Proceedings of the 3rd Fungal Genomics, 4th Fumonisin, and 16th Aflatoxin Elimination Workshops

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Introduction: Aflatoxin and Fumonisin Elimination and Fungal Genomics Workshop – 2003

Mycotoxin contamination continues to be a food and feed safety problem in the U. S. and throughout the world. Medical concerns associate mycotoxin consumption to a variety of human clinical syndromes in one or more geographical areas. The research results presented at this workshop were exciting developments in our continual struggle to develop the practical knowledge and technologies that will enable us to eliminate mycotoxins as practical problems for the producing industries. Along with the new developments, however, comes sadness that active members of the research group do retire. Anne Desjardins read a poem paying homage to Ron Plattner who recently retired from USDA-ARS after many, many years of productive work in the field of mycotoxicology. Ron's contributions to our understanding of fumonisins and other *Fusarium* toxins have been immense, and he will be sorely missed.

Fungal Genomics
Significant progress has been made since the first workshop two years ago. The EST projects in progress at that time are now nearing completion generating voluminous data on the genetics of *F. verticillioidies* and *Aspergillus flavus*, which can now be utilized through the use of microarrays. In addition, funds are available to carry out the whole genome sequencing of *A. flavus* through university/government collaboration. Fungal genomics is very important in helping us understand how fungal genes and regulatory processes become functional and can be targeted to control toxin formation or fungal development. Additionally, use of genomics information will help us (1) rapidly identify at the molecular level how natural products become toxin inhibitors as in the case of gallic acid, (2) understand fungal ecology, e.g., how do atoxigenic strains survive in the field and what makes a particular strain more aggressive, and (3) understand the complex host-pathogen interactions, particularly during changes in microclimate or macroclimate of the fungus. *F. verticillioides* was the unanimous choice as the next high priority candidate for genome sequencing when resources are located, now that the *A. flavus* and *F. graminearum* genome sequencing projects have been fully funded. A research proposal to sequence the genomes of *F. verticillioides* and *F. oxysporum* has been submitted with the goal of comparing the sequences of these two fungi with the already available genome of *F. graminearum* in order to address questions about species evolution, pathogenicity, and secondary metabolism.

Fumonisins
The health effects of fumonisins continue to be scrutinized, particularly by the medical community because of concerns regarding interactions with dietary folic acid and possible association with spina bifida. Research results included a three year survey of fumonisin levels in corn from the highlands (>1700 m) and lowlands (<360 m) of Guatemala, which showed that fumonisins and *Fusarium verticillioides* in highland corn were quite rare in contrast to their common occurrence in corn from the lowlands. Preparation of corn-based foods using alkali processing (nixtamalization) reduced fumonisin in the final product in contrast to baking and frying, which had little effect. Recent investigations with a model system for biosynthesis of fungal polyketides (like fumonisin) identified two mutants of FUM5, a polyketide synthase in *F. verticillioides*. Gene deletion studies helped to determine the function of several genes of the FUM gene cluster and identified all four genes involved in adding the tricarballylic acid moieties to the fumonisin backbone. Microarray analysis of *F. verticillioides* has identified potential candidate genes having roles in regulating fumonisin biosynthesis under different environmental conditions. High fumonisin levels in corn grown in the Southeast may have an environmental basis since drought stress and high humidity come together at and after flowering, an

environment often found in the Southern coastal areas. North Carolina corn considered clean had high levels of fumonisin when analyzed.

Aflatoxins
Control and ultimate elimination of aflatoxin contamination continues to be a major goal of the research of many of the scientists attending this meeting. That research can be divided into five major areas of emphasis, and some of the highlights in each of those areas are summarized briefly below.

Crop Management and Handling, Insect Control, and Fungal Relationships
Plant stress, either from too densely planted corn or low nitrogen, was found to contribute to higher levels of mycotoxins in corn which suggests that cultural practices can help control mycotoxin production. A pear-ester kairomone disrupted codling moth mating in walnut orchards and thus should reduce infection with *Aspergillus* molds and thus aflatoxin in tree nuts. During analysis of pistachio nuts, particle size distribution, sub-sample size, and method of homogenization (wet slurry or dry) all contributed to variance in aflatoxin. The wet slurry method, which appears to increase extraction of aflatoxin, is important in analysis of products being exported to Europe where wet slurrying is commonly used. A high speed optical method for sorting corn (at a rate of 250 bu/hr) reduced mycotoxins by over 80% while removing only 5% of the incoming product. A sorter may miss some kernels since kernel orientation and/or kernel velocity as it passes through the machine are important to the accuracy of the method. The primary cost, besides the capital investment for the sorting equipment, is the rejected product, which is usually lost. There is already some limited use of optical sorters for aflatoxin, mostly for food grade white corn.

Crop Resistance – Genetic Engineering
Presentations included the development and identification of potential anti-*flavus* peptides and genes, and new generations of peptides with enhanced stability, specificity and activity. Lytic peptides inhibit fungal growth, and although there are still some technical problems associated with D4E1, a new generation of synthetic peptides is being designed. Transgenic plants (either cotton or tobacco) that express the lytic peptide D4E1 or a bacterial chloroperoxidase have enhanced resistance to fungal pathogens *in vitro* and *in vivo*. Biolistic transformation of plastids increases the expression of genes in transgenic cotton and should result in multiple copies of the anti-*flavus* gene per plant, and consequently higher levels of expression. Since there is no apparent silencing mechanism within chloroplasts, gene expression levels should be stable. Gene expression in chloroplasts will be followed by exploration of protein expression in other types of plastids. To achieve enhanced resistance to *A. flavus* infection in peanut, intellectual property issues must not limit transgenic distribution, particularly of promoter elements and selectable markers. The ubiquitin promoter used to express anti-*flavus* genes in peanut should drive expression in most tissues throughout the plant life. Mercury resistance through the MerB gene product was chosen as a marker because it is the intellectual property of the University of Georgia, plant tissues are sensitive to mercury compounds, and appropriate levels for selection have already been identified.

Crop Resistance - Conventional Breeding
There was significant discussion and excitement regarding marker assisted selection (MAS) relating to conventional breeding. Much success has been achieved in research to develop markers for reduced aflatoxin in corn. The hope is that breeding programs will be able to use MAS to develop resistance with no decline in yield.

Microbial Ecology
The effectiveness of biocontrol strains with corn depends greatly on the environmental conditions present in different regions of the United States. In warmer climates, corn is planted early and cool

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nighttime temperatures may not be conducive for growth and sporulation by nonaflatoxigenic strains after application to soil. In southern Texas, cotton is the dominant crop during the warmest part of the growing season and, therefore, is critical in influencing *A. flavus* populations. Treatment of corn with biocontrol strains alone in southern Texas would not be effective because large amounts of aflatoxigenic *A. flavus* are contributed by infected cotton. However, treatment of cotton may have a carry over effect on corn in reducing aflatoxin contamination. Further north, where the incidence of infection by *A. flavus* is lower, treatment of corn would be expensive and not economically feasible. Aflatoxin levels in Arizona cottonseed vary considerably from year to year following treatment with nonaflatoxigenic *A. flavus,* and the effectiveness of the treatment is best measured by the proportion of biocontrol strain to wild-type strains in soil and cotton. The pyrrocidine antibiotics (newly recognized group of pyrrolizidine-like compounds) from *Acremonium zeae* were shown to be antagonistic to *A. flavus* and *F. verticillioides*. The fungus has been detected in whole symptomatic corn kernels from wound-inoculated ears of a commercial hybrid grown in central Illinois; in certain years these compounds may be important in limiting aflatoxin and fumonisin contamination in the northern corn-growing regions of the United States. Wounded pistachio nuts on the tree sprayed with the yeast, *Pichia anomala,* reduced colonization by *A. flavus* and other fungi under stress conditions. Population changes in soil and on leaf surfaces followed application of nonaflatoxigenic strains of *A. flavus*.

Natural Products for Prevention of Fungal Invasion and /or Aflatoxin Biosynthesis
Gallic acid is an exciting, potent natural compound present in the walnut seed coat and inhibitory to aflatoxin. There is essentially no gallic acid present in the kernel ("meat") of the walnut without the seed coat, and aflatoxin production appears to be suppressed so long as the seed coat is present. Varying levels of gallic acid in different walnut lines apparently are due to structural differences in the hydrolyzable tannins present, and their levels may be amenable to manipulation by conventional breeding. Inhibition of aflatoxin by gallic acid may be reversible if gallic acid is removed. No other compounds in walnut are as inhibitory to aflatoxin biosynthesis as gallic acid. No field work has been done as yet, although we know that walnuts have much lower levels of aflatoxin contamination than pistachios or almonds, which have little or no gallic acid in the seed coat. The regulatory approval process for a transgenic approach to increase expression of an endogenous compound such as gallic acid was discussed, and the major question is whether or not it must go through the same approval process as a foreign gene. It is hoped that regulatory requirements would be less than for introduction a foreign gene into a genome.

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3rd Annual Fungal Genomics Workshop

Moderator – Emory Murphy, Georgia Peanut Commission

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Towards Elimination of Fumonisin from Maize – A Comparative Analysis of *Fusarium verticillioides* Expressed Sequence Tags (EST)

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Fumonisins are a family of polyketide-derived mycotoxins produced by the maize pathogen *Fusarium verticillioides*. This fungus can be found growing endophytically in most, if not all, maize fields in the US. But under some conditions, it can cause disease at any stage of maize plant development including ear, root and stalk. In addition, fumonisins can be found in all qualities of maize, from exceptional to poor. Fumonisin ingestion is associated with several animal diseases and is epidemiologically associated with human esophageal cancer in some regions of the world. We are working to limit maize crop losses, due to physical damage as well as the presence of fumonisins, by understanding toxin biosynthesis and pathogenesis. The identification of genes expressed under different fungal growth conditions has given scientists important clues to understand biological processes.

We, in collaboration with The Institute for Genomic Research (TIGR), have sequenced the 5'ends of *F. verticillioides* cDNAs derived from seven different growth conditions. Growth conditions of three libraries were similar but differed in length of time (i.e. 24, 48/72, and 96 hours) the fungus was allowed to grow on fumonisin production media (e.g. GYAM). The overall goal for these three libraries was to identify differentially expressed genes important to fumonisin biosynthesis. Growth conditions of remaining four libraries included a plant (maize) component. The overall goal for this set of libraries was to identify differentially expressed genes important for fungal/plant interactions. The first library of this set was prepared from *F. verticillioides* spores germinated for 10 hours in water extracts of maize seedlings, the second library was prepared from the fungus grown on excised maize seedling roots and shoots, the third was prepared from the fungus grown on developing maize kernels and the fourth was a subtracted library prepared from fungal culture with or without 2-benzoxazolinone (BOA), an antimicrobial compound produced by maize.

A total of 55,150 ESTs have been generated which represent 10,539 unique ESTs. 50,432 ESTs align to generate 5,816 tentative consensus (TC) sequences leaving 4,716 sequences as singletons. Comparison of each collection, or library, against the total collection is beginning to identify subsets of genes that may play a specific role in fumonisin biosynthesis as well as fungal plant interactions. For example, there are 756 unique ESTs within the 48/72-hour GYAM Library and 888 unique ESTs within the 96 hour FPM Library. Almost all of the fumonisin cluster (*FUM*) genes are represented in the 48/72 hour GYAM Library (13/15) while all *FUM* genes are represented in the 96 hour GYAM Library. No *FUM* genes are represented in the 24-hour GYAM Library. We are in the process of developing a DNA microarray containing a complete set of all the unique genes to identify differentially expressed genes from different growth conditions. Both experimental systems will help identify important regulatory genes whose characterization may lead to the development of new fungal or fumonisin control strategies in the field.

Aspergillus flavus EST and Microarray in Identifying Target Genes for Controlling Aflatoxin Contamination

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Aflatoxins are the most carcinogenic natural toxins produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus*. The biosynthesis of aflatoxins is a multi-enzymatic process encoded by over two dozens corresponding genes specific for this secondary metabolic process in *A. flavus* and *A. parasiticus*. Studies on the molecular mechanism of aflatoxin B_1 biosynthesis have led to the identification and sequencing of a completed aflatoxin pathway gene cluster of 70 kilobase pairs in length consisting of at least 25 identified genes including a positive regulatory gene as transcription activator. In addition, a well defined sugar utilization gene cluster consisting of four genes adjacent to the aflatoxin pathway gene cluster has also been identified. A nitrogen utilization gene cluster consisting of two genes has also been identified somewhere in the fungal genome. In order to better understand the molecular mechanism and regulation of aflatoxin biosynthesis, plant-fungal interaction and evolutionary biology of these toxigenic fungi, the *A. flavus* Expressed Sequenced Tag (EST) project has been carried out at the USDA-RS, Southern Regional Research Center (SRRC).

The strain of *A. flavus* used in this project was wild-type aflatoxin-producing strain NRRL 3357. A total of 7,214 unique EST sequences have been identified from a normalized cDNA library. These ESTs represent 70%-80% of the total genes within the *A. flavus* genome. The *A. flavus* gene index has been constructed. Among the 7,214 unique ESTs, 3,728 tentative consensus (TC) sequences are assembled and 3,486 singleton sequences are identified from 22,324 usable sequences obtained. Among the genes identified, many are rare copy genes potentially involved in secondary metabolism and gene regulation. All known aflatoxin biosynthetic genes have been identified among the sequenced clones in the library. Within the unique ESTs, we have identified many of the genes that may be involved directly or indirectly in aflatoxin formation. The genes of interest can be summarized in the following four categories: 1) aflatoxin biosynthetic pathway genes; 2) regulatory genes that have the potential to regulate aflatoxin production or signal transduction, e.g. genes encoding DNA-binding proteins, RNA-binding proteins, zinc-finger proteins, transcription regulators, transducins, cAMP receptors, protein kinases etc.; 3) genes that have the potential to contribute to fungal virulence or pathogenicity; 4) genes involved in fungal development. The latter two categories of genes could be involved in processes such as sporulation, conidiation, hyphal growth and hydrolytic activities. Some unique genes in the EST library show sequence homology to genes encoding hydrolytic enzymes, such as amylase, cellulase, pectinases, proteases, chitinase, chitosanases, pectin methylesterases, endoglucanase C precursor, glucoamylase S1/S2 precursors, *β*-1,3-glucanase precursor, 1,4-*β*-D-glucan cellobiohydrolase A precursor, glycogen debranching enzyme and xyloglucan-specific endo-*β*-1,4-glucanase precursor. Such hydrolytic enzymes could be highly expressed virulence factors during invasion of *A. flavus* into crops and, if so, have the potential to be useful targets for inhibiting aflatoxin production or for antifungal growth through genetic engineering. Microarray containing all of these unique genes (TC + singleton) is being constructed. Functional genomics studies using microarray under different conditions are underway. The application of EST/Microarray technologies will provide vital information for developing new strategies for control of aflatoxin contamination of crops.

Conserved Candidate Regulatory Sequence Motifs in the Aflatoxin Biosynthetic Genes of *Aspergillus parasiticus*, *Aspergillus flavus*, and Other Related Fungi

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With the availability of sequenced and annotated fungal genomes, we have directed our research focus to employing large-scale functional analysis of these genomes. We are particularly exploring signaling and transcriptional regulatory components and circuits controlling virulence in fungal pathogens. Transcriptional regulation is mediated through the binding of transcriptional regulators to specific DNA sites typically upstream of the transcription initiation site. We are using mutagenesis and gene expression profiling via DNA microarrays to identify the regulatory proteins and their circuits. This report summarizes our initial efforts to build and employ analysis tools for identifying the *cis*-acting transcription factor binding sites.

Functional gene regulatory motif sequences are conserved across genes and genomes due to selective pressure imposed by the requirements of function. Nearby sequences not restrained by functional requirements more rapidly diverge in sequence. Candidate regulatory sequences are identified by this sequence conservation. We have constructed a software tool, TIGR GenomeSlicer, which efficiently recovers targeted regions, particularly potential promoter regions, from sequenced and annotated genomes for subsequent analysis. We couple the output from this software with publicly available *Ab-initio* motif search software (AlignACE, http://atlas.med.harvard.edu/). We have applied these tools to identifying candidate regulatory motifs in negatively regulated stringent response genes in *Myxococcus xanthus* as a simple initial model. After this methods validation model, we have applied these tools to the potential promoter regions of the aflatoxin biosynthetic genes in *Aspergillus parasiticus*. With the availability of sequenced orthologues of genes from *A. flavus*, *A. nidulans*, *A. niger*, *A. fumigatus*, and *A. oryzae*, these tools will bring tremendous power to the identification of strong candidate *cis*-acting regulatory sequences in *Aspergillus* genomes.

The *Aspergillus flavus* Sequencing Project

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Funding was received from Microbial Genome Sequencing Project, USDA National Research Initiative, to completely sequence the genome of *Aspergillus flavus*. Gary Payne and Ralph Dean, the principal investigators of the grant, will collaborate with USDA/ARS/SRRC and The Institute for Genomic Research (TIGR) to complete a 6 X coverage of *A. flavus* strain NRRL 3357. The project will begin November 1, 2003. Sequencing and automated annotation will be done at TIGR under the supervision of Dr. William Nierman. USDA/ARS/SRRC in New Orleans, LA, is providing its Expressed Sequence Tag (EST) genomic database for *A. flavus* as a "matching" resource towards the complete genomic characterization of *A. flavus,* and they are also providing additional funds for finishing the sequence and closing the gaps. Jiujiang Yu at USDA/ARS/SRRC is leading the USDA/ARS EST program. As a member of the International *Aspergillus* Genomics Steering Committee, he will be representing the *Aspergillus flavus* community in the larger *Aspergillus* community.

Sequence assemblies will be released after three months and will be available on the TIGR website (www.tigr.org). The sequence will be updated monthly and electronic annotation will be updated after each 1X coverage. Data will be downloaded also to CIFR (Center for Integrated Fungal Research) at NCSU for manual annotation and data mining using DeCIFR (www.cifr.ncsu.edu). Progress on the sequencing project and manual annotation can be found at www.Aspergillusflavus.org.

Success in obtaining funding for this project was the result of the strong *A. flavus* community that is composed of producers, commodity groups, food and feed industries, and researchers interested in the control of aflatoxin contamination in food crops. The breadth of research on *A. flavus* and control of aflatoxin contamination can be seen at www.Aspergillusflavus.org. To ensure a close linkage with the *A. flavus* community and with the larger fungal community, a nine member Steering Committee will aid in the oversight of the project and facilitate the dispersal of information gained from this study. The members of the steering committee are: Gary Payne, Ralph Dean, Jiujiang Yu, William Nierman, Nancy Keller, Heather Wilkinson, Charles Woloshuk, Gregory May, and Joan Bennett. Drs. T. E. Cleveland and Deepak Bhatnagar, who established the *A. flavus* ESTs sequencing project, will work closely with the Steering Committee.

Once this project is completed, the *A. flavus* genome will be completely characterized, which should reveal vulnerability in critical genetic processes in the fungus that could be interrupted to control aflatoxin contamination, which causes hundreds of millions of dollars in crop losses during years of severe outbreaks. The available sequence will also have a broader impact by aiding in our understanding of the evolution of mycotoxin production and the identification of fitness and pathogenicity factors in mycotoxin-producing fungi.

Targeting Stress-Response Genes for Control of Mycotoxin Biosynthesis in *Aspergillus*

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Extremely low levels (1-2 ppb) of aflatoxin contamination can result in rejection of agricultural commodities for human or animal consumption, especially with major importers of U. S. peanut, cottonseed, corn, fig and tree nut products. In view of progressively tightening restrictive thresholds on contamination levels, to avoid significant economic losses it is paramount that methods are developed to prevent even slight levels of aflatoxin contamination in these products.

We are targeting certain stress-response genes in aflatoxigenic aspergilli as an approach to developing methods for eliminating aflatoxin biosynthesis. These genes appear to play a significant role for inducing biosynthesis of aflatoxin. For instance, our research unit recently discovered constituents in hydrolyzable tannins that completely shutdown aflatoxin biosynthesis. The most active constituent is gallic acid. To study the mode of action of gallic acid's anti-aflatoxigenic activity we began to examine specific genes involved in stress-responses of fungi. By using deletion mutants of *Saccharomyces cerevisiae*, as a model system, we found the mode of action of gallic acid appears to be as an antioxidant. For example, the negative effects on yeasts lacking the antioxidative stress gene *cta1* exposed to hydrogen peroxide were reversed when the same mutants were treated with gallic acid. This finding suggests gallic acid may be countering oxidative stress-response induced biosynthesis of aflatoxin in *Aspergillus*. Examination of another deletion mutant lacking the signal transduction gene *sho1,* which induces downstream expression of at least four antioxidative stress genes, showed similar results as to those of the *cta1△* mutant. These findings have encouraged us to identify other stress-response genes upstream from the gene cluster of the aflatoxin biosynthetic pathway. Such stress-response genes may be responsible for signal transduction, inducing upregulation of transcription factor(s) further downstream that, in turn, initiate aflatoxin biosynthesis.

In a further effort to discover genes in mycotoxigenic fungi involved in induction of toxin biosynthesis, we are searching the new bioinformatics EST database of *A. flavus* developed by the Food and Feed Safety Research Unit, USDA-ARS, Southern Regional Research Center, New Orleans, LA. Based on results using the model yeast system, described above, we have identified orthologs of yeast MAP kinase pathway and antioxidative stress-response genes in the *A. flavus* EST database. In order to study the *A. flavus* orthologs directly, we are developing a vector system wherein these genes are recombined in the yeast cell. The system includes a vector having an origin of replication, selectable markers, a yeast centromere and cloning sites for insertion of exogenous DNA. We see this new fungal functional genomics system as being a complement to approaches involving microarray analysis. Our approach, like microarrays, is a high throughput analysis, but can rapidly screen individual, specifically targeted genes.

Molecular Characterization of Resistant Mechanisms to Aflatoxin Contamination and Generation of ESTs and Microarray Chips for Analysis of Gene Expression in Peanut

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Cultivated peanut exhibits a considerable amount of variability for morphological traits and for resistance to insects and diseases. However, previous research with molecular markers has detected little variation at the DNA level. Gene discovery and marker development are needed in cultivated peanut. The development of new technology using EST (expressed sequence tag) and EST-derived SSR (simple sequence repeat) should enable research to finally measure and exploit variation at the DNA level in the cultivated peanut species. We have generated about 2000 ESTs from two cDNA libraries of peanut, immature pods of A13 (tolerant to drought stress and preharvest aflatoxin contamination) and leaf tissues of peanut line C34-24 (resistant to leaf spots and tomato spotted wilt virus). This was the first report on peanut EST generation and functional genomics, and 1345 ESTs have been released to GenBank (CD037499 to CD038843) and produced microarray chips for gene expression analysis. We have successfully used these ESTs for gene identification and EST-derived SSR marker development.

In the laboratory screening of the germplasm from China and India, the differences of peanut kernel infection by *Aspergillus flavus* were significant. Several Chinese and Indian lines had lower *A. flavus* colonization than Georgia Green. Evidence supports that trypsin inhibitor is associated with the resistance in the laboratory, and the concentration and activity of TI in resistant genotypes were higher than in susceptible genotypes. The induction of chitinase and *β*-1-3-glucanase by infection were evidenced in resistant more than in susceptible genotypes, and the roles of chitinase and *β*-1-3-glucanase were possible in association with the resistance/susceptibility to aflatoxin formation in peanut.

PANEL DISCUSSION: 3rd Annual Fungal Genomics Workshop

Panel Chair: Deepak Bhatnagar

Panel Members: Thomas Cleveland, Gary Payne, Jiujiang Yu, Bill Nierman, Bruce Campbell, and Baozhu Guo

It was evident from the presentations that significant progress has been made since the first workshop held two years ago in Phoenix. At that time two *Aspergillus* EST projects (one by Gary Payne and another by Nancy Keller) and one on *Fusarium* by Charles Woloshuk were underway, and the two large scale ARS EST projects (on *Aspergillus flavus* and *Fusarium verticilliocides)* were just getting started in collaboration with The Institute for Genomics Research. All these EST projects are now nearing completion and the volumes and volumes of data generated on the genetics of *F. verticilliodes* and *A. flavus* are expected to be utilized shortly through the use of microarrays.

In addition to these projects, as Gary Payne pointed out, now funds are available to carry out the whole genome sequencing of *A. flavus* (not yet on *F. verticillioides*). In addition to sequencing projects of interest to the group here, a number of other whole genome sequencing projects on *F. graminearum*, *A. niger, A. nidulans, A. oryzae*, *A. terreus* and *A. fumigatus* are nearing completion.

So, what does all this mean in controlling toxin formation:

(1)   From Bill Nierman's talk we have learned that we will have a better understanding of how genes become functional in the fungi of our interest. This will be important as we look at the cascades of regulatory processes governing both toxin synthesis and fungal development.

(2)   It was evident from Jiujiang Yu's talk that there are numerous regulatory factors that could potentially be targeted to control toxin formation or fungal development, especially the ones involved in giving the fungus the ability to invade crops. (Charles Woloshuk, later in the morning, talked about what some of these factors may be doing in fumonisin biosynthesis as well.)

(3)   Another use of the information derived from genomics will be to measure "cause and effect." When we identify a natural product that inhibits either toxin production or fungal growth, we will be able to rapidly identify at the molecular level what is causing this inhibition, much like what Bruce Campbell was able to show in the case of gallic acid. Similarly, we could possibly analyze why a resistant crop variety in "resistant." In addition to all this, Peter Cotty and Kenneth Ehrlich will use the genomics information to understand fungal ecology, e.g. how do atoxigenic strains survive in the field and what makes a particular strain more aggressive than its cousins in the field. By comparison between *A. flavus* and *A. fumigatus* genomics we will be able to figure out why one (*A. flavus*) poses an agricultural problem and the other one is a human pathogen. And such information will be very valuable to mycologists such as Bruce Horn.

(4)   By using genomics information, we could enhance our ability to identify mycotoxigenic fungi, as Pat Dowd indicated in his presentation during the Fumonisin Workshop.

(5)   Finally, we will be able to better understand the host-pathogen interactions (as Ed Cleveland indicated in his talk). Once we have microarrays available for all the crops, similar to the one Baozhu Guo talked about for peanuts, then, along with the fungal microarrays, we can very rapidly study the complex interactions between the crop-host and the fungus. We will be able to use these observations to even predict what would happen to these interactions during changes in microclimate or macroclimate of the fungus.

In other words, when the potential of the genomics tools is fully utilized, we should be well on our way to solving the mycotoxin contamination problems.

The other items that were raised during discussion included the genomics issues for the future:

(1)   Specifics for the use of the *A. flavus* and *F. verticilliodes* EST information on understanding gene function in these fungi were discussed. A comparison of the *A. flavus* and *A. oryzae* genomic databases (at ARS/SRRC) could provide information on the traits in *A. flavus* that allow it to survive in the field and infect crops. Similarly, a comparison of the *F. verticillioides* EST collection against the *F. graminearum* genome sequence (at ARS/NCAUR) could be helpful in understanding plant-fungal interactions.

A more detailed discussion centered on which fungal genome should be the next one to be sequenced (of interest to this group, now that *A. flavus* and *F. graminearum* genome sequencing projects have been fully funded). *F. verticillioides* was the unanimous (and obvious) choice as the next high priority candidate for genome sequencing. Charles Woloshuk informed the group that a proposal entitled "Comparative Genomics of Plant Pathogenic *Fusarium* Species" will be submitted (Dec. 15, 2003) to the Microbial Genome Sequencing Program sponsored by NSF and USDA. The proposal requests funds to sequence the genomes of *F. verticillioides* and *F. oxysporum*. The overall goals will be to compare the sequences of these two fungi and the already available genome of *F. graminearum* to address questions about species evolution, pathogenicity, and secondary metabolism (mycotoxin biosynthesis). The proposal is being submitted by the Center for Genome Research/MIT in Cambridge, MA, with representative members of the *F. oxysporum* and *F. verticilliodes* research communities.

