplasm provided from Spain and China. Several peanut lines are also selected with very low fungal colonization in the laboratory and low aflatoxin levels in the field cage studies (2 year). A peanut linkage mapping population has been developed from Tifrunner (resistance to TSWV and leaf spots) × GT-C20 (low aflatoxin and resistance to bacteria wilt) and will be used linkage map construction and QTL studies.

Maize microarray, both cDNA and oligo arrays, have been used to study the gene expression profiles in response to drought stress and Aspergillus infection. Maize lines used in these studies were GT-A1-1, Tex6, A638, B73, M017, L0964, L01016, Tex205 and Tex202. Ten cross-talking genes have been identified and will be used in gene expression analysis among more inbred lines, hybrids and RILs. We have been developing EST database as tools and resources for peanut community to gain genomic information and knowledge and discover DNA-markers and genes. With the first batch of ESTs submission to public domain-GenBank in 2003, we used this information to characterize some peanut transcripts in response to peanut leaf spot disease and Aspergillus infection and drought stress using peanut cDNA microarray. Peanut seed ESTs will be completed soon with over 20,000 ESTs from 6 cDNA libraries at R5, R6, and R7 stages of Tifrunner and GT-C20.

Progress Toward Identifying New Sources of Genetic Variation Associated with Reduced Levels of Aflatoxin Accumulation in Maize

Thomas Brooks[1], Matthew Krakowsky[2], W. Paul Williams[1], and Gary Windham[1]
[1]USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [2]USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA

Abstract not submitted.

Proteomic Identification of Maize Cob Proteins that Potentially Confer Resistance to Aflatoxin

Dawn Luthe[1], Olga Pechanova[1], Bela Peethambaran[1], Tibor Pechan[2], Susan Bridges[3], Leigh Hawkins[4], Gary Windham[4] and W. Paul Williams[4]

[1]*Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS;* [2]*Life Sciences and Biotechnology Institute, Mississippi State University, Mississippi State, MS;* [3]*Department of Computer Science, Mississippi State University, Mississippi State, MS;* [4]*USDA-ARS Corn Host Plant Resistance Research Unit, Mississippi State University, Mississippi State, MS*

In this study, we determined the proteome of the developing maize cob and silks 21 days after silking. Using 2-D gel electrophoresis and Multi Dimensional Protein Identification Technology (MudPIT), we identified approximately 1600 cob and 900 silk proteins. In addition, we compared the cob proteome of resistant (R- Mp313E) and susceptible (S- SC212m) inbreds and inoculated and uninoculated ears using Differential In Gel Electrophoresis (DIGE). DIGE analysis revealed interesting differences in the protein composition between R and S lines. In general, R contained more antioxidant enzymes, small heat shock proteins and enzymes involved in phenolic metabolism, whereas the S contained more chitinases and a different set of protein in phenolic metabolism. The sets of proteins induced at 10 and 35 days after inoculation also differed considerably between R and S cobs. Similar types of results were found for silk proteins. The proteomic approach will allow us to select protein and genes for marker selected breeding programs, in addition to providing clues about the mechanisms of alfatoxin resistance in developing ears.

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

M.W. Olsen[1], P.J. Cotty[2] and S. Husman[3]
[1]*Department of Plant Sciences,* [2]*USDA–ARS,* [3]*Cooperative Extension, The University of Arizona, Tucson, AZ*

In Arizona, southern Texas and the Imperial Valley of California, aflatoxins are always a concern in cottonseed used for animal feed. Aflatoxin contamination of cottonseed occurs in two phases, the first when *A. flavus* infects developing bolls through wounds or cracks, and the second when mature seed is exposed to both conducive temperatures and moisture. Rank cotton, dense canopies, dew, and late irrigations increase the severity of second phase contamination. At present there are no assays available to compare susceptibility of cotton cultivars to second phase contamination under field conditions. Development of such an assay is the first objective of this project. Another objective is to determine if seed hardness and seed coat fragility are related to aflatoxin contamination. Deliberate wetting of open bolls using a mist system is being used to simulate moisture effects.

In 2004 trials, bolls were misted three times for one day each. Humidity was increased over non-treated controls, but not to an acceptable level, and aflatoxin contamination of seed was very low to non-detectable in all samples. In 2005 trials, two different experiments were done. The first was a continuation from 2004 of investigations of the timing of wetting on aflatoxin contamination. There were four treatments with eight replications each: no wetting, wetting early at first boll opening, wetting late when most bolls were open, and wetting both early and late. Plots were all planted with DP449BR. The second experiment was a variety trial in which eight replications of four varieties, Hammer (CPCSD high yielding, thin-coated seed variety), DP455BR, ST5599BR and PHY470WR were wetted both early and late.

Field plots are established at The University of Arizona Maricopa Agricultural Center (MAC), and a misting system for increasing humidity in the crop canopy was installed. Water was pumped from the irrigation ditch through two inch pipe throughout the field to 20×20 ft^2 plots. The misting system consisted of 12 five-gal/hr brass foggers spaced about three feet apart in a 10×10 ft^2 grid of ½ inch PVC pipe. Based on 2004 humidity data, misters were raised to 2 ft above ground and misting volume increased. Humidity and temperature of treated plots was monitored using Hobo data loggers to quantify impact of wetting regimen on canopy environment. Treatments consisted of two multiple day misting periods in August-September (early) and October (late). Cotton was harvested in early November and immediately ginned.

Harvested seed will be tested for aflatoxin, and the data used to continue design of a field based screening technique. Ultimately a model will be developed relating wetting period and temperature to aflatoxin content. This will contribute both to development of the screening technique and to a better understanding of the second phase of contamination. A model will be useful to growers in assessing crop aflatoxin risk prior to harvest and to researchers in development of alternative aflatoxin management strategies.

Corn Hybrids with Exotic Germplasm and Low-Aflatoxin

Wenwei Xu[1], Jinfen Zhang[1], Gary Odvody[2], and W. Paul Williams[3]
[1]Texas A&M University, Lubbock, TX; [2]Texas A&M University, Corpus Christi, TX; [3]USDA/ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS

Aflatoxin contamination of corn is a chronic problem in the southern United States. We observed that drought tolerant corn hybrids produced a higher yield and had much less grain mold than susceptible hybrids under drought conditions. The objective of this study was to determine if genetic improvement of abiotic stress tolerance and corn earworm resistance can reduce the aflatoxin risk in this region. Ten Texas Experimental Agricultural Station (TAES) experimental hybrids and five commercial checks (Pioneer hybrids 34K77 and 31B13, Garst 8285, Triumph 1416, and DK XL269) were grown in Lubbock, Halfway, Corpus Christi, and Beeville in Texas and Mississippi State, MS in 2003 and 2004. In Lubbock and Halfway, plants were inoculated one week after silking by injecting *A. flavus* conidia into silk channels. In Corpus Christi, Beeville, and Mississippi State, corn kernels colonized by *A. flavus* were distributed between all rows when the first hybrid was at the mid-silking stage to provide the increased and uniform aerial dissemination of conidia. In all cases, the inoculum was from a high aflatoxin-producing *A. flavus* strain (NRRL3357). A limited late planting date was used in Corpus Christi, Beeville and Mississippi State to encourage severe drought stress at later stages of maturity. The tests used a randomized complete block design with nine replications at Corpus Christi and Beeville, four replications in Lubbock and Halfway, and three replications in Mississippi. Ears from each plot were hand-harvested. All ears were threshed and agronomic data were recorded including grain yield. Grain samples were ground in a Romer mill and aflatoxin B_1 assay was done on 50 g sub-samples of the finely ground material for each composite replication using the Vicam immunoassay/fluorometer system.

Two-year results showed that S1W × CML343 and S2B73 × NC300 had significantly lower aflatoxin than the control hybrids. Hybrid B110 × SGP3 had high yielding and high aflatoxin in most environments in two years. In 2003, the aflatoxin level in S1W × CML343, S2B73 × NC300 and P31B13 was 49, 31, and 161 ppb respectively at Corpus Christi, TX. The aflatoxin of the Mississippi State test in 2003 was generally low and not significant among the entries. The results in 2004 were in general consistent with the results in 2003. In 2004, the aflatoxin levels in S1W × CML343, S2B73 × NC300, and P31B13 (CK) was respectively 5.3, 16.7, and 70.0 ppb at Corpus Christi; 10.1, 9.4, 5.8 and 33.3 ppb at Mississippi State. In 2004, Lubbock had the rainfall and temperatures favorable for corn growth and development. The aflatoxin levels under well-watered and drought stressed test in Lubbock were similar, although the average grain yield of the 14 entries declined from 184 bu/a in well-watered condition to 126 bu/a in drought stressed condition. The aflatoxin levels in S1W × CML343, S2B73 × NC300, and P31B13 was 90.0, 30.5, and 260.0 ppb under well-irrigated conditions (mean of 14 hybrids as 134.8 ppb), while under drought conditions were 93.5, 33.5, and 240 ppb (mean of 14 hybrids as 120.5 ppb). These low aflatoxin hybrids yielded well in comparison to the checks. For example, the average yield of S1W × CML343 in Halfway and Lubbock in 2003 and 2004 was 228 and 220 bu/a while P31B13 produced 222 and 245 bu/a. The S1W × CML343 is a white hybrid. In other field trials in south Texas in 2005, this hybrid produced 119 and 157 bu/a at Weslaco and Ganado in comparison to 111 and 138 bu/a of P31B13. These results indicate that breeding for drought tolerance and earworm resistance is a promising approach to reduce aflatoxin contamination in corn grown in Southern environments. Some of our experimental hybrids have comparable yield yet significantly low aflatoxin in comparison to the commercial hybrids. The TAES experimental hybrids and their parental lines have at least 25% tropical germplasm and were selected for drought and heat tolerance, CEW resistance and overall agronomic performance. They have tight husk, good ear tip coverage, significantly lower grain mold and less ear injuries by corn earworm.

Computational Tools for Protein Identification and Gene Ontology Annotation of the Maize Proteome

Susan M. Bridges[1], Julia E. Hodges[1], Gregory Bryce Magee[1], Nan Wang[1], Dawn S. Luthe[2], and W. Paul Williams[3]

[1]*Department of Computer Science and Engineering, Mississippi State University, Mississippi State, MS;*
[2]*Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS;*
[3]*USDA ARS Corn Host Plant Resistance Research Unit, Mississippi State, MS*

The Corn Host Plant Resistance Research Unit, USDA-ARS, Mississippi State University in collaboration with Dr. Dawn Luthe, is conducting proteomics studies to determine the effects of biotic and abiotic factors on *Aspergillus flavus* infection and aflatoxin accumulation in maize. Computational support has provided scientists with tools to improve protein identification rates and provide efficient and informative annotation of the proteome.

Characterization of the maize proteome of the developing ear under different conditions has the potential to reveal the fundamental processes that confer resistance in some cell lines. Advances in proteomics have been made possible by high-throughput methods for gel electrophoresis and new technologies for mass spectrometry such as LC/MS/MS. We have previously reported the development of the PIE database of translated ESTs and have shown that use of this database for protein identification for a 2-dimensional gel experiment with cob proteins results in identification of 87.5% of the spots compared to a 56% identification rate with the NCBI database non-redundant green plant database. The PIE database pipeline has been parallelized and now runs on a high performance cluster, making it possible to rapidly generate updated databases.

Additional tools have been developed to streamline the protein identification process and to provide the Gene Ontology annotation of the identified proteins. The multi-dimensional protein identification technology (MudPIT) can be used to separate many hundreds to thousands of peptides in a single experiment. The results obtained from Sequest analysis of MudPIT experiments can be quite challenging to analyze, particularly when the database used for queries is highly redundant. This is the case when using translated ESTs because many correspond to the same protein or to closely related proteins. Scientists typically must integrate information from several repetitions and data sources to determine confidence in an identication. The PepSort tool was developed to assist with this type of analysis. The tool combines multiple reps selecting the best score for each peptide based on a user specified scoring system. Potential protein duplicate identifications are collected and presented to the user simultaneously so the user can select the best identification and eliminate duplicates. Scores and counts for peptides are updated automatically when duplicates are removed.

We have deployed the MaizeGO database, as part of Agbase www.agbase.msstate.edu, a curated, open-source, Web-accessible resource for functional analysis of agricultural plant and animal gene products. Four tools have been developed as part of AgBase to support Gene Ontology annotation of proteins from high throughput experiments: GO Retriever, GO annotator, GO Profiler and GO Slim Viewer. GO Retriever provides a first pass retrieval of GO annotations that are currently published for resources accessed by the AgBase database. GO Annotator is used to supplement the annotations provided by GO Retriever by providing predictions of GO annotations for proteins based on homology with annotated proteins based on user-selected BLAST parameters. GO Profiler provides an overview of GO associations available for a user-specified species including the number of GO associations and the number of annotated proteins. The GO Slim Viewer provides an overview of the membership in GO categories of a protein data set using categories defined in a GO Slim. Output is in a form that can be easily imported into Excel for formatting as a pie chart.

Progress in Breeding Peanut for Resistance to Preharvest Aflatoxin Contamination and Drought

C.C. Holbrook[1], B.Z. Guo[1], P. Timper[1], D.M. Wilson[2], D. Sullivan[1], E. Cantonwine[2], and C. Kvien[2]
[1]USDA-ARS, Tifton, GA; [2]University of Georgia, Tifton, GA

Aflatoxin contamination costs the U.S. peanut industry over $20 million annually. The development of peanut cultivars with resistance to preharvest aflatoxin contamination (PAC) would reduce these costs. We have developed field screening techniques that can measure genetic differences in aflatoxin contamination, and have used these techniques to identified 11 core accessions that have shown at least a 70% reduction in PAC in multiple environments. We have also identified significant reduction in PAC in peanut genotypes with drought tolerance. These sources of resistance to PAC have been entered into a hybridization program. They have been crossed with cultivars and breeding lines that have high yield, acceptable grade, and resistance to tomato spotted wilt virus (TSWV). Due to the large environmental variation in PAC, it is not feasible to examine these breeding populations until late generations when there is less heterozygosity and adequate seed are available for field testing using multiple replications. We have examined numerous breeding populations and have identified several families and individual breeding lines that have relatively low PAC, relatively high yield, and acceptable levels of resistance to TSWV. However, much faster breeding progress could be achieved through the development and use of indirect selection techniques. We are exploring this with studies on mechanisms of resistance to PAC and attempting to develop molecular markers for resistance. The most promising mechanisms we have identified thus far are resistance to drought and resistance to the peanut root-knot nematode. We have developed several late generation breeding lines with resistance to drought. These lines have exhibited reduced aflatoxin contamination in multiple environments. Recently, we have developed late generation breeding line with resistance to TSWV and the peanut root-knot nematode that appear to have agronomically acceptable yield and grade. Testing is ongoing to determine if these lines will have reduced aflatoxin contamination.

Searching for New Resistance and Control Measures of Aflatoxin in Corn

Steven Moore[1], Hamed Abbas[2], and Mark Millard[3]

[1]*Louisiana Agricultural Experiment Station – Louisiana State University Agricultural Center, Alexandria, LA;* [2]*USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS;* [3]*North Central Regional Plant Introduction Station – Iowa State University, Ames, IA*

The mission of aflatoxin research in the LSU AgCenter is to reduce or eliminate aflatoxin contamination in corn. Specific objectives include identifying new germplasm with improved resistance, using glufosinate to reduce aflatoxin contamination, and evaluating the use of atoxigenic *Aspergillus flavus* strains. In the 2005 Southeastern Regional Aflatoxin Test (SERAT) in Central Louisiana, numerous hybrids from multiple breeding programs were identified that had higher yields and lower aflatoxin than commercial checks, indicating that progress is being made in developing commercially viable cultivars. Tx 807 (PI619430), TZI18 (PI506253), Haiti 33 (PI483902), ARIP69 (PI218189) and CML 43 (PI595535) had lower aflatoxin than the Mp313*B73 and Tex6*B73 resistant checks in the 2005 screening trial. A corn line from Sundance Genetics had the lowest aflatoxin contamination in material analyzed from the 2005 screening trial up to now. Glufosinate treatment lowered aflatoxin contamination in the non-Liberty Link corn hybrid 'N83-Z8' but seemed to have little or no effect in Liberty Link corn hybrid 'N83-N5'. Glufosinate lowered aflatoxin more when applied sooner after mid-silk than when applied later after mid-silk. The average aflatoxin contamination in corn sprayed with 4.25 and 8.5 ounces of glufosinate were significantly lower than the control and reduced aflatoxin by about 45% in ear-inoculated treatments and by about 86% in ground-inoculated treatments. Glufosinate application to corn threatened by aflatoxin contamination appears to be a hopeful economic tool for producers. More field-plot and field-scale research is needed to confirm benefits and determine best practices. There appeared to be a small but beneficial effect of applying the atoxigenic strain 'K-49' on reducing aflatoxin in corn. Atoxigenic strains may be a useful tool in reducing aflatoxin but much work remains to find the most effective strains and to determine field application strategies.

Development of Aflatoxin-resistant Maize Inbreds and Identification of Potential Resistance Markers through USA-Africa Collaborative Research

Robert L. Brown[1], Zhi-Yuan Chen[2], Abebe Menkir[3], and Thomas E. Cleveland[1]

[1]*Southern Regional Research Center, USDA-ARS, New Orleans, LA;* [2]*Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA;* [3]*International Institute of Tropical Agriculture, Ibadan, Nigeria*

A research collaboration between the Southern Regional Research Center (SRRC) and the International Institute of Tropical Agriculture (IITA) was initiated in 1998. The purpose of this collaboration is to develop aflatoxin-resistant maize inbreds for use in West and Central Africa and for use in the U.S. as well. Another objective is to identify markers in the maize lines generated through this collaboration to facilitate marker-assisted transfer of resistance traits. 35 S_6 temperate lines and 91 S_4 tropical lines have been advanced to S_7 and S_5 respectively. 44 S_5 lines showing good agronomic traits were sent to the U.S. for analysis using the kernel screening assay (KSA). 44 additional new inbreds from different crosses were screened in the U.S.; thus far 6 show levels as low or lower than resistant inbred, MI82. Most advanced inbreds with desirable agronomic characteristics were planted in the dry season to generate hybrids to test for agronomic performance in at least 2 locations in 2005. New inbred lines from this program, once resistance and agronomic traits are confirmed, will be released as sources of genes for resistance to U.S. breeding programs and will be used for development of hybrids and synthetics in African national programs. Basic genetic information will also be generated using near isogenic lines with contrasting levels of aflatoxin to develop a breeding strategy for pyramiding different alleles that confer resistance to mycotoxins. Proteome analysis of pairs of closely-related S_4 lines have demonstrated, as in a previous study employing different germplasm, constitutively-expressed stress-related proteins that are associated with resistance. A previously undescribed beta-1, 3-glucanase, also associated with resistance, was identified in this investigation and cloned. Glyoxalase 1 and PR 10 proteins, previously identified through proteomics as associated with resistance, are being investigated in RNAi gene silencing studies. Seed has been produced from RNAi transformations and is being characterized for gene expression levels and for aflatoxin accumulation. New RNAi studies will focus on trypsin inhibitor and serine/threonine kinase.

PANEL DISCUSSION: Crop Resistance — Conventional Breeding

Panel Chair: Don White

Panel Members: Javier Betrán, Kerry Mayfield, David Kendra, Tom Gradziel, Baozhu Guo, Tom Brooks, Dawn Luthe, Mary Olson, Wenwei Xu, Susan Bridges, Corley Holbrook, Steve Moore, Paul Williams, and Bob Brown

Summary of Presentations: Presentations included in the conventional breeding session varied greatly in approach and goals. Several projects are concentrating on identification of new sources of resistance mostly from exotic germplasm. Other projects are combining identification of sources of resistance with applied breeding programs that are concentrating on low aflatoxin as well as agronomic characteristics that will be required before resistant varieties will be used by farmers. Some are using molecular marker assisted back crossing to move chromosome regions associated with resistance from agronomically poor inbreds into commercially acceptable inbreds. Other projects are using genomic and proteomics approaches in an attempt to better understand the nature of resistance.

Tremendous progress has been made in the breeding of resistant peanuts. With peanuts it is necessary to have grade, virus resistance and other traits before varieties can be commercially used. With corn, a number of projects are developing hybrids with emphasis on resistance to aflatoxin production coupled with yield, resistance to root and stalk lodging, and other agronomic characteristics. The projects on corn have joined forces to evaluate hybrids produced in everyone's project and compare them with commercially used corn hybrids. With tree nuts research has concentrated on avoidance of insect damage which is highly correlated with penetration of Aspergillus species and the production of aflatoxin.

Summary of Panel Discussion: There was discussion on how we can utilize data developed with genomics and proteomics in applied breeding programs. The panel agreed that it will take some time before genomics approaches can be directed toward applied breeding programs. It was suggested that the opportunity exists with corn to look at genomics and proteomics data associated with chromosome regions where molecular markers have shown to be associated with genes for resistance. That would enhance the possibility of finding specific genes, especially from the resistant inbred line Mp313E. There was also discussion of exactly how Hazard Analysis Critical Control Point (HACCP) relates to resistance. It was pointed out that HACCP is actually a highly structured and systematic way to address important breeding goals. Also, there was discussion on the use of Liberty herbicide on non-Liberty Link corn hybrids to reduce aflatoxin. Basically the herbicide Liberty is applied after pollination on hybrids susceptible to the herbicide. This is been shown to reduce aflatoxin. It was suggested that the mode of action of Liberty herbicide is production of ammonia which is known to reduce aflatoxin.

There was general agreement among the panel that outstanding progress has been made with respect to resistance to aflatoxin production.

Multilocation Evaluation of Aflatoxin Accumulation in Yellow Maize Hybrids

Cody McKee, Tom Isakeit, Gary Odvody, Kerry Mayfield, Javier Betrán
Texas A&M University, College Station, TX

A major obstacle in maize production across the Southern U.S. and other parts of the world is accumulation of aflatoxin, a known carcinogen in both humans and livestock produced by *Aspergillus flavus*. Aflatoxin contamination is difficult to evaluate in the field because of varying amounts of source inoculum and dependency on favorable environmental conditions. Texas is an excellent area for examining aflatoxin accumulation because of the tendencies for abiotic stresses such as drought and high temperatures. Our objectives were to 1) estimate the responses of these hybrids to aflatoxin across a range of environments; 2) identify the hybrids within each group that exhibited the lowest levels of contamination; 3) analyze the relationship between agronomic performance and aflatoxin accumulation; and 4) determine how much genotype×environment interaction (GEI) affect these traits. In the past, our program has examined aflatoxin accumulation at three environments in Southern Texas, Weslaco, Corpus Christi and College Station. Concern has been raised that this is not sufficient to examine genotype×environmental effects. Therefore, during 2005 twenty five hybrids, 20 experimental testcrosses with inbreds LH195 and LH210 and 5 commercial hybrids (P31B13, P32R25, BH8913, DKC69-72 and W4700), were evaluated under inoculation with *A. flavus* in eight locations representing the maize producing regions of Texas. Aflatoxin concentration was 407 ng g^{-1} at College Station, 86 ng g^{-1} at Weslaco, 1094 ng g^{-1} at Corpus Christi, 131 ng g^{-1} at Castroville, 315 ng g^{-1} at Wharton, 407 ng g^{-1} at Granger, 209 ng g^{-1} at Bardwell, and 274 ng g^{-1} at Prosper. Overall, we found that the commercial hybrids had higher grain yields than the experimental hybrids, with P31B13 yielding the highest at 6.4 Mg ha^{-1}. However, experimental hybrids, especially testcrosses with LH195, were less susceptible to aflatoxin accumulation than commercial hybrids. Hybrid Tx-LAMA2002-42/LH195 had the lowest average aflatoxin accumulation across locations at 154 ng g^{-1}. Aflatoxin concentration was positively correlated with % of ear rot and test weights and negatively correlated with grain yield and 1000 kernel weight. We observed a significant GEI for both aflatoxin concentration and grain yield. Therefore, multiple locations are necessary for estimating agronomic performance and response to aflatoxin of maize hybrids.

Southern East Regional Aflatoxin Test (SERAT)

Michael Clements[1], Paul Williams[1], Steve Moore[2], Matthew Krakowsky[3], Baozhu Guo[3], Don White[4], Wenwei Xu[5], Tom Isakeit[6], Tom Brooks[1], Gary Windham[1], Hamed Abbas[7], James Perkins[8], Daniel Gorman[9], Quinton Raab[10], Keith Arnold[10], David Smith[11], and Javier Betrán[6]

[1]*USDA–ARS, Mississippi State, MS;* [2]*Louisiana State University, Alexandria, LA;* [3]*USDA–ARS, Tifton, GA;* [4]*University of Illinois, Urbana, IL;* [5]*Texas A&M University, Lubbock, TX;* [6]*Texas A&M University, College Station, TX;* [7]*USDA–ARS, Stoneville, MS;* [8]*Monsanto Company Crop Protection, Waterman, IL;* [9]*Pioneer – Dupont, Cairo, GA;* [10]*BH Genetics, Moulton, TX;* [11]*Zea Sage, Sycamore, IL*

Aflatoxin contamination of corn grain is a chronic problem for growers in the southeast United States. For several years, research groups at Louisiana, Mississippi, Georgia, Illinois and Texas have been screening corn germplasm for response to aflatoxin contamination at specific locations. Although several sources of resistance have been identified and released, at present, there are no elite inbred lines resistant to aflatoxin that can be used directly in commercial hybrids. Aflatoxin accumulation is severely affected by the environment. Genotype by environment interaction (GEI) is normally significant with genotypes showing different relative response across environments. A testing network of environments across major growing areas affected by aflatoxin has been established to identify the most consistent stable sources of resistance. SERAT is a multilocation and multistate regional test of the most promising germplasm from each breeding program. Participants provide seed of a few hybrids and a testing location. Evaluations are conducted under inoculation with *A. flavus* following the protocols commonly used by each research group. In addition to aflatoxin, grain yield and other agronomic traits such as maturity, lodging, grain moisture, test weights, etc. are recorded. In 2004, SERAT tests were conducted at six locations: Alexandria, LA; Tifton, GA; Starkville, MS; Urbana, IL; Halfway, TX; and Weslaco, TX. The silk channel inoculation method was used at all locations except Urbana and Alexandria, where inoculation with a pinboard was used, and Starkville, where inoculum was injected through husk leaves into the side of the ear. Aflatoxin concentration was variable across locations. Average aflatoxin was 730 ng g^{-1} at Alexandria, 54 ng g^{-1} at Tifton, 392 ng g^{-1} at Starkville, 182 ng g^{-1} at Halfway, 21 ng g^{-1} at Urbana and 652 ng g^{-1} at Weslaco. Significant GEI was observed for both aflatoxin and grain yield. Principal component analysis of aflatoxin concentrations suggested different response of hybrids to the different locations. In 2005, SERAT tests were conducted at 9 locations: Alexandria, LA; Tifton, GA; Starkville, MS; Urbana, IL; Halfway, TX; and Weslaco, TX; Ganado, TX; Mistic, GA; and Claxton, GE. Similar inoculations techniques as in 2004 were used. Tests at Mistic and Claxton, GA were not inoculated. Average aflatoxin was 275 ng g^{-1} at Alexandria, 587 ng g^{-1} at Tifton, 118 ng g^{-1} at Ganado, and 134 ng g^{-1} at Weslaco. Aflatoxin concentrations for the rest of the locations are being quantified. Grain yield was also variable across locations. Average grain yield was 88 bu/a at Weslaco, 166 bu/a at Mistic and Claxton combined, 117 bu/a at Ganado, 131 bu/a at Tifton, 129 bu/a at Starkville, 118 bu/a at Halfway, and 116 bu/a at Alexandria. As in 2004, highly significant GEI was observed for grain yield with environments discriminating differently testing hybrids. Response of materials from different programs was variable, in that hybrids showed desirable expression for different traits such as aflatoxin, grain yield and standability. This suggests possibilities of combining positive traits by crossing germplasm from different programs. In general, experimental hybrids have shown less susceptibility to aflatoxin but less grain yield than commercial checks. Locations discriminated corn hybrids differently for both aflatoxin content and grain yield. SERAT has promoted collaboration and identified complementary germplasm from different programs. With this collaborative regional testing, we expect to identify the most stable sources of aflatoxin resistance, assess their consistency across different environments and treatments, characterize their agronomic performance, increase the collaboration among research groups in different states, and to assess the magnitude and nature of genotype × environment interaction for aflatoxin.

Evaluation of CIMMYT Germplasm for Response to Aflatoxin Production in the Southern USA

Dan Jeffers[1], Matt Krakowsky[2], Paul Williams[3], Javier Betrán[4]
[1]CIMMYT, Mexico D.F.; [2]USDA-ARS, Tifton, GA; [3]USDA-ARS, Mississippi State, MS; [4]Texas A&M University, College Station, TX

The demand for safe, nutritious corn requires efforts to develop improved hybrids with better food processing and nutritional qualities. Exotic white and yellow lines represent a source of genes for quality traits. The International Maize and Wheat Improvement Center (CIMMYT), has developed germplasm more tolerant to abiotic stresses and resistant to biotic stresses, for targeting aflatoxin prone areas especially in Africa. Our objective was evaluate the response of selected CIMMYT white and yellow corn inbreds and hybrids to aflatoxin contamination in Southern USA, and to determine the genetic variability that exists for resistance to *Aspergillus* ear rot in CIMMYT germplasm for use by US investigators, CIMMYT, and their network of partners in developing countries who need maize varieties with improved grain quality and storability. Twenty five CIMMYT yellow hybrids, twenty five white hybrids, twenty four white inbreds and twenty eight yellow inbreds were or are currently being evaluated in Texas, Mississippi and Georgia. These inbreds and hybrids were selected based on low levels of *Aspergillus flavus* ear rot infection under field evaluations using artificial inoculations in Mexico. U.S. evaluations in 2005, were artificially inoculated with *Aspergillus flavus* isolate NRRL3357 two weeks after flowering using the silk channel inoculation method in Starkville, MS and the colonized kernel method in Weslaco, TX. Quantification of aflatoxin was conducted using the Vicam Aflatest (Watertown, MA). There were significant differences for aflatoxin content in both inbreds and hybrids. White and yellow inbreds were evaluated in Starkville, MS. Some genotypes did not flower early enough for inoculations, and no aflatoxin data was collected. Average aflatoxin concentration was 608 ng g^{-1} for white inbreds and 451 ng g^{-1} for yellow inbreds. White quality protein maize (QPM) inbred CML142 and yellow QPM inbred CLQ-G2507 had aflatoxin levels below the resistant checks, Mp313E and Mp717, respectively. Average aflatoxin concentrations for white hybrids were 148 ng g^{-1} in Starkville, MS and 17 ng g^{-1} in Weslaco, TX. Environmental conditions were not conducive for *Aspergillus* infection and aflatoxin production with the colonized kernel inoculation technique used in Weslaco. Several white hybrids (e.g., CML341 × CML254, CML341 × CML495) had lower aflatoxin than the most resistant check. Average aflatoxin concentrations for yellow hybrids were 83 ng g^{-1} in Starkville, MS and 38 ng g^{-1} in Weslaco, TX. Yellow hybrids CML-451 × CL-02844 and CL-02450 × CML454 had lower aflatoxin levels than resistant checks in Starkville, MS. Less variation for aflatoxin levels was observed in Weslaco, TX. Overall, significant differences for aflatoxin concentration were observed for both CIMMYT white and yellow maize inbreds and hybrids. Genetic variation, based on differences in the production of aflatoxins, was observed among exotic CIMMYT germplasm, where some CIMMYT inbreds and hybrids had aflatoxin concentrations similar to the most resistant checks. Multilocation and multiyear evaluations would be needed to select the most promising germplasm to introgress in U.S. breeding programs.

Phenotypic and Genotypic Characterization of a RIL Maize Mapping Population for Aflatoxin and Secondary Traits

Melanie Edwards, Monica Menz, Tom Isakeit and Javier Betrán
Texas A&M University, College Station, TX

Aflatoxin is a mycotoxin produced by *Aspergillus flavus* that is toxic to both humans and livestock. Breeding efforts to produce commercial hybrids resistant to aflatoxin would be enhanced by better understanding of the genetic components involved. A recombinant inbred line (RIL) population was developed using phenotypically divergent parental inbreds CML176 and Tx811 to map quantitative trait loci (QTL) for response to aflatoxin and root lodging. This population of 162 S6 RILs, was evaluated in replicated trials in two Texas locations, Weslaco and College Station under inoculation. Root lodging, aflatoxin concentration, maturity, endosperm texture, and kernel integrity were all measured for the population. Heritability for each trait, and phenotypic and genotypic correlations of secondary characteristics to aflatoxin, were estimated from variance components. The mapping population showed significant differences and broad ranges for all agronomic traits studied, with the offspring showing transgressive segregation for each of the traits. The trials had severe root lodging as a consequence of heavy winds before or around flowering time. Lines had significant differences for root lodging with CML176 being more susceptible than Tx811. Mean aflatoxin concentration was higher at College Station (292 ng g^{-1}) than Weslaco (214 ng g^{-1}). Overall mean for the population across locations was 250 ng g^{-1}. The population mean for kernel integrity was 2.6 (in a visual scale from 1 to 5), which was closer to the average for Tx811 (2.8) than that of CML176 (1.7). The population mean for endosperm texture was 2.2 (in a visual scale from 1 to 5), intermediate between the means for parental lines Tx811 (2.7) and CML176 (1.6). Kernel Integrity was the trait that was most highly genotypically correlated to aflatoxin concentration (0.847 at College Station, 0.705 at Weslaco). CML176 has a lower kernel integrity rating (thus more intact kernels) than Tx811, as well as having a lower aflatoxin concentration. Endosperm texture was also highly correlated to aflatoxin concentration (0.456 at College Station, 0.492 at Weslaco), although less so than kernel integrity. The flinty endosperm of CML176 is correlated to a lower aflatoxin concentration than floury endosperm like that of Tx811. Heritability estimates at Weslaco were high for all studied traits. Heritability for aflatoxin concentration was 0.32 at College Station and 0.66 at Weslaco. The two traits correlated to aflatoxin accumulation, endosperm texture and kernel integrity, were both highly heritable (0.85 and 0.76, respectively) and also easy to select for in the field. This provides possibilities for future selection indices that may expedite selection for aflatoxin resistance and provide a more inexpensive initial selection criterion. The population was then genotyped using simple sequence repeat (SSR) markers, and marker data compared to phenotypic data to ascertain associations between loci and response to aflatoxin or root lodging by using single marker analysis in SAS. In preliminary analysis, several markers were significantly associated with root lodging *per se* (umc2163, umc2180, umc1124) and with response to aflatoxin (hpi072, phi087). Additional genotyping and marker analysis is undergoing.

Expression of LOX Pathway Genes in Corn Embryos Associated with *Aspergillus flavus* Resistance

Alberto Camas[1], L. Lopez[1], P. Williams[2] and D.S. Luthe[1]
[1]*Department of Biochemistry and Molecular Biology, Mississippi State University, MS;* [2]*USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State University, MS*

The accumulation of aflatoxin, a mycotoxin produced by the fungus *Aspergillus flavus* during maize grain fill continues to do be a problem. Because most aflatoxin problems develop in the field. The best strategy for eliminating mycotoxin production is to develop preharvest host resistance to aflatoxin contamination. USDA-ARS scientists at Mississippi State University have contributed to this intense field research by releasing several corn inbreds as a source of resistance to kernel infection by *A. flavus*. However, incorporating resistance from these sources into commercial hybrids rquires identification and characterization of factors shown to be associated with resistance. The ability to identify molecular markers associated with resistance would help to advance the breeding program and provide clues about the mechanisms of resistance. We found several lox pathway genes associated with corn resistance. By using QT-PCR we compared *lox*, *aos*, and *opr* expression levels of mature and immature embryos from corn inbreds and different hybrids between resistant and susceptible inbred genotypes. The differences in gene expression between resistant and susceptible genotypes could be related to the plant resistance mechanisms. Therefore we propose some of the LOX pathway genes as potential molecular markers that could contribute to get commercially available and agronomically acceptable corn lines.

Breeding for Increased Resistance to *Fusarium verticillioides* in Maize

Magen Starr[1], Leilani Robertson[1,2], James Holland[1,3] and Gary Payne[2]
[1]*Department of Crop Science, North Carolina State University, Raleigh, NC;* [2]*Department of Plant Pathology, North Carolina State University, Raleigh, NC;* [3]*USDA, ARS, Plant Science Research Unit, North Carolina State University, Raleigh, NC*

Fusarium ear rot in maize is most often the result of colonization by *Fusarium verticillioides* (formerly *F. moniliforme*) which produces fumonisin, a mycotoxin responsible for diseases such as leukoencephalomalacia (LEM) in horses, and pulmonary edema (PES) in hogs, and is correlated with esophageal cancer in humans.