4th Annual Fumonisin Elimination Workshop

Moderator – Gerald Donaldson, Texas Corn Producers Board

Insect Management for Reduction of Mycotoxins FY 2003 Report

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Predictive Monitoring: A sampling technique was developed to augment predictions made by a computer program used to forecast mycotoxin presence in Midwest corn. Samples of different tissues taken from corn plants during 2000-2003 and analyzed using PCR, indicated material that accumulated in leaf axils (primarily anthers and pollen) was a good source of material to detect the presence of *Fusarium* fungi potentially involved in mycotoxin production. There was considerable variability from field to field and year to year. In both 2002 and 2003, *F. proliferatum* and *F. verticillioides* were relatively uncommon compared to *F. subglutinans*. Visible moldiness of leaf axil material was a relatively good indicator of the presence of *Fusarium spp.* fungi. Conversion of the predictive computer program from a DOS to Windows version has been completed. An additional portion to help farmers make economic decisions on treating for pests or early harvest is under development. Values predicted for fumonisin for 2002 (a relatively hot, wet year during the growing season in Central Illinois) were relatively low compared to actual field values. Collected data was used to adjust the program without adversely affecting predictions for prior years, which had been fairly accurate. Although low levels of aflatoxin were predicted for the Central Illinois area, no aflatoxin was detected in fields surveyed. However, some grain loads were rejected by elevators in the area near the highest predicted values. When weather data from the areas where high levels of aflatoxin occurred in 2002 were used in the program, predictions of over 100 ppm resulted in some cases. In 2003, no aflatoxin was predicted and fumonisin levels were predicted to be low in general (< 2 ppm). Analysis is pending. In the third year of a study on trap types and pheromone sources in Bt vs. non-Bt sweet corn, a commercial source of traps was found to be as effective as prior traps developed by USDA. Also, a commercial source of the dusky sap beetle pheromone was found to be as effective as USDA-produced material. Thus, effective commercial materials are now completely available for monitoring the dusky sap beetle (which is also associated with mycotoxin problems).

Insect Resistance/Plant Transformation: Lines of tobacco that produce an activated form of the maize RIP have now had plants identified that appear to have the gene in homozygous state. This will allow crosses with other plants having different resistance genes (tobacco anionic peroxidase) to determine gene combination effects. Plant allelochemicals responsible for insect resistance in silks of Tex6 are still being determined, as are insect resistance chemicals produced in high peroxidase plants. Constructs with maize peroxidase clones potentially involved in fungal resistance based on pIs similar to isozymes associated with resistant material have been produced and await expression in maize lines for evaluation. A construct incorporating a gene coding for an analog of a novel protein previously demonstrated to be toxic to caterpillars is being introduced into maize for evaluation. Plants containing a mutated regulatory gene introduced transgenically that allows for accumulation of phenylpropanoids and other resistance materials appears to be more resistant to some insect feeding, such as by fall armyworms, but has lower productivity than wild-type plants. Floral tissue from a mutant plant line was found to cause high mortality to cabbage loopers and corn earworms, and to significantly reduce feeding and growth of fall armyworms. Responsible bioactive compounds from the floral tissue are being evaluated. Constructs produced from different transposition sources designed to increase precision of gene insertion have been produced and are being evaluated.

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A Survey of Fumonisins in Maize in the Highlands and Lowlands of Guatemala 2000-2002

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Maize samples were collected from highlands (> 1700 m) and lowlands (< 360 m) of Guatemala in 2000 to 2002. Samples were analyzed for fumonisin B1 (FB1) by high performance liquid chromatography (HPLC) in 2000 and 2001 and by LC ion trap mass spectrometry (LCMSMS) in 2002. The detection limit for HPLC and LCMSMS methods were 0.3 ppm and 0.05 ppm, respectively. The LCMSMS method also detected FB2 and FB3. Samples in 2002 that had detectable FB1 but were < 0.3 ppm were assigned a value of 0 ppm. The mean FB1 level (2000 to 2002) in maize from the lowlands (1.2 ±0.3 ppm, n=205) was significantly higher than the maize from the highlands (0.26 ±0.18 ppm, n = 142). The incidence of FB1 positive samples was significantly greater in the lowland maize (109/205) compared to that from the highlands (13/142). In the maize from the highlands, 9% of the samples contained ≥0.3 ppm FB1 with the highest being 7.3 ppm, whereas, in the lowland samples 53% were ≥0.3 ppm FB1 and 2.4 % were ≥10 ppm with one sample being 21 ppm. Analysis of the samples from 2002 by LCMSMS revealed that most FB1 positive samples contained FB2 and FB3 at a ratio of 1:0.4:0.3. In addition, approximately 92% (104/113) of the samples from the lowlands in 2002 contained detectable levels of FB1, whereas, all but 5 (5/92) of the samples from the highlands contained no detectable 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 (PMTDI) recommended by the WHO/FAO Joint Expert Committee on Food Additives (2 μg total fumonisins/kg bw) with 52% of the maize samples (24%=2.6 μg total fumonisins/kg bw/day; 20% = 7.8 μg total fumonisins/kg bw/day; 6%=25.2 μg total fumonisins/kg bw/day; 2% = 57.7 μg total fumonisins/kg bw/day). However, even the highest estimated intake is below the no observable adverse effect level (200 μg fumonisin B1/kg bw/day based on renal toxicity in rat) upon which the PMTDI was calculated (support: USDA FAS grant X01-4510-62-751071-4; ILSI NA Technical Committee on Food Toxicology and Safety Assessment).

Field Performance of Corn Grown from *Fusarium verticillioides*-inoculated Seed

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Fusarium verticillioides is an important fungus occupying dual roles in the corn plant. The fungus functions as an endophyte, a fungal/host interaction beneficial to the growth of some plants. At other times, the fungus may function as a mycotoxin-producing pathogen. The advantages and/or disadvantages of the endophytic relationship must be established in order to target the level for controlling diseases and mycotoxins. One possibility could be to ensure seed corn is fungal free prior to planting; however, corn and *F. verticillioides* may exist in a symbiotic, mutualistic relationship until external abiotic and/or biotic factors impinge on the stability of the relationship. Targeting seed borne infections as a control site can not be designated until the advantages and/or disadvantages of such a corn-*F. verticillioides* interaction is evaluated under field conditions.

Reciprocal inoculations were made with two isolates of *F. verticillioides*, RRC 374 and RRC PAT, on two corn genotypes, Silver Queen and GT-MAS:gk. The former genotype is a commercial hybrid sweet corn and the latter an experimental dent corn resistant to aflatoxin production by *Aspergillus flavus*. Experiments were conducted during the growing seasons of 1997, 1998, and 1999 in irrigated sites at the Georgia Coastal Plain Experiment Station, Tifton, Ga.

Plant growth was compared among years, between corn genotypes within each year, and among plants grown from non-inoculated and *F. verticillioides*-inoculated seed. Significant differences in growth occurred among the three years of the study and between the two corn genotypes. However, no consistent significant differences occurred among mature plants based on different seed inoculations. Consequently, seed borne *F. verticillioides* infections were not detrimental to the performance of the corn plant under field conditions existing during this study. We propose that the first line of defense for preventing *F. verticillioides* disease and mycotoxin production should focus on the corn plant by ensuring proper growth conditions and adequate storage conditions for plant products.

Identifying QTLs for Fumonisin Accumulation and Ear Rot Resistance in Maize

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Maize inbred lines GE440 and NC300 were identified in preliminary studies as potential sources for resistance to fumonisin accumulation and *Fusarium* ear and kernel rot. Two mapping populations, GE440 x FR1064 and NC300 x B104, were created to identify the loci associated with ear rot and fumonisin accumulation resistance. Our research is focused on answering two key questions: (1) Do some QTLs (Quantitative Trait Loci) for ear rot resistance also confer resistance to fumonisin accumulation? and (2) Are QTLs consistent across populations?

In 2002, the GE440 x FR1064 population was grown at Mt. Olive, NC, and Haubstadt, IL, and the NC300 x B104 population was grown at Clayton, NC. In 2003, both the GE440 x FR1064 population and the NC300 x B104 population were grown at Plymouth, NC, and Clayton, NC. Populations were replicated twice both years at all locations. Primary ears were inoculated with a mixture of three isolates each of *F. verticillioides* and *F. proliferatum*. Inoculated ears were rated for the percentage of kernels rotted. The grain was then ground, bulked by plot, and evaluated for fumonisin concentration using ELISA.

The GE440 x FR1064 segregating progeny differed significantly for ear rot, with a heritability of 0.66. Genotypic effects were significant for both ear rot resistance ($p < 0.0001$) and for fumonisin accumulation resistance ($p < 0.0001$) across locations. G x E interaction was not significant for fumonisin accumulation resistance ($p = 0.0591$), but was significant for ear rot resistance ($p < 0.0001$). Heritability on a family mean basis for fumonisin accumulation resistance was estimated as $h^2 = 0.34$. The NC300 x B104 population is segregating for both traits, with genotype being significant for both ear rot resistance ($p < 0.0001$) and for fumonisin accumulation resistance ($p < 0.0001$). Heritability on a line-mean basis for fumonisin accumulation resistance was estimated as $h^2 = 0.70$.

SSR markers are being used to fingerprint both populations. To date we have identified five potential QTLs for ear rot resistance and two potential QTLs for fumonisin accumulation resistance in the GE440 x FR1064 population. Our results suggest that QTL mapping for both ear rot resistance and fumonisin accumulation resistance should be possible, since significant variation was observed in both populations. After our mapping project is complete, we hope to be able to associate QTLs with either ear rot resistance or fumonisin accumulation resistance. We will also determine if some QTLs for ear rot resistance also confer resistance to fumonisin accumulation and whether QTLs are consistent across populations.

Microarray Analysis Reveals Genes with Similar Expression as *FUM* Genes in *Fusarium verticillioides*

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Studies on the *FCCl* gene of *Fusarium verticillioides* have linked the regulation of FB_1 biosynthesis with pH. By growing the wild type and *FCC1* mutant at pH 3 and pH 8, two major phenotypic differences (FB_1 and conidiation) clearly distinguish the two strains. Specifically, the wild type produces FB_1 only at pH 3 but conidiates under both pH conditions, and the *FCC1* mutant produces detectable FB_1 and abundant conidia only at pH 3. *FCC1 is* similar to type-C cyclin UME3 in *Saccharomyces cerevisiae,* which pairs with the kinase UME5 to phosphorylate the C-terminal domain of the RNA polymerase II. UME3 is reported to regulate the expression of genes involved in carbon metabolism, filamentous growth, meiotic development, amino acid and purine biosynthesis, and stress responses. The phenotype of the *FCCl* mutant indicates that FCCl is involved in both development (conidiation) and secondary metabolism (fumonisin biosynthesis).

To identify genes with similar expression patterns to fumonisin biosynthetic *(FUM* genes, a microarray of 733 DNA sequences was probed with RNA isolated from the wild type and *FCC1* mutant. Experiments included a comparison of gene expression in the wild type and *FCC1* mutant when grown on maize kernels and at two pH conditions (pH 3 and 8). In the maize experiment, 67 ESTs were found to be significantly different by ANOVA. Sixteen out of the 17 *FUM* ESTs on the array were among this group. Statistical analysis of data obtained from microarrays probed with RNA isolated from the two fungal strains grown at pH 3 and pH 8 indicated that 15 ESTs that represent eight *FUM* genes were expressed differentially. Also, 19 ESTs were found to exhibit the same expression pattern as these eight *FUM* genes; namely, greater expression in the wild type than the *FCC1* mutant at pH 3, greater expression in the wild type at pH 3 than the wild type at pH 8, and greater expression in the *FCC1* mutant at pH 3 than in the *FCC1* mutant at pH 8. Among this group were six ESTs representing genes with known functions.

The study has provided us a list of potential candidate genes for future studies to determine their functional roles in fumonisin biosynthesis. The results of this study also illustrate the effect of *FCC1* on transcription under different growth environments.

The Effects of Cooking on Fumonisins: Overview and Recent Studies

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Fumonisins are mycotoxins found in corn and corn-based foods. Nixtamalization (cooking in alkaline water) extracts fumonisins from the corn, and other cooking methods have been found to variably reduce fumonisin concentrations in foods. However, the extent to which fumonisin-matrix binding or the formation of unknown, but biologically active fumonisin derivatives occurs in cooked products is not known. If novel or matrix-bound fumonisins are present in significant amounts, then routine chemical analysis might underestimate fumonisin concentrations in and toxicity of cooked food products. Bioassay is an approach for investigating the effect of cooking or other chemical/physical treatments on the fate of fumonisins in food matrices that circumvents analytical uncertainty.

Corn, masa made from the corn by nixtamalization, and tortilla chips made from the masa were extracted (5 grams each, extracted 3 times with 50 ml acetonitrile/water 1:1), and the extracts combined, dried, and redissolved in 1 ml DMSO. Vero cells were grown to confluence in 1 ml wells and then exposed to the redissolved corn, masa, and tortilla chip extracts (4 μl/ml medium) for 48 hr. The corn extract inhibited ceramide synthase activity (a fumonisin-specific effect) as shown by a significant increase in the amount of the enzyme's sphingoid base substrate sphinganine (Sa) (mean=132 pmole/well) and the increase in the ratio of sphinganine to sphingosine (Sa/So) in the cells. In contrast, the amount of Sa found in the cells dosed with the masa, baked tortilla chip or fried tortilla chip extracts (mean Sa=14-24 pmole/well) remained low, as did Sa/So (0.17-0.28). Fumonisin B_1 concentrations (HPLC analysis of the extracts) of the masa and chips (3.6-6.7 μg/g) were likewise reduced 80-90% compared to the corn (32 μg/g).

In a second experiment, baked cornbread, pan-fried corn cakes, and deep-fried corn fritters were made from cornmeal that had been spiked (5% w/w) with *Fusarium verticillioides* culture material. The cooked products were then mixed with rodent chow and fed to male rats (n=5) for 2 weeks at high (20% w/w spiked cornmeal equivalents = 1% w/w culture material equivalents) or low (2% spiked cornmeal equivalents) doses. Positive control groups were fed diets containing 20% or 2% w/w of the uncooked spiked cornmeal and a negative control group was fed a diet to which sound corn only was added. Toxic response to the baked cornbread, pan-fried corn cake, deep-fried fritter and positive control diets was similar and characterized by decreased body weight gain (high-dose only), decreased kidney weights, and the apoptotic kidney and liver lesions typically caused by fumonisins in rats. Fumonisin concentrations (fumonisin B_{1+2}) in the high-dose pan-fried corn cake diet (92 ppm) was 30% lower than those of the high-dose baked cornbread (132 ppm), deep-fried fritter (120 ppm) and positive control (131 ppm) diets.

Taken together, the results indicate that nixtamalization of corn reduces both the fumonisin concentration and fumonisin-like biological activity of the masa product and that baking and frying had no significant effect on the biological activity of fumonisins in corn-based foods. These experiments also provided no indication that unknown, biologically active fumonisins were formed during cooking.

Genetic Engineering of Polyketide Biosynthesis in *Fusarium verticilliodes*

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Filamentous fungi are rich sources for polyketide natural products including valuable medicines. These products are synthesized by iterative modular polyketide synthases (PKSs). It remains unclear how the single-module PKSs control the highly diverse product structure. The objective of the experiments is to establish a model system for studying the biosynthetic mechanism of fungal polyketides. We have taken a genetic approach to manipulate several PKS genes involved in the biosynthesis of mycotoxins. These PKSs have a similar size and almost identical domain arrangement, yet synthesize polyketides with a chain length varying from 4 to 42 carbons. A gene disruption followed by functional complementation using a heterologous domain/module could yield new products, which may subsequently reveal information on the biosynthesis mechanism of fungal PKSs. Through point-mutation and domain-replacement, we have generated mutants for two domains of FUM5, a PKS required for synthesizing the important food polluting mycotoxin fumonisins in *Fusarium verticillioides*. HPLC-ELSD and LC-ESMS analysis revealed that *Fusarium* mutated on the active site of methyltransferase domain of FUM5 produced demethylated fumonisins, demonstrating the feasibility of the genetic approach to engineering the biosynthesis of fungal polyketides. When the ketoacyl synthase domain of FUM5 was replaced by a heterologous domain from PKS1 required for T-toxin synthesis in *Cochliobolus heterostrophus,* the mutants produced fumonisins. Similar results were observed when the methyltransferase domain of FUM5 was replaced by the corresponding domain of PKS1. These results show that these domains are interchangeable and functionally complementary. The studies open a new approach to investigate the biosynthetic mechanism for these economically significant metabolites found in filamentous fungi.

Esterification of Tricarballylic Acid Side Chains to the Fumonisin Backbone Requires the Activity of Four *FUM* Genes

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The fungus *Fusarium verticillioides* can cause stalk and ear rot of maize. *F. verticillioides* can also contaminate maize with the polyketide derived mycotoxins called fumonisins. A number of animal diseases have been associated with ingestion of maize contaminated with fumonisins. Equine leukoencephalomalacia and porcine pulmonary edema have been correlated with fumonisin as well as cancer in laboratory animals. In an effort to reduce or eliminate fumonisins from food products, we are interested in understanding the molecular genetics required for the production of fumonisins by *F. verticillioides*.

Recently, a gene cluster comprised of 15-coregulated transcripts was described in *F. verticillioides*. Predicted amino acid sequences of the genes in the cluster reveal activities that could be required for fumonisin production. To determine the role in fumonisin production, if any, of each of the 15 *FUM* genes, we have disrupted or deleted each of them individually and have assessed the resulting mutants for fumonisin production.

Deletion of a number of genes has led to the characterization of fumonisin pathway intermediates. For example, *FUM3* which encodes a dioxygenase is required for the hydroxylation of carbon 5 of the fumonisin backbone. Similarly, *FUM13*, which encodes a short chain dehydrogenase/reductase, is required for the reduction of a keto group to a hydroxyl group on carbon 3 of the fumonisin backbone. Here we describe the affects of individual deletions in *FUM7*, *FUM10*, *FUM11* and *FUM14* of the *FUM* gene cluster.

Deletions in *FUM10* and *FUM14* resulted in the accumulation of hydrolyzed forms of fumonisin B$_3$ and fumonisin B$_4$. These hydrolyzed forms of fumonisin lack the tricarboxylic acid molecules usually attached to carbons 14 and 15 of the fumonisin backbone. Deletion of *FUM11* resulted in the accumulation of half-hydrolyzed forms of fumonisin B$_3$ and fumonisin B$_4$. Deletion of *FUM7* resulted in the accumulation of a fumonisin-like molecule with a molecular weight of 718. This compound is consistent with a molecule with additional carbon-carbon double bonds in the tricarboxylic acid portion of the molecule. Taken together, these results indicate that these four genes are all involved in the addition of the tricarballylic acid molecules.

Deletion analysis has allowed us to more fully understand the biosynthetic pathway leading to fumonisin production. We have been able to identify stable intermediates in the biosynthetic pathway, and in conjunction with the individual deletion mutants, we have been able to more precisely order the biosynthetic pathway and assign particular roles to specific *FUM* cluster genes.

PANEL DISCUSSION: 4th Annual Fumonisin Elimination Workshop

Panel Chair: Ron Riley

Panel Members: Pat Dowd, Ron Riley, Ida Yates, Leilani Robertson, Charles Woloshuk, Ken Voss, Xiangcheng Zhu and Robert Butchko

The 4th Fumonisin Elimination workshop was moderated by Gerald Donaldson of the Texas Corn Producers Board. There were eight presentations covering a wide variety of topics ranging from the development of novel methods for the detection and monitoring of toxigenic *Fusarium* species in developing corn to the efficacy of processing methods to reduce fumonisins in foods.

Summary of Presentations: The first speaker was Pat Dowd who reported on the use of a predictive molecular method for monitoring *Fusarium* in the field. The method was used to augment predictions made by a computer program for forecasting mycotoxin presence in Midwest corn. Ron Riley reported on the results of a three year survey of fumonisin levels in corn from the highlands (>1700 m) and lowlands (<360 m) in Guatemala. The results showed that the occurrence of fumonisins and *Fusarium verticillioides* in highland corn was quite rare, whereas, in corn from the lowlands they were both quite common. Ida Yates summarized the results of field studies comparing the effects of *F. verticilliodes* infection on the performance of two corn genotypes. The seed borne infection was not detrimental to the performance of either genotype. Leilani Robertson has identified two maize inbred lines as potential sources of resistance to both fumonisin accumulation and ear and kernel rot. Promising quantitative trait loci (QTLs) for ear rot resistance and resistance to fumonisin accumulation have been identified. Charles Woloshuk reported the results of microarray analysis in *F. verticillioides* that identified potential candidate genes having roles in regulating fumonisin biosynthesis under different environmental conditions. Ken Voss showed that preparation of corn-based foods using alkali processing (nixtamalization) reduces fumonisin in the final product, whereas, baking and frying have little effect. In addition, cooking does not increase the toxicity of the final products indicating that unknown, biologically active fumonisins were not produced during processing. Xiangcheng Zhu reported on recent investigations to develop a model system for studying the biosynthesis of fungal polyketides (like fumonisin). Two mutants have been identified for FUM5, a polyketide synthase in *F. verticillioides* and it was shown that domains from other fungi could be interchanged and the resulting genes retained functionality. Robert Butchko reported on studies of gene deletion studies to determine the function of FUM7, FUM10, FUM1 and FUM14 of the FUM gene cluster. It was found that all four genes were involved in the addition of the tricarballylic acid moieties to the fumonisin backbone.

Summary of Panel Discussion: Following the presentations there was the reading of a poem by Anne Desjardin paying homage to Ron Plattner, who recently retired from USDA-ARS after many, many years of productive work in the field of mycotoxicology. It was generally agreed that Ron's contributions to our understanding of fumonisins and other *Fusarium* toxins has been immense and that he will be sorely missed. Following the tribute to Ron, Charles Woloshuk and other panel members were asked to comment on the practical implications of their molecular work as it pertains to reducing fumonisins in corn. Charles pointed out that understanding the factors that regulate toxin production will lead to the identification of molecular targets in both the fungus and the plant that can be used to improve resistance to fumonisin accumulation and can also be used to predict what environmental factors are most important in regulating fumonisin production in the field. Robert Butchko and

Xiangcheng Zhu added several comments along the same line and pointed out that identification of the genetic basis of toxin production and resistance are key to developing control strategies and will also assist in developing molecular markers to be used in breeding for resistance. Don White asked Ken Voss and Ron Riley to discuss the current status of the studies suggesting a possible link between human neural tube defects (NTD) and fumonisin exposure in China. Ken briefly summarized recent studies and concluded that there is currently no convincing evidence that the fumonisins can cause NTD in humans; however, NTD have been produced in mice, and fumonisins can inhibit the transport and alter the processing of folate. Folate deficiency is a known risk factor for NTD in China and elsewhere. Leilani Robertson and Ida Yates were asked why the fumonisin levels are always high in corn grown in the southeastern US. There was no easy answer, but it was pointed out by Don White and others that maybe it was an artifact of the fact that corn is more intensely monitored in southern states, whereas, in the Midwest, the corn goes from the field to the elevator and is not analyzed until it reaches its final destination. Don White added that fumonisin seems to be more of a problem when environmental conditions of drought stress and high humidity come together at and after flowering. That type of environment may be found in isolated areas of the Midwest in some years; however, it is often found in the coastal areas, especially in the Southern states. Charles Woloshuk raised the question about the correlation between ear and kernel rot and fumonisin levels. Leilani commented that in North Carolina you could have 30 ppm FB_1 and no evidence of rot. Charles expressed skepticism and Anne Desjardin agreed. Don White supported Leilani's observation and added that the proof was in the fact that the growers rate the corn as clean and yet high levels of fumonisin can be found when analyzed. Gary Payne agreed with Don and stated that even when aflatoxins and fumonisins are high there are cases where there is no evidence of rot or symptoms and that that has been the case for many years! Drought conditions alone do not seem to favor high levels. The question of the correlation between the degree of rot and fumonisin levels was left unresolved. The discussion session ended with Pat Dowd's updating the current status of the predictive computer program that has been used successfully and is in the process of being converted to a more user-friendly format that will help farmers make decisions aimed at reducing aflatoxin and fumonisin levels in harvested corn in the Midwest.

Members of the Maize 12-Oxophytodienoic Acid Reductase Gene Family are Induced by *Fusarium verticillioides*

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12-oxo-phytodienoic acid reductases (*OPR*s), enzymes of the biosynthetic pathway which convert linolenic acid to jasmonic acid, belong to a family of flavin-dependent oxidoreductases. *OPR*s control the flow of metabolites from the 18-carbon group of α-linolenic acid derivative 12-oxophytodienoic acid to the precursor of the natural jasmonic acid. Jasmonic acid has been demonstrated to inhibit aflatoxin biosynthesis in *Aspergillus* spp. Previous study by a microarray RNA profiling technique showed that maize transcripts of certain *OPR* genes are induced by *Fusarium verticillioides* spores. To further our understanding of the function of maize *OPR*s (*ZmOPR*s) in response to infection with *F. verticillioides*, we identified and cloned four maize *OPR* cDNAs. Northern blotting analysis showed that expression of *ZmOPR* genes is differentially regulated in leaves by defense-related signaling molecules such as jasmonic acid, salicylic acid, abscisic acid, and ethylene. *ZmOPR1* or/and *2* are inducible by salicylic acid and not by jasmonic acid, ethylene or abscisic acid. In contrast, *ZmOPR*3 transcripts accumulated after treatment with all the hormones tested as well as in response to wounding. Accumulation of *ZmOPR1* or/and *2* mRNAs was strongly induced in both cell suspension cultures and in silks in response to infection with *F. verticillioides* spores. Furthermore, *ZmOPR1* or/and *2* transcript levels were induced in silks of an inbred line resistant to *Fusarium* ear rot but not in susceptible lines. These data suggest that these *OPR* genes may be involved in resistance-associated defense responses.

Discontinuous Distribution of Fumonisin Biosynthetic Genes in the *Gibberella fujikuroi* Species Complex

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Fumonisins are carcinogenic mycotoxins that frequently contaminate maize. Most studies on fumonisin production have focused on *Fusarium verticillioides* because of the widespread occurrence of this fungus on maize. However, production has also been reported in several other *Fusarium* species in the *Gibberella fujikuroi* (*Gf*) complex and in *F. oxysporum*. The *Gf* complex consists of at least 29 closely related *Fusarium* species and includes several important plant pathogens and mycotoxin producers, including *F. circinantum*, *F. proliferatum* and *F. subglutinans*. Recently, a fumonisin biosynthetic gene (*FUM*) cluster was identified in *F. verticillioides*. In the current study, we surveyed 27 species of *Fusarium* for the presence of four *FUM* cluster genes and for fumonisin production.

Strains of each species were subjected to Southern blot analysis with hybridization probes prepared from fragments of the *FUM1*, *FUM8*, *FUM12* and *FUM19* genes. These four genes are distributed across the entire length of the *FUM* cluster and they all function in fumonisin biosynthesis. Among species within the *Gf* complex, *FUM* genes were detected only in *F. anthophilum*, *F. fujikuroi*, *F. globosum*, *F. proliferatum*, *F. nygamai*, and *F. verticillioides*. These six species include at least one member of each of the three major clades delineated by O'Donnell et al. (1998, *Mycologia* 90:465-493) in the *Gf* complex. Fumonisin production was also detected in these same species except for *F. anthophilum*. Among the species outside the *Gf* complex, *FUM* genes and fumonisin production were detected only in *F. oxysporum* strain O-1890.

We also conducted a phylogenetic analysis using nucleotide sequences from the *FUM1* and *FUM8* coding regions from the 7 species with the genes. The relationships inferred from this analysis were similar to those inferred from previous analyses of the *Gf* complex that used the 28S rDNA, mitochodrial small subunit rDNA and β-tubulin genes (O'Donnell et al. 1998, *Mycologia* 90:465-493). However, unlike the previous analyses the *FUM1*/*FUM8* analysis indicated that species in the Asian and American clades of the complex were more closely related to one another than either was to the African clade. The analyses by O'Donnell et al. indicate that the Asian and African clades are more closely related to each other than either is to the American clade.

The results of this study indicate that *FUM* genes are discontinuously distributed in the *Gf* complex. The detection of *FUM* genes only in species known to produce fumonisins indicates the inability of some species within the *Gf* complex to produce fumonisins results from the absence of *FUM* genes rather than the presence of genes that are either not functional or not expressed. Similarities in phylogenetic relationships inferred from *FUM1*/*FUM8* and previous analyses suggest that the discontinuous distribution of *FUM* genes in the *Gf* complex arose, at least in part, via differential inheritance of the genes as species evolved. The presence of *FUM* genes in only 7 of the 27 species examined suggests that the potential to produce fumonisins is limited to relatively few species.