Previous studies in maize have demonstrated that there is genetic variation for resistance to Fusarium ear rot in field maize (King and Scott, 1981; Clements et al., 2004) but have revealed no evidence of complete resistance. A high genetic correlation between ear rot and fumonisin in two maize populations (Robertson et al., 2005) suggests that selection against ear rot should result in reduced susceptibility to fumonisin contamination. Fumonisin content has a higher heritability, so direct selection against fumonisin content is predicted to be theoretically more efficient than indirect selection against ear rot for reducing susceptibility to fumonisin contamination. However, fumonisin assays require much more time and money than ear rot measurements. Therefore, selection against ear rot is hypothesized to be more practically efficient at identifying lines with reduced susceptibility to fumonisin.

A specific objective of this project is (1) to backcross genes conferring resistance to Fusarium ear rot and fumonisin contamination from the agronomically poor inbred line GE440 to the susceptible elite line FR1064, and (2) to test the effectiveness of selection against ear rot at reducing susceptibility to fumonisin contamination in that population.

Quantitative Expression Analysis of Adversity Resistance Genes in Corn Germplasm with Resistance to Preharvest Aflatoxin Contamination

M. Luo[1,2], D. Davis[3], W. Xu[4], D. Lee[2], and B.Z. Guo[1]

[1]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]University of Georgia, Department of Crop and Soil Sciences, Tifton, GA; [3]University of Missouri–Columbia, Division of Plant Sciences, Columbia, MO; [4]Texas A&M University, Lubbock, TX

Aflatoxin contamination of corn in the field is known to be influenced by numerous factors. Drought stress is conducive to *Aspergillus flavus* infection and aflatoxin accumulation. Drought tolerant germplasm could reduce preharvest aflatoxin contamination. The goals of this project are to understand the changes of gene expression in response to drought stress using maize microarray and to identify the biochemical pathways and important genes associated with resistance to *A. flavus* and drought tolerance. In this report, we are reporting the development of a set of gene/probes in assessment of maize germplasm with drought tolerance and *A. flavus* resistance. In our 2004 maize microarray study, we found the quantitative difference in gene expression under drought stress, and the resistance by induction using BTH was not significantly improved. Based on the gene expression analysis and reported data, we selected 119 genes, including two reference genes, with adversity resistance to test gene differential expression in six maize lines, A638, B73, LO1016, LO964, MO17 and Tex6, using real-time RT-PCR. The drought stress was applied at 25DAP (day after pollination) for stressed plots. Corn ears at 35 DAP were harvested and only kernels were used for gene expression analysis in response to drought stress using the designed primers. We are interested in the genes related with adversity resistance, particularly drought tolerance and fungal resistance. Microarray is a powerful tool to select important genes in response to abiotic and biotic stresses. For the last two years, we have been using maize microarray in searching for genes/pathways associated with these two traits and we found that the differential expressions of the majority genes are quantitative. The real-time RT-PCR data of the selected genes indicate that the repeatability of this method is high. The genes related with signal pathways had high C(T) cycles, and 86 genes had detectable and repeatable changes in response to drought stress or among the selected maize lines. A638, TEX6 and LO964 had more up-regulated genes in comparison with B73. If B73, LO1016 and MO17 were used as reference lines, respectively, there were 10 cross-talking positive genes which can be selected from A638, TEX6 and LO964. These genes are related with drought response and disease resistance.

Peanut PR Protein, ß-1,3-glucanase, Induction by *Aspergillus flavus* and Copurification with a Conglutin-like Protein

X. Liang[1], B.Z. Guo[2], and C.C. Holbrook[3]
[1]*Guangdong Academy of Agricultural Sciences, Guangzhou, China;* [2]*USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA;* [3]*USDA-ARS, Crop Genetics and Breeding Unit, Tifton, GA*

Aflatoxin contamination of peanut has been identified as the most important health problem facing the peanut industry. Infection of peanut (*Arachis hypogaea* L.) seeds by *Aspergillus flavus* and *A. parasiticus* is a serious problem that can result in aflatoxin contamination in the seeds. Breeding resistant cultivars would be an effective approach to reduce aflatoxin accumulation. The objective of this study was to investigate the expression of pathogenesis-related (PR) protein β-1,3-glucanase and the isoform patterns in peanut seeds inoculated with *A. flavus*. Peanut genotypes, GT-C9 and GT-C20 (both resistant to *Aspergillus flavus* infection), and Georgia Green and A100 (both susceptible to *A. flavus* infection), were used in this study. The activities of β-1,3-glucanase were similar in the un-infected seeds of all genotypes, but increased significantly in the resistant genotypes after inoculation in comparison with the susceptible genotypes. An in-gel (native PAGE) enzymatic activity assay of β-1,3-glucanase revealed that there were more protein bands corresponding to β-1,3-glucanase isoforms in the infected seeds of resistant genotypes than in the infected seeds of susceptible genotypes. Both acidic and basic β-1,3-glucanase isoforms were detected in the IEF gel. Thin layer chromatography (TLC) analysis of the hydrolytic products from the reaction mixtures of the substrate with the total protein extract or individual band of native PAGE revealed the presence of enzymatic hydrolytic oligomer products. The individual bands corresponding to the bands of β-1,3-glucanase isoforms Glu 1-5 were separated on the SDS-PAGE resulting in two bands, 10-kDa and 13-kDa, respectively. The sequence of the 13-kDa major protein band showed a high degree of homology to conglutin, a storage protein in peanut seeds. Conglutin is reported as a peanut allergen, *Ara h2*, and has trypsin inhibitor function. Our data provide the first evidences for peanut having β-1,3-glucanase activities and the association with the resistance to *A. flavus* colonization in peanut seeds.

Corn Husk Characteristics Potentially Associated with Resistance to Aflatoxin Contamination of Grain: A Preliminary Study

M.J. Clements and W.P. Williams
USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS

Feeding damage from several insect pests on corn ears contributes to conditions that favor severe Aspergillus and Fusarium ear rots and severe mycotoxin accumulation in grain. Husk characteristics that serve as barriers to insect movement often are negatively associated with insect feeding damage to ears, and therefore are thought to serve as mechanisms of resistance to disease development and mycotoxin accumulation in grain. Typically, husk tightness has been quantified subjectively either shortly after silking or after plants have dried down and are ready for harvest. Rationale for timing of husk tightness evaluations is not available in literature. Our objective is to identify a plant growth stage or seasonal period at which differentiation of husk tightness among various corn genotypes is maximized. We examined four methods of evaluating husk tightness over six sampling periods in replicated trials at Mississippi State University in 2005. Force required to remove husk leaves from the ear was measured mechanically (gauged pull) and subjectively (subjective pull). Force required push a 3-mm dia. steel rod longitudinally along the ear between husk leaves and kernels was measured mechanically from the tip to the shank end of the ear (adhesion from the tip) and from the shank to the tip end of the ear (adhesion from the shank). Greatest differentiation of husk tightness among genotypes was observed between 28 to 35 days post mid-silk for the four methods evaluated. Gauged pull at 28 days post mid-silk provided greatest range of data, and good resolution to differentiate the four genotypes tested; however, all four methods warrant further examination as potential methods of quantifying husk tightness. This study will be repeated in 2006 along with, potentially, an evaluation of husk tightness among F2:3 families associated with resistance or susceptibility to aflatoxin contamination in grain in another study. Information on timing of husk tightness evaluations will optimize studies aimed at differentiating genotypes with tight or loose husks and the identification of quantitative trait loci associated with husk tightness.

Chalcone Synthase, a Gene that Influences Both Drought Response and Aflatoxin Accumulation in Maize

M. Gerau, D. Bush, D. Davis, C. Morriss, and G. Davis
Division of Plant Sciences, University of Missouri–Columbia, Columbia, MO

Drought stress increases aflatoxin contamination in maize. Identification of the genes that mediate this effect will facilitate development of stable, low aflatoxin accumulation maize lines. Roots are the first organ to sense soil drying. Identification of genes that are involved in early root response to soil drying is an important first step towards reducing drought stress and the associated increases in aflatoxin level. Towards this end, we identified thirty-seven root architecture QTL in the maize Intermated B73 × Mo17 mapping population. Both well-watered and water-stressed root architecture was studied. Several of the QTL correspond to genes for abscisic acid (ABA) biosynthesis. ABA has long been linked to drought response. Abscisic acid levels have been implicated in mediating reactive oxygen species damage. Prior QTL analysis of aflatoxin accumulation in maize identified a QTL that coincides with the chalcone synthase gene in maize. Chalcone synthase (*c2*) is a polyketide secondary metabolite produced by the maize plant which is the rate limiting step in anthocyanin pigment production and serves as a branch point to several other flavonoid compounds. Subsequently, the role of this gene in aflatoxin was confirmed in independent studies. Naringenin, a product of the *c2* gene has also been shown to reduce *Aspergillus flavus* growth *in vitro*. The *c2* gene has also been implicated in changes in root branching under water stress by QTL and mutant analysis. This gene represents a potential target for reducing drought stress related increases in aflatoxin levels and understanding the mechanism by which they occur. In other plants, naringenin has been shown to increase branching by affecting polar auxin transport. Studies are underway to determine whether auxin is involved in mediating the drought associated increase in aflatoxin accumulation in maize.

18ᵀᴴ ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 2: MICROBIAL ECOLOGY

Moderator: Phil Wakelyn, National Cotton Council

Effect of Fungal Competition on the Colonization of Wounded Peanut Seeds by *Aspergillus* section *Flavi* from Natural Soil Populations

B.W. Horn
USDA-ARS, National Peanut Research Laboratory, Dawson, GA

The effect of fungal competition on the colonization of wounded peanut seeds by *Aspergillus* section *Flavi* species in soil was examined. Viable peanut seeds were wounded and inoculated with cultivated soils differing in composition and density of *Aspergillus* species, then incubated for 14 d at different temperatures and seed water activities. Maximum percentages of seed colonization by section *Flavi* species occurred at 22–37 °C and a seed water activity of 0.92–0.96. Under these conditions, competitive saprotrophic ability of section *Flavi* was high and approximately 50% of the peanut seeds with a propagule of *A. flavus* or *A. parasiticus* at the wound site became colonized. Wounds inoculated with soil were initially colonized by *A. terreus* (2–4 days), which was then quickly overgrown by section *Flavi* species and *A. niger* (> 4 days). Further successional changes in the peanut mycobiota were not observed except for the appearance of *Eupenicillium ochrosalmoneum* sporulating on the heads of section *Flavi* species. A significant interactive effect (P < 0.0001) was observed between soil densities of *A. flavus* and soil densities of other, potentially competing species within section *Flavi* (*A. parasiticus*, *A. caelatus* and *A. tamarii*). Colonization of seeds by *A. flavus* decreased as soil densities of competing section *Flavi* increased. Soil densities of section *Flavi* species and *A. niger* showed a similar interactive effect (P < 0.0001). Therefore, competition among Aspergilli is responsible for suppressing seed colonization by individual section *Flavi* species. Other species in the genera *Aspergillus*, *Penicillium* and *Fusarium* were capable of invading peanut seeds primarily when soils contained low densities of section *Flavi* species (< 50 CFU/g) or when combinations of temperature and seed water activity were suboptimal for section *Flavi*.

Transfer of Aflatoxin Biocontrol Technology: Results of First Commercial Use in Peanuts

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

A method for biological control of aflatoxin contamination of peanuts has been developed through several years of research. Biological control is achieved by introducing a dominant population of a nontoxigenic strain of *Aspergillus flavus* into the soil of the growing peanut crop, and the applied strain competitively excludes toxigenic strains in the colonization of peanuts during periods of late-season drought. A significant accomplishment in the development of this technology was the development of a unique formulation technique in which conidia of the nontoxigenic strain are coated onto the surface of hulled barley, which serves as a carrier for delivery of the fungus to the field and also as a substrate for further proliferation of the fungus after application. ARS patents for this technology were licensed for commercialization in 2002, and the biocontrol product, afla-guard®, received EPA section 3 registration as a biopesticide in 2004. This made possible the first commercial use of the product on approximately 5000 acres of peanuts during crop year 2004 in southeastern Alabama and southwestern Georgia. To determine the efficacy of afla-guard® in large-scale use, soil samples from representative treated and untreated fields were dilution plated to determine *A. flavus* populations and toxigenicity. In addition, farmers' stock peanut samples were collected at buying points in each area of use and analyzed for aflatoxin. Finally, treated and untreated peanuts that had been stored separately for several months were analyzed after shelling to determine aflatoxin in shelled lots prior to sale.

Application of afla-guard® changed the composition of *A. flavus* soil populations from 71.1% toxigenic strains in untreated fields to only 4.0% in treated soils. Analyses of farmers' stock peanuts being delivered at seven different locations showed a consistent reduction in aflatoxin contamination in peanuts from fields treated with afla-guard®. Over all locations, aflatoxin averaged 78.9 ppb in untreated peanuts compared with 11.7 ppb in treated peanuts, an 85.2% reduction. Peanuts from treated and untreated fields were stored together in separate warehouse bins at two different locations. Aflatoxin analyses at the Unadilla, GA location showed a mean aflatoxin concentration in all shelled edible lots from untreated fields of 36.2 ppb compared with a mean of 0.9 ppb in lots from treated fields. At the Dawson, GA storage location, aflatoxin means for shelled lots were 7.2 and 2.2 ppb for untreated and treated peanuts, respectively. For shelled lots to be sold to a manufacturer, an official aflatoxin analysis of the lot must show the lot to contain ≤15 ppb of aflatoxin. If the lot contains >15 ppb, costly remilling and blanching must be carried out to try to reduce the level to 15 ppb or less. In analyses of shelled untreated lots from the Unadilla warehouse, 48.4% of those lots tested at >15 ppb compared with no such lots from the treated peanuts. At the Dawson location, 15.8% of shelled lots from untreated fields contained >15 ppb compared with no lots of treated peanuts. This translates to a reduction in net shelled stock value for untreated peanuts of 13.0 and 4.3% for Unadilla and Dawson, respectively. Using the European Union tolerance of 4 ppb for total aflatoxins, those reductions in value for untreated peanuts were 19.1 and 11.6%, respectively. Converting the value changes to a farmers' stock ton basis, the differences in net farmers' stock value for the peanuts stored in Unadilla were $51.55 and $72.78 per ton, respectively, for the 15 and 4 ppb tolerances. Equivalent differences for the Dawson peanuts were $11.33 and $39.18, respectively. These data illustrate the economic benefits that can be gained from use of the biocontrol agent to control aflatoxin in peanuts.

Atoxigenic Strain Technology for Aflatoxin Control in Cotton

Larry Antilla[1] and Peter J. Cotty[2]
[1]*Arizona Cotton Research & Protection Council Phoenix, AZ;* [2]*Agricultural Research Service, USDA, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ*

Atoxigenic strains of *Aspergillus flavus* have been investigated as biological control agents for the mitigation of aflatoxin contamination of a variety of commercial crops since the late 1980's. One such strain, *Aspergillus flavus* AF36, occurs naturally in Arizona soils and has repeatedly been demonstrated to have the ability to competitively exclude aflatoxin producing fungi and thereby reduce aflatoxin contamination of cottonseed. The Arizona Cotton Research & Protection Council and the Agricultural Research Service established a partnership to develop commercial scale AF36 production and evaluate commercial scale methodologies for utilizing atoxigenic strains in the late 1990s. The goals of this unique cooperative venture were to: a) develop area-wide management strategies for the Arizona cotton industry; b) obtain EPA registration of AF36, thereby facilitate its maximum utilization on cotton; c) systematically evaluate effects of variability in grower utilization of AF36 treatments on efficacy in aflatoxin management, and d) develop standard operating procedures and manufacturing protocols which would allow other interested grower groups or cooperatives to readily access this public sector technology. Field evaluation studies were the subject of increased attention during the 2004 treatment season. Based on observations from 2003, it appeared that light soils with reduced moisture carrying capacity had a tendency to support poor growth of AF36 and thereby poor efficacy if applications were made prior to adequate canopy closure and hence reduced humidity levels at the soil surface. Studies suggested that direct exposure of inoculated wheat seed to sunlight has a deleterious effect on fungal survival. At the very least, seeds exposed to direct sunlight were in a moisture deprived environment and therefore less capable of sporulation. In an effort to correct this situation in 2004, a test was arranged with a cooperating gin to maximize conditions for AF36 survival. The test involved three participating growers with acreage totaling 648. A seasonal employee was hired to coordinate all treatments to exact program specifications. All fields were tracked to 80% canopy closure or above. Irrigation schedules were carefully recorded so that AF36 could then be applied by tractor using Gandy boxes and drop tubes 24 hours or less prior to irrigation onset. Post treatment evaluations indicated that near 100% of the applied AF36 product (colonized wheat seed) sporulated. At harvest, fourteen fields from the block were tested for AF36 and the highly toxigenic S strain. AF36 displacement levels on the crop ranged from 71–100% with an average of 82.4% for all fields. Corresponding S strain levels ranged from 0 to 7.5% with an overall average of 0.6%. This represented a significant change in the fungal community ratio for the principal farmer in the test area where pretreatment background soil analyses on five fields averaged 46% S strain and only one percent AF36. The dramatic effects associated with increased grower attention to detail during treatments with atoxigenic strain AF36 established the need for a retrospective analysis of commercial applicator practices in relationship to the highest and lowest levels of displacement of aflatoxin producers by AF36 following treatment to crops. To this end, a standard questionnaire was developed to characterize and quantify producer practices and observations associated with AF36 applications. The information requested from growers related to application, irrigation, pesticide use, crop characteristics, and weather and pest conditions (bird, rodent and insect). In addition, a soil type database was initiated to further elucidate the relationship between product performance and the broad range of extrinsic factors influencing such. Area-wide program statistics for Arizona in 2004 are as follows: a total of 23,439 acres were treated representing twelve separate geographical areas in the State. Ten gins participated and a total of 851 separate commercial fields were treated. A total of 775 soil samples were collected and analyzed with average pretreatment AF36 and S strain levels of 27% and 26%, respectively. A total of 125 post application crop samples taken at harvest 2004 averaged 65% AF36 and 3% S strain. Areas that experience particularly large benefits from AF36

Managing Aflatoxins in Cotton-Corn Rotations

Peter J. Cotty
USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ

Abstract not submitted.

Antilla & Cotty, continued

applications continue to be those that treat relatively large acreage in the same geographical areas over multiple years. Two such ginning communities in Yuma County have, through 2004, completed two or more years of coordinated program activities. Results of treating more than 7,900 acres combined have produced an average AF36 level on the crop of 81.5% with a corresponding S strain average of 4.5%. The ability of AF36 to carry over from one crop year to the next in situations where treatments are not applied annually has been routinely demonstrated in numerous program settings. The most striking example of this phenomenon was documented in the Mohave Valley in northwestern Arizona in 2004. AF36 treatments of 930 and 2,700 acres in 2002 and 2003 respectively produced average displacement levels of 81% AF36 in the soil and 85% on the crop. No treatments were made in the Mohave Valley during 2004. Despite the fact that no Mohave treatments were made in 2004, AF36 still composed 82% of the *A. flavus* resident in Mohave Valley soils previously treated. Modified fungal community structure was maintained throughout the 2004 crop season and 68% of the *A. flavus* on the 2004 Mohave Valley cottonseed was AF36 after ginning. Future efforts will focus on continued characterization of optimum treatment delivery systems and on improved manufacturing and formulations.

Aflatoxin Control in Pistachios: Biocontrol Using Atoxigenic Strains

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

For the past several years, we have investigated the use of atoxigenic strains (strains not able to produce aflatoxins) of *Aspergillus flavus* as biocontrol agents to reduce aflatoxin contamination of pistachios in California. This approach has been very successful in commercial cotton fields in Arizona where the atoxigenic strain AF36 has substantially reduced aflatoxin contamination of cottonseed. In 2001 and 2002, the three promising atoxigenic strains A564, A815, and AF36 (this strain, the same as used in Arizona cotton fields, was only applied in 2002) were applied in a flood-irrigated research pistachio orchard. In early summer for both years, wheat seeds infected with these strains were applied to the orchard floor at the rate equivalent to 10 lbs/acre. No atoxigenic strains have been applied in this orchard since 2002. In order to determine the survival and spread of the atoxigenic strains, soil samples were collected on 30 August, 2004. The density of *A. flavus*/*A. parasiticus* in the soil did not significantly differ among treatments. Most of the *A. flavus* isolates from the soil in the treated areas belonged to the atoxigenic strain applied there (80.3 to 96.7% of the isolates, depending on strain) even though the atoxigenic strains had not been applied since 2002, demonstrating that the atoxigenic strains persist well in pistachio orchard soil. The applied strains were detected in the untreated areas at low levels (2.4 to 4.9% of the isolates, depending on strain), suggesting only slight movement of the applied atoxigenic strains to untreated areas. In 2005 additional soil samples were collected on 29 August and are currently being evaluated.

Starting in 2003, the atoxigenic strain AF36 was applied in a different research pistachio orchard that was irrigated by microsprinklers. In 2004 wheat seeds infected with AF36 were applied after collecting soil samples on 6 July. On 12 September, samples of leaves, early split nuts, and soil were collected. On the following day, the nuts were harvested, and samples collected from the harvested nuts. In late summer the density of *A. flavus*/*A. parasiticus* in soil was not significantly different between treated areas and untreated areas. The incidence of AF36 among *A. flavus* isolates increased from before applying the wheat to late summer in the treated areas (from 0.6% to 25.8% and from 42.4% to 70.1% for areas treated for one year and two years, respectively) but not in the untreated areas (6.1% and 1.1%). The incidence of AF36 on leaves did not significantly differ between areas treated with AF36 (19.1% and 27.2%) and untreated areas (17.5%). No kernel decay by *A. flavus* was found in over 600 early split nuts, suggesting that applying AF36 does not significantly increase decay of the nuts. 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. In 2005 wheat infected with AF36 was applied on 29 June after collecting soil samples. Leaf, nut, and additional soil samples were collected in late summer of 2005 and are currently being evaluated.

The incidence of atoxigenic strains among *A flavus* isolates occurring naturally in commercial pistachio orchards in California was determined. All three atoxigenic strains, AF36 (5.3%), A564 (1.7%), and A815 (1.0%) were detected among 794 isolates of *A. flavus* from commercial pistachio orchards. A new study was initiated that will determine the natural incidence of 15 additional atoxigenic *A flavus* strains in commercial pistachio orchards. Preliminary results show that these other atoxigenic strains occur at very low levels in commercial pistachio orchards.

Aflatoxin Control in Figs: Biocontrol and New Resistant Cultivars

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

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 2004 we applied the atoxigenic strain AF36 for a second year in a drip-irrigated Calimyrna fig orchard. On 6 July, wheat seeds infected with AF36 were applied at the rate of 41.2 g wheat/tree (equivalent to 10 lbs/acre). On 26 August, we collected noncaprified figs from the orchard floor. As in 2003, the applied atoxigenic strain AF36 was detected colonizing and sporulating on the noncaprified figs. On 15 September, leaf, fruit, and additional soil samples were collected. The soil had a higher density (107.3 cfu/g soil) of *A. flavus*/*A. parasiticus* in the areas under the drip lines where infected wheat had been placed than in the middles (11.0 cfu/g) or under the drip lines in the untreated areas (0.3 cfu/g). In late summer, all of the *A. flavus* isolates obtained from the soil under the drip lines in the areas treated in 2003 and 2004 belonged to the strain AF36 compared to only 47.3% of the isolates from the untreated areas. Furthermore, the incidence of AF36 in the middles was 92.3%, suggesting that there was movement of AF36 from the applied areas under the drip lines to the middles. The density of *A. flavus*/*A. parasiticus* and the incidence of AF36 on leaves did not differ significantly between treatments. No decay by *A. flavus* was found in 900 dried figs that were examined, 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.

In 2005 we did not apply any atoxigenic strains in this orchard. However, we did collect samples in order to determine the survival and spread of the previously applied atoxigenic strains. On 26 August, we collected noncaprified figs from the orchard floor but observed no colonies of *A. flavus* on them. On 7 September, leaf, fig, and soil samples were collected and are currently being evaluated.

The incidence of atoxigenic strains among *A. flavus* isolates occurring naturally in commercial fig orchards in California was determined. A total of 322 isolates of *A. flavus* from commercial fig orchards were evaluated, and all three atoxigenic strains AF36 (7.8%), A564 (0.6%), and A815 (0.6%) were detected. In addition, a new study was initiated that will determine the natural occurrence of 15 additional atoxigenic *A. flavus* strains in commercial fig orchards.

New fig selections have been developed by a University of California breeding program that produced selections having Calimyrna ancestry but with figs that had smaller eyes (the opening to the interior of the fig) than Calimyrna figs and did not need to be pollinated by the fig wasp. The most promising selection, previously named 6-38W but now given the name Sierra, has been released to commercial fig growers. In 2004 Sierra figs had substantially smaller eye diameter of dried fruit (1.5 mm) than the commercial cultivars Adams (2.3 mm) and Calimyrna (2.2 mm) but the same as Conadria (1.5 mm). Furthermore, the incidence of decay by *Aspergillus* sect. *Flavi* of the dried figs of Sierra was substantially lower (0.0%) than that of Calimyrna (5.7%). The figs of the new fig cultivar Sierra have consistently had substantially less fungal decay, including decay by aflatoxin-producing fungi, than Calimyrna figs.

Identification of Bacterial Antagonists of *Aspergillus flavus* from California Almond Orchards

Jeffrey D. Palumbo, James L. Baker and Noreen E. Mahoney
USDA-ARS, Western Regional Resarch Center, Albany, CA

Bacterial populations from two California almond orchards were evaluated for their potential as biological control agents against *Aspergillus flavus*. Bacteria were isolated from washes of almond flowers, immature fruits and mature fruits by direct plating. Isolated strains were screened for antagonistic activities against *A. flavus* strain Papa827, a *nor* mutant strain that accumulates norsolorinic acid under aflatoxigenic conditions. Inhibition of growth and aflatoxin production (visualized by the orange pigmentation of norsolorinic acid) by bacterial isolates was assessed using agar- and liquid-based coculture assays. Initial screens identified 338 isolates with antifungal phenotypes and were studied further. Of these, 147 isolates inhibited growth of *A. flavus*, and 24 isolates inhibited aflatoxin production. These isolates were further characterized by examining their production of extracellular chitin- and yeast cell wall-hydrolyzing enzyme activities. Bacterial strains were identified by 16S rDNA sequence analysis and by nutritional analysis using the Biolog microbial identification system. Several genera were identified, including *Bacillus*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, and several plant-associated enteric and non-enteric bacteria. Because of their relative frequency of isolation, as well as their antifungal activities and resistance to environmental stress, *Bacillus* isolates appear to be most promising for development for biocontrol of *A. flavus* on almonds.

Biological Control of *Aspergillus flavus* by a Saprophytic Yeast Strain in Tree-Nut Orchards: Progress in 2005

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

The fungus, *Aspergillus flavus,* produces aflatoxin which is the most potent carcinogen known. This mycotoxin is very hazardous to the health of both human and animal. National economic losses are in the billions of dollars per year due to aflatoxin contamination of agricultural commodities including pistachio. Resistant pistachio hybrids are not available. Thus biological control is a viable and environmental-friendly approach to manage pre-harvest aflatoxin contamination.

The yeast, *Pichia anomala* strain WRL-076 inhibited aflatoxin production of *A. flavus* by 99% in a bioassay protocol (Hua et al., Appl Environ Microbiol 1999; 65: 2738–2740). This particular yeast was demonstrated to reduce spore production of both toxigenic and aflatoxigenic isolates of *A. flavus* in pistachio flowers and nut-fruits as well as in almond and pistachio leaves in the lab experiments (Hua, Acta Hort 2002; 591: 527–530; Hua, IOBC Bulletin 2004; 27: 291–294).

The saprophytic fungus, *A. flavus,* infects plants through wounds. Aflatoxin contamination is well documented to be associated with wounding in corn, peanuts, cotton and tree-nuts before harvest. Two experiments were conducted in a commercial orchard in the summer of 2005 in collaboration with D.E. Parfitt and Brent Holtz, University of California Davis. Nut-fruits of pistachio were individually wounded with a dissecting needle. Four treatments were applied. Branches of nut clusters were sprayed with water; sprayed with an aqueous suspension of yeasts at 5×10^7 cells/ml; sprayed with an aqueous suspension of yeasts at 5×10^7 cells/ml and two hours later sprayed with spore suspension of *A. flavus* at 1×10^3 cells/ml; or sprayed with a spore suspension of *A. flavus* at 1×10^3 cells/ml. Four trees were randomly selected for each treatment. Nut-fruits were harvested 3–5 weeks after spraying. The data show that *P. anomala* WRL-076 reduced the frequency of *A. flavus* colonization by 4 to 10 times and decreased the total propagules of *A. flavus* by 80 to 99% in comparison to nut-fruits not sprayed with the yeast.

We demonstrated that *P. anomala* could grow at low water activity (a_w). PEG (polyethylene glycol) 8000 was used to adjust medium a_w to 0.96, which mimicked a water stress condition of −5.62 MPa. The yeast cells formed a film and inhibited the growth of *A. flavus* inoculated to the medium. Aflatoxin contaminations of corn, peanut, cotton and tree nuts are known to be associated with water stress. This trait makes the species very suitable as a biological control agent against *A. flavus* under water stress conditions.

Seventeen media of different composition were evaluated for the production of *P. anomala* to generate high numbers of yeast cells. The bioassay protocol was used to assess each medium for the production of yeast cells competent in inhibiting the growth and aflatoxin production of *A. flavus*. Two media showed good yield of yeast cells and supported the production of competent *P. anomala* for biological control of *A. flavus*.

Cultural Conditions Promoting Chitinase Production in *Gliocladium catenulatum*

David F. Kendra[1], Michael J. Muhitch[2], Amber Anderson[1] and Cesaria E. McAlpin[1]
[1]*Mycotoxin Research Unit, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL;*
[2]*Rochester College, Rochester Hills, MI*

Gliocladium catenulatum is a known mycoparasite of several fungal genera including *Aspergillus* and *Fusarium*. The use of mycoparasites to control fungal plant pathogens either as direct biocontrol agents or as novel sources of antifungal compounds is increasing in importance to serve as environmental alternatives to chemical control. The antagonistic activity of biocontrol agents is attributable to one or more complex mechanisms including the production of antibiotic metabolites, competition for nutrients, induction of systemic acquired resistance, increased nutrient availability to the host plant and production of cell wall hydrolyzing enzymes, including chitinase and β-1,3-glucanase. *Gliocladium catenulatum* is known to produce several secondary metabolites with antimicrobial activity, including verticillin and glisoprenin however there is no report of chitinolytic activity. In this paper we report the cultural conditions required to produce chitinase in minimal media supplemented with chitin. Optimal production of extracellular chitinase was observed in a liquid medium previously developed for culturing *Fusarium chlamydosporum* when cultured at 25 °C for 21 days and a slightly acidic pH. Chitinase activity was repressed if the colloidal chitin medium was amended with sucrose, glucose, cellulose or starch and eliminated by xylan or lactose. The nitrogen source significantly influenced chitinase activity with KNO_2 supporting the best production.

PANEL DISCUSSION: Microbial Ecology

Panel Chair: Bruce Horn

Panel Members: L. Antilla, P.J. Cotty, J.W. Dorner, M. Doster, S.S. Hua, D.F. Kendra, J.D. Palumbo

Jeffrey Palumbo was asked to address a potential problem with biological control in almond orchards; namely, that biocontrol bacteria and aflatoxin-producing fungi have different temperature and water activity requirements. He replied that most of the work thus far has been performed in the laboratory and that future field trials should indicate how different environmental conditions affect the ability of bacteria to inhibit aflatoxin production.

Larry Antilla was asked how early application of nonaflatoxigenic *A. flavus* can be effective if the cotton canopy is not fully developed. He replied that application is early in the Yuma region of Arizona because canopy closure is a month ahead of cotton-growing regions further to the west. A fair amount of canopy and associated shading are required for maximum sporulation of the biocontrol fungus. Peter Cotty added that at 6% relative humidity during the period of application (middle of June to the middle of July), 18–20% moisture in the wheat grain containing the biocontrol fungus is difficult to attain. Soil type is also a factor: cotton canopy may be more important in sandy soils than in clay soils, which are better at retaining moisture. Furthermore, native toxigenic populations of *A. flavus* are increasing in soil during canopy formation and if the biocontrol application is delayed too long, effective displacement of toxigenic strains may not be possible. Donald Wicklow mentioned that *A. flavus* sclerotia applied to Georgia crop fields germinated under the crop canopy but not in the exposed regions between the rows.

Peter Cotty was asked to describe the fate of highly contaminated cottonseed after arrival at the oil mill. He stated that when the seeds are crushed, aflatoxins are retained in the meal and are not present in the oil. However, a profitable oil mill must market the high-value meal, which is rich in protein. Mills often segregate seeds before crushing to keep aflatoxin concentrations in meal below 20 ppb; seed lots that exceed this limit are marketed in Mexico. The hulls of cottonseed typically do not contain aflatoxins but during processing, they may become contaminated with aflatoxins from dust and seed fragments.

Joe Dorner was asked if there was any incentive for growers to use biocontrol in peanuts. He replied that there is little incentive for the growers under the current peanut program because farmers' stock peanuts are examined for visible *A. flavus* but are not analyzed for aflatoxins. Even if aflatoxins are present, the grower is not penalized because peanuts without visible *A. flavus* (seg 1) have a guaranteed government loan price regardless of aflatoxin concentration. Seg 1 peanuts are later analyzed for aflatoxins after they are shelled and sized in commercial shelling plants. Therefore, most of the economic burden for aflatoxins is borne by the shelling companies. There is some evidence that afla-guard® suppresses other peanut fungal diseases such as white mold (*Sclerotium rolfsii*). An increase in peanut yield resulting from reduced disease incidence may provide incentive for peanut growers to utilize biocontrol.

David Kendra was asked about the chitosan used in his examination of chitinase production by *Gliocladium catenulatum*. He replied that chitosan is deacetylated chitin and that the amount of acetylation on the backbone of the molecule, an important feature in the activity of chitinase, can be regulated. Chitosan can be maintained at a high molecular weight with varying degrees of acetylation. Crab shell chitin, in contrast, contains impurities and requires considerable clean up.

The panel in general was asked whether biocontrol fungi induce resistance in crop plants. Sylvia Hua answered that the induction of resistance gene products after spraying corn silks with yeast might be detected through analysis of messenger RNA and protein products. Bruce Horn added that because crops are invaded by *A. flavus* at low water activities, phytoalexins which require high water activities would not be an important form of resistance. Joe Dorner also mentioned that infection levels remain

the same in untreated and treated crops and, therefore, an increase in host response would not be expected.

Mark Doster was asked whether cyclopiazonic acid (CPA) has ever been detected in pistachio nuts. He replied that CPA has not been detected but that the nuts have only recently been examined for this mycotoxin. Because CPA-producing fungi, such as *Aspergillus tamarii*, occur in pistachio nuts, low levels might be expected. In a follow-up question concerning the correlation between high aflatoxin contamination and low-yield years for pistachio nuts, Doster stated that naval orangeworm damage is more extensive during low-yield years, which may account for increased aflatoxin contamination.

Peter Cotty was asked whether populations of *A. flavus* S strain would rebound if applications of nonaflatoxigenic *A. flavus* were discontinued. He replied that fields require continuous treatment. Stable biocontrol populations cannot be maintained because of the large influx of toxigenic strains from surrounding fields. Since the S strain often comprises 70–80% of *A. flavus* populations in soils, this particular morphotype is well adapted to its environment. Another question posed for Cotty concerned the reasons why sorghum does not become highly contaminated with aflatoxins. Sorghum has a complicated mycobiota and the high incidence of Dematiaceous fungi may competitively inhibit invasion by aflatoxin-producing fungi. In areas of Africa where both sorghum and corn are planted, corn becomes highly contaminated with aflatoxins whereas sorghum contains low levels of the mycotoxin.

For the final topic of discussion by the panel, Joe Dorner was asked about the procedures for sampling peanuts for aflatoxins. In unshelled farmers' stock peanuts, a 2-kg sample is removed and examined only for visible A. flavus. These samples show extreme variability in aflatoxin levels when tested for experimental purposes. After shelling, peanuts are analyzed for aflatoxins by removing three 48-pound samples from each 22-ton lot. In the first sample, peanuts that test for aflatoxins at ≤8 ppb and >45 ppb are accepted and rejected, respectively, without further testing. If the aflatoxin concentration falls within this range, the second sample is analyzed. If the mean of first and second samples is ≤12 ppb, the peanut lot is accepted; if the mean is >23 ppb, the lot is rejected. Finally, if the mean aflatoxin level falls within this range, the third sample is analyzed and the mean of all three samples must be ≤15 ppb for acceptance. A follow-up question concerned the fate of peanut lots that have been rejected because of aflatoxin content. The sheller may have the seeds blanched and re-color sorted. Removal of the skins during blanching exposes discoloration in peanuts and as a consequence, color sorting becomes more effective. If the blanched peanuts still fail the aflatoxin analysis, the seeds are used for oil.