Evaluation of White Food-grade Dent Corn Hybrids for *Fusarium* Ear Rot and Fumonisin Concentration

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Fusarium ear rot of corn caused by *Fusarium verticillioides* (syn. = *F. moniliforme*) and *F. proliferatum* is of concern due to the production of fumonisin mycotoxins. In 2002, 30 commercial white and 3 yellow food-grade dent corn hybrids were evaluated for *Fusarium* ear rot and fumonisin concentration in grain. Ears were inoculated by injecting a spore suspension down the silk channel and into the side of the ear one and two weeks after pollination. A competitive direct ELISA was used to determine fumonisin concentration. Hybrids differed significantly for both fumonisin concentration and *Fusarium* ear rot (P < 0.0001). Spearman's rank correlation for fumonisin concentration and *Fusarium* ear rot was r = 0.54 (P = 0.0009). Fumonisin concentration of individual hybrids, averaged over replications, ranged from 4.7 to 59.6 ppm. White and yellow food-grade dent corn hybrids had average fumonisin concentrations of 23.6 and 19 ppm, respectively. Both the white and yellow food-grade dent corn hybrids had an average of 6% of the ear rotted.

Evaluation of Two Seed Treatments for Control of Fumonisin in Corn Grain

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Fusarium ear rot of corn caused by *Fusarium verticillioides* (syn. = *F. moniliforme*) and *F. proliferatum* is of concern due to the production of fumonisin. The objective of this study was to determine the effect of two seed treatments on fumonisin concentration in corn grain. Seven commercial hybrids were planted in 12 locations throughout the U.S. (Illinois (4), Texas (3), Mississippi (2), Indiana, Iowa, and North Carolina) in 2002. The experimental design was a split plot with three replicates with hybrids as main plots and seed treatments as subplots. The seed treatments were Captan 400 @ 1.76 fl oz/cwt + Allegiance @ 0.1 fl oz/cwt, GB83 @ 7.1 oz/cwt (commercial formulation of *Bacillus mojavensis*, RRC 101 (ATCC 55732) Patent No. 5,999,117 by C.W. Bacon and D.M. Hinton, 1999), and a nontreated control. Seed treatments were applied by Gustafson LLC. Locations, hybrids, and the location by hybrid interaction were all significantly different for fumonisin. Mean concentration of fumonisin across all hybrids was greater than 4 ppm in five locations with a range of 0.5 ppm in Texas to 34.6 ppm in North Carolina. Neither seed treatment affected fumonisin concentration.

16th Annual Aflatoxin Elimination Workshop

Session 1:

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

Moderator: Scott Averhoff, National Corn Growers Association

Atoxigenicity in *Aspergillus flavus* Induced by the Gallic Acid Moiety of Walnut Hydrolyzable Tannins

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

A. flavus NRRL 25347, originally isolated from pistachio, was shown to produce an extracellular tannase, capable of hydrolyzing the tannin to produce glucose, gallic acid, and ellagic acid. An *in vitro* time-course experiment of aflatoxin production by *A. flavus* grown on Vogel's media N (VMN) over 11 days showed that gallic acid was a potent inhibitor of aflatoxin at 0.2% incorporation, reducing levels to ca. 8% of control, whereas ellagic acid had little effect at the same level. Ellagic acid appeared to produce a slight time-lag in growth of the fungus which may account for the small reduction in aflatoxin production. Commercial tannic acid, a hydrolyzable tannin containing only gallic acid moieties, completely suppressed aflatoxin production at 0.4% incorporation.

Treatment of walnut seed coat tissue with anhydrous methanolic HCl yielded methyl gallate and ellagic acid, the levels of which could be measured by reverse phase HPLC. Gallic acid levels in seed coat of 'Tulare' and the variety 'Chico', which is susceptible to aflatoxin formation, were determined on a biweekly basis throughout the growing seasons 2002 and 2003. Levels in 'Tulare' were significantly higher, and were maintained throughout the growing season, whereas those in 'Chico' declined steadily as the nuts matured. At maturity, 'Tulare' had a gallic acid content 1.5-2 times higher than the level in 'Chico'.

Within a group of tree nut seed coats tested, gallic acid content correlated inversely with ability to produce aflatoxin. English walnut cultivars collected at maturity for the 2003 season had gallic acid levels ranging from 1.4-3.4% in the order: 'Chandler' < 'Chico' < 'Serr' < 'Payne' < 'Hartley' < 'Tehama' < 'Tulare' < 'Red Zinger'; the black walnut species *J. hindsii* 'Rawlins' and *J. nigra* 'Thomas' had levels of 1.0 and 1.1%, respectively. 'Kerman' pistachio seed coat had 0.5% gallic acid, but 'Nonpareil', 'Mission', and six other almond varieties, only had trace levels (<0.1%). The hydrolyzable tannins in pistachio are structurally similar to those in walnut, differing primarily in that they do not generate ellagic acid on hydrolysis; they therefore consist entirely of gallate esters of polyols.

The evidence indicates that hydrolyzable tannins have a potent effect on production of aflatoxin by *A. flavus*. In walnuts this atoxigenicity is phytochemically induced by biosynthesis and maintenance of high levels of tannins throughout the growing season. Gallic acid, produced *in situ* by a fungal tannase, is the specific tannin component responsible for suppression of aflatoxin biosynthesis by the fungus whereas the other common moiety, ellagic acid, has only a very minor effect. Gallic acid should be amenable to enhancement of levels by conventional breeding or genetic manipulation. Furthermore, this readily available natural product may reveal the role of aflatoxin in the ecology of *A. flavus* by providing a useful tool to study the effects of down-regulating specific genes in the biosynthetic pathway.

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

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

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

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

The first candidate gene involved in the early stages of GA biosynthesis, that is, DAHP synthase, has been cloned by RACE-PCR. DAHP synthase catalyzes the first reaction in the shikimic acid pathway and controls carbon flow towards the aromatic amino acids. Northern anaylsis was performed to determine the levels of DAHP synthase transcript accumulation over the 2002 growing season. An inverse correlation between DAHP synthase expression and gallic acid accumulation was detected.

We have also been investigating the dehydrogenase responsible for gallic acid synthesis. We have developed an enzyme activity assay that enables us to visualize gallic acid production on a native protein gel. The activity levels of the gallic acid synthesizing enzyme increase during the early stages of seed coat development and decline towards the end of the growing season (i.e. August through September). Importantly, these levels correlate with the levels of gallic acid accumulation detected in the Tulare seed coat. In order to determine the sequence of this gene, a cDNA library has been constructed from Tulare pellicle mRNA. Currently, forty thousand clones have been picked and archived. EST sequencing reactions have been initiated at 1000 clone intervals; ultimately 5000 clones will be sequenced from the library. Multiple sequence alignment and motif search analysis will be performed to identify candidate genes involved in GA metabolism.

Inhibitory Seed Proteins as Markers for Resistance to Aflatoxin Accumulation

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A goal of our research program is to identify compounds that are toxic to *Aspergillus flavus*, clone the genes encoding these compounds, and use DNA sequence data to develop markers that can be used in marker assisted breeding programs aimed at developing genotypes of corn with resistance to aflatoxin accumulation. Previous research from our labs led to the identification of two inhibitory compounds in corn inbred Tex6, one that inhibits fungal growth and one that inhibits aflatoxin production but not fungal growth. The growth-inhibiting compound was identified as a chitinase. Primers derived from a partial peptide sequence of the chitinase were used to clone the chitinase gene from Tex6. Two chitinases similar to chitA and chitB were cloned using the primer pairs. These sequences were used to select primers to amplify a 524 fragment for use as a probe in marker assisted breeding programs. We also have begun the initial purification of the aflatoxin biosynthesis inhibitor (ABI). This protein, which is present in mature corn seeds, has a molecular weight between 80,000 and 100,000 Daltons. Partial purification of the protein by gel filtration and DEAE chromatography yielded a preparation that inhibits aflatoxin biosynthesis at 48 ug/mL but that has no effect on fungal growth. This protein will be purified to homogeneity, partially sequenced, and the sequence used to develop DNA primers specific for the gene. Once the gene is cloned, DNA specific primers will be developed for use in a marker assisted breeding program.

Potential Use of Naphthalene Acetic Acid to Inhibit Sporulation of *Aspergillus parasiticus* and its Possible Mechanism of Action

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Previous studies have shown that 50mM of Naphthalene Acetic Acid (ANA) inhibited growth and aflatoxin production in *Aspergillus parasiticus* by 75 and 97%, respectively, while sporulation was stimulated around 300%. Higher concentrations (100mM) of the same compound caused total inhibition of growth and sporulation. The most interesting observation was realized when *A. parasiticus* spores were cultivated in potato dextrose agar amended with 100 mM concentrations of ANA. Spores only reached the swollen stage and were unable to form germ tubes. ANA was capable of arresting the germination process up to 12 hrs when it was added at different intervals of time. To find out the possible mechanism, the transcription of the flbA and aflR genes was evaluated. The transcription of both genes was blocked by ANA.

Interactions between *Aspergillus flavus* and Corn Earworm on Insect Resistant Maize

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Maize is a preferred host of the corn earworm, *Helicoverpa zea* Boddie, and is the primary ear feeding pest of the crop in the MS Delta. It is unknown if this insect is an important vector or only a facilitator of *Aspergillus flavus* infection in maize. Understanding the relationship between the fungus, insect, and plant may be useful when designing methods to reduce the production of aflatoxin in maize grain. The objectives for this study were to: 1) examine the association between corn earworm damage to maize ears and aflatoxin levels in corn earworm resistant and susceptible maize hybrids, and 2) to study the relationship between *A. flavus* and corn earworm. Corn earworm resistant hybrids and their susceptible isolines were grown at Stoneville, MS in 2001 and 2002. Four treatments, nested as sub-plots within each plot, were applied at maize silking. The treatments consisted of all combinations of spraying the silks with *A. flavus* and artificially infesting the silks with corn earworm. At plant maturity, entries were harvested and ppb aflatoxin was determined by using an ELISA. There was no interaction between the sprayed and sprayed-with-insects treatments ($F = 1.28$, df = 1,19, $P = 0.2719$) because a high percentage (72%, n = 89 ears in 2001 and 82%, n = 108 ears in 2002) of ears from the sprayed only treatment were infested with feral corn earworm, therefore, data from the sprayed and sprayed-with-insects treatments were combined. For both years, most insect resistant maize entries (not DK 626Bt) had less aflatoxin when compared to their susceptible isolines for the sprayed treatment. Quantification of insecticidal proteins and a silk diet bioassay demonstrated that DK 626Bt was not resistant to corn earworm. Results showed that growing corn earworm resistant hybrids reduced the incidence of aflatoxin in harvested maize grain.

These results indicate that corn earworm is associated with increased aflatoxin in maize, if we assume the insect resistance factors do not have anti-fungal properties. To test this assumption, insecticidal protein Cry1Ab was incorporated into PDA media and the media was then infested with *A. flavus.* Cry1Ab is expressed in maize genetically transformed with *Bacillus thuringiensis*. After three days, we found a reduction in fungal growth when the PDA media contained 4.0 and 20.0 ug of Cry1Ab/ml. Therefore, the reduction in aflatoxin levels we discovered in transgenic maize may be due, in part, to the antifungal properties of the insecticidal protein, Cry1Ab, expressed in the silks and developing seed. To understand if the insect plays a role in the fungal infection of maize seed, we quantified *A. flavus* from various insect tissues that were collected from larvae harvested from *A. flavus* sprayed maize ears. Larvae were dissected under sterile conditions and *A. flavus* propagules were determined on modified dichloran-rose bengal medium for the exterior of the insect (cuticle) and internal organs. There were significant differences among insect tissue entries for *A. flavus* ($F = 25.19$, df = 3,22, $P < 0.0001$). The foregut and hindgut had significantly more *A. flavus* propagules than either the cuticle or midgut. These results indicate that corn earworm larvae can potentially distribute a high infective dose of *A. flavus* to the maize ear as the larvae feed through the maize silk channel and on the developing seed. Future research will examine fungal development within the insect, mechanisms of insect vectoring of *A. flavus*, and anti-fungal properties of *Bt* maize seed.

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

Panel Chair: Gary A. Payne

Panel Members: R. J. Molyneux, R. M. Muir, D. Guzman-de-Peña, C. A. Abel

Four speakers in this session presented information on natural products that inhibit either growth or aflatoxin production by *Aspergillus flavus*. One speaker presented information of the effect of insect resistant maize on aflatoxin production. The natural compounds reported ranged from small molecular weight compounds such as gallic acid and naphthalene acetic acid to an 80 Kd protein. In the panel discussion the speakers were asked several questions. Below are the questions asked and the answers provided.

Q: The gallic acid is present in the seed coat. How much would need to be in the meat of the walnut?
A: There is essentially no gallic acid present in the kernel of the walnut without seed coat ("meat"). However, we have preliminary information that aflatoxin production is suppressed so long as the seed coat is present. There may be some sort of signal induced by *A. flavus* exposed to seed coat that affects all fungal growth in the vicinity. We are actively exploring this question.

Q: Are the differences in free levels of gallic acid in the different walnut lines due to changes in the activity of the esterase in the pathway?
A: We believe that the gallic acid levels in the different walnut lines are due to structural differences in the hydrolyzable tannins present. The initial product of tannin biosynthesis is pentagalloyl glucose, from which gallate ester moieties can be removed by hydrolysis or undergo oxidative dimerization leading to ellagic acid. A reduction in either of these processes would result in higher gallic acid levels from the action of the fungal esterase. The gallic acid levels would, therefore, seem to be amenable to manipulation by conventional breeding.

Q: Is the inhibition of aflatoxin by gallic acid reversible if gallic acid is removed?
A: This is a good question but one which we have not yet investigated, although we certainly plan to do so. I suspect that the inhibition will prove to be reversible.

Q: Have you found other compounds in walnut inhibitory to aflatoxin biosynthesis?
A: None that is as potent as gallic acid. We have previously published research (J. Agric. Food Chem. 2000, 48, 4418-4421) showing that some of the naphthoquinones present in walnut hulls can reduce aflatoxin levels at certain concentrations, although they also enhance aflatoxin production at other concentrations.

Q: Have you done any experiments inoculating different walnut varieties with *A. flavus* to test for the effect of gallic acid on resistance in the field?
A: No. We have considered this question but it is very difficult to design an experiment with appropriate controls. For example, it would be difficult to rehydrate the nuts in a consistent manner so that fungal growth could occur. Furthermore, it would be also be problematic to inoculate the nuts in shell in a sterile manner. The best "field" evidence of resistance is the fact that walnuts have much

lower levels of aflatoxin contamination than pistachios or almonds, which have little or no gallic acid in the seed coat.

Q: Does purple corn have higher gallic acid?
A: I cannot answer this. I believe that the purple color comes from anthocyanins, flavonoid-derived pigments. Therefore, there may be a certain amount of condensed tannins, i.e. gallate esters of flavonoids, which could yield some gallic acid by action of the esterase. I am not aware of any evidence of hydrolyzable tannins in corn.

Q: Did you look at the morphology of cultures treated with ANA (NAA)?
A: I did look at them. The cultures remained in the stage at which NAA is added. That is, if NAA is added in the germling stage the fungus will stay in the germling stage even if you incubate it for 5 days. Similarly, if the fungus is a 12 hour-colony it will retain that morphology. One thing that we did observe was that if the germlings were treated with NAA and incubated for 72 hrs they remained as tiny colonies that did not sporulate even if incubated in fresh media without NAA for 5 days.

Q: Does NAA act by delaying aflatoxin biosynthesis?
A: The compound inhibits aflatoxin synthesis. If you incubated the fungus for several days at a molarity of NAA that cannot be overcome by fungal growth, aflatoxin synthesis is inhibited.

Q: Did you test other synthetic hormones besides NAA?
A: Yes, we used Indole-3-butyric Acid, IBA potassium salt, and NAA (potassium salt).

Q: Why did you test NAA?
A: NAA is an auxin, and auxins are most active in young developing tissues, such as shoot meristems and young leaves and fruit. Because penetration of corn in the field occurs through the silks, we wanted to know if different hormone levels may favor fungal infection.

Q: Will NAA be stable if mixed in corn flour?
A: We have not tried corn flour but we have tried corn grain and ground corn grain.

Q: What is your thinking about NAA and possible applications?
A: It can be used to protect grains, nuts during storage or transport overseas to inhibit fungal growth, sporulation and decrease the risk of aflatoxin contamination. We have generated data with higher concentrations and we are evaluating the activity at these concentrations. Cinvestav is seeking legal registration.

Q: Did you look at the morphology of cultures treated with ABI?
A: The cultures were not obviously different; however, we are examining the cultures for more subtle effects.

Q: Does ABI act by delaying aflatoxin biosynthesis?
A: We have not yet looked at the effect of ABI on the kinetics of aflatoxin production.

Q: How does your chitinase's activity compare to that of other chitinases?
A: It is difficult to compare activities because different assays have been used. Extrapolations indicated that our chitinase is more active that Chit A.

Q: Were there any structural features that might help stabilize your chitinase?
A: Good point. We have not looked at this.

Q: There are several chitinases in maize. How does the one you identified compare with those?
A: Chit 1 shows a high degree of similarity to Chit A and Chit B.

Q: Have you tested the effect of Cry1b on aflatoxin production in the fungus?
A: No, but this should be done.

Q: What was the source of Cry1Ab? Was it purified or culture filtrate? What might be its mechanism of inhibiting fungal growth?
A: The Cry1Ab was purified from a culture of Cry1Ab-transformed *E. coli*.

Q: Does a transgenic approach to increase expression of an endogenous compound have to go through the same approval process as a foreign gene?
A: I'm not sure. It seems logical that if you are amplifying the expression of a trait or turn on genes that would help promote the expression of a trait, I don't believe there would be the same restrictions as introducing a foreign gene into a genome. I believe it would be similar to breeders who use mutation to develop a trait, e.g. "low-satsoy" soybean.

Q: When you dissected the corn earworms fed on silks did you find any that had *A. flavus* in the organs that were fed uninoculated silks?
A: I did not dissect any corn earworms from uninoculated silks during my study. Last summer (2003), I collected feral corn earworm larvae from corn ears in the MS Delta and positively identified *A. flavus* in the GI tract of 26% of the larvae (n= 100). I may do more work on this in the future looking at the fungus on feral adult and larval corn earworms.

Q: How far do the insects migrate?
A: It is commonly believed that the majority of our corn earworms migrate from Mexico, Texas, and Louisiana. There is a population that overwinters locally and infests corn but this number is believed to be small.

Inhibition of Aflatoxin Biosynthesis by Gallic Acid

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Tree nut research has demonstrated that aflatoxin (AF) biosynthesis appears to be inhibited by gallic acid. During invasion by *Aspergillus flavus*, fungal tannase releases gallic acid (GA) from the hydolyzable tannins present in the pellicle of walnut and the hull of pistachio. Studies have shown that GA content of the seed coat or hull correlated inversely with the ability of the fungus to produce AF. The mechanism by which GA inhibits AF biosynthesis is not clear. Using a molecular biological approach we have shown that growth of *A. flavus* in the presence of GA results in the inhibition of transcription of AF biosynthetic genes. Growth of *A. flavus* on solid medium supplemented with 0.2% GA or 0.4% tannic acid resulted in 95% or greater inhibition of AF production but did not affect fungal growth. The inhibition appears to be at the level of transcription of AF biosynthetic genes as GA at 0.25% was shown to almost completely inhibit expression of *nor1* and *ver1* genes and only slightly reduced the levels of *aflR*. However, the transcriptional level of a housekeeping gene was not affected. Due to the fact that *aflR* was still being expressed in the presence of GA, another transcriptional activation factor, that perhaps interacts with *aflR* and is required for efficient transcription of AF biosynthetic genes, may be down-regulated by a signaling pathway involved in the oxidative stress response.

We have cloned the tannase gene from *A. flavus* and constructed a disrupted version of it in an attempt to generate tannase "knockout" strains of *A. flavus*. The ability of a tannase mutant to successfully invade and produce AF on a resistant tree nut variety will add support to the theory that release of GA from tannins by the action of tannase is responsible for the resistance phenotype observed in certain varieties of tree nuts. It is known that GA is an antioxidant and it may be suppressing mechanisms in the fungus that regulate responses to oxidative stress which has been shown to activate fungal AF biosynthesis. If this proves true then potential control strategies should focus on increasing the tannin content of susceptible crops or identify other molecules that can interfere with oxidative stress signaling pathways involved in regulation of AF biosynthesis.

Completed Sequence of the Aflatoxin Pathway Gene Cluster in *Aspergillus parasiticus*

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Aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFG_1, AFB_2, and AFG_2) are secondary metabolites produced primarily by the filamentous fungi *Aspergillus flavus* and *A. parasiticus*. Due to the risk of aflatoxin contamination of foods and feed on human health and livestock, research on the natural occurrence, identification, characterization, biosynthesis and genetic regulation of aflatoxins, as well as prevention and control of aflatoxin contamination of food and feed have been carried out in great detail. By mapping overlapping cosmid clones in *A. parasiticus* and *A. flavus*, it was demonstrated that at least nine aflatoxin pathway genes from early stage (*nor1* gene) to later stage (*omtA* gene) of the biosynthetic pathway were clustered. In an effort to define and characterize the genes in the complete aflatoxin pathway cluster and elucidate their function, an expanded search for additional aflatoxin pathway genes extending in both directions of the originally identified cluster in *A. parasiticus* was performed. We sequenced additional cosmid clones on both 5' and 3' ends; identified four sugar utilization genes adjacent to the aflatoxin gene cluster; identified and sequenced five (5) additional ORFs (genes) by blast search sequence analysis; sequenced all of the intergenic regions of the previously cloned genes to close the gaps by PCR strategy; and identified and validated the exon and intron sequences by RT-PCR. The entire aflatoxin biosynthetic pathway gene cluster which consists of a total of 25 genes or ORFs within the 70 kb DNA region in *A. parasiticus* was established. The gene density, on average, is about 2.8 kb for each gene. Among these genes there are three large transcripts of about 5-7 kb each for the fatty acid synthase alpha (5.8 kb) and beta (5.1 kb) subunits (FASa and FASb) and the polyketide synthase (PKS, 6.6 kb). Excluding these three large genes, the average size of the other 22 genes is about a little over 2 kb for each gene. In the 5' end of the cluster sequence, an approximate 2 kb DNA region with no identifiable ORF was located. This sequence presumably marks the end of this cluster in the 5' orientation since no intergenic region within the aflatoxin cluster is larger than 1 kb. The 3' end of the cluster is delineated by a well-defined sugar utilization gene cluster containing four genes. The corresponding enzymes of many aflatoxin pathway genes have been identified and characterized within the aflatoxin biosynthetic gene cluster in *A. flavus* and *A. parasiticus*. Over the years as the genes involved in aflatoxin biosynthesis were cloned, they were named based on either the substrates their products acted upon or their proposed enzymatic activity. This has led to some confusion, so we here have renamed them systematically according to the conventional gene nomenclature of *Aspergillus*. They are: *aflA* (*fas-2*), *aflB* (*fas-1*), *aflC* (*pksA*), *aflD* (*nor-1*), *aflE* (*norA*), *aflF* (*norB*), *aflG* (*avnA*), *aflH* (*adhA*), *aflI* (*avfA*), *aflJ* (*estA*), *aflK* (*vbs*), *aflL* (*verB*), *aflM* (*ver-1*), *aflN* (*verA*), *aflO* (*omtB*), *aflP* (*omtA*), *aflQ* (*ordA*), *aflR* (*aflR*), *aflS* (*aflJ*), *aflT* (*aflT*), *aflU* (*cypA*), *aflV* (*cypX*), *aflW* (*moxY*), *aflX*, *aflY*. The function of these pathway genes and newly identified transcripts (open reading frames) and their expressional regulation are reported or proposed.

Purification of a Maize Aflatoxin Inhibitor

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The development of maize cultivars with durable resistance to aflatoxin accumulation requires that plant genes influencing fungal growth and aflatoxin production be identified and linked to molecular markers to facilitate breeding. Several kernel characteristics such as fatty acid and pericarp wax and the relative levels of antifungal proteins are thought to regulate the maize seed-*Aspergillus flavus* interaction. We are using a simple liquid bioassay to identify maize kernel proteins from the resistant inbred Tex6 that contribute to the resistant phenotype. Previously, Huang et al. (Phytopathology 87:622-627, 1997) identified inhibitory activity to growth and aflatoxin production in Tex6 kernels. A protein responsible for growth inhibition was subsequently purified and identified as a chitinase (Moore et al., Phytopathology, in press, 2003). We are attempting to purify a protein that inhibits aflatoxin biosynthesis (termed Aflatoxin Biosynthesis Inhibitor). To date, selective $(NH_4)_2SO_4$ precipitation, size exclusion chromatography, and DEAE anion exchange chromatography have yielded enriched preparations of ABI. Additional purification steps are in progress.

Use of the Yeast *Saccharomyces cerevisiae* as a Model System for High Throughput Screening of Genes Involved in Oxidative Stress Response Induction of Aflatoxin Biosynthesis

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Potent anti-aflatoxigenic activity was discovered in the pellicle (paper-like covering) of kernels of the commercial walnut variety, 'Tulare' (*Juglans regia* L.). The active components of the pellicle were found to be mainly the gallic acid constituent of hydrolysable tannins. Gallic acid almost completely inhibits biosynthesis of aflatoxin and its precursors (*e.g.*, norsolorinic acid, *etc.*). The mode of action of the acid is associated with antioxidant modulation of the oxidative stress-response of the fungus. Yeast cells treated with tannic or gallic acid or methyl gallate recover from severe oxidative stress caused by exposure to hydrogen peroxide. We postulate that various metabolic activities during growth and development of *A. flavus* result in high oxygen demand. This high demand, and additional reactive oxygen radicals produced during plant-pathogen interactions, as well as intracellular metabolism are probable sources of oxidative stress in *A. flavus*. Our study shows antioxidant compounds in hydrolysable tannins affect this oxidative stress-response. To further understand the oxidative stress-response in *A. flavus*, we identified forty-three orthologs of yeast genes involved in signal transduction and antioxidative stress defense through an EST (Expressed Sequence Tag) database search. Molecular characterization and functional complementation of these genes in yeast and *Aspergillus* are currently underway to discover new approaches to control aflatoxin biosynthesis during pathogen infection.

Phytoalexin Production by the Peanut Plant at Early Stages of Development

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Stilbene phytoalexins are produced by the peanut plant as a defense response to exogenous stimuli, particularly when challenged by fungal invasion. The plant's ability to produce phytoalexins under unchallenged conditions has not been reported. Phytoalexins possess antifungal activity against *Aspergillus, Penicillium* and *Cladosporium* species, and are suggested to play an important defensive role at early stages of plant development. The purpose of this work was to characterize phytoalexin production by different parts of the developing peanut plant. The concentrations of stilbene phytoalexins varied from 10 ng/g of *t*-resveratrol in the hypocotyl to 12 μg/g of the major unknown stilbene in the root mucigel. At least 5 new stilbenes were detected in the mucigel; their structural elucidation is in progress. Two major bound phenolic acids, ferulic and *p*-coumaric, were present in high concentrations in the root, the hypocotyl and the shoot; *p*-coumaric acid was found in cotyledons. Three different peanut genotypes (Georgia Green, Valencia and Virugard) produced the same set of stilbene phytoalexins and bound phenolic acids. The mucigel contained low-polar stilbenes, but none of the known phytoalexins such as the arachidins and *t*-resveratrol. The hydrophobic nature of the mucigel stilbenes suggests that they may have an affinity for fungal membranes, the usual site of phytoalexin action. Besides lubricating the root and enhancing soil quality, the peanut root mucigel may serve as an active protective barrier for soil fungi due to the presence of phytoalexins. Although ferulic and *p*-coumaric acids are typically associated with dormancy, these phenolic compounds also may protect the plant against pathogenic fungi.