Influences of Crops and Geographic Features on Communities of Aflatoxin-producing Fungi

Ramon Jaime[1] and Peter J. Cotty[2]
[1]*Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ;* [2]*USDA-ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ*

Aflatoxins are produced by asexual fungi belonging to *Aspergillus* section *Flavi*. Soils in areas where contamination is common contain diverse communities of aflatoxin producing fungi. Communities of section *Flavi* differ by region in both species composition and aflatoxin producing potential. *A. flavus* can be divided into two morphotypes (the S and L strains) based on morphological, genetic, and physiologic criteria. The strain S produces numerous small sclerotia (average diameter <400 μm) and high levels of aflatoxins, while the strain L produces fewer, larger sclerotia (average diameter >400 μm) and variable levels of aflatoxin, ranging from none to high levels. The strain S of *A. flavus* has been reported as a natural soil inhabitant in several areas worldwide including Southeast Asia, South America and North America. Since cottonseed is a preferred feed for dairy cows and aflatoxin B_1 in feed is transferred to the milk in the slightly modified form aflatoxin M_1, dairies typically pay a premium for clean cottonseed. Thus, in areas where aflatoxin contamination is common, aflatoxin content is the most important factor determining seed value. Both *A. flavus* community structure and aflatoxin contamination present spatial variation. However, relationships of contamination to fungal community structure in soils have not been described. The objectives of the current study were to: 1) spatially analyze *A. flavus* communities in soils of South Texas; 2) evaluate influences of crop rotation on community structure of *A. flavus*; and 3) determine relationships of *A. flavus* community structure to soil texture.

The structure of *A. flavus* communities residing in soils of South Texas was determined by analyzing 326 soil samples from 152 fields located from the Rio Grande Valley in the south to Fort Bend county in the north in the springs of 2001 to 2003. The previous season crop was identified for most sampled fields. Soil samples were oven dried at 48 °C for 48 hours before processing. *A. flavus* was isolated from soil by dilution plating onto a modified Rose Bengal agar. *Aspergillus* section *Flavi* colonies were sub-cultured on 5/2 agar (5% V8 juice and 2% agar) for 5 to 7 days at 31 °C and assigned either to the *A. flavus* S or L strains, *A. tamarii* or *A. parasiticus* on the basis of colony characteristics and isolate morphology. Colony forming units (CFU/g), Percent of the S strain (Percent S), and percentages of clay, silt and sand were analyzed by Analysis of Variance and Variance Components Analysis using General Linear Models, Pearson's Correlation Analysis, and geostatistics.

Aspergillus flavus communities in soils of South Texas differ significantly among regions in both community density (CFU/g) and S strain (Percent S) incidence. Quantities of *A. flavus* were greater in areas of the Upper Coast than in areas of the Costal Bend. On the other hand, average S strain incidence was lower in areas of the Rio Grande Valley than in areas of either the Coastal Bend or Upper Coast. Small geographic scales (within and among fields) explained most of the variance for CFU/g (93.9%) and 68% of the variance for Percent S, while large geographic scales (among regions) only influenced Percent S explaining 24% of the variance. Most of the variation (75.9% to 90.7%) for the soil texture variables clay, silt and sand occurs among fields and among areas. Fields where the previous crop was corn had higher CFU/g (1,485) compared to either cotton (566) or sorghum (157). On the other hand, fields previously cropped to cotton had higher Percent S (28.6%) than those to corn (17.0%). Fields previously cropped to sorghum were intermediate between those cropped to cotton and corn. Pearson's correlation analyses showed significant positive correlations between percent of clay and both Percent S and CFU/g, and significant negative correlation between percent of sand and both Percent S and CFU/g. The results of this study demonstrate that both crop rotation and soil texture influence both population density of *A. flavus* and incidence of the S strain.

Aflatoxin Contamination of Maize in Africa

Claudia Probst[1], Henry Njapau[2], and Peter J. Cotty[1,3]
[1]*Division of Plant Pathology and Microbiology, The University of Arizona, Tucson, AZ;* [2]*Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD;* [3]*USDA-ARS, The University of Arizona, Tucson, AZ*

Aflatoxins are carcinogenic and teratogenic metabolites produced by several *Aspergillus* species, including *Aspergillus flavus* and *Aspergillus parasiticus,* during infection of a variety of crops either prior to or after harvest. Aflatoxin-producing fungi vary widely in many characteristics and both ability to infect and decay crops and aflatoxin-producing capacity differ among aflatoxin-producing fungi. Thus, the potential of these fungi to contaminate crops with aflatoxin also varies. Determining the most important causes of a contamination event requires considering both the aflatoxin-producing potential of the fungi present and the frequencies with which they occur in the contaminated crop. Maize (*Zea mays*) is highly susceptible to infection by aflatoxin-producing fungi. Such infections can lead to severe contamination with aflatoxin, resulting in diminished crop values and increased health risks for animals and humans. Negative health consequences for humans caused by ingestion of aflatoxin contaminated foods include impaired growth, cancer and death. Many countries have set maximum allowable levels for aflatoxins in order to limit health risk. However, these standards have little influence on ingestion of aflatoxins by most poor, small-scale farmers in Africa.

During January to June 2004, 317 cases of acute aflatoxicosis in Eastern and Central provinces of Kenya were identified, with a case-facility rate of 39%. The epidemic was caused by ingestion of maize with aflatoxin concentrations up to 4,400 ppb. Although aflatoxins have been associated with lethal food poisoning in Kenya three times since 1981, the fungi contaminating the maize with aflatoxins have not been characterized. We analyzed 103 maize samples collected during the 2004 outbreak from the most affected districts in order to identify the most important aflatoxin-producing fungi. A total of 1,223 *Aspergillus* Section *Flavi* isolates were recovered from the maize and characterized. Over 97% of the Section *Flavi* isolates were *A. flavus* and the reminder (2.3%) were *A. parasiticus*. No other aflatoxin-producing fungi were detected. *A. flavus* can be delineated into two morphotypes, the S and L strains, which differ in fruiting habit and aflatoxin-producing ability. The majority (73%) of the *A. flavus* isolates belonged to the S strain, which was not previously known in Africa. S strain isolates produced much greater quantities of aflatoxins than the L strain isolates (665 μg aflatoxin B_1/g mycelium, n=117; versus 40 μg aflatoxin B_1/g mycelium, n=30). Of the fungi examined, only *A. parasiticus* produced G aflatoxins. Both the S and L strain *A. flavus* produced only B aflatoxins. Incidence of the S strain increased with average aflatoxin content from 69% in samples with <20 ppb total aflatoxins to 94% in samples with >1,000 ppb total aflatoxin. Indeed, the S strain occurred in the poisonous maize at higher proportions than previously observed on any crop from any location, worldwide. The distinct ecology of the S strain should be taken into account during development of methods to prevent future aflatoxicoses in Kenya. This causal agent should be the target of long-term preventative measures.

Influences of Herbicides on Release of Atoxigenic Strains

Nicholas P. Garber[1] and Peter J. Cotty[2]
[1]*Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ;* [2]*USDA–ARS, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ*

Abstract not submitted.

Screening of Atoxigenic *Aspergillus flavus* Isolates for Ability to Inhibit Aflatoxin B$_1$ Production by Toxigenic *Aspergillus flavus*

A. Jha, R. Sweany and K.E. Damann
Dept. of Plant Pathology & Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA

Biological control of aflatoxigenic *Aspergillus flavus* using atoxigenic isolates of the same fungus has been demonstrated in cotton and peanuts. In order to select potential biocontrol isolates for corn, a collection of 41 atoxigenic isolates of *A. flavus* were individually evaluated for ability to inhibit aflatoxin B$_1$ production by a single toxigenic isolate in a suspended disc assay. Eight isolates completely inhibited aflatoxin production whereas 4 others were highly inhibitory. These selected isolates will be applied to corn in a field test to determine their ability to prevent aflatoxin contamination.

18ᵀᴴ ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 3: CROP RESISTANCE — GENETIC ENGINEERING

Moderator: Keerti Rathore, Texas A&M University

Gene-based Antifungal Strategies in Peanut

Ye (Juliet) Chu[1], Paola Faustinelli[1], Laura Ramos[1], Kanniah Rajasekaran[2], Jeff Cary[2], Corley Holbrook[3], Peggy Ozias-Akins[1]

[1]*Department of Horticulture, University of Georgia Tifton Campus, Tifton, GA;* [2]*USDA-ARS, SRRC, New Orleans, LA;* [3]*USDA-ARS, Coastal Plain Experiment Station, Tifton, GA*

Genetic mechanisms leading to antifungal strategies for aflatoxin reduction in peanut are being investigated. These include genetic engineering as well as mutation breeding approaches. Peanut genetic engineering is relatively inefficient compared with many other crops, testing for aflatoxin reduction in the field takes several years, and acceptance of transgenic crops is tenuous in some parts of the world. Therefore, in addition to antifungal strategies based on genetic engineering, we are placing increasing emphasis on specific gene mutations that could affect resistance traits. Three objectives have been the focus of our research over the past year. The first has been to characterize transgenic peanut containing an anti-apoptotic gene from human, Bcl-xl. The second has been to explore the use of a mutant population generated for discovery of gene function for aflatoxin reduction goals. The third has been to test allergen gene promoter ability to drive expression of reporter genes.

The putative antifungal gene Bcl-xl encodes for an anti-apoptotic protein from humans. Bcl-xl has been transformed into tobacco and was shown to confer broad-spectrum fungal pathogen resistance as well as resistance to tomato spotted wilt virus (Dickman et al., PNAS 2001; 98: 6957). After microprojectile bombardment of embryogenic cultures, we recovered 42 independent hygromycin-resistant lines of peanut from which 309 plants were regenerated. Almost 90% (269) were PCR positive for Bcl-xl. Several lines have been tested by western blotting for protein expression and by RT-PCR for RNA expression. Plants that are PCR positive for presence of the gene also have been RT-PCR positive indicating that expression at the RNA level was occurring. Fewer lines have shown detectable protein expression either as a result of low expression levels or low sensitivity of the western. One line that produced progeny and was segregating for the transgene was examined in more detail. It has been speculated that Bcl-xl can function as a stress-related protein; therefore, we have tested the tolerance of transformed plants to paraquat as a quick assay for protein activity. One progeny plant that consistently showed expression of Bcl-xl on western blots also consistently showed a higher level of tolerance to paraquat than either the background genotype, Georgia Green, or the progeny that no longer contained the transgene. Other plants that showed expression according to RT-PCR results, but inconsistent western blot results, were either intermediate in tolerance to paraquat or similar in response to the non-transgenic control. It is possible that a threshold level of expression is required for efficacious alleviation of stress due to herbicide treatment.

For the second objective of mutation breeding, the goal is to produce a TILLING population that can be screened for lipoxygenase mutants or any other gene suspected to enhance aflatoxin production. TILLING stands for Targeting Induced Local Lesions IN Genomes (McCallum et al., Plant Physiol 2000; 123: 439), is a mutation strategy, and was first tested in *Arabidopsis*. TILLING can identify mutants based on screening with gene sequence rather than for phenotype. Leaf samples have been collected from ~1500 M2 plants mutagenized with ethylmethane sulfonate and DNA has been extracted from 384 of these to use for TILLING. The TILLING technique is being tested with an allergen gene, *ara h 2*, for which we have generated sufficient genomic sequence for this purpose. Two copies of the *ara h 2* gene are present in peanut, one from the A genome and one from the B genome. Gene-specific primer sets have been designed for TILLING so that mutations in each copy of *ara h 2* can be screened separately.

Characterization of allergen gene sequence has allowed the isolation of promoters that may be useful for antifungal gene expression, particularly when expression is to be targeted to the developing seed. The allergen promoter sequences have been fused with a reporter gene (β-glucuronidase or GUS) and tested for transient expression after bombardment of immature cotyledons. GUS expression was observed with both a 1 kb and 2 kb upstream fragment from *ara h 2*. The 2 kb fragment contains a methyl jasmonate response element; therefore, the effect of this signaling molecule on expression of the gene is being studied.

Transgenic Peanuts with Enhanced Resistance to *Aspergillus flavus*

Arthur K. Weissinger
Department of Crop Science, North Carolina State University, Raleigh, NC

Abstract not available. Please see page 16 of the introduction for a summary.

Identification, Characterization and Antifungal Activities of Silk Proteins in *Aspergillus flavus* Resistant and Susceptible Corn Inbreds

Bela Peethambaran[1], Gary L. Windham[2], Leigh Hawkins[2], Paul Williams[2] and Dawn S. Luthe[1]

[1]*Biochemistry and Molecular Biology Department, Mississippi State University, MS;* [2]*USDA–ARS, Corn Host Plant Resistance Unit, MS*

Research in our laboratory is focused on eliminating aflatoxin contamination in maize (*Zea mays* L.) by increasing resistance to *Aspergillus flavus* infection during ear development. Because it has been postulated that the fungus enters the ear via the silks, we are investigating the proteome of silk proteins in maize inbreds that are resistant or susceptible to aflatoxin contamination and /or *A. flavus* infection. We hope to identify proteins that directly contribute to the resistance phenotype or proteins/genes that can be used for marker-assisted selection in breeding programs. Control silks were collected from Mp313E, Mp420 (resistant), Tx601 (intermediate resistance) and Sc212M, Mp339 (susceptible) 21 and 25 days after silk emergence (DAS). Infested ears were inoculated with *A. flavus* at 15 (DAS) and were collected 21 DAS and 25 DAS. Silk proteins were extracted and analyzed by 2-dimensional gel electrophoresis (2-DE). Gel images were analyzed by PD Quest software (BioRad) and comparison were made among inbred and between inoculated and uninoculated samples. MALDI-TOF mass spectroscopy and LC/MS/MS were used to identify common silk proteins and those that consistently differed among resistant and susceptible lines, or inoculated and uninoculated ears. Selected candidate genes sequences were investigated for polymorphism and its RNA expression was also studied. Agar plate assays using GFP-tagged *A. flavus* were used to study the resistance potential of proteins extracted from the resistant and susceptible genotypes. The growth of *A. flavus* in the presence of the silk protein extracts was determined by measuring GFP fluorescence and ergosterol content.

Silencing the Expression of RAP Genes in Maize and the Effect on Host Resistance against *Aspergillus flavus* Infection and Aflatoxin Production

Zhi-Yuan Chen[1], Robert L. Brown[2], Thomas E. Cleveland[2], and Kenneth E Damann[1]

[1]*Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA;* [2]*Southern Regional Research Center, USDA-ARS, New Orleans, LA*

Aflatoxins are carcinogens produced mainly by *Aspergillus flavus* during infection of susceptible crops such as maize (*Zea mays* L.). Although resistant maize genotypes have been identified, the incorporation of resistance into commercial lines has been slow due to the lack of selectable markers. Recently, resistance-associated proteins (RAPs) have been identified by comparing constitutive protein profiles between resistant and susceptible maize genotypes using proteomics. Preliminary characterization of some of these RAPs suggest that they play a direct role in host resistance, such as pathogenesis-related protein 10 (PR-10), or an indirect role, such as glyoxalase I, through enhancing the host stress tolerance. However, direct evidence for their involvement in kernel resistance were lacking.

In the present study, an RNA interference (RNAi) gene silencing technique was used to silence the expression of these genes. RNAi silencing is a posttranscriptional, sequence-specific RNA degradation process. It is triggered by a double stranded (ds) RNA, leading to the degradation of homologous RNA encoded by endogenous genes, and transgenes. A binary vector containing all the key elements needed to generate a dsRNA structure was constructed using Gateway technology. Two inverted repeats of parts of the coding region of *glx-I* and *pr-10* were integrated into the vector through site-specific recombination. The resulting constructs (GLX-I RNAi vector and PR-10 RNAi vector) were then transformed into immature maize embryos using both bombardment and *Agrobacterium* infection.

Thirty two out of 38 and 11 of 15 callus clones of, *glx-I* and *pr-10*, respectively, representing independent transformation events were confirmed to be positive for transformation through PCR. The extent of gene silencing in transgenic callus tissues varies from one to another ranging from 20% to over 99%, and depends on the RNAi constructs based on real-time PCR. It appears that callus clones generated from PR-10 RNAi vector had more dramatic interference in the expression (with an average of over 90% silencing) than that observed in callus clones regenerated from GLX-I RNAi vector (with an average of 50% silencing). The RNAi silenced transgenic maize seeds have also been obtained from plants regenerated from *Agrobacterium* transformed callus clones. The number of kernels per ear also varies significantly, from as few as 2 to as many as 196. For each construct, kernels from 8 ears were germinated and genomic DNA was isolated. PCR confirmation of transformation using seedling genomic DNAs found that only two out of 8 and one out of 8 were negative, for GLX-I and PR-10, respectively. Kernel screening assay of the transgenic maize kernels demonstrated a significant increase in susceptibility to *A. flavus* colonization and aflatoxin production in some of silenced transgenic lines compared with non-silenced control kernels, suggesting their direct involvement in aflatoxin resistance in maize.

Genetic Engineering of Cotton for Resistance to Phytopathogens including *Aspergillus flavus*

Kanniah Rajasekaran[1], Mauricio Ulloa[2], Bob Hutmacher[3], Jeff Cary[1], Jesse M. Jaynes[4] and Thomas Cleveland[1]

[1]*USDA, ARS, SRRC, New Orleans, LA;* [2]*USDA-ARS, Western Integrated Cropping Systems Research Unit, Cotton Enhancement Program, Shafter, CA;* [3]*UC Davis, CA;* [4]*Tuskegee University, Tuskegee, AL*

Fertile, transgenic cotton plants expressing the synthetic antimicrobial peptide, D4E1, were produced through *Agrobacterium*-mediated transformation (Rajasekaran et al. 2005). PCR products and Southern blots confirmed integration of the D4E1 gene, while RT-PCR of cotton RNA confirmed the presence of D4E1 transcripts. *In vitro* assays with crude leaf protein extracts from T0 and T1 plants confirmed that D4E1 was expressed at sufficient levels to inhibit the growth of *Fusarium verticillioides* and *Verticillium dahliae* compared to extracts from negative control plants transformed with pBI-d35SΩ-*uid*A-nos (CGUS). Although *in vitro* assays did not show control of pre-germinated spores of *Aspergillus flavus*, bioassays with cotton seeds *in situ* or *in planta*, inoculated with a GFP-expressing *A. flavus*, indicated that the transgenic cotton seeds inhibited extensive colonization and spread by the fungus in cotyledons and seed coats. *In planta* assays with the fungal pathogen, *Thielaviopsis basicola,* which causes black root rot in cotton, showed typical symptoms such as black discoloration and constriction on hypocotyls, reduced branching of roots in CGUS negative control T1 seedlings, while transgenic T1 seedlings showed a significant reduction in disease symptoms and increased seedling fresh weight, demonstrating tolerance to the fungal pathogen. Field evaluation of T2 progeny for Fusarium wilt (*Fusarium oxysporum* f.sp. *vasinfectum* (FOV) Atk. Sny & Hans) race 1 was carried out in sandy soil that also exhibited presence of root-knot nematodes (*Meloidogyne incognita*). R2 progenies of four independent transformation events expressing the antifungal peptide D4E1, a transgenic control entry with the GUS marker gene and the original non-transgenic variety (Coker 312), along with commercial Acala (*G. hirsutum*) and Pima (*G. barbadense*) cultivars were included in the field evaluation. Entries were planted in a randomized complete block design with four replications on 10 feet long plots. Plant survival rate, foliage damage symptoms, vascular root staining, presence of root-knot, and agronomic data have been collected for these entries. Preliminary observations indicated that the transgenic entries showed a healthy, higher germination stand (up to 68%) than the controls (43%). We hope to complete the initial field evaluation this year (2005) and the promising lines will be re-tested for pathogen resistance including pre-harvest-resistance to *Aspergillus flavus*.

Rajasekaran K, Cary JW, Jaynes JM, Cleveland TE. Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants. Plant Biotechnology Journal 2005; 3: 545–554.

PANEL DISCUSSION: Crop Resistance — Genetic Engineering

Panel Chair: Arthur Weissinger

Panel Members: Z-Y Chen, P. Ozias-Akins, B. Peethambaran, K. Rajasekaran

Panel summary not submitted due to illness of the panel chair. Please see page 16 of the introduction for an overview of the session's presentations.

18ᵀᴴ ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 4: CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

Moderator: Pat O'Leary, Cotton Incorporated

Update on Validation and Distribution of a Computer Program for Predicting Mycotoxins in Midwest Corn

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

Since the last report, the predictive computer program as been further validated in 2003 and 2004. A correlation coefficient of 0.85, after one outlier was removed, was obtained for commercial field samples from 2000–2003 (which included a few dozen different fields and hybrids). Samples were predicted to have low levels of fumonisin in 2004 (less than 1 ppm) and this is what occurred in all fields sampled. No aflatoxin was predicted to occur in either 2003 or 2004, and none was found. In 2005, the program predicted a high probability that *A. flavus* inoculum would be present at silking, which was communicated to farmers present at a field day in late June. Continued predictions indicated low levels (from 30 to less than 20 ppb), depending on weather conditions at different locations. Insect levels were very low throughout most of the season. Ears in dry areas were monitored by both PFD and popcorn company representatives, and also brought in to elevators for aflatoxin determinations by farmers. Kernels with BGYF, and in one case, visible *A. flavus* colonization, were encountered. A meeting with elevator operators indicated some samples were testing positive for aflatoxin at 20 ppb from several different areas, and scattered rejected loads occurred through late September. Samples were taken in fields under study, and results of analyses are pending. As indicated previously, cooperation between USDA and Illinois Central College resulted in a Windows version with an added Help section, and a "custom" module that allows one to customize if outlier hybrids are encountered provided prior years' data, including mycotoxin levels, are available. A "beta" version has been demonstrated via network conferences to different companies (contact Kate O'Hara, Technology Information Officer, to arrange a demonstration: kate@ncaur.usda.gov). An additional economic decision-making module is partly written. Additional plans are to validate the program for popcorn, which is widely grown in Central Illinois (samples were taken in 2005) and other food-grade corn as information becomes available. A finalized version of the initial program may be put on a website within the next year or two, but commercial interest may alter this schedule. Overall, hybrids and cultural management presently used by farmers, coupled with more recently identified factors, such as use of Bt versions of preferred hybrids, early planting to escape caterpillar damage in milk stage, scouting for damaging insects including use of lures and traps identified in project research, fungal monitoring using leaf axil material, and the predictive computer program indicating when mycotoxin-producing fungi occur, or specific mycotoxin levels that may occur without intervention by the farmer, could be rationally combined into a management plan.

Mechanisms of Preharvest Aflatoxin Contamination in Peanut Infected by Root-Knot Nematodes

Patricia Timper[1,] Corley Holbrook[1], and Dave Wilson[2]
[1]USDA ARS, Tifton, GA; [2]Department of Plant Pathology, University of Georgia, Tifton GA

Infection of peanut by root-knot nematodes (*Meloidogyne arenaria*) can lead to an increase in aflatoxin contamination of kernels when the plants are subjected to drought stress during pod maturation. It is not clear whether the increased aflatoxin contamination is primarily due to greater invasion of the galled pods by toxigenic *Aspergillus* spp. or whether root galling is also involved. Nematode damage to the pods and/or roots may also delay pod maturity. Small, immature peanuts are more prone to aflatoxin contamination than are mature, undamaged peanuts. Our objectives were: 1) to determine the contribution of root and pod galling caused by root-knot nematodes to the increase in aflatoxin contamination, and 2) whether nematode infection increases the percentage of immature peanuts.

A greenhouse experiment was conducted in which pods and roots were physically separated. Pod set was restricted to soil-filled pans (41 cm dia. × 10 cm depth), while the roots grew underneath the pan into a pot. Root-knot nematodes (RKN) were applied to the root zone of half the plants, the other plants did not receive nematodes in the root zone. Plants received three pod treatments after pod set: one application, two applications, and no application of RKN. The treatments were arranged in a completely randomized design with 12 replicates/treatment. Conidia of *Aspergillus flavus* and *A. parasiticus* were added to each pan when the plants started to flower. Plants were subjected to drought stress 40 days before harvest. The results were similar among the two trials of the experiment so the data was combined for analysis. Adding nematodes to the pod zone had no effect on aflatoxin concentrations in the peanut kernel. However, the lack of an effect may have been to due to the low occurrence of galling on the peanut hulls. In pots where nematodes were added to the root zone, 50 to 80% of the root system was galled. Adding nematodes to the root zone increased (P = 0.003) aflatoxin concentrations in the peanut kernels from 37 ppb in the control to 67 ppb.

A field microplot study was conducted in 2003 and 2004 to determine whether infection of peanut by RKN increases the percentage of immature kernels. Half of the 12 plots were inoculated with nematodes at two different times (at plant and after pegging) and the other half were not inoculated with nematodes. All plots were inoculated with *A. flavus*/*A. parasiticus*. Drought was induced 5 to 6 weeks before digging. In both 2003 and 2004, the presence of nematodes did not increase aflatoxin concentrations in the peanuts. In 2003, plants infected with RKN produced a greater (P = 0.0001) percentage of immature kernels than uninfected plants; however, in 2004 nematodes had no effect on the percentage of immature kernels even though root galling and yield reductions were greater in 2004 than in 2003.

In summary, infection of peanut roots by the peanut root-knot nematode increases aflatoxin contamination of the kernels. Nematode damage to the roots results in greater drought stress which may result in greater susceptibility to aflatoxin contamination. The contribution of pod galling and immature kernels on the increase in aflatoxin levels in nematode-infected peanut are still unclear.

Experimental Use of the Pear Ester Kairomone to Improve Codling Moth Control in Walnuts

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

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

Various control tactics are being investigated using the pear-ester kairomone to directly manage both adult and larval CM in walnut orchards. The primary control tactics being researched are using the kairomone to augment mating disruption of male moths and "attract and kill" "bait-sprays" targeting neonate larvae. Due to the implementation of the Food Quality Protection Act of 1996 the most effective and inexpensive insecticides for codling moth control, the organophosphate (OP) insecticides, will in the very near future be highly restricted or completely banned from use. Also, the current alternative control materials, both insect growth regulating (IGR) and biological-viral insecticides and pheromone mating disruptants, and their required application rates are much higher or prohibitive in cost. Thus, both insecticidal and pheromone mating disruption alternative strategies must be made more effective, affordable, and acceptable for control use. Our goal and hypothesis is that the pear-ester kairomone will act to improve the control efficacy and diminish the amount of insecticide and pheromone disruptant required to control damaging populations of CM in walnut orchards. This is based on our prior reported research that mating disruption be more effective using the combination of pheromone and kairomone over the current pheromone-alone tactic. Also, neonate CM larvae are highly attracted to the kairomone, thus bait-sprays of kairomone+insecticide might attract - kill target larvae more effectively.

Trécé, Inc. has developed a micro-encapsulated (MEC) sprayable formulation of the kairomone. The sprayable MEC-kairomone was tank-mixed as an adjuvant with reduced rates of insecticides and applied as a full-coverage spray by handgun-sprayers. Four insecticides tested were: two OPs choropyrifos and phosmet, an IGR methoxyfenozide and a CM granulosis virus. Bait-spray trials were conducted in a 20 acre walnut orchard, using a completely randomized block design of eight single-tree replicates per treatment. Treatments were the insecticides alone compared with treatments of insecticide+MEC adjuvant. Six application sprays were applied (June−mid-September). Controls were 20 random picked un-sprayed trees. Occurrence and degree of nut damage was evaluated by nut drop, pre-harvest canopy infestation, and harvest nut knock-down sampling. Results were very encouraging, with the MEC-kairomone adjuvant reducing CM damage by 83% and 90% for the OP insecticides and 54% and 47% for the IGR and viral insecticides. Navel orangeworm damage was also significantly reduced.

These studies show promise that the kairomone can improve insecticide efficacy and contribute to new IPM tactics for CM and NOW and the integrated reduction of aflatoxin incidence.

Liberty Link and Urea on Aflatoxin and Fumonisin Levels in Corn

H. Arnold Bruns and H. K. Abbas

USDA-ARS-MSA, Crop Genetics and Production Research Unit, Stoneville, MS

An experiment was done to ascertain if Liberty herbicide {glufosinate-ammonium [2-amino-4-(hydro xymethylphosphinyl)-ammonium salt]} or urea $[CO(NH_2)_2]$ would reduce fungal growth of *Aspergillus flavus* Link ex Fries and *Fusarium verticillioides* (Sacc.) Nirenberg (synonym = *F. moniliforme* J. Sheld.), and thus their respective mycotoxins, aflatoxin and fumonisin, in pre-harvest corn (*Zea mays* L.). Four corn hybrids, two genetically modified to be resistant to Liberty herbicide and two non-genetically modified, were planted at Stoneville, MS in 2001, 2002, 2003, and 2004 in a randomized complete block with a split-plot arrangement of treatments replicated four times. The experiment was furrow irrigated. Individual plots were two rows 9 m long, spaced 102 cm apart, and included one of the following treatments: 1) untreated non-inoculated check; 2) untreated inoculated check; 3) 0.23% v:v Liberty:water; 4) 1.13% v:v Liberty:water; and 5) 0.075 molar solution of urea. Twenty ears selected at random in each sub-plot were inoculated with a pin bar, using a culture of F3W4 *A. flavus*. *Fusarium verticillioides* was allowed to infect naturally. Among years, inoculated ears averaged 153.6 mg/Mg to 257.3 mg/Mg more aflatoxin than non-inoculated ears. Neither Liberty nor urea reduced aflatoxin or fumonisin contamination. Hybrids did not differ in yield or aflatoxin contamination but one brand had less fumonisin (3.7 mg/kg and 2.3 mg/kg) than the other (7.5 mg/kg and 6.9 mg/kg). Grain yields were less in 2004 (6.9 Mg/ha) than 2001 (8.8 Mg/ha) or 2002 (9.0 Mg/ha).

PANEL DISCUSSION: Crop Management and Handling, Insect Control and Fungal Relationships

Panel Chair: Pat Dowd

Panel Members: Patricia Timper, Douglas Light, and Arnold Bruns

Panelists described various studies. Pat Dowd indicated that the mycotoxin prediction program developed for Midwest corn had done well with fumonisins from 2000–2004, and predicted the aflatoxin which occurred in central Illinois in 2005. Patty Timper indicated higher levels of aflatoxin when nematodes were added to the root zone, but effects on pod galling varied. Doug Light reported that the pear ester had been microencapsulated and when combined with reduced rates of different insecticides, often provided significantly better rates of control than standard rates of corresponding insecticides alone. Arnold Bruns indicated a multiyear study where Liberty herbicide was applied to corn ears at black layer did not result in any significant reductions of aflatoxins.

In response to questions, Pat Dowd indicated that the program was designed for Midwest corn, but may have application for other areas, depending on how similar hybrids were (no one from seed companies provided further insight). He indicated that the aflatoxin predictions had not been communicated widely because upper ARS management was concerned about the limited area the program had been validated and potential effects on the market. He also indicated that a group was interested in helping with validation over a wider area.

In response to questions, Patty Timper indicated that the root and pod zones were watered separately, so the pod zone could remain under drought while the root zone could be watered to keep the plants alive. She also indicated that the mechanism of nematode involvement in tall fescue toxic endophytes was not clear yet.

In response to questions, Doug Light indicated that the microencapsulated granules were effective for 2–3 weeks, and the twist on attractants were effective for a month.

Anthocyanins from Petunia Floral Structures that Inhibit Corn Earworm Development

Eric T. Johnson[1], Patrick F. Dowd[1], Mark A. Berhow[2]
[1]Crop BioProtection Research Unit, USDA-ARS, National Center for Agricultural Utilization Research, Peoria IL; [2]New Crops and Processing Research Unit, USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL

Our previous studies identified a series of anthocyanins other than cyanidin (which is found in corn) with significant activity against corn earworms. We used three different lines of petunia that had alternating colored and blue/purple sectors (subsequently found to be due to a combination of malvidin and petunidin glucosides) to examine the effects of anthocyanins in an intact plant system. Larvae typically produced less damage on the colored vs. white sectors of the petunia flowers, and larvae that fed on the colored sectors typically weighed significantly less that those feeding on the white sectors. A combination of petunidin and malvidin glucosides used in a low protein diet (to simulate the nutritional content of the flowers) at an approximate natural concentration of 1000 and 2000 ppm respectively, also produced larvae that were significantly smaller than those feeding on solvent control diet.

Ground-Based Remote Sensing for Rapid Selection of Drought and Aflatoxin Resistant Peanut Genotypes

D.G. Sullivan[1] and C.C. Holbrook[2]

[1]*USDA-ARS Southeast Watershed Research Laboratory, Tifton, GA;* [2]*USDA-ARS Crop Genetics and Breeding Research Unit, Tifton, GA*

In the Southeastern U.S., peanut producers are challenged by long growing seasons and periodic drought. The continued development of drought and aflatoxin resistant peanut *Arachis hypogaea* L.) cultivars is essential to maintain productivity under less than ideal growing conditions. Remote sensing of canopy reflectance is a well-established method of evaluating crop condition, and thus shows promise as a new technique for the rapid selection of drought and aflatoxin resistant peanut genotypes. The objective of this study was to evaluate ground based reflectance measurements to more accurately quantify small differences in genotype response to drought conditions. In April 2004 several small plots (2 m × 2 m) were established at the Gibbs Farm research facilities in Tifton, GA. Treatments consisted five peanut genotypes encompassing a range of drought tolerance and yield characteristics arranged in a completely randomized block design. Drought conditions were simulated beginning 90 days after planting and maintained through harvest. Once drought conditions were established, a handheld radiometer was used to acquire twice weekly reflectance measurements in the visible and near infrared regions of the spectrum. Coincident with remotely sensed data collection standard visual ratings and soil water content (0–15cm) were acquired. Seasonal measurements included aflatoxin and yield measurements. Our data indicate that remotely sensed data provide more specific and timely estimates of genotype response to drought, and could be used to enhance breeding progress of drought and aflatoxin resistant peanut varieties.

Correlations Between Biotic Stresses and Aflatoxin Contamination in Maize

Matthew Krakowsky[1], Xinzhi Ni[1], and Richard Davis[2]
[1]*USDA-ARS, Crop Genetics and Breeding Research Unit, Tifton, GA;* [2]*USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA*

Aflatoxin, a toxin produced by the fungus *Aspergillus flavus*, is the most potent carcinogen found in nature. Aflatoxin contamination of maize is a chronic problem in the southern US, where high temperatures, water stress, and insect damage produce conditions conducive to infection of maize by *A. flavus*. The purpose of this research was to determine the relationship between two biotic stresses, leaf feeding by the fall armyworm (FAW), *Spodoptera frugiperda*, and root feeding by the root-knot nematode (RKN), Meloidogyne incognita, and contamination of grain with aflatoxin. In the first experiment, five hybrids (four commercial and one aflatoxin resistant) were grown in a split-plot design with whole plots representing FAW artificially-infested or non-infested conditions and split-plots representing a hybrid. FAW damage was evaluated at seven and fourteen days after infestation. In the second experiment, three commercial hybrids were grown in a randomized complete-block design in a field with high population densities of RKN. A fumigant nematicide was used to create plots with minimal nematode damage to compare to non-fumigated plots with a high level of nematode damage. Early (pre-plant), mid, and late (at harvest) season nematode population levels were estimated based on soil samples. Correlations between plant damage, plant stress, and yield and aflatoxin contamination can be used to evaluate the significance of particular biotic stresses on aflatoxin contamination of maize and determine the focus of genetic improvement and crop management programs.

18TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

SESSION 5: DETECTION, EXTRACTION, AND ANALYSIS OF AFLATOXINS; POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS

Moderator: Tom Wedegaertner, Cotton Incorporated

Distribution of Aflatoxin in Non-irrigated Peanuts

Thomas F. Schatzki and Martin S. Ong
Western Regional Research Center, Agricultural Research Service, USDA, Albany, CA

The B_1 and total aflatoxin distribution in Georgia florunner peanuts has been measured. Sample distributions were measured in approximately 400 small samples each of Jumbo, Medium and Small Oil Stock (OS) sublots, containing 20, 10, and 5 kernels, respectively. Results were converted to single kernel probability density (SKD) distributions, *f(ln c)*, using methods previously published (J Agr Food Chem 1995; 43: 1561–1565). This constitutes the first direct small sample experimental establishment of *f(ln c)* in peanuts. All three sublots show evidence in the SKD of peaks at about $c = 10^5$, $5*10^3$, and a partial peak at $c < 5*10^2$ ng/g. The first and last of these are similar to peaks seen in tree nuts. The experimental distributions were also fitted to the 2-parameter negative binomial distribution (NBD), first suggested for peanuts by Whitaker and Wiser (J Amer Oil Chem Soc 1969; 46: 377–379). The NBD follows the *c*-average of the experimental data quite well, but does not represent any of the peaks and valleys. Thus it can not be used to deduce information of nut physiology or growing conditions, which were so successfully deduced for tree nuts by non-parametric methods. The NBD might suffice to estimate sample mean and variance at sample sizes drastically different from the sample sizes measured, but this would require measurement of samples of varying sizes from the same lot. Such data is presently not available. Comparison of the results obtained here with those obtained by Whitaker et al. (J AOAC Int 1994; 77: 659–666) on a large set of similar lots of Georgia florunners but with much larger sample size (2000 to 6000 pods). When the latter results were reduced by use of the NBD to the same size as measured here, a somewhat degraded, again peak-less, fit was obtained. This degradation was almost certainly due to the sample variance which was 30–700% larger in the Whitaker case. These results suggest, but do not prove, that the NBD is capable of spanning a very large sample range. Proof would require large and small samples from the same lot. Both the present and the Whitaker data show a cut-off of contamination occurring slightly above $5*10^5$ ng/g noted in all tree nuts previously measured. This is the first test of the NBD to small samples.