Session 2:
Crop Science -- Conventional Breeding
Moderator: Bob Klein, California Pistachio Commission

Identification, Development and Characterization of Corn Germplasm to Reduce Aflatoxin Contamination

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Aflatoxin (AF) is a potent toxin and carcinogen produced by the fungus *Aspergillus flavus* Link:Fr. that limits corn marketability, causing economic losses because of risk to animal and human health. Aflatoxin is a chronic problem for growers in the southeast United States. Our goal is to identify and develop corn inbreds with resistance factors that can reduce the risk of AF. We are searching for new sources of AF resistance by screening diverse maize germplasm. The most promising lines are validated and characterized through multi-location testing and genetic studies. White and yellow, subtropical and tropical, temperate, Quality Protein Maize, and high and low oleic/linoleic maize inbreds were evaluated *per se* along with hybrids for response to field AF accumulation under *A. flavus* inoculation in replicated experiments at three locations in South and Central Texas between 2001 and 2003. Quantification of AF was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest[TM]). Significant differences were detected in all the experiments except at College Station in 2002, where ground inoculation with *A. flavus*-colonized kernels and environmental conditions were not conducive for AF. Aflatoxin content averages and ranges were high enough to discriminate among testing genotypes. Response to AF contamination of testing genotypes varied substantially among environments (i.e., high genotype by environmental interaction). However, multi-year and multi-location testing has permitted the identification of lines with consistently low susceptibility. Yellow inbreds CML323, Tx772, CML288, NC300, FR2128, CML338, CML161 and experimental lines TxX69's have shown reduced levels of AF in inbreds and hybrids. White inbreds CML176, CML269, CML78, Tx807, and TX110 (in some hybrid combinations) are the most promising lines to contribute AF-resistance factors. Most of these lines have shown good agronomic performance in southern areas. This aspect can facilitate the combination of AF-resistance factors into elite genetic backgrounds suitable to produce commercial hybrids.

Progress in the Identification and Characterization of Maize Resistance Traits against Aflatoxigenic Fungi

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The main thrust of our research has been to identify resistance markers in maize germplasm demonstrating resistance to aflatoxin contamination by *Aspergillus flavus*. Our aim is to use this information to enhance host resistance in maize lines with superior agronomic characteristics. Our primary strategy has been to identify, using comparative proteomics (resistant vs. susceptible germplasm), resistance-associated proteins (RAPs) and their corresponding genes, further characterize the proteins/genes, and perform confirmation studies to determine their actual role in resistance. Using this approach, we've identified a number of RAPs (stress-related and antifungal) in U.S. lines, and characterized trypsin inhibitor, glyoxalase and pathogenesis-related (PR-10) proteins. To evaluate their role in resistance, we are currently designing an RNAi gene silencing vector and vector constructs involving genes encoding the above three proteins, for maize transformation.

Rapid progress made in an aflatoxin-resistance breeding program at IITA-Nigeria has advanced lines by selfing and selecting for agronomic characteristics, ear rot resistance, and aflatoxin-resistance, to the S5 generation (98% homogeneity). One group of lines originate from crosses involving 75% U.S. and 25% African genetic backgrounds (temperate), and a second group are from crosses involving 50% of each (tropical). Recently, S4 lines of the temperate materials were screened at SRRC for aflatoxin accumulation using the kernel screening assay (KSA). Five different closely-related pairs of lines, with individuals within each pair differing significantly in aflatoxin levels, were identified during screening, and were subsequently subjected to proteome analysis. Results show both qualitative and quantitative kernel embryo protein differences between resistant and susceptible members of the closely-related pairs. Also, as seen previously within U.S. lines, several stress-related proteins were expressed at higher levels in African resistant lines than in susceptible ones. However, two protein spots were higher in susceptible lines in 3 of 5 pairs of these lines. Two "new" RAPs not seen in previous investigations, serine/threonine kinase and a glucose/ribitol dehydrogenase, were identified in this study. Dehydrogenase is higher in resistant lines in 3 of 5 pairs. Also, globulin 2, protein spot #1158 only, is unique to resistant lines in 4 of 5 pairs. Thus, rapid progress made in breeding at IITA has facilitated the advancement of proteomics investigations at SRRC, by providing closely-related germplasm differing in aflatoxin levels. Evaluating these for resistance-associated differences should prove more fruitful than evaluating lines from very diverse genetic backgrounds, which, heretofore, was standard protocol.

The aim of future studies will be to identify protein differences expressed in kernel endosperm tissue of S4 temperate lines and characterize selected proteins identified from both embryo and endosperm tissue. Other planned investigations include studying polymorphism of RAP genes, and performing promoter analysis of selected genes. Previously-observed phenomena and recent preliminary studies indicate a link between kernel water relations and resistance. We will, therefore, conduct kernel physiology studies to clarify this potential linkage.

Distribution of Aflatoxin in Resistant and Non-Resistant Maize Populations

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New genes are needed to develop corn hybrids with resistance to *Aspergillus flavus* and/or aflatoxin biosynthesis. Currently there is no commercial hybrid marketed with advertised resistance and the resistance that is available would not likely keep aflatoxin levels in grain below that needed for commercial sale in a high-incidence year such as 1998. The end-goal of this research is to find new resistant genes by screening global collections of corn germplasm. Before large-scale screening can proceed, several procedural questions must be resolved, including; (1) How many ears of each accession should be tested to confidently assess resistance? (2) Will self-pollination be necessary? (3) How does artificial inoculation with *A. flavus* spores affect assessment? (4) Can ears be bulked? A 2-year field study was funded in 2002 to answer these questions and to determine the frequency distribution of aflatoxin and fumonisin resulting from factorial treatments of inoculated/non-inoculated (*A. flavus*) and self-pollinated/open-pollinated populations of susceptible 'B73' and resistant 'Tex6' inbreds. A split-plot design with 20 replications was used with inbred as main plot and pollination/inoculation treatment as sub-plot. Harvested ears were rated for quality, fungal growth, bright greenish-yellow fluorescence, aflatoxin (ppb), and fumonisin (ppm). Inoculating with *A. flavus* greatly increased fungal growth, BGYF, and aflatoxin. Fungal growth and aflatoxin were greater in the susceptible line. Aflatoxin increased while fumonisin decreased when plants were self-pollinated. The correlation coefficients of aflatoxin with BGYF and fungal growth were 0.93 and 0.87, respectively. Fumonisin was not affected by any variable other than pollination method. There were no significant interactions between inbreds and inoculation or pollination methods (Type III SS). The distribution of aflatoxin was greatly skewed toward low aflatoxin under natural infection, following a Poisson type distribution pattern. Aflatoxin was more normally distributed when inoculated, coinciding with over a three-fold decrease in error. These results indicate that time-consuming self-pollinating techniques will not be necessary to assess resistance, and that inoculating with *A. flavus* spores to help insure a sufficient concentration of aflatoxin for effective screening will not inhibit distinguishing superior lines with resistant genes. Inoculating with *Aspergillus flavus* spores helped reduce error. The field experiment was repeated using B73 and Mp313 in 2003. The ultimate objective is to determine confidence intervals for aflatoxin readings based on sample size (ear number).

Analysis of Breeding Corn Lines with Resistance to Preharvest Aflatoxin Contamination and Drought Stress

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Aflatoxin contamination of corn in the field is known to be influenced by numerous factors, several of which cannot be controlled by the producer. In the Southeastern U. S., insect populations, such as corn earworm, are extremely large every year, and drought stress is conducive to *Aspergillus flavus* infection and aflatoxin elaboration. The development of "southern hybrid-type" corn with good husk coverage, insect and aflatoxin resistance, and with acceptable yield will help southern corn growers produce a quality, profitable crop. The objectives were to conduct field evaluation and comparison of the selected single-cross hybrid combinations for yield and aflatoxin contamination among inbred lines selected from GT-MAS:gk population and lines from China, CIMMYT/Mexico, and Spain with hard kernel character and drought tolerance, and to identify and understand genes/pathway using micrroarray analysis to study gene expression as influenced by drought stress, *A. flavus*, and the interactions.

Single-cross hybrid field tests in Georgia and Texas have shown that several crosses have good yield potential for the Southeastern region in comparison with local commercial hybrids. A cooperative test conducted by voluntary cooperators was initiated and named as SERAT (South East Regional Aflatoxin Test) to conduct a region wide cooperative test to determine agronomic performance and aflatoxin resistance of developing corn cultivars. This year, there are 4 participating cooperators from TX, LA, and GA.

We also used microarray analysis of gene expression to study the effects of drought stress, *A. flavus* infection, SA induction on gene expression in developing corn ear/kernels. Genomic research of gene expression analyses in the developing ears/kernels will help identify and understand the function of genes that control significant biological processes and agronomic performance under stress of drought and fungal infection, which are crucial in the development of genetic approaches for control of preharvest aflatoxin contamination. The data analyses of gene expression are in progress to identify the genes/pathways involved in plant response to the stresses.

Use of Molecular Markers to Create Commercially Usable Corn Hybrids with Low Aflatoxin in Grain

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The goal of this research is to produce commercially usable corn inbreds that in hybrid combinations have measurable levels of resistance. The created resistant inbreds will be used directly to produce resistant commercial hybrids or as superior sources of resistance which along with the information on molecular markers associated with genes for resistance will clearly demonstrate to commercial corn breeders the methods for converting their proprietary susceptible inbreds into resistant inbreds. Molecular markers have numerous advantages including the ability to recover the agronomically superior traits of the susceptible inbred, avoiding the necessity of aflatoxin analyses in each generation of breeding, and employing a common technology used by many companies.

We have identified several sources of resistance and with several know the inheritance of resistance and the molecular markers associated with resistance. With inbred line Mp313E we are crossing resistance on chromosome 4L into the commercially used inbred FR1064. Chromosome 4L has been shown by USDA/ARS studies in Mississippi to carry one or more Quantitative Trait Loci (QTL) for resistance. In 2002 we evaluated lines that were either two or three backcrosses to FR1064 and homozygous for chromosome 4 region from Mp313E that would have 87.5% or 93.75% similarity to the commercially used FR1064. Environmental conditions in 2002 were extremely favorable for toxin production and FR1064 and related lines had $\geq$ 1000 ng/g whereas the resistant recoveries were $\leq$ 300 ng/g. We also are developing commercially usable lines using resistance from Tex6, which is an unreleased inbred line developed at the University of Illinois. For Tex6 we are selecting chromosome segments for lower aflatoxin located in chromosome bins 5.01 and 10.05-.07. With Tex6 recoveries we have lines where Tex6 was crossed with B73 and backcrossed twice to B73, selfed three times, found to be homozygous for the QTL on chromosomes 5 and 10, then crossed with FR1064, backcrossed to FR1064, and selfed twice. These early generation lines were evaluated in a study where B73 had 1,206 ng/g, FR1064 had 1,010 ng/g, Tex6 had 64 ng/g and families in the backcrossing study ranged from 95 to 292 ng/g with an average of 165 ng/g. At this time we do not have complete marker data on those families; thus, some of the more susceptible may not have all the QTL associated with resistance. Also in 2003 we evaluated three testcross hybrids between early generation resistant inbred of the cross between FR1064 and Mp313E backcrossed two times to FR1064 selfed twice and homozygous for chromosome 4L QTL from Mp313E. Testcross hybrids were made with the commercially used LH182. The resistant hybrids had between 20 and 160 ng/g with an average of 85 ng/g compared with other commercial hybrids in the test that were $\geq$ 200 ng/g.

In 2003 we have 78 early generation resistant lines in evaluation at Urbana, IL, and 39 testcross hybrids with FR4310 evaluated in Urbana, one location in Texas, and a fewer number in Mississippi. Agronomic characteristics of the testcross hybrids look very favorable.

We also are developing lines with QTL for resistance from both Mp313E and Tex6. The pyramided resistance will likely approach the level necessary to achieve complete control in most situations. With significant funding we will continue backcrossing and could be commercially testing resistant hybrids in 3 to 5 years.

Molecular Characterization of Resistance to Aflatoxin Accumulation in Mp313E

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Aflatoxin is a carcinogenic and toxic compound produced by the fungus *Aspergillus flavus* that can be found at detrimentally high concentrations in maize grain. Screening procedures have led to the discovery of sources of resistance to aflatoxin accumulation in maize, but poor associated agronomic characteristics and complex inheritance have limited transfer of resistance to elite inbreds. This study was undertaken to identify QTL associated with reduced aflatoxin accumulation and aid in transfer of regions containing them. Initial studies identified one QTL in chromosome 4L which significantly effected aflatoxin build up in multiple years in a mapping population derived from the cross Mp313E × Va35. The subsequent study consisted of 210 $F_{2:3}$ families derived from a cross between the resistant inbred Mp313E and the susceptible inbred B73. Plots were evaluated in replicated trials in four environments for resistance to aflatoxin accumulation. A genetic map consisting of 85 SSRs and covering 1553 cM was used for performing composite interval mapping (CIM). CIM was used to identify 3, 2, 4, and 3 QTL regions within the tests MSU2000, Stone2000, MSU2001, and MSU2002, respectively. QTL tended to be additive in nature with the Mp313E parent contributing to reduced aflatoxin concentration in all but one case. Two QTL regions, on chromosomes two and four, were significant in at least three environments. A QTL on chromosome two (bins 2.05 - 2.07) represented by marker bnlg371 was located on chromosome two and accounted for 7% to 18% of variation in aflatoxin levels depending on environment. A QTL on chromosome four (bins 4.06 - 4.08) and represented by marker bnlg2291 explained from 8% to 16% of phenotypic variance. This QTL has been noted in earlier studies while the QTL on chromosome 2 is new. Genotypic class means of families selected for homozygosity in flanking markers at these two loci differed by greater than 50% in each environment with respect to aflatoxin levels. Identified QTL confirm important regions influencing aflatoxin accumulation previously identified and present new ones of equal effect.

Breeding Peanut for Resistance to Preharvest Aflatoxin Contamination and Drought Tolerance

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This is a long term research project designed to result in the development of peanut cultivars with resistance to preharvest aflatoxin contamination (PAC). The initial objectives were to develop large-scale screening techniques and to identify sources of resistance to PAC in peanut germplasm. Screening techniques were developed, and fifteen sources of resistance were identified. These sources of resistance were then used in a hybridization program to combine this resistance with acceptable agronomic performance. Several hundred $F_{4:5}$ breeding lines were created and tested for resistance. Six breeding lines were identified that have relatively low aflatoxin contamination and relatively high yield when subjected to late season drought stress. We also documented an association between drought tolerance and reduced aflatoxin contamination, and are using drought tolerance as an indirect selection technique for resistance to aflatoxin contamination. We evaluated other breeding lines that were not developed specifically for reduced aflatoxin contamination and/or improved drought tolerance. Three of these advanced breeding lines have exhibited significantly higher yield and significantly lower aflatoxin contamination in comparison to the standard check cultivar, Georgia Green.

Aflatoxin Suppression is not Associated with Gallic Acid Metabolism in Almond though Large-scale Field Trials Support Effective Control through Integrated Endocarp-kernel Based Resistance

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Research objectives in 2003 included genetic and biochemical studies of: a) the toxin suppression in almond, b) the integrity and genetic manipulation of endocarp based resistance to insect vectors of *Aspergillus flavus*, and c) the breeding and field testing of advanced almond selections combining resistance to preharvest aflatoxin contamination with high horticultural quality. A comprehensive survey of California and European almond varieties as well as related species and/or species crosses including *Prunus persica, P. mira, P. webbii, P. fenzliana, P. argetia, P. bucharica,* and *P tangutica*, has failed to identify promising sources of known toxin suppression (gallic acid) compounds. Backcross breeding lines derived almond x *P. persica* interspecific hybrids have shown up to 90% suppression of toxin production in almond kernel tissue following controlled inoculations, and while the mechanism remains unknown, it does not appear to be associated with gallic acid compounds. Genetic analysis of both fungal and insect resistance is being pursued through the construction of cDNA libraries from susceptible and resistant almond pellicles and embryos. A large number of EST sequences have now been analyzed with additional sequences being identified and posted monthly on the CUGI web site. Genes discovered through this approach could be used indirectly as markers to improve breeding efficiency and as candidate genes for genetic engineering. Current research on improving recovery of transgenic almonds includes the development of binary vectors for the chemically inducible regeneration genes PGA6 and the ESRI.

Increasing attention is being focused on identifying factors predisposing infected almond kernels to the infrequent but potentially high levels of aflatoxin formation. A phenomenon of pellicle ink-staining has been shown to be associated with contamination by *A. niger, Cladosporium* and other weak pathogens. Asymmetries in kernel development have been found to be associated with pellicle contamination by these fungi. The relations of these kernel asymmetries and within-kernel differences in *A. flavus* contamination and aflatoxin formation are presently being studied.

Large-scale testing of breeding selections containing endocarp and kernel based resistance to insect and/or fungal damage continue to show efficacy under a wide range of field conditions in grower trials in the upper mid- and lower Sacramento Valley as well as upper and mid-San Joaquin Valley. Additional advanced selections combining resistance with improved horticultural quality are presently being propagated for grower testing in the southern San Joaquin Valley beginning in 2004.

Third generation breeding lines possessing resistance to preharvest aflatoxin contamination, expressed either as a suppression of toxin formation following successful infection and/or protection of the developing and maturing kernel from insect/fungal damage, are now being recovered in breeding progeny. These breeding selections also combine high horticultural quality and tree productivity with the range of new insect and pest resistance traits characterized by these aflatoxin resistance studies.

PANEL DISCUSSION: Crop Resistance – Conventional Breeding

Panel Chair: C. Corley Holbrook

Panel Members: F. Javier Betran, Tom Brooks, Robert L. Brown, Tom M. Gradziel, Baozhu Guo, C. Corley Holbrook, Steven H. Moore, and Donald G. White

Summary of Presentations: The moderator of this session was Bob Klien who is with the California Pistachio Commission. Excellent presentations were given to provide state-of-the-art information on conventional breeding efforts to develop resistance to aflatoxin contamination in corn, peanut, and almond. The development of host resistance is a key strategy for effective control of aflatoxin contamination.

Several groups reported on research to identify, characterize, and develop corn germplasm to reduce aflatoxin contamination. New sources of resistance were reported, and research was presented defining some possible mechanisms of resistance. Molecular marker assisted selection of QTL's for low aflatoxin contamination is being utilized in corn breeding programs. Research was also reported to develop expressed sequence tags (EST's) for use in selection programs.

Results for peanuts focused primarily on the development of breeding lines that have relatively low aflatoxin and relatively high yield when subjected to late season drought stress. Previous research had documented an association between drought tolerance and reduced aflatoxin contamination, and drought tolerance is being used an indirect selection technique for resistance to aflatoxin contamination. Research is ongoing to attempt to associate EST's with drought tolerance and/or reduced aflatoxin contamination in peanut.

In almonds, resistance to aflatoxin contamination is being pursued through the integration of fungal pathogen and insect vector resistance. Breeding lines exhibiting low aflatoxin contamination have been developed using this approach. This material is currently undergoing large-scale field testing.

Summary of Panel Discussion: The discussion that followed the presentations focused on ways to more rapidly advance breeding progress. Specifically, panel members were asked what was the most critical missing element to developing resistant cultivars for their crops. Representatives for all crops indicated a need for more basic information which could lead to a better understanding of the interaction of *Aspergillus* with these crop species. The need for higher levels of resistance in corn was expressed, as was the need for more and larger breeding populations to enhance the opportunities for selection. Breeding progress in peanut could be enhanced by the development of molecular markers for drought tolerance and/or reduced aflatoxin contamination.

It was noted that there was much more discussion on marker assisted selection (MAS) in this section on conventional breeding than there had been previous years. Much success has been achieved in research to develop markers for reduced aflatoxin contamination in corn. The hope is that breeding programs will be able to use MAS to develop resistance with no decline in yield.

This led to a good deal of discussion on the likelihood of having commercial corn hybrids with resistance to aflatoxin contamination. Much progress has been made towards that goal; however, the concern was expressed that companies would never be able to advertise this trait as a selling point.

Aflatoxin Contamination of Groundnut: Conventional Breeding for Resistance

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Aflatoxin contamination of groundnut or peanut (*Arachis hypogaea* L.) is a widespread serious problem in most groundnut-producing countries where the crop is grown under rainfed conditions. The semi-arid tropical environment is conducive to preharvest contamination when the crop experiences drought before harvest, whereas in the wet and humid areas, postharvest contamination is more prevalent. Breeding for resistant cultivars is one of the possible means of reducing aflatoxin contamination. These cultivars will be of great value as no cost input to the farmers in both developed and developing countries. At ICRISAT research on breeding for resistance to aflatoxin contamination started in 1976. In the beginning, resistance to *in vitro* seed colonization by *Aspergillus flavus* (IVSCAF) received maximum attention. The importance of resistance to natural seed infection and aflatoxin production later was recognized and received increasing attention. Sources for resistance to seed infection and aflatoxin production were identified. We have developed and tested several hundred breeding lines for resistance to IVSCAF and seed infection and yield. Some of the breeding lines have seed infection and colonization equal to or less than the best resistant control cultivar J 11, and high-yield potential across seasons/years, and locations. Of these ICGV 88145 and ICGV 89104, and ICGV 91278, ICGV 91283 and ICGV 91284 have been released as improved germplasm. ICGVs 91278, 91283, and 91284 have performed well in evaluations in Thailand and Vietnam. ICGV 87084, ICGV 87094, and ICGV 87110, bred at ICRISAT Center for resistance to seed infection, were also found to be resistant in Senegal, Niger, and Burkina Faso in West Africa. ICGV 91278 and ICGV 91284 are also doing well in West Africa. We are attempting to enhance levels of resistance to aflatoxin contamination and combine it with the rust and late leaf spots resistance and good agronomic background. One way to achieve greater success in conventional breeding efforts is to modify screening techniques to suit screening of segregating generations and select resistant plants/progenies. The genetics of resistance mechanisms has not been clearly established. The information on allelic relationship among various sources for each resistance trait is crucial and will enable breeders to pyramid the non-allelic genes for each resistance mechanism. There is an urgent need to locate germplasm sources in the core and mini-core collections with stable, high levels of resistance to different mechanisms. Unfortunately, the progress made so far in conventional breeding has not been able to produce cultivars that are free from aflatoxin contamination. The recourse to biotechnology to provide transgenic protection to groundnut against infection by aflatoxin-producing fungi may help in obtaining groundnuts free from aflatoxin.

Functional Genomics of *Arachis hypogaea* L. for Understanding Host Peanut and *Aspergillus* Interactions

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Expressed sequence tag (EST) libraries for cultivated peanut (*Arachis hypogaea* L.) were developed from two cDNA libraries constructed using mRNA prepared from immature pods of peanut line A13 (tolerant to drought stress and preharvest aflatoxin contamination) and leaves of peanut line C34-24 (resistant to leaf spots and tomato spotted wilt virus). Randomly selected cDNA clones were partially sequenced to generate a total of 1825 ESTs, 769 from C34-24 cDNA library and 1056 from A13 cDNA library, in which 536 and 769 unique ESTs were identified, respectively. The results of BLASTx search showed that 52.8% of the ESTs from leaf tissue and 78.6% of the ESTs from the pod tissue have homology to known gene function. There are about 27.3% and 22.1% ESTs matching homologous sequences in dbEST of GenBank based on BLASTn algorithm which have unknown functions. These ESTs were queried against MIPS functional catalogue criteria and sorted according to putative function into 15 categories. We have released 1345 ESTs to GenBank under accession numbers CD037499 to CD038843. We have successfully used these ESTs for gene identification and EST-derived SSR marker development. We have arrayed about 400 unigenes of adversity resistance on glass slides for gene expression analysis and characterized 44 EST-derived SSR markers for cultivated peanut, in which over 20% SSR produced polymorphic markers among 24 cultivated peanut genotypes. The microarray chips with the 400 unigenes with putative functions of adversity resistance are in process for gene expression analysis using mRNA probes from different lines under different challenges of drought stress or fungal infection to identify genes related to the abiotic or biotic stresses. This initiative research could contribute considerable information to peanut functional genomics in novel gene discovery and marker development. Further characterization of these adversity resistance genes may explain the resistant mechanisms functioning in these two peanut lines.

Report on Microarray Analysis of Gene Expression of Corn in Response to *Aspergillus flavus* and Drought Stress

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Previous research has shown the possible relationship between drought tolerance and reduced preharvest aflatoxin contamination in corn (*Zea mays*). The goals of this project are to better understand global gene expression under drought stress and *Aspergillus flavus* infection at different stages of seed development, and to identify the related biochemical pathways and important genes of resistance to *A. flavus*. The inbred lines of GT-A1-1 and B73 were used in this study. We are interested in the genes expressing at the late development phase of corn kernel under water deficit stress and *A. flavus* challenge. Transcriptional profiles of corn kernels at 25 days after pollination (DAP) were compared under different conductions including normal development, water deficit stress, *A. flavus* challenge, and salicilic acid (SA) inducement. The latest version of maize micorarray chips-Master Unigene from Maize Gene Discovery Project were used in the study, which has about 20,736 unique ESTs in each slide. Microarray scanner- ScanArray Express was used to produce and analyze the microarray image. The result shows: in the 25 DAP corn kernels of GT-A1-1, there are 1203 up-regulated and 1357 down-regulated genes expressed under drought stress. There are 620 genes in normal condition and 682 genes in drought condition expressed after *A. flavus* inoculation.

To analyze the genes that express both in drought stress and *A. flavus* challenged corn, the genes expression profilings were compared under these two conditions. Four hundred sixty-three spots in chips appeared significant positive signal both in comparison. These genes include in metabolism, energy, protein destination, secondary metabolism, transcription, cellular biogenesis, cellular transport etc. Some genes were found to belong to cell communication and signal transduction such as MAP3k-like protein kinase, receptor protein kinase, serin / threonine kinase, calmodulin etc. The genes in defense and cell rescue were also found such as early drought induced protein, heat shock protein, pathogenesis related protein, polygalacturonase, stress-induced protein sti 1, abscisic acid induced protein, etc. To identify the genes related to *A. flavus* resistance, gene expression profiling was studied in the *A. flavus* inoculation under drought stress and SA inducement, and about 685 and 1174 genes were found to express, respectively. These primary results and the further identification are still in process.

Relationship of Antifungal Trypsin Inhibitor in Peanut Resistant and Susceptible to *Aspergillus flavus*

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Peanut is frequently subjected to contamination by aflatoxin produced by *Aspergillus spp*. The objectives of this study were to study the relationship of constitutive expression of trypsin inhibitor (TI) in peanut seeds and resistance to *A. flavus* infection.

The differences of seed infection by *A. flavus* among 13 genotypes were significant. Yueyou 20 and Zhanque48 showed high resistance to *A. flavus* infection. A trypsin inhibitor in peanut seeds was purified by acetone fractionation, followed by passing through a DEAE-Sephadex A50 ion-exchange column. The purified inhibitor consisted of two subunits with molecular weight of 10.3 and17 kDs, respectively. The inhibition of germination and hyphal growth of *A. flavus* were observed in protein concentration 10 µg/mL on V8 juice agar medium. The relationship between the TI concentration/activity and resistance to *A. flavus* infection was compared. The concentration and activity of TI in resistant genotypes were significantly higher than that in susceptible genotypes.

Differential Induction of Antifungal Protein Chitinase and *β*-1-3-Glucanase by *Aspergillus flavus* in Different Peanut Lines

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Chitinase and *β*-1-3-glucanase are believed to be important PR-proteins in defending plants against pathogens. They can protect plants from fungal infection by their direct lytic action on fungal cell walls or by releasing oligosaccharide signal molecules that can activate a variety of plant defenses.

The differential expression of chitinase and *β*-1-3-glucanase in response to infection by *Aspergillus flavus* was evaluated in mature kernels of both resistant and susceptible peanut genotypes. The increase of endo-chitinase activity was found after seeds were treated with *A. flavus* in both resistant and susceptible genotypes. The enzyme activity was considerably higher (4 fold) in the resistant genotypes at 3-4 days after inoculation, but 3-fold in susceptible genotypes. No significant differences in the activities of exo-chitinase were observed in both resistant/susceptible genotypes. *A. flavus* inoculation led to an increase of *β*-1-3-glucanase activity in both resistant and susceptible genotypes. In resistant genotypes, the *β*-1-3-glucanase activity doubled one day after and increased by 10-fold five days after inoculation, but it was significantly lower in susceptible genotypes.

In native PAGE, one band corresponding to endo-chitinase isoforms was detected in both susceptible and resistant genotypes. But the new band occurred in resistant genotypes one day after inoculation, but three days after inoculation in susceptible genotypes. The isoform patterns of *β*-1-3-glucanase showed that 8 new isoform bands were detected in resistant genotypes after inoculation, while 5 bands were detected in susceptible genotypes. The new bands corresponding to *β*-1-3-glucanase were tested the first day after inoculation in resistant genotypes and the fourth day in susceptible genotypes. Chitinase in Yueyou 20 seed treated with *A. flavus* was purified by precipitation with $(NH_4)_2SO_4$ at a saturation of 60% followed by affinity chromatography on a column of regenerated chitin. The purified enzyme showed a single band on SDS-PAGE and the molecular weight was estimated as 31 kDa. The purified chitinase can significantly inhibit spore germination and hyphal extension of *A. flavus in vitro*. The results above implied that the two hydrolyses play an important role in resistance to *A. flavus* infection in peanut seeds.

Reaction to Aflatoxin Contamination among Peanut Germplasm Lines with Resistance to Bacterial Wilt

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Peanut is an important oilseed as well as cash crop for farmers in China. Bacterial wilt (BW) caused by *Ralstonia solanacearum* has been among the major constraints to peanut production in central and south China. As a soil-born bacterial disease, BW is very difficult to control, and the only feasible management approach is planting BW-resistant peanut cultivars. In most cases, BW-resistant cultivars are essential for production in the heavily-infested regions. China has a large collection of BW-resistant peanut germplasm and the incidence of BW in the fields has been reduced drastically due to the improved resistant cultivars. However, the warm and moist weather in the areas with bacterial wilt disease is also favorable for infection of *Aspergillus flavus*, *A. parasiticus*, and aflatoxin contamination. Therefore, all the BW-diseased areas are also suffering from serious aflatoxin contamination in central and south China. Genetic improvement for resistance to aflatoxin contamination based on the BW resistance is crucial to comprehensive management of both constraints, and the diversified BW-resistant germplasm has made this possible. By root cross-inoculation of *R. solanacearum* and *A. flavus* in the late growth stage of peanut, it was found that infection of *R. solanacearum* to immature pods could encourage pre-harvest invasion of *A. flavus* and increase aflatoxin contamination, but the reaction varied among BW-resistant genotypes. Several BW-resistant peanut genotypes were grown on natural BW nursery with high inoculum pressure of *R. solanacearum* in Hongan and disease-free field in Wuhan and tested for their natural contamination with aflatoxin. The preliminary results showed that the peanut lines with high latent infection/colonization of *R. solanacearum* and/or poor drought tolerance had higher aflatoxin contamination. Thirty-one lines with various BW resistance levels were investigated for their resistance to seed invasion of *A. flavus* and to aflatoxin production. From replicated experiments for seed resistance to invasion, it was found that Xiaohongmao had similar seed resistance to invasion as J11. It was interesting to note that Xiaohongmao has the highest oleic content and the smallest pod size among the BW-resistant genotypes. From experiments for resistance to aflatoxin production, two BW-resistant genotypes, Taishan Zhenzhu and Zhonghua 6, were found to have lowest aflatoxin contents after inoculation of *A. flavus*. It was also interesting to note that Taishan Zhenzhu was the BW resistance parent of Zhonghua 6. It was concluded that it would be possible to improve resistance to aflatoxin contamination based on the resistance to bacterial wilt disease.

Aflatoxin Accumulation in Maize Inbreds and Hybrids

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Preharvest aflatoxin (AF) contamination is one of the main limitations for corn production in Texas. In the USA, grain with more than 20 ppb (ng/g) of aflatoxin B_1 is banned from interstate commerce, and if it contains more than 300 ppb, it cannot be used as livestock feed. There are no elite inbreds fully resistant to AF that can be used directly in commercial corn hybrids. Our objective was to measure aflatoxin accumulation of yellow corn inbreds and hybrids, evaluated under inoculation in three locations in South and Central Texas: College Station (CS), Weslaco (WE), and Corpus Christi (CC) during 2001, 2002 and 2003. An alpha-lattice design with 3 or 4 reps was used. Drought and heat stress were induced by late planting and limited irrigation. Inoculation was with a conidial suspension of *Aspergillus flavus* (isolate NRRL3357) injected 6-10 days after midsilk (the nonwounding silk channel inoculation technique) at CS and WE during 2001 and at CS during 2003. At CS and WE during 2002, at WE during 2003, and at CC during all three years, inoculation was done by placing *A. flavus* colonized corn kernels on the soil surface between treatment rows around mid-silk stage. At harvest, infected ears were husked, rated for kernel integrity and visible fungal colonization, shelled, ground with a mill, and evaluated for AF. AF was quantified with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest[TM]). Significant differences among inbreds were detected in all three seasons at the WE location. During 2001, the average AF was 1343.17 ppb with a range of 452.5 ppb to 2750.0 ppb. The inbreds showing less susceptibility were CML289, Tx601Y and Tx732. During 2002, the average AF was 532.76 ppb, with a range of 26.75 ppb to 1905.9 ppb. The inbreds showing less susceptibility were Tx772, NC300 and CML338. During the 2003 season, the average AF was 491.03 ppb, with a range of 10.5 ppb to 2575 ppb. The inbreds showing less susceptibility were TxXQ69-B4, TxXQ69-B2 and Tx772. AF accumulations among inbreds varied from year to year, with no consistencies over the three years. Significant differences among the hybrids were detected in all locations except in CS in 2002. The average AF in 2001 was 131.5 ppb at CS, 530.7 ppb at WE and 1682.2 ppb at CC. The less susceptible hybrids at WE were Tx601Y/NC300 and CML161/CML170 and at CC DK668 and RX889. Average AF in 2002 was 20.4 ppb at CS, 220.13 ppb at WE, and 925.0 ppb at CC. The hybrids with the lowest AF across locations were FR2128/NC300, TX770/CML288 and NC300/CML288. The average AF in 2003 was 186.8 ppb at CS, 217.49 ppb at WE and 40.73 ppb at CC. Less susceptible hybrids were CML338/Tx772 and B104/Tx772 at CS and FR2128/NC300 and CML338/NC300 at WE. Both inoculation techniques were effective to induce AF but the levels and ranges were higher with the silk channel technique than by using colonized kernels technique. The relative aflatoxin accumulation in hybrids changed substantially with the location and was not consistent. Nevertheless, yellow corn inbreds such as CML288, NC300, FR2128, CML338, Tx772, and CML161 had less AF in several hybrids and locations. The less-susceptible hybrids to AF involved subtropical or exotic adapted inbreds with hard endosperm. In general, their hybrids were more resistant to AF than current commercial hybrids.

Aflatoxin Accumulation and Associated Traits in Maize Inbreds and their Testcrosses

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Preharvest aflatoxin (AF) contamination is one of the main limitations for corn (*Zea mays*, L.) production in the southern USA, causing enormous economic losses and posing a risk to animal and human health. The objectives of this study were (1) to estimate aflatoxin accumulation and expression of associated traits in QPM inbreds and their testcrosses, (2) to compute their repeatabilities and correlations, and (3) to study the relationship between inbred lines and their testcrosses for aflatoxin accumulation. Forty-eight inbreds and their testcrosses with Tx804, a soft endosperm *o2* temperate inbred, were evaluated in three locations in south and central Texas: College Station (CS), Weslaco (WE), and Corpus Christi (CC) during year 2002. The inbreds were developed in Texas from CIMMYT QPM populations 65, 66, 69, 70, Pools 26, 33 and 34, and from a Temperate x Tropical High-Oil population. Inbreds Tx802, CML161 and Tx804 were used as checks in the inbred line evaluation. Hybrids Pioneer Brand 31B13 and 32R25, Dekalb DK668, Asgrow RX897, high lysine hybrids (OP) from Crow's Hybrid Company, SR470, SL53 and SR660, and 5 QPM hybrids were added to the hybrid experiment to complete a 60 entry trial. An alpha-lattice field experimental design with 4 reps at CS and WE and 3 reps at CC was used. In addition to aflatoxin, the following traits were measured: grain yield as hand harvested dried grain weight expressed in Mg/ha, female flowering (FF) as days from planting to 50% of the plants in one plot with emerged silks, visual rating for kernel integrity (1 = all ears without splits kernels or damaged by insects to 5 = most of the ears with splits and/or insect damage), endosperm texture as visual rating from 1 (flint = round crown kernel and vitreous appearance) to 5 (dent = kernels with pronounced dentation and high proportion of floury endosperm). Average AF accumulation for inbreds was 286.3 ng/g at WE, and for testcrosses was 596.5 ng/g at CC, 325.1 ng/g at WE, and 105.1 ng/g at CS. Flinty orange inbreds developed from CIMMYT Population 69 were the least susceptible to AF accumulation both in inbreds and testcrosses at all locations. Repeatabilities for AF (R = 0.67) and its logarithmic transformation (R = 0.92) in inbreds were relatively high compared with previous estimations. Repeatability estimates for AF in testcrosses were 0.38 at CS, 0.62 at WE, 0.66 at CC, and 0.54 across locations. AF in testcrosses was positively correlated with endosperm texture (0.67) and kernel integrity (0.60), and negatively correlated with grain yield (- 0.30) and silking date (- 0.50). The association between the expression of traits in inbreds and AF in testcrosses was relatively high for endosperm texture (R^2 = 0.62), silking date (R^2 = 0.44), kernel integrity (R^2 = 0.39), and AF (R^2 = 0.28 for ng/g and 0.6 for log ng/g). Less AF accumulation was associated with flinty endosperm texture, better kernel integrity, greater grain yield, and later maturities. It seems plausible to select for associated traits having high heritabilities and strong correlation with aflatoxin, in addition to low aflatoxin accumulation in inbreds and hybrids to reduce the risk of aflatoxin contamination.

Progress Toward Developing Stress-Tolerant and Low-Aflatoxin Corn Hybrids for the Southern States

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Aflatoxin contamination of corn, caused by *Aspergillus flavus*, is a chronic problem in Texas and the southern United States. A hot and dry environment and corn earworm (*Heliocoverpa zea*) feeding increases the aflatoxin level. Drought and heat tolerant corn have less grain molds under drought stress. We believe that genetic improvement of drought tolerance, heat tolerance, and corn earworm resistance can reduce the aflatoxin risk in Southern environments.

Experimental hybrids and commercial checks were grown at Corpus Christi and the High Plains in Texas. Aflatoxin was measured only at Corpus Christi. The Corpus Christi tests were planted late (early April) to encourage severe drought stress at later stages of maturity. The tests used a randomized complete block design with nine replications. When the first hybrid was at mid-silk, corn kernels colonized by a high aflatoxin-producing isolate of *A. flavus* (NRRL3357) were distributed between all rows at 1 kg dry seed equivalent per 9.4 m. This provided the increased and uniform aerial dissemination of conidia which favored greater infection by the high aflatoxin- producing isolate but without any physical injury to ears and kernels. Ears from each plot were hand-harvested. All ears were threshed and agronomic data were recorded including grain yield. To reduce aflatoxin assay costs, grain from the original replications were systematically pooled to three composite replications and analyzed for aflatoxin content. Replications 1, 2, 3 were pooled to form composite rep 1, and reps 4-6 and 7-9 to form composite reps 2 and 3, respectively. All grain from each composite replicate was initially ground in a Romer mill at the coarse grind setting. After thoroughly mixing the ground kernels, a 200 g sub-sample was ground again at the finest grind setting on the mill. Aflatoxin 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. Standard ANOVA was performed to test for differences among hybrids. Natural log transformation of aflatoxin data was made before data analysis.

The average aflatoxin levels were 398 ppb, 1276 ppb, and 849 ppb for the tests in 2000 (20 hybrids), 2001 (14 hybrids), and 2002 (12 hybrids), respectively. In the three years, our hybrid Tx202 x CML343 consistently had low aflatoxin contamination (120 – 207 ppb) which was 69% to 92% lower than the check Pioneer hybrid P31B13. In 2003, aflatoxin concentrations at Corpus Christi ranged from 31 to 240 ppb with a mean of 97 ppb. Four experimental hybrids had significantly lower aflatoxin (31 to 54 ppb) than the check 31B13 (161 ppb). The experimental hybrids were developed by the corn-breeding program of the Texas Agricultural Experiment Station (TAES) in Lubbock. The low-aflatoxin TAES hybrids yielded equally or significantly higher than the checks at Corpus Christi and in the High Plains. For example, S1W x CML343 yielded 14,288 kg/ha while 31B13 produced 13,946 kg/ha under irrigated conditions at Halfway, TX. The TAES experimental hybrids and their parental lines were selected for drought and heat tolerance, CEW resistance, and overall agronomic performance. They have tight husk, good ear tip coverage, significantly lower grain mold, and less ear injuries by corn earworm. Results indicate that breeding for drought tolerance and earworm resistance is a promising approach to reduce aflatoxin contamination in corn grown in Southern environments.

Using Genotype by Environmental Interactions to Characterize Corn Hybrids for Resistance to Aflatoxin

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Erratic aflatoxin contamination in corn (*Zea mays* L.) poses difficulty in separating more resistant hybrids from less resistant ones in cultivar field trials. The objective of this research was to identify superior corn hybrids using statistical techniques where the aflatoxin concentration in each hybrid was regressed on the mean aflatoxin concentration of all hybrids in the corresponding environment, i.e., the environmental index. This procedure generally allows a much more powerful test than traditional ANOVA techniques when used to determine the yield and agronomic responses of individual cultivars across environments. Twenty-three commercial corn hybrids were produced in two replications at four Louisiana locations across a 2-year period. Ten ears per plot were inoculated with *Aspergillus flavus* (90×10^9 spores/ml) after silking. The inoculated ears were harvested at maturity along with 10 non-inoculated ears. The ears were rated for *A. flavus* growth, shelled, and kernels ground to a meal. Aflatoxin concentration was determined. Although aflatoxin concentration varied greatly among the environments, there were no significant differences among hybrids via traditional analyses of variance and means separation techniques (P=0.05). There also was no significant interaction between inoculation treatments and hybrids. Inoculated and non-inoculated data were pooled and aflatoxin concentration in each hybrid was regressed on the corresponding mean aflatoxin concentration of all hybrids across all environments. Hybrids had highly significant interactions with the environments, and individual hybrid responses differed significantly. Outliers were identified at P=0.001 and removed, which improved fit (R^2) in 21 of the 23 hybrids. Graphic displays of the interactions clearly revealed superior hybrids. Measuring the genotype by environmental interaction for aflatoxin in commercial corn hybrid performance trials provided a ready means for removing excessively high outliers and increased the ability to identify superior hybrids compared with traditional analyses of variance and means separation techniques.

An Integrated Data Management System Supporting Aflatoxin Studies

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An on-going project in the corn host plant resistance research unit of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) at Mississippi State University is intended to determine the effects of biotic and abiotic factors on *Aspergillus flavus* infection and aflatoxin accumulation on maize and to develop maize cell lines with resistance to the insect damage, *A. flavus* infection, and aflatoxin accumulation. To support this project, researchers in the Intelligent Systems Laboratory of the Department of Computer Science and Engineering at Mississippi State University have developed an integrated database system with data modeling and management capabilities. The intent of this database system is to provide the biological scientists with a complete set of information regarding their experiments. This means that it is necessary to store not only the raw data, but also descriptive data (metadata) about the devices, methods, and software tools used, the parameters that were defined, etc., as well as the experimental results. The information stored in the database should provide users with enough information to be able to recreate the experiments if desired. This database differs from other existing databases in its comprehensiveness and completeness.

The database consists of five major classes of data: germplasm data, field data, quantitative trait loci (QTL) analysis data, proteomics data, and weather data. The germplasm data consists of information about the various inbreds and mapping populations used in the experiments. The field data specifies on which farm (and which block of plants on a given farm) the experiments were conducted along with the field experiment results. The QTL analysis data provides information about the markers, screening gel images, mapping population gel images, genotypes, and QTL analysis results. The proteomic data includes data sample information, sample processing information (i.e., 2D gel images), mass spectrometry data, and mass spectrometry analysis results (i.e., protein database search results). The weather data includes information about air temperature, moisture levels, wind speed, wind run, solar radiation, rainfall, pan evaporation and soil temperature.

A biologist must be able to integrate these different types of data for a complete analysis. The database system provides a tool that can be used to efficiently archive and manage the data. The database system's capabilities will be extended to provide data mining capabilities in addition to the data management capabilities.

A user-friendly web-based interface has been designed to provide quick and flexible access to the database system for investigators. This database system has been designed to be compatible with several existing database system such as the Maize Genetics and Genomics Database (MaizeGDB, http://www.maizegdb.org/) and the PEDRo proteomics database model (http://pedro.man.ac.uk/) so that users may import data from these external databases and export data to them.

Using Genetic Algorithms to Derive Environmental Indices Correlated with Aflatoxin Levels

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Infection by *Aspergillus flavus* and the resulting production of aflatoxin is a major problem in maize in the southern U. S. and other warm climates. Some cultivars of maize are more resistant to *Aspergillus* infection than others. Environmental conditions affect infection rates, but the relationship appears to be quite complex. It is not clear which environmental factors (i.e. temperature, rainfall, etc) are most important, how these factors should be combined (minimum, maximum, average) nor which time periods are most critical. The goal of the present work is to develop a method for deriving an environmental index that correlates with aflatoxin levels in maize and that can be used to study the differences in responses of resistant and susceptible varieties.

Data available for this study include aflatoxin levels at maturity, middle silk (flowering) date, and environmental data from 1998 to 2002. Two aflatoxin-resistant maize single crosses, Mp313E × Mp420 and Mo18W × Mp313E, and two susceptible single crosses, Mp339 × SC212m and GA209 × SC212m, were grown in replicated field trials conducted at Mississippi State, MS, in 1998-2002 and at Stoneville, MS, in 1999. Approximately 7 days after mid-silk, the top ears in each plot were inoculated with an *A. flavus* spore suspension using the side needle technique. Ears were harvested approximately 60 days after inoculation. After the ears were dried and shelled, the grain was ground and analyzed for aflatoxin using the Vicam Aflatest system.

In our approach, we use a genetic algorithm to evolve the specification of an environmental index. An index specification is represented as a population of randomly initialized artificial chromosomes. Each chromosome represents the environmental variable to be measured, the period of time over which it should be measured (beginning date relative to flowering and length of the interval), and how the values should be combined over the interval (maximum, minimum, average). The correlation of the index specified by each chromosome and measured aflatoxin levels is used to evaluate the "fitness" of each chromosome. This correlation is computed using linear regression. More fit chromosomes have a higher probability of being selected for the next generation. Mutation and crossover operations are applied at each generation. A population size of 100, a crossover rate of 0.6, a mutation rate of 0.1, complete replacement, roulette wheel selection, and 150 generations were used for most experiments. We have conducted experiments that consider all environmental variables simultaneously and experiments that consider the environmental variables one at a time. Considering the environmental variables individually worked much better with the limited amount of data available.

Experiments using this approach have been conducted with field data for two susceptible and two resistant lines. The results show that, for all of the four pedigrees, air temperature, rainfall, and pan evaporation are the most important environmental factors affecting aflatoxin levels. The time intervals identified as important for rainfall usually precede those selected for temperatures. Information about the intervals over which the environmental variables are most important may provide useful insights for future work.

Effects of Pollen Genotype on *Aspergillus flavus* Infection and Aflatoxin Accumulation in Maize

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Aflatoxin contamination of grain caused by *Aspergillus flavus* is a major problem of maize production in the United States. Aflatoxin is a chronic problem in the South and occurs sporadically in the Corn Belt. Planting maize hybrids with genetic resistance to aflatoxin contamination is one way to potentially reduce or eliminate toxin levels in grain. Germplasm lines with resistance to aflatoxin accumulation have been developed and released, and are available for use in both public and commercial breeding programs. Most evaluations for aflatoxin resistance are conducted in field studies consisting of open-pollinated plants. It is not known whether the aflatoxin resistance of the pollen parent affects aflatoxin accumulation in ear-bearing plants artificially inoculated with *A. flavus*. The purpose of this investigation was to compare the effects of pollen source on aflatoxin contamination of grain of hybrids with varying levels of aflatoxin

Experiments were conducted on the R. R. Foil Plant Science Research Center, Mississippi State, MS. Commercial hybrids used as ear-bearing plants were TV2100, an aflatoxin susceptible hybrid, and RX938, a moderately resistant hybrid. Five hybrids used as the pollen source for the crosses were Mp313E X Mp715, RX938, TV2100, GA209 X SC212M, and Silver Queen. The degree of aflatoxin susceptibility and resistance varied greatly among these five hybrids. Ear-bearing plants were hand pollinated with pollen from each pollen source. Pollinated ears were inoculated 14 days after pollination using the side-needle technique. Ears were harvested 65 days after pollination and aflatoxin contamination was determined using the Vicam Aflatest. The five pollen source hybrids were allowed to open pollinate and the ears were inoculated 14 days after midsilk. The ears were harvested and aflatoxin quantified as described above. *A. flavus* kernel infection of hand pollinated and open pollinated ears was determined by plating kernels on Czapek agar amended with NaCl.

The five pollen parents varied significantly in levels of aflatoxin contamination and in *A. flavus* kernel infection. Silver Queen, a sweet maize hybrid, had the highest level of aflatoxin accumulation with 3253 ng/g. Mp313E X Mp715, an aflatoxin resistant hybrid, had the lowest level of aflatoxin accumulation among the five open pollinated hybrids with a level of 22 ng/g. Silver Queen had a significantly higher kernel infection percentage among the five open pollinated hybrids. Mp313E X Mp715 had the lowest percent kernel infection. Aflatoxin contamination was significantly higher in ears of TV2100 pollinated with pollen from the resistant hybrid, Mp313E X Mp715, than when pollinated with pollen from the four other hybrids. There were no differences ($P = 0.05$) in aflatoxin contamination of ears of TV2100 pollinated using pollen from RX938, TV2100, GA209 X SC212M, or Silver Queen. Pollen source had no affect on *A. flavus* infection of kernels from ears of TV2100 (the ear bearing plant). Aflatoxin contamination was highest in ears of RX938 pollinated with pollen from Mp313E X Mp715, RX938 and GA209 X SC212M. Aflatoxin contamination in ears of RX938 when pollinated with pollen from Mp313E X Mp715 was significantly higher than when pollinated with pollen of Silver Queen or TV2100. *A. flavus* infection of kernels from ears of RX938 was not affected by pollen source.

Although pollen source may affect aflatoxin contamination, results indicate that the primary factors responsible for resistance are most likely controlled by the genotype of the ear-bearing plant.

Investigation of Gene Effects on Aflatoxin Concentration in a Segregating Maize Population

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One approach toward enhancing resistance to *Aspergillus flavus* infection and aflatoxin accumulation in corn (*Zea mays* L.) involves testing of candidate genes. Candidate genes associated with environmental stresses including drought stress, heat stress, insect damage, and nutrient deficiency were chosen for study. Gene sequences were also contributed from other laboratories. Selections included loci coding for basal endosperm specific proteins, heat and drought-stress related oxidases, pathogen specific signaling peptides, proteins involved in insect resistance, and water-stress related proteins. Genetic markers (normally microsatellites) were collected that are known to be associated with the gene of interest. When these were not available, primers were created based on published sequence data. Markers were first screened over inbreds differing in aflatoxin build up in order to identify size polymorphisms. Polymorphic markers were then added to the genetic map in a $F_{2:3}$ mapping study for further analysis. Primer-derived sequences not obviously differing in length were then sequenced to determine if single nucleotide polymorphisms exist between resistant and susceptible inbreds. These can be used to create functional markers for addition to the mapping study. Finally, single marker analysis followed by composite interval mapping are being used to statistically measure the effect a gene of interest may have on aflatoxin accumulation. To date, the gene sequences *glb2*, *mir3*, and putative gene sequence *hmc1a* have had specific sequence regions amplified from their inbred parents Mp313E and B73. These have been screened for size polymorphism and await further marker development. Amplified fragment size differences are not readily apparent between resistant and susceptible inbreds for many of the markers studied thus requiring specific sequencing to identify alleles. Marker umc1631 associated with the *Lox1* gene does appear to be linked to QTL on chromosome 4 influencing aflatoxin accumulation and is a likely candidate. Other candidates await sequencing in order to study further.

Oh516 as a Source of Resistance to *Aspergillus* Ear Rot and Aflatoxin Production in Corn

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Aspergillus ear rot of corn (Zea mays L) produced by *Aspergillus flavus* Link:Fr. is of economic concern due to the production of aflatoxin B_1. Our objective was to determine the usefulness of inbred line Oh516 as a source of resistance to *Aspergillus* ear rot and aflatoxin production by determining the types of gene action for low levels of aflatoxin, ear rot and bright greenish yellow fluorescence (BGYF). We also further investigated selection based on low levels of BGYF as a method to indirectly reduce the concentration of aflatoxin in grain. Lastly, we estimated heritabilities and predicted gain from selection for low levels of aflatoxin, ear rot and BGYF using both the BCP_1S_1 families and those same families test crossed with LH185 at three different locations. In 2001 and 2002, grain from the resistant inbred Oh516 (P_1), the susceptible inbred B73 (P_2), and the F_1, F_2, F_3, BCP_1, BCP_2, and BCP_1S_1 generations were evaluated for aflatoxin concentrations, *Aspergillus* ear rot, and BGYF following inoculation. In 2002, families from the BCP_1S_1 generation were test crossed with LH185 and evaluated for aflatoxin concentrations, *Aspergillus* ear rot and BGYF following inoculation at Urbana, IL, Ganado, TX, and Batesville, TX. Dominance is important for low levels of BGYF and low concentrations of aflatoxin in grain. The correlation coefficients between aflatoxin concentration and BGYF in 2001 and 2002 were 0.67 and 0.57, respectively. Heritabilites for low levels of aflatoxin were greatest at Ganado, TX (29.1%). We determined that inbred Oh516 is a source of resistance that will have utility in improving commercially used corn inbreds for resistance. Molecular markers associated with resistance in inbred Oh516 are currently being located.

Session 3:
    Microbial Ecology
    Moderator: Phil Wakelyn, National Cotton Council

A Good Endophyte of Maize: *Acremonium zeae* Antibiotics Inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*

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The maize endophyte *Acremonium zeae* Gams and Sumner is antagonistic to kernel rotting and mycotoxin producing fungi *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests for antagonism and interferes with *A. flavus* infection and aflatoxin contamination of preharvest maize kernels. Chemical studies of an organic extract from maize kernel fermentations of *A. zeae* NRRL 13540, which displayed significant antifungal activity against *A. flavus* and *F. verticillioides*, revealed that the metabolites accounting for this activity were two newly reported antibiotics pyrrocidines A and B. Pyrrocidines were detected in fermentation extracts for eleven NRRL cultures of *A. zeae* isolated from maize kernels harvested in Illinois (4/4 cultures), North Carolina (5/5) and "USA" (2/2), but were not detected in two corn kernel isolates from Georgia. Pyrrocidine B was detected by LCMSMS in whole symptomatic maize kernels removed at harvest from ears of a commercial hybrid that were wound-inoculated in the milk stage with *A. zeae* NRRL 13540 or NRRL 13541. The pyrrocidines were first reported from the fermentation broth of an unidentified filamentous fungus, LL-Cyan-426, isolated from a mixed Douglas Fir hardwood forest on Crane Island Preserve, Washington, in 1993 [H. He et al., Tetrahedron Letters 43:1633-1636, 2002]. It was reported that pyrrocidine A exhibits potent activity against most Gram-positive bacteria, including drug-resistant strains, and was also active against the yeast *Candida albicans*. In an evaluation of cultural antagonism between isolates of *A. zeae* in pairings with *A. flavus* NRRL 6541 and *F. verticillioides* NRRL 25457, *A. zeae* NRRL 6415 produced the strongest reaction, inhibiting both organisms at a distance while continuing to grow through the resulting clear zone at an unchanged rate. Maximum colony diameters for *A. zeae* NRRL 6415 and NRRL 13540, on potato dextrose agar after 14 days, were attained within the range of 25-30 C, with less growth recorded at 15 C and 37.5 C, and no growth at 5 C. Potential interactions between *A. zeae* and other maize endophytes are considered and the significance of these interactions relative to the aflatoxin and fumonisin contamination of preharvest maize is presented. This is the first report of natural products from *Acremonium zeae*.

Relationship between *Aspergillus* Soil Population Density and Infection of Wounded Peanut Seeds

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Soil is the source of primary inoculum for *Aspergillus flavus* and *A. parasiticus*, fungi that produce the highly carcinogenic aflatoxins in agricultural commodities. Aflatoxigenic fungi commonly invade peanut seeds during maturation and the highest concentrations of aflatoxins are found in insect-damaged seeds. A laboratory assay was developed in which sterile, viable peanut seeds were wounded and inoculated with different soils (n = 20) from cultivated and fallow fields and from forested land. The effect of fungal density in soil on percent seed infection best fits a logarithmic function showing a rapid rise in infection with increasing fungal density followed by saturation at a particular level of infection. Coefficients of determination (r^2) were significant (P < 0.001) for all species in section *Flavi*: *A. flavus* L strain (0.70), *A. flavus* S strain (0.91), *A. parasiticus* (0.55), *A. tamarii* (0.85), *A. caelatus* (0.59) and *A. alliaceus* (0.83, linear function). Species from other sections in the genus, *A. niger* (0.75) and *A. terreus* (0.93), showed similar logarithmic regressions. Other species of fungi were rare on peanut seeds when inoculated with cultivated soils and they were detected only on seeds inoculated with soils containing extremely low densities of section *Flavi* species and *A. niger*.