Inhibition of *Aspergillus flavus* Aflatoxin Biosynthesis by Antioxidant Phytochemicals Occurring in Tree Nuts

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

Walnuts in general and the cultivar 'Tulare' in particular have been shown to be exceptionally resistant to aflatoxigenesis. The resistance factors, located solely in the seed coat or pellicle but not in the kernel, have been shown to be complex hydrolysable tannins. These tannins consist of a glucose core, esterified by gallic and hexahydroxydiphenic acid moieties. *Aspergillus flavus* possess a tannase capable of hydrolyzing the tannins into their component parts. The phenolic moities, gallic acid and ellagic acid (derived from hexahydroxydiphenic acid by spontaneous lactonization) also exhibit antiaflatoxigenic activity but are less potent than the parent tannins. It has been postulated that aflatoxigenesis is a consequence of oxidative stress on the fungus and that compounds capable of relieving oxidative stress should be capable of reducing or eliminating aflatoxin production. This hypothesis has been tested by using *Saccharomyces cerevisiae* as a model fungal system to examine functional genomics of oxidative stress responses. Singular gene deletion mutants of *S. cerevisiae*, exhibited alleviation of oxidative stress induced by treatment with peroxide when tannic, gallic or caffeic acids were present.

In order to extend this finding to *A. flavus* and to elucidate structure-activity relationships, a number of antioxidant phenolic compounds known to occur in tree nuts were tested *in vitro* for their ability to inhibit aflatoxin production in the presence and absence of peroxide-induced oxidative stress. Compounds tested were: the hydrolysable tannins, pentagalloyl glucose and 3,5-digalloyl quinic acid, together with quinic acid itself; the flavonoid, catechin; and caffeic, chlorogenic, ellagic, 4-hydroxybenzoic and 3,4-dihydroxybenzoic (protocatechuic), gallic and vanillic acids. As a model for the anacardic acids present in pistachio hulls, the commercial antioxidant, lauryl gallate, was also tested.

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

Ryann M. Muir[1], Elizabeth Ingham[1], Sandra Uratsu[1], Gale McGranahan[1], Charles Leslie[1], Noreen Mahoney[2], and Abhaya Dandekar[1]

[1]*Department of Pomology, University of California, Davis, CA;* [2]*USDA-ARS, Western Regional Research Center, Albany, CA*

Abstract not submitted.

Inhibition of Aflatoxin Production by Compounds in Corn Seeds

G.A. Payne[1], R.A. Holmes[2], and R.S. Boston[2]
[1]*Department of Plant Pathology North Carolina State University, Raleigh, NC;* [2]*Department of Botany, North Carolina State University, Raleigh, NC*

We have identified compounds present in kernels of the resistant maize inbred Tex6 that influence aflatoxin (AF) biosynthesis and fungal growth in bioassays. In earlier studies a growth inhibitor was purified and determined to be a chitinase (Moore *et al.*, Phytopathology 2004; 94: 82–87). Additional characterization of kernel seed extracts has revealed the presence of two non-proteinaceous compounds that influence AF biosynthesis and growth: Aflatoxin Biosynthesis Inhibitor-1 (ABI-1) and ABI-2. *A. flavus* cultures grown in the presence of ABI-1 exhibit reduced mycelial mat formation, total biomass and AF biosynthesis. Initial biochemical characterization of ABI-1 indicates that it is a non-proteinaceous, heat labile small molecule. Relative to ABI-1, ABI-2 has less influence on fungal growth and a more pronounced effect on AF biosynthesis. ABI-2 is a heat stable, non-proteinaceous compound. Several lines of evidence suggest that ABI-2 belongs to the inositol polyphosphate class of molecules. This is not surprising as maize seeds store high levels of inositol hexakisphosphate (phytic acid) and other inositol polyphosphate precursors. LC/MS analysis of ABI-2 containing fractions revealed enrichment in inositol polyphosphates. Furthermore low phytic acid 1-1 mutant maize kernels have reduced ABI-2 activity and treatment of kernel extracts with phytase (a phosphatase) enhances ABI-2 activity. However, pure phytic acid had no inhibitory effect, suggesting that ABI-2 may be a biosynthetic precursor of phytic acid. Preliminary real time RT-PCR measurements of AF biosynthetic and regulatory gene transcription suggest that ABI-1 and ABI-2 both suppress transcription of pathway genes, but act differently on other regulatory genes. These observations show that seeds of host plants contain compounds that target gene transcription in *A. flavus*.

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

Panel Chair: Russell Molyneux

Panel Members: Gary Payne, Thomas Schatzki

Summary of Panel Discussion: Gary Payne was asked about the nature of the two compounds isolated from corn seeds that inhibit aflatoxin production. Previous work had indicated that these could be proteins. However, based on mass spectrometric evidence, the molecular weight of one was estimated to be 695, even though size exclusion chromatography would suggest a much higher molecular weight compound. The odd-numbered molecular weight would suggest a nitrogen-containing compound and the chromatographic behavior could be explained by structural features that promote bonding to the column matrix.

Russell Molyneux was asked about the rationale for antioxidant compounds present in walnuts to suppress aflatoxin biosynthesis. If aflatoxins are produced in the fungus in response to oxidative stress, how do they protect the fungus from such stress? It was hypothesized that the furanoid double bond can absorb reactive oxygen species in a similar manner to its metabolic epoxidation by P-450 enzymes in mammalian systems. The question as to whether or not juglone could also relieve oxidative stress was also posed. Juglone itself, a quinone, cannot do so because it is fully oxidized but it is an artefact produced by damage to walnut hulls, in which it exists as the glycoside of its reduced quinol form. This quinol would be a potent antioxidant but cannot protect the kernels directly as it only exists in the hulls. There was discussion as to whether pistachios and almonds have antioxidants similar to those in walnuts. Pistachios definitely have structurally similar hydrolysable tannins, although at lower levels, whereas almonds do not. However, almonds contain condensed tannins which may provide some degree of antioxidant protection.

Identification of Two Maize Seed Compounds that Influence Aflatoxin Biosynthesis

Robert A. Holmes[1], Norman J. Glassbrook[2], Rebecca S. Boston[1], and Gary A. Payne[3]

[1]*Department of Botany, North Carolina State University, Raleigh, NC;* [2]*School of Biosciences, Cardiff University, Cardiff, Wales;* [3]*Department of Plant Pathology, North Carolina State University, Raleigh, NC*

Reduction of aflatoxin contamination of maize and other crops would be facilitated by the identification of host compounds that influence secondary metabolism in the aflatoxigenic fungi *Aspergillus flavus* and *A. parasiticus*. We report the isolation of two non-proteinaceous compounds from kernels of the maize inbred Tex6 that inhibit aflatoxin biosynthesis in *A. flavus*. Aflatoxin Biosynthesis Inhibitor-1 (ABI-1) inhibits growth at high concentrations but also influences aflatoxin biosynthesis. LC-MS analysis of ABI-1 containing fractions has yielded several candidate molecules which we will verify using additional purification steps and LC-MS. The second inhibitory compound, ABI-2, inhibits aflatoxin biosynthesis and suppresses conidiation, but does not affect mycelial mass. ABI-2 activity does not accumulate in maize kernels until late in kernel development (later than 28 days after pollination). LC-MS analysis of ABI-2 containing fractions shows that inositol hexakisphosphate (phytic acid) is the major component, with other inositol polyphosphates present. Kernels from *low phytic acid 1-1* mutant lines have less inhibitory activity than wild-type kernels. Treatment of inhibitory fractions with phytase (a phosphatase) enhances inhibition of aflatoxin biosynthesis. However, pure phytic acid and some other inositol polyphosphate isomers have no strong inhibitory effect. Likewise, treatment of pure phytic acid with phytase does not result in inhibition of aflatoxin biosynthesis. Thus, ABI-2 activity appears to be associated with the production of phytic acid, but not phytate per se.

# A New Peanut Phytoalexin with Stilbene and Tetronic Acid Moieties

V.S. Sobolev[1], S.T. Deyrup[2], and J.B. Gloer[2]
[1]USDA, ARS, National Peanut Research Laboratory, Dawson, GA; [2]The University of Iowa, Department of Chemistry, Iowa City, IA

A new pigmented, low molecular weight metabolite has been isolated from peanut (*Arachis hypogaea*) kernels challenged by a soil fungal isolate. The structure of the new compound, termed ST-1, was elucidated by ^{1}H and ^{13}C NMR, MS, and UV spectrometry. The ST-1 molecule bears stilbene and tetronic acid moieties and represents an unusual class of compounds. The only known ST-1 analog was isolated from heartwood of *Pericopsis elata*. Both *A. hypogaea* and *P. elata* belong to the family *Leguminosae*. Like all peanut stilbene phytoalexins, ST-1 naturally exists as the *trans*-isomer, but can be converted into the corresponding *cis*-isomer by exposure to UV/visible light radiation. ST-1 may be responsible for the yellow color that is often observed in high-water-activity peanut kernels challenged by fungi.

   The molecular origin of the new metabolite is unknown; however, the number of carbon atoms corresponds to that of stilbenes suggesting that ST-1 is a degradation product of the coexisting stilbene, *trans*-Arachidin-1. The *ortho*-dihydroxy moiety in Arachidin-1 might be oxidatively dissimilated by bond fission to produce a *cis-cis*-muconic acid derivative. Reduction of the enol system in the latter derivative to the dihydromuconic acid derivative followed by lactonization would give ST-1. Although the sequence in the above scheme is speculative, the proposed steps seem to be likely, based on analogs in the literature.

   Production of ST-1 in peanuts was elicited by different soil fungi, including toxigenic and nontoxigenic *A. flavus* and *A. parasiticus*, as well as by *A. niger* and *A. caelatus*. The new metabolite is suggested to be an important representative of a new class of peanut phytoalexins since its production often exceeds production of major known stilbenes. The biological activity of the new compound is the subject of future investigation.
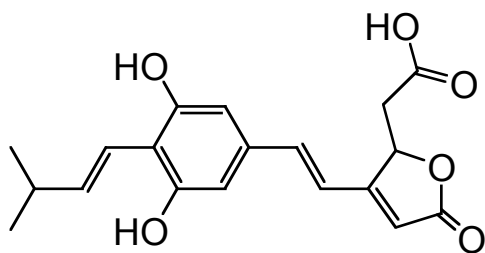
ST-1 pigment from *Arachis hypogaea*
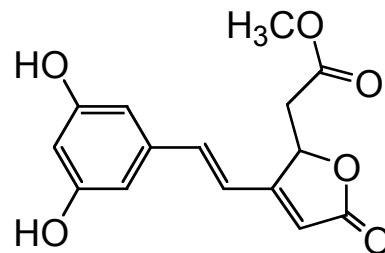
But-2-enolide from *Pericopsis elata*

Examination of Error Components Associated with Quantification of Aflatoxin in Ground Corn Grain with In-house CD-ELISA

M.J. Clements[1], G.L. Windham[1], C.M. Maragos[2], W.P. Williams[1], T.D. Brooks[1], L.K. Hawkins[1], and H.M. Gardner[1]

[1]*USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS;* [2]*USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL*

Genetic resistance is generally considered to be the most desirable means of minimizing aflatoxin accumulation in corn grain prior to harvest; however, variation associated with grain sampling and subsampling techniques, and quantitative analytical protocols greatly impedes accurate classification of genotypes as resistant or susceptible to aflatoxin accumulation in grain. Some widely accepted analytical protocols for aflatoxin quantification in grain include liquid chromatography (LC), thin-layer chromatography (TLC), immunoaffinity column assay (ICA), enzyme-linked immunosorbent assay (ELISA), and high-performance liquid chromatography (HPLC). Each of these protocols involves several components that may contribute substantial cost or variation to quantification of aflatoxin among replicated analyses of the same subsample. Our objective was to examine error components and inputs associated with grain sampling and an in-house competitive-direct ELISA (CD-ELISA) for quantification of aflatoxin in corn grain. Understanding of error components associated with the CD-ELISA will lead to management decisions that minimize cost and maximize data quality.

The CD-ELISA was developed around a monoclonal antibody produced at the USDA-ARS Mycotoxin Research Unit, Peoria, IL. Aflatoxin concentration in corn grain determined with CD-ELISA correlated significantly ($P < 0.0001$, $r = 0.87$) with aflatoxin concentration in grain determined with the widely accepted Vicam AflaTest. Preliminary analysis of error components revealed that greatest variation in aflatoxin concentration from sampling and laboratory assay was explained by 8-gram subsamples of ground grain (56.1%), followed by sub-subsamples of extract solution (24.1), and sub-sub-subsampling (19.8%). An increase in subsample number from 1 to 3 decreased LSR of entry means by approximately 30%, whereas increasing replicates of the assay or increasing sub-subsamples of extract solution had much less of an effect on reducing LSR of entry means. Increasing subsample size from 8 to 20, 50, 100, or 150 grams was not beneficial in reducing variation in aflatoxin concentration associated with subsampling of ground grain. Sensitivity of the assay when run with three subsamples and a 300-fold dilution of subsample extract is approximately greater than or equal to 4 ng aflatoxin per gram of ground corn. The CD-ELISA compares very favorably with the widely accepted Vicam AflaTest, therefore the assay may be used to differentiate corn genotypes that are susceptible or resistant to aflatoxin accumulation in grain.

Using Hyperspectral Technology to Measure Fungal Growth and Assess Mycotoxin Contamination of Corn

Z. Hruska[1], H. Yao[1], K. DiCrispino[1], K. Brabham[1], D. Lewis[1], J. Beach[1], R.L. Brown[2], and T.E. Cleveland[2]
[1]*Institute for Technology Development, Stennis Space Center, MS;* [2]*USDA-ARS, Southern Regional Research Center, New Orleans, LA*

The ultimate aim of the current project is to develop a rapid, non-destructive hyperspectral imaging methodology to measure spectral signatures associated with fungal infection and mycotoxin contamination of corn kernels. The Institute for Technology Development (ITD-Stennis Space Center, Mississippi) has developed a patented, low cost, and portable tabletop hyperspectral imaging system with imaging capability in the visible and near-infrared range (400 and 1000 nm). The instrument is ideal for conducting laboratory-based experiments, but can also be adapted to field applications. In preliminary experiments the hyperspectral sensor distinguished very different and very similar corn varieties based on their spectral signatures as well as identified unique spectral signature for *Aspergillus flavus* (*A. flavus*) and determined that it is readily distinguishable against any background or surrounding surface and among other mold strains.

The next logical step was to characterize the *A. flavus* signature once it infects corn and investigate possible changes in the spectral pattern of *A. flavus* on corn affected by daily growth. It was also important to compare fungal growth with an established chemical assay such as the kernel-screening assay in order to determine if mycotoxin contamination on corn correlates with the spectral pattern of *A. flavus*. Therefore, the objective of the present experiment was to observe any changes in the spectral signature of *A. flavus* on corn kernels over an eight-day growth period. In addition, the daily aflatoxin production was measured and correlated with any observed change in the spectral signature.

A corn line that has been found to consistently produce a robust toxin response was used for aflatoxin infection. Corn kernels were inoculated with *A. flavus* and placed into a 30 °C/100% humidity incubator for 8 days. The kernels were infected in an inoculum made from *A. flavus* (AF13) cultures at a dilution of 4×10^6 spores/ml. Five dishes (1 control and 4 treatment reps) each containing 4 kernels, were imaged each day to establish a daily *A. flavus* growth pattern on corn. VNIR imaging began on the first day of growth, 24 hours after inoculation. Following VNIR imaging, kernels from each day were placed into a 60 °C oven for 2 days to terminate further mold growth in preparation for the kernel screening assay (KSA). Dry kernels were processed for the KSA according to the protocol developed at SRRC. The imaging data were processed and analyzed using image analysis methods and algorithms developed for corn at ITD.

The results suggest that there is a significant spectral signature change between the growth on Day 1 and the growth on the remaining days throughout the spectra (450–900 nm). A significant signature difference between Day 2 and the remaining days exists from 450 to 781 nm. The results also revealed a correlation between toxin level and average reflectance at specific bands throughout the spectra. Establishing correlation between hyperspectral data and the chemical kernel-screening assay over a growth period is the first step toward quantifying toxin on corn kernels.