Nearly all wounded seeds became infected with one or more species from section *Flavi* at > 400-500 CFU/g of soil. An estimated 1-3 propagules at the wound site were sufficient for 50% infection of peanut seeds. Maximum percent infection by individual species often occurred at considerably less than 100% despite high soil densities. The order of species according to maximum infection levels approximated the frequency at which species are found in peanuts in the field: *A. flavus* > *A. parasiticus* > *A. tamarii* and *A. caelatus*. Competition by *A. niger* and other members of section *Flavi*, as evidenced by high densities in soil, may be responsible for limiting infection by aflatoxigenic species.

Commercial Production and Deployment of Atoxigenic Strains of *Aspergillus flavus* in Arizona Cotton

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Aspergillus flavus AF36 belongs to a vegetative compatibility group that has been shown to uniformly lack the ability to produce aflatoxins. AF36 has the ability to competitively exclude aflatoxin-producing fungi and thereby reduce aflatoxin contamination of cottonseed. In greenhouse, field-plot, and commercial field tests the efficacy of *Aspergillus flavus* AF36 has repeatedly been demonstrated. Applications of *Aspergillus flavus* AF36 reduce the average aflatoxin producing potential of fungal communities within both treated and nearby fields. Treatments have multi-year benefits. Large-scale commercial field studies conducted in Arizona over multiple years have demonstrated *Aspergillus flavus* AF36 to be a useful tool in the long-term management of aflatoxin contamination of cottonseed. A partnership between the Arizona cotton industry through the Arizona Cotton Research and Protection Council and the USDA, Agricultural Research Service seeks to develop technology for commercial production and utilization of AF36 in Arizona. The goals of this partnership have been and continue to be: 1. Development of area-wide management strategies; 2. Optimization of application and agronomic practices to achieve maximum single season and long-term reductions in the aflatoxin content of Arizona cottonseed; 3. Development of processes that grower cooperatives and other grower-run organizations can use to produce needed quantities of atoxigenic strain material; 4. Compilation of information required by EPA for full registration of *Aspergillus flavus* AF36. The fourth year of treatments under this collaboration was completed in 2002 with the treatment of 16,558 acres in nine treatment areas extending from the Mohave, Parker and Gila Valleys on the far western edge of Arizona to the Maricopa-Stanfield area in the south central portion of the state. Because a section 3 registration was not granted by EPA for the 2002 season, applications of AF36 continued to be limited to a maximum of 20,000 acres under an Experimental Use Permit. Improved standardization of production had the highest priority in AF36 facility development in 2002. Modifications in the incubation chamber's thermostatic control system facilitated development of optimum temperature regime for large-scale wheat seed colonization. Computerized temperature controls are being developed to maintain more precise management of simultaneous drying of multiple 2,000-pound batches. A Heat-seal packaging system was installed to provide improved product delivery options for farms incapable of handling bulk delivery containers. During the 2002 crop year, a total of nine organized treatment areas representing eleven cotton gins and 629 fields were established in Mohave, La Paz, Yuma, Maricopa and Pinal Counties in Arizona. A combined total of 16,558 acres received AF36 applications. Analysis of the 2002 crop revealed cumulative effects of large-scale AF36 treatments. *A. flavus* communities on crop samples from 271 randomly selected treated fields averaged 70.4% AF36 and 1.8% S strain. This is in contrast to pretreatment *A. flavus* soil compositions that averaged <4% AF36 and >50% S strain. Incidences of atoxigenic strain AF36 increased in four of five areas treated in 2002 compared to 2001 levels: Buckeye (57/41% AF36), Stanfield (78/70% AF36), Parker (56/43% AF36), Gila Valley 65/56% AF36), Roll/Texas Hill (80/82% AF36). First-year treatments to the southern half of the Mohave Valley (northwest Arizona) were particularly effective in 2002. For the past ten years prior to AF36 treatment, annual production of seed below 20 ppb in the Mohave Valley ranged from 35-56%. Following treatment of 933 acres in 2002, 86.3% ginned seed was below 20 ppb. The incidence of AF36 on the crops from six randomly selected fields averaged 91% presence. Efforts continue to focus on improvements in both timing of applications and delivery systems.

Biocontrol of *Aspergillus flavus* by Saprophytic Yeast: Progress from Laboratory Bioassay to Field Trial

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The bioassay developed for screening effective yeast to inhibit both the growth of the *Aspergillus flavus* and aflatoxin production has been used to select a few species of yeast strains as biocontrol agents. One particular yeast, *Pichia anomala* WRL-076, was tested for its antagonistic activities to reduce spore production of both toxigenic and atoxigenic strains of *A. flavus* in pistachio flowers and nut-fruits as well as in almond and pistachio leaves. These experiments were conducted in the lab using sterilized plant material. We monitored the growth and viable number of *A. flavus* spores on yeast-sprayed and unsprayed pistachio fruits, leaves, flowers and almond leaves. In plant samples inoculated with this yeast, the level of spore production was reduced by 60 to 80% compared to the control samples in the absence of yeast.

A research permit for field tests of the yeast has been authorized by the Department of Pesticide Regulation, California Department of Food and Agriculture. Field evaluation of the efficacy of the biocontrol agent*, P. anomala* WRL-076, was carried out at the Wolfskill Experimental Farm in Winters, CA. Pistachio nut-fruits on the tree were individually wounded with a dental needle and sprayed with an aqueous suspension of yeasts at 3×10^7 cells/mL on July 10, 2003. The wounded nut-fruits without yeast-spray were used as controls. Wounded nut-fruit was hand-picked from the tree and immediately placed to a special agar medium on August 8. The experimental nut-fruits were incubated at 28°C for eight days. Colonization and fungal growth on wounded nut-fruits were visually scored with the aid of a microscope. Ninety five percent of the wounded nut-fruits (control) showed obvious fungal growth. In contrast nut-fruits sprayed with the effective yeast showed much less fungal growth and 35% of the nut-fruits did not have any visible fungal growth. The viable fungal counts on individual nut were enumerated by standard microbiological techniques. Colonization of *A. flavus* in yeast-sprayed and wounded nut-fruits was 2-3 fold lower than the control. Furthermore the total spore numbers of *A. flavus* on yeast-sprayed nut-fruits had decreased by 90% in comparison to the control. The field bioassay indicated strongly that *P. anomala* strain WRL-076 could protect the wounds from the infection and growth of *A. flavus* and other fungi.

In conclusion, *P. anomala* WRL-076 sprayed to the wounds of nut-fruits inhibited the colonization of *A. flavus* and other fungi as well as the growth of these fungi under stress conditions. Further field experiments are warranted. Collaborative field trials with researchers and grower cooperatives will be established.

Aflatoxin Control in Figs: Biocontrol and New Resistant Cultivars

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In 2002 we repeated the biocontrol experiment done in 2001. Besides the two promising atoxigenic strains (A564 and A815) of *Aspergillus flavus* used in 2001, an additional atoxigenic strain AF36 was used. On 17 June, wheat seeds infected with these strains were applied using the same methods as in 2001 (41.2 g wheat/tree, equivalent to 10 lbs/acre). On 19 August, we collected noncaprified figs from the soil surface. Almost all *A. flavus* isolates (97%) obtained from the noncaprified figs belonged to one of the applied atoxigenic strains. On 2 October, leaf and additional soil samples were taken. The soil had a low density of *A. flavus* (ranging from 12.0 to 17.6 cfu/g soil, depending on treatment). The AF36 strain increased in the area where wheat with AF36 was applied from 0.0% of the *A. flavus* isolates before the wheat was applied to 43.9% of the isolates 3 months later. The atoxigenic strains A564 and A815 were detected at about the same level in the areas where they were applied (80.0 and 85.4% of the *A. flavus* isolates, respectively). The higher levels of A564 and A815 in the soil compared to that of AF36 are probably due to A564 and A815 also having been applied the previous year. The density of *A. flavus* on leaves did not differ significantly between treatments (ranging from 0.012 to 0.023 cfu/cm^2, depending on treatment). The atoxigenic strains were detected on the leaves at a relatively high frequency (between 55.0 to 78.6% of the isolates belonged to one of the applied atoxigenic strains, depending on treatment). Evaluations of soil samples collected in May 2003 showed that all three atoxigenic strains persisted well through the winter.

In late spring 2003, the research fig orchard was converted from flood irrigation to drip irrigation. On 9 July, wheat seeds infected with the atoxigenic strain AF36 were applied to the area beneath the drip emitters at the same rate as in 2001 and 2002 (41.2 g wheat/tree, equivalent to 10 lbs/acre). Soil collected just prior to application of the wheat had a low density of *A. flavus* (ranging from 0.5 to 10.5 cfu/g soil, depending on treatment), and the incidence of AF36 ranged from 5.9% of the *A. flavus* isolates in the nontreated area to 37.4% in the areas treated with AF36 in 2002. On 21 August, we collected noncaprified figs from the soil surface and observed colonies of *A. flavus* on them. Depending on the treatment, 0.0 to 13.3% of the noncaprified figs had *A. flavus*. On 10 October, leaf and additional soil samples were taken and are currently being evaluated. Isolates of *A. flavus* from commercial fig orchards were evaluated and both the AF36 strain (6.2% of 97 isolates evaluated) and the A564 strain (1.0%) were detected among isolates but not the A815 strain.

New fig selections have been developed by a breeding program that is attempting to produce a Calimyrna-type fig that has the advantages of Calimyrna figs but not the disadvantages of having high levels of decay and aflatoxin contamination. In 2002 the most promising selection, 6-38W, had substantially smaller eye diameter of dried fruit (0.9 mm) than Adams (2.3 mm) and Calimyrna (3.3 mm) but about same as Conadria (0.8 mm). Furthermore, the incidence of decay by *Aspergillus* spp. of the dried figs of 6-38W (1.0%) was substantially lower than that of Calimyrna (5.0%) and Conadria (5.1%). In 2003 the new selection 6-38W had substantially smaller eye diameter of ripe figs still on the tree (0.8 mm) than Conadria (1.3 mm), Adams (3.3 mm), and Calimyrna (4.5 mm). In addition, 1,000 dried figs of 6-38W and each of the commercial fig cultivars (Calimyrna, Conadria, and Adams) have been collected from commercial orchards and are being evaluated for fruit decay. The new fig selection 6-38W continues to show promise in reducing aflatoxin contamination compared to Calimyrna figs.

Aflatoxin Control in Pistachios: Biocontrol Using Atoxigenic Strains

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In 2002 three promising atoxigenic strains (AF36, A564, and A815) of *Aspergillus flavus* were tested as biocontrol agents. On 1 July, wheat seeds infected with these strains were applied at the rate equivalent to 10 lbs/acre in a research pistachio orchard. Soil collected on 23 September in the nontreated area had substantially lower density of *A. flavus*/*A. parasiticus* (10 cfu/g) than the treated areas (43 to 90 cfu/g, depending on strain). Almost all of the *A. flavus* isolates from the soil in the treated areas belonged to the applied atoxigenic strain (93.3 to 98.3% of the isolates, depending on strain). All three strains were detected in the untreated area. The density of *A. flavus* on leaves did not differ between treatments. The atoxigenic strains were detected on the leaves, ranging from 42.4 to 62.8% of the isolates (depending on treatment). In 2003 no inoculum was applied in this orchard, but soil samples were collected on 19 August and are currently being evaluated. Isolates of *A. flavus* from commercial pistachio orchards were evaluated, and all three atoxigenic strains AF36 (6.3% of the 430 isolates evaluated), A564 (2.1%), and A815 (1.9%) were detected.

In 2003 we initiated a biocontrol experiment in a research pistachio orchard that was irrigated by microsprinklers. On 1 July, wheat seeds infected with the atoxigenic strain AF36 were applied. On 23 September, leaf and additional soil samples were taken and are currently being evaluated. Also, early split nuts were collected and found to have very low incidences of decay by *A. flavus* (0.0 and 0.5% of the early split nuts for nontreated areas and areas treated with AF36, respectively).

In 2002 and 2003 various studies besides the biocontrol studies were done. One study investigated the infestation by navel orangeworm (an important factor in aflatoxin contamination) of nuts with no or little shell staining. The results from 2001 suggested that size might be used in addition to shell stain to remove infested nuts. In order to confirm these results, we obtained nut samples for 24 commercial orchards from a processor and are currently evaluating these samples. Because the citrus flat mite can damage the hull (which is possibly favorable for navel orangeworm infestation), nut samples were collected from three orchards with high levels of mite damage and are currently being evaluated. A study investigating the aflatoxin contamination of over 400 "library" samples obtained from a processor found that nuts from a second harvest is almost twice as likely to be contaminated with aflatoxin as the initial harvest; certain counties tended to have more aflatoxin contamination than others; and nuts harvested early had approximately half the incidence of aflatoxin contamination of late harvested nuts.

We completed a two-year study on the relationship of factors that might contribute to aflatoxin contamination in commercial orchards. Soil, leaf, and nut samples were collected from 10 orchards. The incidence of early split nuts in an orchard was correlated with the incidence of normal nuts with split shells, which suggests that practices that enhance normal shell splitting might also increase formation of early split nuts. The amount of dust on the leaves was correlated with the density of *A. flavus*/*A. parasiticus* on the leaves (r^2=0.26). The density of aflatoxin-producing fungi on leaves was poorly correlated to that in soil, suggesting that using leaves and using soil will predict different levels of aflatoxin contamination. In 2003 we initiated a study comparing five orchards with a history of high aflatoxin contamination to five orchards with a history of no aflatoxin contamination. Soil, leaf, and nut samples have been collected from these 10 orchards and are being evaluated.

PANEL DISCUSSION: Microbial Ecology

Panel Chair: Bruce Horn

Panel Members: Donald Wicklow, Bruce Horn, Peter Cotty, Larry Antilla, Sui Sheng Hua, Mark Doster, and Themis Michailides

Summary of Presentations: Phil Wakelyn of the National Cotton Council moderated the session. Panel chair Bruce Horn stated in his introductory remarks that five of the talks dealt with biological control involving reduction of aflatoxins and that the other two talks by Donald Wicklow and Bruce Horn addressed aspects of fungal competition, an important component to biological control. Horn further expressed his opinion that research on biological control, in addition to the practical applications, has led to increased knowledge about the ecology and population biology of aflatoxin-producing fungi. Donald Wicklow presented data showing that *Acremonium zeae* is antagonistic to *Aspergillus flavus* and *Fusarium verticillioides* due to production of the antibiotics pyrrocidines A and B. Pyrrocidine B was detected in whole symptomatic corn kernels from wound-inoculated ears of a commercial hybrid grown in central Illinois and may be important in limiting aflatoxin and fumonisin contamination in the northern corn-growing regions of the United States during years when conditions are not optimal for *A. flavus* and/or *F. verticillioides*. Bruce Horn followed with a summary of his experiments in which sterile, viable seeds were wounded and inoculated with soils with different populations of *Aspergillus* species. The relationship between fungal density in soil and seed infection by species in section *Flavi* was best described by a logarithmic function, and only a few propagules at the wound site were required for high levels of infection. Peter Cotty's presentation indicated that aflatoxin levels in Arizona cottonseed varied considerably from year to year following treatment with nonaflatoxigenic *A. flavus* and that the effectiveness of the treatment was best measured by the proportion of biocontrol strain to wild-type strains in soil and cotton. Geostatistical analyses of aflatoxin content in cotton from southern Texas revealed regions with recurrent patterns of low and high contamination. Larry Antilla detailed recent improvements in solid-state fermentation for producing wheat colonized by nonaflatoxigenic *A. flavus*. He also summarized data showing shifts in soil population composition following application of the biocontrol strain to soil as well as reductions of aflatoxin levels in cottonseed from those areas. Sylvia Hua presented the results of her research showing the inhibitory effects of the yeast *Pichia anomala* on sporulation by *A. flavus* on tree nuts under laboratory conditions. Wounded pistachio nuts on the tree sprayed with the yeast had reduced colonization by *A. flavus* and other fungi under stressed conditions. Biological control to reduce aflatoxins in figs was the theme of Mark Doster's presentation. He presented data showing population changes in soil and on leaf surfaces following application of nonaflatoxigenic strains as well as recent research on the development of new fig varieties that may be resistant to aflatoxin contamination. The final presentation was by Themis Michailides who summarized studies on preventing aflatoxin in pistachios through biological control and described attempts to correlate mite and naval orangeworm damage with extent of aflatoxin contamination.

Summary of Panel Discussion: Peter Cotty was asked about the possibility of using nonaflatoxigenic *A. flavus* on corn for biological control of aflatoxins. He felt that the effectiveness of biological control with corn depends greatly on the environmental conditions present in different regions of the United States. In warmer climates, corn is planted early and the cool nighttime temperatures may not be conducive for growth and sporulation by nonaflatoxigenic strains after application to soil. In southern

Texas, cotton is the dominant crop during the warmest part of the growing season and, therefore, is more important than corn in influencing *A. flavus* populations. Treatment of corn alone in southern Texas would not be effective because of the large amounts of aflatoxigenic *A. flavus* contributed by infected cotton. However, treatment of cotton may have a carryover effect on corn in reducing aflatoxin contamination. Further north, where the incidence of infection by *A. flavus* is lower, treatment of corn would be expensive and not economically feasible.

Donald Wicklow was asked if there was any evidence that *Acremonium zeae* is systemic in corn plants. He stated that Donald Summer reported in 1988 that *A. zeae* moved up the corn stalk at about the same rate as *F. verticillioides*. In Nebraska, irrigation of corn favors *A. zeae* whereas dryland corn contains more *F. verticillioides*. Later in the session, Wicklow stated that a pharmaceutical company first reported the discovery of pyrrocidines from an unidentified species of *Cylindrocarpon* isolated from Crane Island Forest Preserve in the state of Washington.

Mark Doster was asked whether the application of nontoxigenic strains to fig orchards would increase the incidence of moldy figs. He responded by stating that populations increased at most only slightly after application of the biocontrol strain and that application of the same biocontrol strain in Arizona did not increase the level of mold in cottonseed. Concern was expressed that during processing, the biocontrol strain could be further disseminated to clean figs and create an increase in postharvest rot. Themis Michailides countered that dried figs are processed with heat that would kill off any residual Aspergilli.

Bruce Horn was asked whether different fungi colonized the wounded peanut seeds when the water activity and temperature were different from the conditions presented in the talk. He stated that species in section *Flavi* invaded peanut seeds over a wide range of water activity and temperature. Water activity in general was more important for invasion by section *Flavi* than temperature; infection was still high at lower temperatures but took longer to develop. *Fusarium* species were present at the highest water activities and the incidence of *Penicillium* species increased at the lower water activities.

DNA Fingerprinting Analysis of Vegetative Compatibility Groups in *Aspergillus caelatus*

Cesaria E. McAlpin,[1] Bruce W. Horn[2], and Donald T. Wicklow[1]
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Forty-three isolates of *Aspergillus caelatus* whose vegetative compatibility groups (VCGs) have been identified were assessed by DNA fingerprinting using a repetitive sequence DNA probe pAF28 cloned from *A. flavus*. Thirteen distinct DNA fingerprint groups or genotypes were identified among the 43 isolates. Twenty-four isolates belonging to VCG 1 produced identical DNA fingerprints and included isolates from the United States and Japan. Four other DNA fingerprint groups had multiple isolates sharing identical fingerprints corresponding to VCGs 2, 3, 12 and 13. Eight of the 13 fingerprint groups corresponding to VCGs 4-11 were represented by a single isolate with a unique fingerprint pattern. These results provide further confirmation that the pAF28 probe can distinguish VCGs of species within *Aspergillus* section *Flavi* based on DNA fingerprint patterns and that the probe can be used for estimating the number of VCGs in a sample population. Most of the *A. caelatus* isolates produced fewer restriction fragments and weakly hybridized with the repetitive DNA probe pAF28 compared to hybridization patterns obtained with *A. flavus*, suggesting less homology of the probe to *A. caelatus* genomic DNA.

Application of Yeast in the Field for Biocontrol of *Aspergillus flavus*

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Prior work in Dr. Hua's lab has shown that selected yeast strains can competitively inhibit the growth of *Aspergillus flavus*. Yeast will be an ideal biocontrol agent, since the strains are selected from naturally occurring isolates. Issues of potential human toxicity should be much less than with other biologically active agents, such as competitive fungi. However, we do not know whether these strains can survive outside, particularly in the hot dry climates of California where pistachio is grown. The present series of experiments was undertaken to begin to explore the extent to which inhibitory yeast strains can survive in the open environment.

Yeast at concentrations of 10^5/mL, 10^6/mL, 10^7/mL, and 10^8/mL were sprayed on individual 'Kerman' pistachio trees in a replicated randomized design. Three spray dates were tested at the various concentrations. Three reps of 20 nuts were harvested from each tree at three dates and assayed for yeast survival. Effects of concentration of sprayed yeast, duration of protection, and number of spray treatments were evaluated.

Preliminary analysis of the results suggest that differences in time of application during the season appeared to have a much larger effect on the residual nut yeast level than the concentration of yeast applied. The number of spray treatments did not appear to be as important as application at the later spray dates. High levels of residual yeast were found on the nuts at the later spray dates and higher concentrations of spray. Future studies will concentrate on time of application and minimum number of sprays needed for protection, at the higher inoculum levels. Methods for improved uniformity of spray application will also be tested.

Aspergillus flavus Community Composition Influences Contamination of Commercial Cottonseed in South Texas

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Regulatory limitations on the quantity of aflatoxins permitted in foods and feeds exist throughout the world. Where contamination is common, diverse communities of aflatoxin-producing fungi reside. Aflatoxin producers are asexual fungi belonging to *Aspergillus* section *Flavi*. Communities of section *Flavi* differ by region in both species composition and aflatoxin-producing potential. The most common aflatoxin-producing species, *A. flavus,* can be divided into two strains based on morphological, genetic, and physiologic criteria. The S strain produces numerous small sclerotia and high levels of aflatoxins, while the L strain produces fewer, larger sclerotia and, on average, less aflatoxin. The *A. flavus* S strain has been implicated as an important causal agent of aflatoxin contamination in several areas worldwide including Arizona.

Cottonseed samples from trucks transporting cottonseed from several gins in South Texas were taken upon receipt at the Valley CO-OP Oil Mill in Harlingen, Texas during the seasons of 1999 to 2001. Most gins were sampled several times during the season. For data analysis, gins were grouped into three different regions, the Rio Grande Valley, the Coastal Bend and the Upper Coast. Six cores (3 to 5 kg each) were taken from each cottonseed truck, mixed, split and subdivided into a single sample of 1 to 1.5 kg. Members of *Aspergillus* section *Flavi* were isolated from cottonseed washings by dilution plating. Isolates were assigned to *A. flavus* S or L strains after subculturing on 5/2 agar for 5 to 7 days at 31 C. The quantities of *A. flavus* on the cottonseed were calculated as colony forming units (CFU) per gram. The percent of *A. flavus* S strain (Percent S) was obtained by dividing the number of S strain isolates by the total number of *A. flavus* isolates. Data on aflatoxin contamination for each of the sampled gins was obtained from the Valley CO-OP Oil Mill in Harlingen, Texas.

A. flavus communities, described by CFU and Percent S, resident in cottonseed from gins in South Texas, vary across regions and seasons. Both the magnitude (CFU) and strain composition (Percent S) of *A. flavus* communities are related to extent of aflatoxin contamination in cottonseed in South Texas. CFU differed significantly both among seasons and among regions. Average CFU across regions was significantly higher for the 1999 season (3077 CFU) than for the 2000 and 2001 seasons with 522 and 733 CFU, respectively. Percent S differed significantly across regions. The Rio Grande Valley region had significantly lower Percent S (10.2 %), compared with the Coastal Bend and Upper Coast regions with 33.9% and 27.1%, respectively. Multiple linear regression analyses indicate that CFU and Percent S are major factors in contamination of cottonseed by aflatoxins. Both CFU and Percent S are included in the regression model for aflatoxin contamination. Geostatistical analyses indicated spatial continuity for both Percent S and CFU, with seasonal variation in the range of influence. Contrasting maps of both Percent S and CFU for the 1999 through 2001 seasons showed recurrent patterns for Percent S, but not for CFU.

Seasonal variation in aflatoxin contamination was driven mainly by environmental influences on the number of *A. flavus* CFU per gram. Spatial variation of aflatoxin contamination was driven mainly by incidences of the *A. flavus* S strain. CFU reflected most of the seasonal variation in aflatoxin contamination, but not variation in aflatoxin contamination between regions. The current results reveal a clear geographic structure in the distribution of the S strain on commercial cottonseed in South Texas. The S strain is markedly less common on the crop from the lower Rio Grande Valley than from the Coastal Bend Area. Temporal variation in the Percent S on cottonseed across the three years of study was not detected. Results are consistent with the S strain being an important causal agent of aflatoxin contamination in South Texas.

Biocontrol of Aflatoxin by Non-toxigenic *Aspergillus flavus* Isolates

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The ability for two nontoxigenic *Aspergillus flavus* isolates (CT3 and K49) to reduce aflatoxin contamination of corn were assessed in a 3 year field study. Experimental design consisted of 6 wheat inoculant treatments: toxigenic isolate F3W4; two non-toxigenic isolates (CT3 and K49); two mixtures of CT3 or K49 with F3W4; and autoclaved wheat control, applied at 20 kg/ha. In 2001 inoculation with the toxigenic isolate increased levels of aflatoxin in corn by 167% compared to the non-inoculated control, while CT3 and K49 inoculation reduced aflatoxin levels in corn kernels by 86% and 60%, respectively. In 2002 the nontoxigenic CT3 and K49 reduced aflatoxin levels 61% and 76%, respectively, but inoculation with the toxigenic isolate had little effect on aflatoxin. In 2001 mixtures of toxigenic and non-toxigenic isolates had little effect on aflatoxin levels, but in 2002 inoculation with mixtures of K49 and CT3 reduced aflatoxin levels 68 and 37%, respectively. In 2003 a low level of natural aflatoxin was observed. However, in 2003 inoculation of corn with mixtures of K49 or CT3 and F3W4 reduced levels of aflatoxin 94 and 65%, respectively, compared to the toxigenic strain alone. Results indicate that nontoxigenic, indigenous *A. flavus* isolates have potential use for biocontrol of aflatoxin contamination in Mississippi Delta corn.

Characterization of Aflatoxin-Producing Fungi Outside of *Aspergillus* Section *Flavi*

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The agriculturally-important species producing aflatoxin (AF) are *Aspergillus flavus* and *A. parasiticus*. Both of these are members of *Aspergillus* section *Flavi*. Several other members of this section also produce AF, including *A. nomius, A. pseudotamarii*, and *A. bombycis*. Sterigmatocystin (ST) is a mycotoxin structurally and biosynthetically similar to AF. It is an intermediate compound in the AF biosynthetic pathway produced by a number of fungi including *Emericella nidulans* and *Aspergillus versicolor*. In recent work with an AF-producing species outside of section *Flavi, Aspergillus ochraceoroseus*, we found that the genes involved in AF production by this species were often quite different from those of known AF and ST producers, but somewhat more similar to those of the *A. nidulans* ST pathway than to those of the *A. parasiticus* AF pathway (Klich *et al*., Mycologia, in press*)*. *A. ochraceoroseus* apparently did not 'pick up' the AF biosynthetic pathway through horizontal transfer of the gene cluster. This led to the current project in which we are screening for new AF producers, examining known producers for common morphological and ecological characters and comparing them phylogenetically. New isolates characterized in this study include *Em. astellata, Em. venezuelensis*, and *A. taiensis*.

Of the strains screened for AF and ST production, only *Em. astellata* 503/512, *Em. venezuelensis* 2520 and *A. taiensis* 1468 produced AF. Initial analysis indicated that *Em. astellata* 1470/1471 also produced AF/ST but additional assays showed that only ST was being produced under the culture conditions used. Until recently, this species was considered to be a variety of *Em. variecolor*, but the isolates of *Em. variecolor* that we examined did not produce AF. After we found that the type of *Em. astellata* produced AF, we obtained other strains to examine for AF/ST production. A second morphologically similar isolate produced AF but the morphologically distinct strains from Cadiz, Spain only produced ST. *A. taiensis*, like *Em. venezuelensis,* has not yet been formally described.

Phylogenetic analysis of these strains was conducted using primers that amplified 421 bp and 642 bp of the *stcE* and *aflR* genes, respectively (based on *A. nidulans*). Bootstrap analysis indicated that *A. taiensis* and *A. ochraceoroseus* were closely related as were *Em. astellata* strains 503 and 512. This was not unexpected as they were all isolated from similar geographic locations. Interestingly, there seemed to be more relatedness between *Em. astellata* 503 and *Em. venezuelensis* 2520 than *Em. astellata* 503 with *Em. astellata* 512 based on *stcE* sequence analysis. Both analyses demonstrated that *Em. astellata* 1471 was more distantly related to *Em. astellata* 503/512 than was *Em. venezuelensis* 2520. This would indicate that *Em. astellata* 1470/1471 require reclassification. Results of beta-tubulin and 5.8S ITS gene sequence analysis (currently underway) should better clarify the relationships of these strains to one another.

Session 4:

Crop Management and Handling, Insect Control, and Fungal Relationships

Moderator: Doug Smyth, Kraft-Planters

Effects of Ultra-High Plant Populations and N-Fertility on Mycotoxins in Corn

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Stress caused by excessive plant populations and low N-fertility can lower grain yields and increase mycotoxin contamination in corn. Irrigated corn was grown at populations of 69,000, 79,000, 89,000, and 99,000 plants ha^{-1} in both 102 cm and 76 cm row spacings at Stoneville, MS. Nitrogen fertility treatments included 112 kg N ha^{-1} pre-plant only, 224 kg N ha^{-1} pre-plant only, and 112 kg N ha^{-1} pre-plant plus 112 kg N ha^{-1} at GS V6. Grain yields were unaffected by population increases in the 102 cm row spacing and tended to decline with increasing populations in the 76 cm row spacing. Yields were lowest in the 76 cm spacing with the 112 kg N ha^{-1} pre-plant only fertility treatment. Both aflatoxin and fumonisin contamination levels were lower at the high N-fertility treatments than at the 112 kg N ha^{-1} pre-plant only treatment. Aflatoxin and fumonisin levels tended to increase with increasing plant populations.

Optical Sorting of Whole Corn Kernels Contaminated with Aflatoxin or Fumonisin

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Near infrared reflectance spectra (500-1700 nm) of aflatoxin contaminated corn kernels were analyzed to determine the optimal pair of filters needed in a high speed sorting operation to remove aflatoxin contaminated corn. Kernels used for this study were collected from ears that were wound-inoculated with *Aspergillus flavus* in the late milk to early dough stage of kernel maturity. At harvest, the ears were picked and shelled by hand, wounded kernels were discarded, and the whole intact non-wounded bright greenish fluorescent (BGYF) and non-BGYF corn kernels were grouped based on visible symptoms of *A. flavus* infection. Heavily molded and light-weight kernels or kernel fragments were not included in this study as they are usually removed during harvesting or by cleaning equipment at grain elevators. After spectra acquisition, aflatoxin was measured in each individual kernel by standard chemical methods.

During high-throughput sorting operations, whole spectra cannot be acquired and processed at rates that are economically feasible. Most commercial sorting machines are able to measure only one spectral band of light while some machines can measure two bands. A method, based on discriminate analysis, was used to select the optimal pair of spectral bands that would identify kernels contaminated with aflatoxin in a bi-chromatic high speed sorter. It was found that, using the wavelength pair of 750nm and 1200nm, more than 97% of the aflatoxin contaminated kernels were correctly classified as containing either high (>100 ppb) or low (<10 ppb) levels of aflatoxin. Additionally, using this pair of wavelengths, 100% of the fumonisin contaminated kernels were correctly classified as containing high (>100 ppm) or low (<1 ppm) levels of fumonisin. Most kernels with intermediate levels of aflatoxin (10 ppb to 100 ppb) and fumonisin (10 ppm to 100 ppm) were classified as un-contaminated.

A commercially available, bi-chromatic, high speed sorter was tested and found to significantly reduce aflatoxin and fumonisin in corn when using the filter pair of 750 and 1200 nm. This sorter has a throughput of approximately 250 bushels per hour (7000 Kg/hr) and a cost of approximately $60,000. Testing was performed with samples grown in Central Illinois and Northeastern Kansas. The Kansas samples were commercially grown and purchased from a grain elevator after the 2002 harvest. In these samples, the sorter was able to reduce aflatoxin and fumonisin levels by 79% and 81%, respectively, from initial levels of 49 ppb aflatoxin and 19 ppm fumonisin, while only rejecting 5.0% of the incoming product. Higher rejection rates and pre-cleaning of the grain did little to improve sorter performance in terms of aflatoxin reductions. In contrast, significantly more fumonisin was removed when the sorter was set to reject more corn. While rejecting 12.7% of the incoming product, fumonisin was reduced by 91%. For naturally infected and commercially harvested samples grown in Central Illinois (2002 harvest), aflatoxin and fumonisin were reduced by 23% and 86%, respectively, from initial levels of 1.2 ppb aflatoxin and 2.1 ppm fumonisin, while rejecting 1.2% of the incoming grain. The sorter has a limited ability to reduce aflatoxin levels in grain when the initial level is low (below 10 ppb), while fumonisin levels were significantly reduced when the initial level exceeded 1 ppm. The sorter was also tested on corn inoculated with *A. flavus* and harvested by hand (1997 harvest) or a Hege field plot harvester (2002 harvest). Aflatoxin and fumonisin in the Hege harvested grain were reduced by 79% and 83%, respectively, from initial levels of 412 ppb aflatoxin and 19 ppm fumonisin, when 8.8% of the incoming grain was rejected. Aflatoxin and fumonisin in the hand harvested grain were reduced by 76% and 79%, respectively, from initial levels of 1301 ppb aflatoxin and 1.7 ppm fumonisin when 5.8% of the incoming grain was rejected. Increasing the reject rate of the sorter did improve reductions of both aflatoxin and fumonisin in these inoculated samples.

Sample Preparation and Pre-sampling of Pistachios

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A theory has been developed to quantify the reduction of sub-sample variance of aflatoxin contamination, which was observed when granular materials are wet-slurried, rather than dry-ground, during subsample homogenization. A coefficient of variation, based on particle size distribution, subsample size and probability of contamination, was predicted. The theory was tested with dry-ground and with wet-slurried pistachios, and excellent quantitative agreement was obtained. (dry-ground: CV predicted 0.215, experimental 0.20; wet-slurried: predicted 0.097, experimental 0.095; source: single sample of eye-core reject pistachios) A 32% increase in the mean aflatoxin concentration was observed as well when wet slurrying was applied (wet slurried: 87 ± 2 ng/g; dry ground 66 ± 4 ng/g). While no statistical explanation for this effect can be found, it is suggested that it is related to physico-chemical binding between the nut matrix and aflatoxin which is (partly) broken by wet slurrying, making the extraction of more toxin possible. Impact on international trade is immediate and also possible on mammal digestion of aflatoxin. Other parameters that may affect slurrying results will be mentioned as well. The authors would like to thank USDA and the California Pistachio Commission for financial support. A paper describing these results has just appeared in J. Agric. Food Chem. 51(10) 6068-6072 (2003).

Advances in Development of Kairomone-Augmented Control Tactics for Codling Moth and *Aspergillus* in Walnuts

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

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

Trécé, Inc. developed a micro-encapsulated (MEC) sprayable formulation of the kairomone that had good residual field-release activity for over a month. The sprayable MEC-kairomone was tank-mixed with either sprayable MEC-pheromone or insecticides and applied by standard orchard fan-sprayers or gun-sprayers. Mating disruption trials were conducted in: 1) 19 replicated walnut orchards, with treatment and standard blocks being 10 acres, and five replicated apple orchards, with three acre blocks. Bait-spray trials were conducted in three walnut orchards using both single tree replicates (six trees/treatment) or eight replicated four-acre blocks. Occurrence and degree of nut damage was evaluated by nut drop, canopy infestation, and will be evaluated by current harvest sampling. Results are encouraging, but await the critical harvest damage assessment of treatments.

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

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

Panel Chair: Tom Pearson

Panel Members: Arnold Bruns, Douglas Light, and Thomas Schatzki

Summary of Presentations: There were a variety of presentations made for control of mycotoxins using both pre-harvest and post-harvest methods. Plant stress either from too densely planted corn or low nitrogen were found to contribute to higher levels of mycotoxins in corn (Bruns). This work suggests that mycotoxins may be controlled somewhat by cultural practices in areas where mycotoxins can be a problem. Codling moth mating was shown to be disrupted in walnut orchards using pear-ester kairomone. This method reduced the spread of insects in the orchard and has an economic cost comparable to other forms of insecticides that are due to be outlawed for environmental reasons (Light). Along with reducing insect damage, this work should reduce infection of *Aspergillus* molds and aflatoxin in tree nuts. Sample preparation and sampling methods for reducing variance in aflatoxin analysis of pistachio nuts were presented (Schatzki). Particle size distribution, sub-sample size, and method of homogenization (wet slurry or dry) all contribute to variance in aflatoxin results. However, sample homogenization using a wet slurry leads to extraction of more aflatoxin; thus, having an impact on exports to Europe where wet slurrying is used to monitor products arriving at their ports. Lastly, a high speed optical method for sorting and removing corn contaminated with aflatoxin and fumonisin was presented (Pearson). Corn was sorted at a rate of 250 bu/hr and reduced mycotoxins by over 80% while only removing 5% of the incoming product.

Summary of Panel Discussion: Don White mentioned the cost for optical sorting of corn was about $0.13/bu for another application. Pearson added that the main cost, besides the capital investment for the sorting equipment, was the rejected product, which is usually lost. If 5% of corn is rejected and lost, then that would correspond to $0.11/bu (at $2.20/bu corn). There are higher throughput machines, which can reduce some capital costs, but the higher throughput machines cost more to begin with. Don Wicklow asked why a sorter may miss some kernels. This can be due to kernel orientation and/or kernel velocity as it passes through the machine. If the kernel is light weight, it may be traveling slower and results with the reject mechanism's missing it. Merle Jacobs asked what was the false positive rate from the sorter, i.e. how many of the rejected kernels actually had aflatoxin? Pearson replied that single kernel mycotoxin analyses were not done on the sorter rejects, but it appeared that about half of the rejected kernels had symptoms of mold damage. Half of the kernels were symptomless and probably false positives, which is typical of most sorting operations. There is some limited use of optical sorters for aflatoxin, mostly for food grade white corn.

Most of the panel discussion centered on Tom Schatzki's presentation about pre-sampling and sample preparation of pistachio nuts for aflatoxin analysis. Peter Cotty remarked that the differences in aflatoxin results for dry homogenization and wet slurring were alarming. The wet slurry method, used in the EU, appears to increase extraction of aflatoxin. It was suggested that American exporters should simply use the EU method and possibly avoid analysis when shipments arrive in Europe. The EU method can be performed here; but, the analysis won't be accepted unless the lab has EU certification for the commodity being shipped. Currently, peanuts are the only commodity that American labs are EU-certified to analyze for aflatoxin. Schatzki added that particle size distribution in the sub-sample is the largest source of aflatoxin variance contributed by sub-sampling.

Root-Knot Nematodes and Preharvest Aflatoxin Contamination in Peanut

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A field study was conducted to determine whether aflatoxin levels in peanut, *Arachis hypogaea*, were correlated with pod and root galling caused by the peanut root-knot nematode. The experiment was conducted under a rain-out shelter containing 24 cement plots (each 2 x 2 m) with the following treatments: root-knot nematodes alone (RKN), *Aspergillus flavus* (Af) alone, RKN + Af, and no nematodes or fungus. Each treatment was replicated six times. Peanut seedlings, either infected with RKN or uninfected, were transplanted into half the plots. Inoculum of Af was sprinkled over the plant canopy at mid bloom. Drought was induced after pod set by covering plots during rain with a fiberglass shelter. Pod- and root-gall indices were determined for all plants in the plot and averaged. Pods from each plot were bulked, shelled, and a subsample of kernels was used to determine aflatoxin concentration and percentage colonized by Af. In this abstract, we report the results from field trials conducted in 2001 and 2002.

Aflatoxin concentrations in kernels were greater in 2002 than in 2001 (1,994 vs 256 ppb; $P \leq 0.0001$). Because there was no interaction between year and any of the other factors, the data for the two years were combined. In plots inoculated with Af , aflatoxin concentrations were high and were not affected by the nematode; however, in plots not inoculated with the fungus, aflatoxin concentrations were greater in the presence of nematodes. In plots without fungal inoculum, there was an increase ($P \leq 0.04$) in aflatoxin contamination of kernels with increasing root and pod galling. Pod galling accounted for 83% of the variation in aflatoxin concentration in 2001; however, the strength of the relationship with aflatoxin was much less for pod galling in 2002 and for root galling in both years (R^2 between 0.43 and 0.57). Colonization of kernels by *A. flavus* increased with increasing pod galling ($P = 0.04$, $R^2 = 0.18$) in 2001 but not in 2002.

The results of this study indicate that infection of peanut by root-knot nematodes can lead to an increase in aflatoxin contamination of peanut kernels when the plants are subjected to drought stress during pod maturation. This interaction was not observed in plots where *A. flavus* inoculum was applied. Perhaps the high inoculum level of the *A. flavus* group and subsequent aflatoxin production in these plots masked the enhancing effects of the nematode.

Leaf Volatile Profiles of Four Commercial Pistachio Varieties

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Aflatoxin contamination of tree nuts (almonds, walnuts and pistachios) arises from infections of susceptible host nuts with aflatoxigenic strains of *Aspergillus flavus* or *A. parasiticus*. Recently aflatoxin found above stringent limits set by the EU has resulted in rejection of entire almond shipments exported from the U.S. to Europe with large attendant monetary losses. Moreover because of aflatoxin contamination the EU banned all pistachios shipments from Iran in 1997. Attacks by insects, especially codling moth and navel orangeworm, afford openings through which microorganisms, including aflatoxigenic and other fungi, can reach nut kernels. Consequently decreasing insect damage would greatly reduce aflatoxin levels in the final product. The U.S. is now the world's second largest producer of pistachios, just behind Iran. Because insects rely primarily on odors to locate mates and hosts for laying their eggs it seemed that a necessary first step is the examination of the volatile emissions from a number of pistachio varieties. The bulk of U.S. pistachio production comes from the single variety, Kerman. We describe the collection and GCMS analysis of headspace volatiles from fresh leaves of *Pistachia vera* including not only the predominant Kerman, but also three other varieties (Ohadi, Kaleh Gjoochi, and Momtaz) collected from a San Joaquin Valley farm whose owners brought plant material from Iran in the 1970's, developed several varieties and presently grow several of them commercially in limited production.

Branches several feet in length were cut from *Pistacia vera* trees at the Orandi Ranch in Bella Vista, CA (Ohadi, Kaleh Gjoochi and Momtaz varieties) or in orchards maintained by University of California, Davis, Wolfskill Experiment Station, Winters, CA (Kerman variety). Leaves (800 g) were placed in a clean 12L flask and purged with purified air that exited through a Tenax trap. After 18 hours the volatiles were rinsed off the Tenax with diethyl ether, concentrated by distillation and analyzed by GCMS on two 60m capillary columns of different polarity, bonded methyl silicone (DB-1) and bonded wax (DB-Wax) phases. Peak identities were established by comparing mass spectra with library spectra, matching retention indices with data that have been collected at WRRC, or using known standard compounds.

In all of the varieties we examined, terpene hydrocarbons are the predominant volatile constituents with limonene and *trans*-β-ocimene alone accounting for 60%-80% of the total volatiles. However, it is the less prominent volatiles that make the differences between the varieties apparent. The profiles of Kerman and Kaleh Gjoochi are the most similar, yet the two varieties are readily distinguishable if the concentrations of myrcene, methyl benzoate, *cis*-3-hexenyl butyrate and *cis*-3-hexenyl benzoate are compared between the two varieties. Ohadi is the only variety in which *trans*-β-ocimene is the predominant volatile component rather than limonene. In addition greater amounts of methyl benzoate, γ-terpenene and *cis*-3-hexenyl benzoate are characteristic of this variety. The Momtaz volatiles are strikingly different from those of the other three varieties; limonene alone accounts for 73% of the total volatiles. But the most conspicuous feature of the Momtaz variety is the presence of at least 22 sesquiterpene hydrocarbons; those of greatest concentration are: aromadendrene, ledene, isoledene calarene, alloaromadendrene and α-gurjunene. In the other three varieties the sesquiterpenes were not detected or appeared only at very low concentrations.

The attractancy or repellency of pistachio leaf volatiles, individual constituents or mixtures, to insect pests, particularly codling moth and navel orangeworm, has yet to be investigated. Such information may lead to new ecologically sound methods of controlling these pests and in so doing reduce *Aspergillus* infection and subsequent aflatoxin formation. Because U.S. pistachio production is based almost entirely on one variety (Kerman) it is important to maintain other varieties such as Kaleh Gjoochi, Momtaz and Ohadi because they preserve genetic diversity.

Comparisons of Ochratoxigenic *Aspergillus lanosus* and *A. alliaceus* Isolates From California Tree Nut Orchards

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In a previous study, our lab found ochratoxin-producing strains of *Aspergillus alliaceus* in a California fig orchard. During surveys in 2002 and 2003 of California tree nut orchards, we isolated a number of ochratoxin-producing *A. alliaceus* and *A. lanosus* strains from pistachio orchard soil. Prior to our discovery *A. lanosus* had only been reported in India, and the type strains are atoxigenic. Both *A. lanosus* and *A. alliaceus* belong to *Aspergillus* subgenus *Circumdati* section *Flavi*, and are closely affiliated based on molecular systematic studies. However, *A. lanosus* can be differentiated from *A. alliaceus* based on a number of physical and morphological traits. Unlike *A. alliaceus*, *A. lanosus* produces bright yellow, floccose colonies, has branched conidiophores, and forms irregular, fused sclerotial bodies. In addition, we observed that mature sclerotia of *A. alliaceus* can possess cleistothecia containing ascopores. No such structures were found in sclerotia of *A. lanosus*. Among field isolates of both species, we identified sclerotial and non-sclerotial strains. A comparison of ochratoxin A production by these isolates showed that sclerotial strains of *A. lanosus* produced 3.8 to 44.8 µg/mL, and sclerotial strains of *A. alliaceus* produced 3.7 to 43.1 µg/mL. Field isolates and type strains of both species that were non-sclerotial did not produce detectable amounts of ochratoxin A. This is the first report describing the production of sclerotia and ochratoxin by *A. lanosus*.

Session 5:
Crop Resistance --- Genetic Engineering
Moderator: Bob Sacher, ConAgra

Improved Synthetic Peptide Technology for Expression in Plants

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Plants do not possess a complex immunoglobulin-based system such as that found in higher vertebrates to defend themselves against attacking microbial pathogens; however, they do have a wide variety of innate host defense mechanisms at their disposal. These include the production of antimicrobial reactive oxygen species (ROS), secondary metabolites, hydrolytic enzymes, and a wide array of antimicrobial proteins and peptides. Recombinant DNA technologies and plant transformation procedures have been used to introduce and express genes encoding these types of antimicrobial agents in plants in an effort to increase host resistance to plant pathogens. Of particular interest has been the identification and characterization of ribosomally-synthesized antimicrobial peptides. Antimicrobial peptides appear to be ubiquitous in nature being found in many organisms, from humans to bacteria. Various plants produce, either preformed or in response to microbial invasion, cysteine-rich antimicrobial peptides such as thionins, defensins, lipid-transfer proteins, and hevein- and knottin-type peptides. Examples of antimicrobial peptides of mammalian and insect origin include bovine or human defensins and protegrins, magainins from amphibians, and cecropins from the giant silk moth, *Hyalophora cecropia*. These peptides have been shown to be effective against a wide array of microorganisms including fungi. Antifungal peptides act either by lysing the fungal cell or by interfering with cell wall synthesis. Cecropin and cecropin analogs have been expressed in transgenic tobacco (*Nicotiana tabacum*) with mixed results regarding pathogen resistance. We were the first in the world to demonstrate enhanced disease resistance and reduced disease severity in transgenic tobacco expressing designed antimicrobial peptides upon infection with the bacterial pathogen, *Pseudomonas olanacearum*. However, tobacco plants expressing a native cecropin did not confer resistance to *Pseudomonas solanacearum*, presumably due to degradation of the peptide by host proteases. The advent of automated peptide synthesizers and combinatorial peptide chemistry has made it possible to rapidly synthesize and screen large numbers of peptides for their ability to inhibit the growth of target microbial pathogens. These linear peptides often can be less than half the size (10–20 amino acids) of their native counterparts and many times more potent without concomitant toxicity to host tissues. We recently reported on the antifungal activity of a 17 amino acid linear, synthetic peptide designated D4E1. This peptide was shown to interact with sterols present in the conidial cell walls and resist degradation by fungal and host proteases. *In vitro* assays with D4E1 demonstrated a minimal lethal concentration needed to kill 100% of germinating conidia of *Aspergillus flavus*, *A. fumigatus*, and *A. niger* of 12.5, 12.5, and 25 µM, respectively. We have been successful in the *Agrobacterium tumefaciens*-mediated transformation and expression of D4E1 in tobacco and subsequently, the demonstration of antifungal activity from crude extracts of the transformed leaves against *A. flavus* and *Verticillium dahliae*. Additionally, *in planta* assays demonstrated reduced disease severity upon inoculation of transgenic tobacco leaves with other fungal pathogens. In further experimentation, we have shown a number of herbaceous and woody species to possess enhanced resistance to bacterial and fungal pathogens when transformed with genes encoding our designed antimicrobial peptides. The use of further designed antimicrobial peptide-encoding genes for the production of plants with improved disease resistance characteristics is a reality and when applied to plants of agricultural import, significant increase in food and fiber would be realized.

Genetic Engineering of Cotton to Confer Resistance to *Aspergillus flavus*

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Our goal is to engineer cotton with genes that will decrease aflatoxin levels. We have continued our efforts to generate transgenic cotton with enhanced resistance to *Aspergillus flavus*. Our optimized protocols for cotton transformation and regeneration can be used either to express novel structural genes or modify expression of existing cotton genes that confer greater resistance to *A. flavus* infection. Currently, our best structural gene candidates that confer resistance to proliferation of *A. flavus in vivo* are: a small lytic peptide gene, a chloroperoxidase gene, and a neutral cotton chitinase gene. During the past year we have expanded our studies to more fully characterize the anti-*A. flavus* activity of the neutral cotton chitinase, and in the near future we plan to identify additional cotton genes and promoters that respond to *A. flavus* infection using a genomics/proteomics approach.

We have isolated and identified several Class I chitinase genes from cotton. Our chitinase purification scheme involves extraction of ethylene treated leaf tissue with citrate buffer, protein concentration, heat precipitation, and purification using a chitin affinity column. With this protocol, we obtain a fraction that contains three different Class I cotton chitinase isoelectric isomers. In preliminary studies, it effectively inhibits *A. flavus* spore germination *in vitro*. We are particularly interested in the predominant component, which is the neutral chitinase. Before we can fully characterize the efficacy of these class I chitinases and individually characterize the activity of each isoelectric isomer, we will need relatively large amounts of protein. To this end, we have been testing ways to improve the yield of the Class I chitinase column bound fraction above our typical yields of 1-2 μg of protein per gram tissue. We have scaled up the protein extraction, used commercially prepared chitin columns, and introduced a molecular sieve column prior to the chitin binding chromatography step. None of these modifications increased the recovery percentage; however, scaling up the extraction did result in increased recovery.

We have also been developing ways to express the cotton neutral chitinase in bacteria and yeast. Although we were able to express a neutral chitinase fusion protein in *E. coli*, it was not active. This may be because modifications such as glycosylation are needed for activity (there is a putative glycosylation site in the cotton neutral chitinase). Yeast expression systems are capable of basic modifications, result in high yields of fusion protein, and many fusion proteins expressed in yeast are active. A neutral chitnase gene was cloned into the *Pichia pastoris* expression vector pPICZα (Invitrogen). An expressed protein of the appropriate molecular weight that strongly cross-reacts with antiserum raised to a chitinase fusion protein was identified. In our most sensitive activity assay (a liquid assay with glycol chitin as the substrate), fractions that contained the yeast-expressed protein as well as the negative control fractions exhibited activity. However, after dialysis of the control and fusion protein fractions, activity was only seen in the fusion protein fraction. We are now using mixing experiments to determine the effect of the elution buffer components on our assay, and on purified fractions of the native cotton chitnase.

If we determine that the yeast-expressed chitinase is active, we will then compare its effectiveness to that of the column-purified cotton Class I chitinase fraction in an *in vitro A. flavus* spore germination inhibition assay. The results of these studies, along with ongoing studies of the cotton neutral chitinase in transgenic *Arabidopsis* and tobacco will help us to evaluate its effectiveness in inhibiting *A. flavus* infection.

Control of Preharvest Aflatoxin Contamination in Cotton-New Antifungal Proteins and Means for Increasing their Expression

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Past work in our laboratory has demonstrated the successful development of transgenic cotton expressing the antifungal peptide D4E1 gene or a bacterial chloroperoxidase (CPO) gene. Biological assays using D4E1 transgenic cotton have shown that plants expressing this gene should be able to better resist invasion by pathogenic fungi, including *Aspergillus flavus*. This is based on *in vitro* antifungal assays using extracts of transgenic cotton leaves, *in situ* assays looking at the growth of a GFP-expressing *A. flavus* in transgenic cottonseed, and *in planta* assays showing improved resistance to *Thielaviopsis basicola* in transgenic tobacco expressing D4E1. Though promising, the antifungal activities observed in transgenic cotton have always been less than that observed in transgenic tobacco expressing these same antifungal genes. To overcome this apparent reduced expression of the transgenes in cotton, we have undertaken experiments designed to increase their expression. Specifically we are utilizing plastid transformation of antifungal genes in an effort to increase copy number of the transgene in the plant as well as to stabilize transcription and protect the gene product from degradation by proteases. Using biolistic transformation technology we have introduced a vector for plastid transformation harboring the CPO or D4E1 gene into tobacco leaf tissues. In addition to carrying the antifungal gene this vector also expresses the gene for spectinomycin resistance. We have successfully regenerated adventitious tobacco shoots under spectinomycin selection that should harbor a copy of the CPO gene in every plastid (homoplasmic state). PCR analysis of the transgenic tobacco DNA demonstrated that the plants did carry the gene for spectinomycin resistance and the CPO gene. Western blots will be performed to compare the levels of CPO being produced in plastid vs. nuclear transformed tobacco tissues.

We have also engineered a synthetic CPO gene in an effort to optimize the codon usage of this gene of bacterial origin so that it will be better recognized by the plant's eukaryotic translational machinery. The G + C content of the bacterial CPO was about 64% while optimum G + C content in plants is about 45%. A totally synthetic, codon-optimized version of the CPO gene was synthesized with a G + C content of about 50% and transformed into tobacco. Initial western blots of six putative CPO-optimized tobacco clones identified one, clone 6a, that produced the CPO enzyme. Comparison of enzyme activity and *in vitro* anti-*A. flavus* activity of 6a leaf extracts indicated that the optimized version was being produced in greater amounts compared to control tobacco expressing the non-optimized CPO gene. More transgenic tobacco plants expressing the optimized CPO gene will be evaluated.

Genetic Engineering for Aflatoxin Reduction in Peanut: Utility of Selectable Markers and Antifungal Genes

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Genetic engineering of peanut can be accomplished in a genotype-independent manner using microprojectile bombardment. The selectable marker gene hygromycin phosphotransferase (*hph*) is highly efficient for recovery of transgenic cell lines after bombardment of embryogenic tissues and selection on the antibiotic, hygromycin. Since *hph* is an antibiotic resistance gene, and the industry is moving away from antibiotic resistance traits, we have sought alternative selectable markers for peanut transformation. The focus of the transformation research is reduction of aflatoxin contamination which results from the invasion of peanut pods with *Aspergillus flavus* or *A. parasiticus*. Therefore, in addition to the improvement of transformation protocols, we are introducing putative anti-fungal genes that have potential to reduce the growth of *Aspergillus* sp.

A nonheme haloperoxidase gene (chloroperoxidase from *Pseudomonas pyrrocinia*) (CPO-P; Rajasekaran et al. 2000, Plant Cell Rep. 19:333) was engineered into a construct containing the *hph* gene, both genes under the control of the CaMV35S promoter. The construct was introduced into embryogenic cultures of peanut by microprojectile bombardment. Over 150 hygromycin-resistant lines have been selected, and plants have been regenerated from many of these lines. The CPO-P transgene was expressed in 90% of the callus lines tested by Northern blot analysis. Extracts of CPO-P-expressing embryogenic tissues showed a significant inhibition of *Aspergillus flavus* growth *in vitro*. Some of the transgenic plants also showed inhibition of *A. flavus* growth when leaf extracts were used to treat pregerminated spores of the fungus. The most resistant lines showed a 60-70% reduction in colony forming units compared with the non-transgenic control.