Participants

Abbas, Hamed K.
USDA, ARS
Crop Genetics & Production Research Unit
P.O. Box 345
Stoneville, MS 38776-0345
662-686-5313
habbas@msa-stoneville.ars.usda.gov

Antilla, Larry C.
Arizona Cotton Research & Protection Council
3721 East Wier Ave.
Phoenix, AZ 85040-2933
602-438-0059
lantilla@azcotton.org

Appell, Michael
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6249
appellm@ncaur.usda.gov

Averhoff, Scott
Texas Corn Producers Board
P.O. Box 999
Waxahachie, TX 75168
972-937-2587
scottaverhoff@hotmail.com

Balint-Kurti, Peter J.
USDA, ARS
Plant Science Research Unit
NC State Dept. of Plant Pathology, Gardner Hall
Raleigh, NC 27695-7616
919-515-3516
peter_balintkurti@ncsu.edu

Bertels, Paul J.
National Corn Growers
632 Cepi Drive
Chesterfield, MO 63005
636-733-9004
bertels@ncga.com

Betrán, Javier
Texas A&M University
Soil and Crop Sciences Dept.
Heep Building TAMU
College Station, TX 77843-2474
979-845-3469
javier-betran@tamu.edu

Bridges, Susan M.
Mississippi State University
Dept. of Computer Science & Engineering
Box 9637
Mississippi State, MS 39762
662-325-7505
bridges@cse.msstate.edu

Brooks, Thomas D.
USDA, ARS
Corn Host Plant Resistance Research Unit
345 Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-7996
tbrooks@msa-msstate.ars.usda.gov

Brown, Robert L.
USDA, ARS, SRRC
Food and Feed Safety Research Unit
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
225-578-1216
rbrown@srrc.ars.usda.gov

Brown, Daren
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6230
browndw@ncaur.usda.gov

Bruns, H. Arnold
USDA, ARS
Crop Genetics & Production Research Unit
P.O. Box 345
Stoneville, MS 38776-0345
662-686-5279
abruns@msa-stoneville.ars.usda.gov

Butchko, Robert
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6073
butchkora@ncaur.usda.gov

Calvo-Byrd, Ana M.
Northern Illinois University
Dept. of Biological Sciences
1425 W Lincoln Hwy
Dekalb, IL 60115
815-753-0451
amcalvo@niu.edu

Camas, Alberto
Mississippi State University
Dept. of Biochemistry & Molecular Biology
Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-7747
ac201@msstate.edu

Campbell, Bruce C.
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan St
Albany, CA 94710-1100
510-559-5846
bcc@pw.usda.gov

Cantrell, Roy G.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2266
rcantrell@cottoninc.com

Carbone, Ignazio
NC State University
Center for Integrated Fungal Research
Dept. of Plant Pathology
Raleigh, NC 27695-7244
919-513-4866
ignazio_carbone@ncsu.edu

Cary, Jeff
USDA, ARS, SRRC
Food and Feed Safety Research Unit
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
504-286-4264
jcary@srrc.ars.usda.gov

Chen, Zhi-Yuan
Louisiana State University
Dept. of Plant Pathology & Crop Physiology
LSU Agricultural Center
Baton Rouge, LA 70803
225-578-1464
zchen@agcenter.lsu.edu

Clements, Michael J.
USDA, ARS
Corn Host Plant Resistance Research Unit
Box 955
326 Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-8023
mclements@msa-msstate.ars.usda.gov

Cotty, Peter J.
USDA, ARS, SRRC
Div. of Plant Pathology & Microbiology
University of Arizona
1140 E. South Campus Drive
Tucson, AZ 85721-0036
520-626-5049
pjcotty@email.arizona.edu

Czika, Carol A.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2270
cczika@cottoninc.com

Damann, Kenneth E.
Louisiana State University
Dept. of Plant Pathology & Crop Physiology
LSU Agricultural Center
Baton Rouge, LA 70803
225-578-1401
kdamann@agctr.lsu.edu

Davis, Georgia L.
University of Missouri-Columbia
Division of Plant Sciences
1-31 Agriculture
Columbia, MO 65211
573-882-9224
davisge@missouri.edu

Dorner, Joe W.
USDA, ARS
National Peanut Research Laboratory
P.O. Box 509, 1011 Forrester Dr. SE
Dawson, GA 39842
229-995-7408
jdorner@nprl.usda.gov

Doster, Mark A.
University of California
Kearney Agricultural Center
9240 S. Riverbend Ave.
Parlier, CA 93648
559-646-6598
mark@uckac.edu

Dowd, Patrick F.
USDA, ARS, NCAUR
Crop Bioprotection Research Unit
1815 N. University St.
Peoria, IL 61604
309-681-6242
dowdpf@ncaur.usda.gov

Eubanks, Mary W.
Duke University
Department of Biology
Durham, NC 27708-0338
919-660-7419
eubanks@duke.edu

Felton, Gary W.
Penn State University
Dept. of Entomology
501 ASI Bldg.
University Park, PA 16804
814-863-7789
gwf10@psu.edu

Georgianna, D. Ryan
NC State University
Center for Integrated Fungal Research and
Department of Plant Pathology
Box 7567
Raleigh, NC 27695-7567
919-515-6995
drgeorgi@ncsu.edu

Gibson, David
Texas Corn Producers Board
4205 N. I-27
Lubbock, TX 79403
806-763-2676
dgibson@texascorn.org

Glassbrook, Norm
NC State University
851 Main Campus Drive
267 Partners III
Raleigh, NC 27606
919-451-5060
njglassb@ncsu.edu

Godshalk, Brent
BASF Plant Science
26 Davis Drive
Research Triangle Park, NC 27709
919-547-2883
godshae@basf-corp.com

Gorman, Daniel P.
Pioneer Hi-Bred
2300 Industrial Park Rd. NE
Cairo, GA 39828
229-378-8240
dan.gorman@pioneer.com

Gradziel, Tom
University of California
Plant Sciences
Davis, CA 95616
530-752-1575
tmgradziel@ucdavis.edu

Guo, Baozhu
USDA, ARS
Crop Protection & Management Research Unit
P.O. Box 748
Tifton, GA 31793-0748
229-387-2334
bguo@tifton.usda.gov

Hammond, Bruce G.
Monsanto Co.
800 North Lindbergh Boulevard
Mail Zone: O3C
St. Louis, MO 63167-0001
314-694-8482
bruce.g.hammond@monsanto.com

Hawkins, Leigh K.
USDA, ARS
Corn Host Plant Resistance Research Unit
325 Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-2707
lhawkins@msa-msstate.ars.usda.gov

Highland, Brett
AgraQuest, Inc
1069 Eisenhower Dr
Nokomis, FL 34275
941-484-4523
bhighland@agraquest.com

Holbrook, Corley
USDA, ARS
Crop Genetics and Breeding Research Unit
P.O. Box 748
Tifton, GA 31793-0748
229-386-3176
holbrook@tifton.usda.gov

Holland, James B.
USDA, ARS
Plant Science Research Unit
Dept. of Crop Science, Box 7620 NC State
Raleigh, NC 27695-7620
919-513-4198
james_holland@ncsu.edu

Holmes, Robert A.
NC State University
Dept. of Botany, Campus Box 7612
Raleigh, NC 27695-7612
919-515-6995
raholme2@unity.ncsu.edu

Horn, Bruce
USDA, ARS
National Peanut Research Laboratory
P.O. Box 509, 1011 Forrester Dr. SE
Dawson, GA 39842-0509
229-995-7410
bhorn@nprl.usda.gov

Hruska, Zuzana
Institute for Technology Development
Building 1103, Suite 118
Stennis Space Center, MS 39529
228-688-2509
zhruska@iftd.org

Hua, Sui Sheng
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan St
Albany, CA 94710
510-559-5905
ssth@pw.usda.gov

Isleib, Thomas G.
NC State University
Dept. of Crop Science, Box 7629
Raleigh, NC 27695-7629
919-515-3281
tom_isleib@ncsu.edu

Jacobs, Merle
Almond Board of California
1150 9th Street Suite 1500
Modesto, CA 95354
209-343-3222
mjacobs@almondboard.com

Jacobus, Carrie A.
NC State University
851 Main Campus Dr.
267 Partners III
Raleigh, NC 27606
919-515-6995
cajacobu@unity.ncsu.edu

Jaime-Garcia, Ramon
University of Arizona
Div. of Plant Pathology & Microbiology
P. O. Box 210036-0036
Tucson, AZ 85721
520-626-7855
rjaime@email.arizona.edu

Jaramillo, Frank
Office of the Texas State Chemist
Reed McDonald Building Room 302
College Station, TX 77836
979-845-1121

Jones, Don C.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2367
djones@cottoninc.com

Kendra, David
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6579
kendrad@ncaur.usda.gov

Kerns, Mike
Monsanto Co.
3302 SE Convenience Blvd.
Ankeny, IA 50021
515-963-0621
mike.kerns@monsanto.com

Keys, Lynda S.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2269
lkeys@cottoninc.com

Kolomiets, Mike V.
Texas A&M University
Dept. of Plant Pathology and Microbiology
2132 TAMU
College Station, TX 77843-2132
979-458-4624
kolomiets@tamu.edu

Krakowsky, Matthew
USDA, ARS
Crop Genetics and Breeding Research Unit
P.O. Box 748
Tifton, GA 31793-0748
229-387-2341
mkrakowsky@tifton.usda.gov

Lee, Robert Dewey
University of Georgia
15 RDC Road RDC-CES-UGA
Tifton, GA 31793
229-386-3432
deweylee@uga.edu

Light, Douglas M.
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan Street
Albany, CA 94710
530-304-9302
dlight@pw.usda.gov

Luo, Meng
University of Georgia
Crop Protection & Management Research Unit
2747 David Rd.
Tifton, GA 31794
229-387-2377
mluo@tifton.uga.edu

Luthe, Dawn
MS State University
Dept. of Biochemistry, Box 9650
Mississippi State, MS 39762
662-325-7733
dsluthe@ra.msstate.edu

Mahoney, Noreen
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan Street
Albany, CA 94710
510-559-5971
nmahoney@pw.usda.gov

Mayfield, Kerry L.
Texas A&M University
Soil & Crop Sciences Dept., MS 2474
College Station, TX 77843-2474
979-845-3469
kerry-mayfield@tamu.edu

Milla, Susana R.
NC State University
Dept. of Crop Science, Box 7629
Raleigh, NC 27695-7629
919-515-3196
susana_milla@ncsu.edu

Molyneux, Russell J.
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan Street
Albany, CA 94710
510-559-5812
molyneux@pw.usda.gov

Moore, Steven
Louisiana State University
Dean Lee Research Station
8105 Tom Bowman Drive
Alexandria, LA 71302
318-473-6524
smoore@agcenter.lsu.edu

Muir, Ryann M.
University of California
Dept. of Pomology, One Shields Ave.
Davis, CA 95616
530-752-5325
ryannmuir@hotmail.com

Murphy, Emory M.
Georgia Peanut Commission
P.O. Box 967
Tifton, GA 31793-0967
229-386-3470
emory@gapeanuts.com

Nichols, Robert L.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2371
bnichols@cottoninc.com

Nielsen, Ray F.
Smart World Organics, Inc.
18744 Titus Road
Hudson, FL 34667
727-697-3661
ray@smartwp.com

Nierman, William
TIGR
9712 Medical Center Drive
Rockville, MD 20850
301-424-4316
wnierman@tigr.org

Nwosu, Victor
Masterfoods, USA
800 High St.
Hackettstown, NJ 7840
908-850-7545
victor.nwosu@effem.com

O'Brian, Greg
NC State University
Dept. of Plant Pathology
851 Main Campus Drive
Raleigh, NC 27695
919-515-6995
greg_obrian@ncsu.edu

Olsen, Mary W.
University of Arizona
Dept. of Plant Sciences
1140 E. South Campus Dr. Forbes 204
Tucson, AZ 85721
520-626-2681
molsen@ag.arizona.edu

Ozias-Akins, Peggy
University of Georgia
Department of Horticulture and NESPAL
P.O. Box 748
Tifton, GA 31793-0748
229-386-3902
pozias@uga.edu

Palumbo, Jeffrey
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan St.
Albany, CA 94710
510-559-5876
palumbo@pw.usda.gov

Payne, Gary
NC State University
Department of Plant Pathology, Box 7567
223 Partners III
Raleigh, NC 27695
919-515-6994
gary_payne@ncsu.edu

Peethambaran, Bela
Mississippi State University
21H Wallace Circle
Starkville, MS 39759-3084
662-341-1293
bp64@msstate.edu

Perkins, James M.
Monsanto Co.
8350 Minnegan Rd.
Waterman, IL 60556-7113
815-758-9524
jim.perkins@monsanto.com

Pritchard, Bethan
NC State University
2124 Gorman St.
Raleigh, NC 27606
919-247-2901
beth_pritchard@ncsu.edu

Proctor, Robert
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6380
proctorh@ncaur.usda.gov

Raab, Quinton J.
B-H Genetics
300 S Lancaster Street PO Box 620
Moulton, TX 77975-0620
361-596-4088
qraab@sbcglobal.net

Rajasekaran, Kanniah
USDA, ARS, SRRC
Food and Feed Safety Research Unit
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
504-286-4482
krajah@srrc.ars.usda.gov

Rathore, Keerti S.
Texas A&M University
Inst. For Plant Genomics & Biotechnology
2123 TAMU Borlaug Center
College Station, TX 77843-2123
979-862-4795
rathore@tamu.edu

Richard, John
Romer Labs, Inc.
1301 Stylemaster Dr.
Union, MO 63084
636-583-8600
johnr@romerlabs.com

Robens, Jane F.
USDA, ARS
National Program Leader Food Safety
Rm 4-2184 GWCC
5601 Sunnyside Avenue
Beltsville, MD 20705-5138
301-504-5381
jfr@ars.usda.gov

Robertson, Leilani A.
NC State University
Dept. of Crop Science
2122 Williams Hall, Box 7620
Raleigh, NC 27695
919-515-4087
larobert@unity.ncsu.edu

Schatzki, Thomas F.
USDA, ARS, WRRC
Plant Mycotoxin Research Unit
800 Buchanan St.
Albany, CA 94710
510-559-5672
tom@pw.usda.gov

Shim, Won-Bo
Texas A&M University
Dept. of Plant Pathology & Microbiology
2132 TAMU
College Station, TX 77843-2132
979-458-2190
wbshim@tamu.edu

Simmons, Mary W.
USDA, ARS
National Program Leader Crop Production and
Protection, Rm 4-2210 GWCC
5601 Sunnyside Ave.
Beltsville, MD 20705
301-504-5560
kws@ars.usda.gov

Smith, David R.
Zea Sage
1834 West Forestview Drive
Sycamore, IL 60178
815-501-1659
dsmith1834@tbcnet.com

Sobolev, Victor
USDA, ARS
National Peanut Research Laboratory
P.O. Box 509, 1011 Forrester Dr. SE
Dawson, GA 39842
229-995-7446
vsobolev@nprl.usda.gov

Starr, Magen R.
NC State University
Dept. of Crop Science, Box 7620
Raleigh, NC 27695
217-377-6883
mrstarr@ncsu.edu

Sullivan, Dana
USDA, ARS
Southeast Watershed Research Laboratory
P.O. Box 748
Tifton, GA 31793-0748
229-386-3665
dgs@tifton.usda.gov

Timper, Patricia
USDA, ARS
Crop Protection & Management Research Unit
P.O. Box 748
Tifton, GA 31793
229-386-3188
ptimper@tifton.usda.gov

Tonos, Jennifer L.
USDA, ARS
Crop Genetics and Production Research Unit
NBCL, 59 Lee Rd. Building B
Stoneville, MS 38776
662-686-5230
jtonos@msa-stoneville.ars.usda.gov

Valentine, Howard E.
American Peanut Council
10625 Big Canoe
Jasper, GA 30143
706-579-1755
pnuttech@alltel.net

Voss, Kenneth A.
USDA, ARS, RBRRC
Toxicology and Mycotoxin Research Unit
950 College Station Road
Athens, GA 30605
706-546-3315
kvoss@saa.ars.usda.gov

Wakelyn, Phillip J.
National Cotton Council
1521 New Hampshire
Washington, DC 20036
202-745-7805
pwakelyn@cotton.org

Walker, Scott
Monsanto Co.
9631 Hedden Road
Evansville, IN 47725
812-867-7183
scott.l.walker@monsanto.com

Wang, Xingfen
NC State University
Dept. of Crop Science, Box 7620
Raleigh, NC 27695
919-515-2704
xwang15@unity.ncsu.edu

Wedegaertner, Tom C.
Cotton Incorporated
6399 Weston Parkway
Cary, NC 27513
919-678-2369
twedegaertner@cottoninc.com

Weissinger, Arthur K.
NC State University
Dept. of Crop Science, Box 7620 NCSU
Raleigh, NC 27695-7620
919-515-2704
arthur@unity.ncsu.edu

White, Don G.
University of Illinois
Crop Sciences Dept., N425 Turner Hall
1102 South Goodwin Ave.
Urbana, IL 61801
217-333-1093
donwhite@uiuc.edu

Wicklow, Donald
USDA, ARS, NCAUR
Mycotoxin Research Unit
1815 N. University
Peoria, IL 61604
309-681-6243
wicklodt@ncaur.usda.gov

Willcox, Martha
2501 Porter St. NW #710
Washington, DC 20008
202-248-2232
mwillcox@earthlink.net

Williams, W. Paul
USDA, ARS
Corn Host Plant Resistance Research Unit
340 Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-2735
wpwilliams@msa-msstate.ars.usda.gov

Windham, Gary L.
USDA, ARS
Corn Host Plant Resistance Research Unit
217 Dorman Hall, Stone Blvd.
Mississippi State, MS 39762
662-325-2594
glwindham@msa-msstate.ars.usda.gov

Wisser, Randall J.
Cornell University
Institute for Genomic Diversity
160 Biotechnology Bldg.
Ithaca, NY 14853
607-255-0761
rjw29@cornell.edu

Woloshuk, Charles P.
Purdue University
Dept. of Botany and Plant Pathology
915 W. State Street
West Lafayette, IN 47907-2054
765-494-3450
woloshuk@purdue.edu

Wra, Lawrence J.
BASF Plant Science
26 Davis Drive
Research Triangle Park, NC 27709
919-547-2004
neal@basf.com

Wu, Minsheng
NC State University
Dept. of Crop Science, Box 7620
Raleigh, NC 27695
919-515-2704
minsheng_wu@ncsu.edu

Wynne, Johnny C.
NC State University
Deans Office
College of Ag and Life Sciences
Raleigh, NC 27695
919-515-2668
johnny_wynne@ncsu.edu

Xu, Wenwei
Texas A&M University
Agricultural Research and Extension Center
Route 3, Box 219
Lubbock, TX 79403
806-746-4015
we-xu@tamu.edu

Yao, Haibo
Institute for Technology Development
976 Maple Creek Dr.
Slidell, LA 70461
228-688-2509
hyao@iftd.org

Yu, Jiujiang
USDA, ARS, SRRC
Food and Feed Safety Research Unit
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
301-795-7570
jiuyu@srrc.ars.usda.gov

Zhang, Jinfen
Texas A&M University
Agricultural Research and Extension Center
Route 3, Box 219
Lubbock, TX 79403
806-746-6101
TSNYJ999@yahoo.com.cn

Zimeri, Anne Marie S.
USDA, ARS, RBRRC
Toxicology & Mycotoxin Research Unit
950 College Station Road
Athens, GA 30605
706-546-3188
azimeri@saa.ars.usda.gov

Author Index

A
Abbas, H. K. 77, 81, 117
Anderson, A. 99
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B
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Chan, K. L. 35, 45
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Chen, Z.-Y. 78, 110
Chu, Y. 107
Clements, M. J. 81, 88, 130
Cleveland, T. E. 35, 36, 37, 39, 45, 78, 110, 111, 131
Cotty, P. J. 73, 93, 94, 95, 96, 102, 103, 104
Cox, J. E. 43
Coy, A. E. 70
Culbreath, A. 70

D
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