Anti-apoptotic genes are regulatory genes that interfere with programmed cell death. Their expression in tobacco and tomato has resulted in high levels of tolerance or resistance to the fungal pathogens, *Sclerotinia sclerotiorum*, *Botrytus cinerea*, and *Cercospora nicotianae* (tobacco; Dickman et al. 2001, PNAS 98:6957), *Alternaria alternata*, *Colletotrichum coccodes*, *Pseudomonas syringae* (tomato; Lincoln et al. 2002, PNAS 99:15217), as well as the viral pathogen, *Tomato spotted wilt virus*. We have begun transformation of peanut with the anti-apoptotic gene, *Bcl-xl*, and have obtained 11 lines from two bombardment experiments that test positive by PCR for the presence of the transgene. Twenty-two additional bombardment experiments are under selection.

Antibiotic resistance is a trait that has low consumer acceptance. We have shown that green fluorescent protein (GFP) can be used for visual selection of stably transformed peanut or as a screenable marker when co-transformed with an antibiotic resistance gene. Visually selected lines that also are antibiotic resistant have been tested for quantitative fluorescence both under selection and without selection and were shown to be roughly equivalent. Since co-transformation with the GFP reporter gene and another gene can reach a frequency of >60%, we are using this approach to test selection with the organomercurial lyase gene, *merB*, that can confer resistance to phenylmercuric acetate.

Expression in Transgenic Peanut of Maize RIP 1, a Protein with Activity against *Aspergillus spp*

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

We have also worked to improve the overall efficiency of peanut transformation through the use of RB 7 matrix attachment regions, which have been shown to enhance gene expression and phenotypic stability in transgenic plants. We have also developed an improved peanut transformation procedure in which DNA is transferred to imbibed peanut embryos from mature seeds, in an attempt to reduce the time in culture, improve fertility of transgenic plants, and reduce labor needed to produce transgenic peanuts. Finally, we are using co-bombardment to transform peanuts. In this procedure two plasmids are introduced into recipient tissue simultaneously. This procedure offers the possibility of removing selectable marker genes from transgenic lines through genetic segregation in progeny of transgenic plants, and so could alleviate fears associated with the presence of genes conferring antibiotic resistance in foods.

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

Transformation incorporating bombardment of dry embryos instead of secondary embryogenic callus appears to offer a significant savings of labor and a slight reduction in overall transformation time. Average time from initiation of cultures to observation of first flower on transgenic plants was 287 ± 29 days for 'Georgia Green' (n = 49), and 296 ± 32 days for 'NCV 11' (n = 50). Transformation with Mod 1 did not appear to alter time in culture, compared with untransformed tissue culture derived checks. Overall transformation efficiency was 9.21 % (43 PCR + events/467 embryos treated) for 'Georgia Green', and 4.79 % (49 PCR + events/1024 embryos treated) for 'NCV 11'.

From 467 treated embryos of cv. 'Georgia Green', 43 events were recovered that were positive for Mod 1 by PCR. Ten of these events were shown by western blot to produce the Mod 1 protein. From 1024 treated embryos of peanut cv. 'NCV 11', 49 PCR positive events were recovered, of which 18 express the Mod 1 protein.

Transformation with the Mod 1 transgene did not appear to reduce fertility. Average pod yield in 49 transgenic plants of cv. 'Georgia Green' was 80 ± 28, while 3 untransformed, tissue culture derived plants of this genotype yielded an average of 63 ± 12 pods. Average pod yield in 50 transgenic plants of cv. 'NCV 11' was 47 ± 17 pods, while 3 untransformed, tissue culture derived plants of this genotype yielded an average of 62 ± 11 pods. This observation is important, because it indicates that Mod 1 protein is not phytotoxic, and is, therefore, well suited for antifungal use in plants.

PANEL DISCUSSION: Crop Resistance – Genetic Engineering

Panel Chair: Caryl A. Chlan

Panel Members: Jeffrey Cary, Kanniah Rajasekaran, Arthur Weissinger, Jesse Jaynes, Peggy Ozias-Akins

Summary of Presentations: The moderator of the session was Bob Sacher, ConAgra. There were five presentations that covered topics associated with development of transgenic cotton and peanuts that have enhanced resistance to *Aspergillus flavus*. Transformation and regeneration procedures have been developed for both of these crops and presented at previous workshops, but this year some new modifications in the peanut transformation protocol were presented. Common themes in the presentations were the development and identification of potential anti-*flavus* peptides and genes, novel selectable markers, sequences that can confer enhanced stability, and vectors free of proprietary concerns for which the investigators will have freedom to operate. The results from analyses of transgenic cotton and peanut plants were also presented.

Lytic Peptides: Jesse Jaynes described strategies for lytic peptide identification and development. Naturally occurring lytic peptides have been identified from many different sources, and are logical candidates as potential anti-pathogen genes. Although early studies of transgenic tobacco that expressed a naturally occurring lytic peptide (cecropin) were not encouraging, subsequent studies of plants that expressed engineered cecropins demonstrated that expression of lytic peptides could enhance pathogen resistance. The effect of structure and composition on the activity of lytic peptides has been determined through analysis of suites of synthetic peptides that incorporate changes in the natural peptide sequence. Based on these studies, new generations of peptides have been constructed with enhanced stability, specificity and activity. *In vitro* and *in vivo* experiments with the engineered lytic peptide D4E1 have clearly shown that lytic peptides inhibit fungal growth. There are still some technical problems associated with D4E1 studies such as difficulty in raising antibodies to it making it difficult to detect and determine expression levels in transgenic plants. A new generation of synthetic peptides is being designed to overcome some of these technical difficulties.

Cotton: Caryl Chlan and Jeffrey Cary presented summaries of genetic engineering projects focused on developing transgenic cotton with enhanced resistance to *A. flavus* infection. This approach is dependent on development of structural gene/promoter combinations that will result in expression of anti-*flavus* proteins or peptides in appropriate tissues either constitutively or specifically in response to fungal infection. Caryl Chlan described studies of a cotton neutral chitinase. This chitinase has been targeted as a potential anti-*flavus* structural gene. In preliminary studies, this chitinase has been shown to inhibit *A. flavus* spore germination *in vitro*. Efforts to improve the purification of this chitinase from cotton tissue were described. This chitinase has also been expressed in a yeast expression system (*Pichia pastoris*) and the expressed chitinase appears to be enzymatically active. *In vitro* studies of the purified native chitinase and the yeast-expressed chitinase will allow determination of minimum inhibitory concentrations needed to directly compare the efficacy of the cotton chitinase to other anti-*flavus* compounds such as the lytic peptide D4E1 and chloroperoxidase.

Jeffrey Cary presented the results of recent studies designed to increase the expression of genes in transgenic cotton. Transgenic plants (either cotton or tobacco) that express the lytic peptide D4E1 or a bacterial chloroperoxidase have enhanced resistance to fungal pathogens *in vitro* and *in vivo*. To further

improve the efficacy of these anti-*flavus* genes in transgenic cotton, they have been working to obtain higher levels of expression. One approach that they have used involves biolistic transformation of plastids. This approach should result in multiple copies of the anti-*flavus* gene per plant, and consequently higher levels of expression, and could be implemented with a variety of anti-*flavus* structural genes. Studies of chloroplast expression of genes in tobacco have shown that there is no apparent silencing mechanism within chloroplasts and, therefore, expression levels should be stable. Results from CPO plastid transformation studies of transgenic tobacco show that the plants do carry the CPO gene, and studies are underway to compare the CPO expression levels between plastid and nuclear transformed tobacco tissues. To specifically enhance CPO expression levels in transgenic cotton, an engineered CPO gene has been developed. The engineered CPO gene has a G + C content that is closer to that for plants, and has been transformed into tobacco. The expression levels of optimized CPO were greater than those observed for the non-optimized CPO.

Peanuts: Peggy Ozias-Akins and Arthur Weissinger described their recent progress towards developing transgenic peanut with enhanced resistance to *A. flavus* infection. It is imperative that once transgenics are developed, their distribution and further development is not limited by intellectual property issues. So, in addition to identifying appropriate structural genes to confer resistance to *A. flavus*, promoter elements and selectable markers that do not have intellectual property constraints and have a high probability of consumer acceptance are needed. At previous workshops, Peggy Ozias-Akins has shown that Green Fluorescent Protein (GFP) can effectively be used as a screenable marker when peanut tissues are co-transformed with an antibiotic resistance selectable marker. Since consumer acceptance of antibiotic resistance markers in transgenic plants is low, an alternate selectable marker, the MerB gene product has been evaluated. Tests of this potential selectable marker are underway to determine the transformation and selection efficiencies. In addition to developing new selectable markers, studies of two different anti-*flavus* structural genes were also summarized. One potential anti-*flavus* gene tested in peanuts was the non-optimized CPO gene. Leaf extracts of transgenic peanut plants that express the non-optimized CPO gene inhibit the growth of *A. flavus in vitro* 60-70% more than the non-transformed control. A second anti-*flavus* gene currently under study is an anti-apoptotic gene that should enhance resistance to pathogen infection by inhibiting the cell's programmed cell death pathway. Tests of transgenic lines confirm the presence of the anti-apoptotic gene, and further studies are underway.

Arthur Weissinger has evaluated the potential of ribosome inhibiting proteins as anti-*flavus* structural gene candidates. Activated RIP 1 from maize (proteolytically processed) inhibits *A. flavus* growth *in vitro*. A synthetic gene that encodes the active form of RIP 1 has been developed and designated Mod 1. Both Georgia Green and NCV 11 peanut cultivars were co-bombarded with plasmid vectors that contained Mod 1 and regulatory regions (anti-*flavus* structural gene expression) and plasmid that contained a gene for hygromycin resistance (selectable marker). When compared to wild type, non-transformed plants, Mod 1 expression did not effect the time needed for development of mature plants, nor did it effect fertility. Consequently, Mod 1 is a good candidate anti-*flavus* structural gene since it is not phytotoxic. To improve the efficiency and stability of peanut transformation, matrix attachment regions that enhance gene expression and confer phenotype stability in transgenic plants were incorporated into the transgenes. Improvements were also made to the transformation method. In the improved procedure, imbibed embryos from mature seeds are co-bombarded with plasmid constructs. By using imbibed embryos, the time in culture and amount of labor is reduced, and the fertility is improved.

Summary of Panel Discussion: Part of the panel discussion focused on the activity of the anti-*flavus* compounds and what was the best approach to get expression that would most likely target *A. flavus* expression in specific tissues or more generalized expression. Art Weissinger indicated that the promoter that is being used to express anti-*flavus* genes in peanut is the ubiquitin promoter. This

promoter should drive expression in most plant tissues, and could combat the growth of *A. flavus* throughout the life cycle of the plant. Additionally, because the plant is expressing the anti-*flavus* compound in most tissues constitutively, there may be significant accumulations of the anti-fungal protein. If this anti-fungal protein is stable, then there may be some post harvest protection as well.

The chloroplast transformation system was also the subject of some discussion. A question was raised as to whether the proteins would be expressed and/or active in non-green tissues such as the seed. Panel members brought up the points that proplastids are present in non-green tissues, and that the pericarp has some plastids. One of the objectives of the chloroplast transformation studies is to first develop expression in the chloroplasts, and then to apply this knowledge to express proteins in other types of plastids. Another potential benefit of using a plastid transformation system may be increased stability of proteins.

There was some discussion about the use of mercury as a selectable marker – why it was chosen and would it present any obstacles to commercialization. Mercury resistance conferred by the MerB gene was chosen because it is the intellectual property of the University of Georgia and its scientists. In addition, plant tissues are sensitive to mercury compounds, and appropriate levels for selection have been identified. Since all the selection for transgenics is performed in the laboratory and in greenhouse trials, mercury containing compounds are not used in the field. If necessary, analysis of field grown plants can be performed with PCR.

The last issue addressed by the panel was the question of how gene copy number effects expression. The peanut transformation protocols use biolistics to transform the plant tissue. To obtain reasonable transformation efficiencies, relatively high concentrations of DNA are used. This can result in multiple copies of the gene integrated into the plant genome. Copy number estimates range as high as 200 copies. This sequence redundancy can lead to silencing. Additional copies of a gene do not necessarily correspond to increased expression. It was suggested that some additive effects can be seen up to about 10 copies. Other transformation methods, such as *Agrobacterium*-mediated transformation can also result in multiple copies of a gene of interest being incorporated into a genome, but the number of copies integrated is much smaller.

Comparative Proteomic Analysis of Maize Silks in *Aspergillus flavus* Resistant and Susceptible Inbreds

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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 Sc212 and Mp339 (susceptible) 21 days after silk emergence (DAS). Infested ears were inoculated with *A. flavus* at 15 DAS and were collected 21 DAS. Silk proteins were extracted and analyzed by 2-dimensional gel electrophoresis. Gel images were analyzed by PDQuest software (BioRad) and comparisons were made among inbreds 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 differ among resistant and susceptible lines, or inoculated and uninoculated ears.

Proteomic Analysis of Maize Rachis Tissue in *Aspergillus flavus* Resistant Inbreds

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Several maize inbred lines resistant to *Aspergillus flavus* infection and aflatoxin contamination have been developed by USDA-ARS scientists at Mississippi State University. Our goal is to identify proteins in these lines that may be involved in resistance to fungal infection by comparing proteins separated by 2-D gel electrophoresis from the rachis (cob) and pericarp tissues of resistant and susceptible inbred lines. These tissues were selected because studies with GFP-tagged *A. flavus* indicated that fungal growth was blocked in rachis of resistant lines. Pericarp is another type of maternal tissue that may be involved in controlling fungal proliferation. Inbreds were tagged at silk emergence and rachis and pericarp samples were collected at various intervals during ear development. We performed the 2-D gel analysis from samples collected 21 days after silking from the resistant lines, Mp313E, Mp420 and Mp715; a line with intermediate resistance, Tx 601; and susceptible lines, SC212M, Mp 339 and B73. Analysis of rachis and pericarp 2-D gels with PDQuest software (BioRad) revealed several hundreds of Coomassie blue-stained proteins in each genotype. There were several proteins specific to resistant and susceptible lines. MALDI-TOF MS will be used to identify the specific proteins that may be correlated with resistance and susceptibility to *A. flavus*.

Catalase3 and Maize Resistance to *Aspergillus flavus*: a Possible Link?

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Zea mays L. infection by *Aspergillus flavus* frequently produces aflatoxin, which reaches the food chain through contaminated kernels. Since *A. flavus* is versatile, prolific and has a broad host range, its management and control pose a major challenge to maize farmers and breeders. Thus far, breeding for resistance has been the most useful means of control although it has not been very effective. Hence, our laboratory is working to find more durable sources of resistance and to study the mechanisms that lead to resistance to fungal growth and/or aflatoxin accumulation. In a mapping population derived from Mp313E (resistant) and Va35 (susceptible), there is a significant QTL for aflatoxin resistance on chromosome 4, near the map location for catalase3 (cat3). Therefore, we are examining the involvement of catalase in the resistance mechanism. Our previous data indicate that catalase specific activity was higher in developing embryos from resistant compared to susceptible lines. The gel activity assay also showed that the catalase isozyme from the susceptible lines migrated faster than that from the resistant lines. We cloned cat3 cDNA from a resistant line (Mp313E) and a susceptible line (Sc212M) and compared their nucleotide and amino acid sequences. Our preliminary data suggest that the mRNA in the susceptible line maybe truncated. We are performing more experiments to verify these results and to analyze the possible role of cat3 in maize resistance to *A. flavus*.

Identification of a Cotton Gene that is Preferentially Expressed in Seed Coat Tissues

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Aflatoxins are toxic and carcinogenic polyketide secondary metabolites produced by the filamentous fungus *Aspergillus flavus* during growth on crops such as corn, cottonseed, peanuts, and tree nuts. Efforts are underway in our lab to develop transgenic cotton that expresses genes for antifungal proteins and peptides that inhibit the ability of *A. flavus* to invade cotton and produce aflatoxins. We are interested in identifying novel promoter sequences of cotton genes able to drive expression of antifungal genes in a tissue-specific manner. We have shown that during invasion of cottonseed, *A. flavus* most often enters the seed at the chalazal end and ramifies along the inner seed coat prior to colonizing the lipid-rich cotyledons. Based on this observation we were interested in identifying cottonseed coat-specific genes and eventually characterizing their promoters. We chose to undertake a proteomics approach to identify cotton genes whose products are localized in seed coat tissues. Promoters could then be cloned and characterized from candidate seed coat-specific genes and used for control of expression of antifungal resistance genes in cotton.

Two-dimensional SDS-PAGE analysis of total cotyledonary and seed coat protein from *Gossypium hirsutum* Coker 312 identified three proteins that appeared to be expressed only in seed coat tissues. Peptide sequencing of the protein demonstrating the highest level of production among the three identified indicated that it was a glutathione-S-transferase (GST). The Gh 312 seed coat GST demonstrated high identity with a *G. hirsutum* fiber GST EST sequence (97%) but also showed low identity with another fiber GST cDNA (16%). This indicated that as in other species, cotton GSTs are present in the genome as a gene family. Southern hybridization analyses did indicate that the seed coat GST may be part of a small gene family. RNA was isolated from tissues at various time points, Northern blotted, and hybridized with a 400 bp probe representing the 5' seed coat GST coding region. Northern hybridization showed that expression of GST is much higher in seed coat tissue than cotyledonary tissue. Also, very little expression is noted in root or leaf tissues. The level of expression of seed coat GST appears to be similar over all time points analyzed for both cotyledonary and seed coat tissues. Use of Quantitative RT-PCR should enable more accurate analysis of the levels of expression of the seed coat GST with respect to other tissues and developmental stages.

Enhanced Fungal Resistance Conferred by the Expression of a Gene Encoding the Synthetic Peptide D4E1 in Transgenic Cotton

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We have demonstrated that the transgenic expression of a linear, amphipathic, synthetic, lytic peptide (D4E1) offers the potential for the control of the aflatoxigenic *Aspergillus flavus* and other microbial phytopathogens. 1) Purified D4E1 controlled *A. flavus* (IC_{50} = 10 μM) and several other microbial pathogens, including vascular pathogens of cotton. 2) Crude protein extracts from leaf tissue of transgenic tobacco plants expressing the peptide significantly reduced the fungal colonies arising from germinated conidia of *A. flavus*, *Fusarium verticillioides,* and *Verticillium dahliae*. 3) Transgenic tobacco plants expressing D4E1 showed increased resistance *in planta* to the anthracnose-causing fungal pathogen, *Colletotrichum destructivum*. 4) Crude extracts from transgenic cotton callus and leaf tissue showed significant control of *V. dahliae*; however, control of *A. flavus* was not significant with leaf extracts from transgenic cotton. 5) *In situ* assays with immature cottonseeds (21-28 dpa), inoculated with a GFP-expressing *A. flavus* strain, showed that the transgenic cottonseeds are capable of delaying and reducing the fungal advance in both seed coat and cotyledons, as measured by the GFP fluorescence. Based on these antimicrobial assays and molecular assays, several transgenic lines (R1) were selected and evaluated for resistance *in planta* to the phytopathogen, *Thielaviopsis basicola* (IC_{50} = 0.5 μM) that causes black root rot in emerging seedlings. The results were compared with control, transgenic seedlings expressing the GUS marker gene only. A greater percentage (>65%) of transgenic progeny seedlings escaped severe damage by *T. basicola* whereas majority of the control seedlings succumbed to the disease. The fresh weight of transgenic seedlings was significantly higher than that of controls.

Construction of a Gene-silencing Binary Vector for Studying the Functions of Aflatoxin Resistance-associated Proteins/Genes in Corn through Genetic Engineering

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Aflatoxins are carcinogens produced by *Aspergillus flavus* and *A. parasiticus* during infection of susceptible crops such as corn. Though resistant corn genotypes and resistance associated proteins (RAP) and their corresponding genes have been identified, the direct involvement of these genes in kernel resistance has been difficult to demonstrate until the recent discovery of RNA interference (RNAi) phenomenon. This posttranscriptional gene silencing (PTGS) is a sequence-specific RNA degradation process. It is triggered by a double stranded (ds) RNA, leading to the degradation of homologous RNA encoded by endogenous genes, and transgenes. This RNAi technology can be used to specifically silence one of those RAP gene candidates at a time and then evaluate the changes of kernel resistance in the silenced transformants. As a first step, we are constructing a binary vector containing all the key elements needed to generate a dsRNA structure. With the incorporation of site-specific recombination Gateway technology in the vector, constructs with different genes of interest can be easily created from a basic vector (pTF102-d35S-attR4-attR3) through site-specific recombination, eliminating the need of time-consuming multiple ligation and cloning steps. We have also successfully transformed immature corn embryos and regenerated corn plants using a blank control vector through collaboration with ISU. RNAi constructs will be used in the transformation as soon as they are available. These RNAi studies should uncover the role of RAP gene(s) play in kernel resistance. Genes confirmed as important to resistance can then serve as markers for marker-assisted breeding.

Using Functional Genomics to Identify the Role of Lipoxygenases in *Aspergillus flavus* and Maize Interactions

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Lipoxygenases (LOX) catalyze the hydroperoxidation of polyunsaturated fatty acids such as linolenic and linoleic acids to produce an array of oxylipins. Recent biochemical and gene expression analyses of resistant versus susceptible corn lines strongly suggested that 9-lipoxygenases (9-LOXs) and their metabolites may be the *Aspergillus* and aflatoxin susceptibility factors in plants that act to increase *Aspergillus* sporulation and aflatoxin production in maize. In contrast, 13-LOXs are hypothesized to increase aflatoxin resistance because its product, jasmonic acid, acts to inhibit aflatoxin biosynthesis. To test these hypotheses directly, the long-range goal of our research is to define unambiguously the role of all LOXs in aflatoxin susceptibility and/or resistance by disrupting all the LOX genes in both susceptible and resistant corn lines by using reverse genetics approach. If these hypotheses are correct, then **(1)** knocking out 9-LOX genes in susceptible inbred lines will result in increased resistance to aflatoxin accumulation and **(2)** inactivating 13-LOX genes will decrease aflatoxin accumulation in resistant lines. To accomplish this goal we initiated a large scale functional genomics project to suppress expression of every member of the maize LOX gene family. We have reported last year the identification of 11 different LOX genes in maize genome. An additional 13-LOX gene was identified this year. In comparison, rice contains at least 14 different genes and Arabidopsis genome encodes only 6 LOX genes. Gene expression analysis by RNA profiling techniques and Northern blotting results suggested that many of the LOX genes are differentially expressed in diverse maize tissues, in response to defense-associated signaling molecules such as JA and ethylene. Up to date, by using reverse genetics strategy, we have identified several *Mu*-element insertional mutant alleles in seven putative 9-LOXs and in one 13-LOX gene. These mutants are being backcrossed into genetic backgrounds that are either resistant or susceptible to aflatoxin and fumonisin accumulation. When near isogenic wild type and mutant LOX lines are generated, we will test them for any changes in resistance to ear rots caused by *Aspergillus flavus* and *Fusarium verticillioides* and accumulation of aflatoxins/fumonisins.

Aflatoxin and Insect Response of Near-isogenic Non-Bt and Cry1A(b) (Mon810 and Bt11) Commercial Corn Hybrids in South Texas in 2001-2002

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Experiment objectives were to use near-isogenic pairs of corn hybrids to assess influence of the Cry1A(b) Bt gene and transformation event on (1) amount and type of insect injury (2) pre-harvest aflatoxin content at maturity, (3) agronomic performance, and (4) to determine potential relationships among these assessments. Seventeen pairs of near-isogenic Cry1A(b) Bt/ NonBt commercial corn hybrids were obtained from seven companies in 2001 and five companies in 2002. Numbers of hybrid pairs for each of the Mon810, Bt11,and 176 transformation events were 8, 7, and 2 for 2001 and 7, 8, and 2 for 2002, respectively, with 13 hybrid pairs common to both years. Hybrids were planted in a randomized complete block design with nine two-row replicates of each hybrid. Near-isogenic Bt and NonBt hybrids were always in adjacent rows with location determined randomly. Outside rows of each hybrid were used for insect evaluation and the inner two adjacent rows were harvested for agronomic and aflatoxin evaluation. Test locations at Texas Agricultural Experiment Stations for both years were two Orelia soil sites approximately 0.5 miles apart at Corpus Christi, TX (CC-IRR, drip irrigation as needed until silking and CC-DRY, dryland with high potential for drought stress) and a dryland location (60 miles N) at Beeville, TX (BEE). Planting dates were 3-4 weeks later than normal to maximize potential for drought stress during later stages of crop development. Target populations were 18-20 K per ac. Planting dates for CC and BEE were March 26 and April 5, 2001, and March 28 and April 3, 2002, respectively. Inoculum was produced by growing a high aflatoxin-producing isolate of *Aspergillus flavus* (NRRL 3357) on autoclaved corn kernels. When the first hybrids reached mid-silk at any site, inoculum was distributed on the soil surface between treatment rows at the rate of 1 kg dry seed equivalent per 200 ft.

Contrast analysis compared each pair of Bt/NonBt means (a<0.05) of the same Bt transformation event. The ANOVA model included Location and Hybrid Type (Bt11, NonBt11, Mon810, NonBtMon810, 176, NonBt176) and the Location by Hybrid Type interaction. Pearson correlation coefficients were determined for variables related to aflatoxin content (log 10 transformation). Insect pest data included whorl injury (0-9 rating / 10 plants), ear injury (average length of injury from tip in cm / 10 ears), and shank injury (proportion of shanks injured / 10). Ears were harvested after kernel moisture in all hybrids was below 15%**.** After agronomic evaluation, threshed grain from the nine replicates of each hybrid were pooled into three composite replicates as follows: Reps (1,2,3), (4,5,6), and (7,8,9). All grain from each composite replicate, usually ninety ears, was ground in a Romer mill and a subsample was analyzed for aflatoxin content (ppb) using the Vicam Aflatest P immunochemical assay. Corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*) were the primary, naturally-occurring insect pest populations causing injury to whorls and ears with the latter causing most injury to ear shanks.

At all locations and years, whorl and ear injury were significantly greater for NonBt hybrids than their Bt11 or Mon810 hybrids. At both locations each year, shank injury (not evaluated at BEE) was significantly greater for NonBt hybrids compared to their Bt11 and Mon810 hybrids except for Bt11 at CC-DRY in 2002. Whorl and ear insect injury ratings were significantly higher in the NonBt hybrids compared to Event 176 Bt hybrids at all sites in 2002 only. In 2001, NonBt hybrids had significantly greater aflatoxin content than their near-isogenic Bt11 and Mon810 hybrids at CC-IRR but differences were not significant at CC-DRY and BEE. In 2002, Bt11 and Mon810 Bt hybrids had significantly higher aflatoxin content than their near-isogenic NonBt hybrids at both CC sites. Conversely, Non-Bt hybrids had significantly greater aflatoxin content than near-isogenic Bt11 and Mon810 hybrids at BEE in 2002. There was a consistent significant positive correlation between aflatoxin (Log 10) and ear injury rating at all sites and years for NonBt hybrids. Yield was significantly greater in Bt11 and Mon810 hybrids than in near-isogenic NonBt hybrids at all locations and years except for BEE in 2001 where overall yields were quite low. The only significant yield differences for Event 176Bt hybrids were at CC-IRR in 2001 where the NonBt hybrids were significantly higher. Yield was negatively correlated with aflatoxin content when the relationship was significant.

Aflatoxin Accumulation in Transgenic Peanut Lines Containing Anti-Fungal Genes

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Seven peanut lines containing anti-fungal transgenes and three checks (Okrun, Georgia Green, and Tifton 8) were evaluated in field plots in Tifton, Georgia for their levels of preharvest aflatoxin contamination (PAC). All plots were inoculated with a mixture of *Aspergillus flavus* and *A. parasiticus*. Drought and heat stress were imposed for the 40 d preceding harvest. In both tests, the transgenic genotypes, K24 and K34, exhibited at least a 50 percent reduction in aflatoxin contamination when compared to their parent genotype, Okrun. This indicates that these transgenic lines may have useful resistance to *Aspergillus*. These lines are being reevaluated in field studies in 2003.

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