Request for Report for Projects Awarded in 2013 and 2014 by

Mississippi Center for Food Safety and Post-Harvest Technology

Title: Detection and Quantification of Gossylpol and Insecticides in Mississippi Cotton Seeds and Their Oil Products

Award year: 2014

PI: Din-Pow Ma

Co-PI: Jiaxu Li

Collaborator:

1. Objectives.

   a. Detect and quantify gossypol and pesticides in three mostly planted cotton varieties in Mississippi and a genetic standard line DES119 using HPLC and ELISA (enzyme-linked immunosorbent assay) methods.

   b. Prepare crude seed oils and quantify gossypol and pesticides in the crude oils from the three Mississippi mostly planted cotton varieties and DES119. Quantify the amounts of gossypol and pesticides with commercial cotton oil products from Mississippi factories.

2. New Accomplishments toward objectives. Please indicate if all objectives listed were completed.

   Have completed the analysis of gossypol and pesticides in three mostly planted cotton varieties (ST5288B2F, DP0912B2RF, and PHY499WRF) and a genetic standard line DES119 using HPLC/UV and HPLC/Mass.

   Have completed the analysis of the insecticide imidacloprid in ST5288, DP0912, PHY499, and DES119 using the ELISA method.

   Haven't completed the analysis of gossypol and the insecticides in cotton seed oils, will complete the analysis by Sept. 30, 2015.

3. Objectives not accomplished and impediments to meeting objectives.

   It is very difficult to obtain cotton seed oil samples from Cottonseed Oil Manufacturers. We are only able to get one seed oil sample from Delta Oil Company.

4. If continuing project, when will new and/or long term objectives be completed?

   One can develop gossypol-free cotton seeds via genetic engineering using RNA interference technology. This will take two years to reach the objectives.

5. Students supported

a. PhDs (% FTE and name): Qing Miao (0.7 FTE)
b. M.S. (% FTE and name)
c. Undergraduate (number of students): One

6. Leveraged Funds: External Competitive Funding Applied and Awarded based on findings from this project.
   a. Applied for:
      i. Funding agency
      ii. Program
      iii. Funding request ($$)
   b. Awarded:
      i. Funding agency
      ii. Program
      iii. Funding awarded ($$)

7. Outputs – In addition to the above, please populate the following sections to be included in a report to be compiled in a FSI Research Accomplishment Booklet. The project report will also be posted in a FSI website to be developed.

Project Summary (Issue/Response)

In this box type 300—400 word project summary in 10 pt font.

Cotton is an important cash crop of global significance. Although cotton is primarily cultivated for its fiber, it is also used for cottonseed oil production. Mississippi is the 5th largest cottonseed-producing state in the United States. Cottonseed production plays a vital role in Mississippi's economy. One of the potential problems consuming cotton seed oil is the presence of the toxic compound gossypol. Gossypol is a natural terpenoid aldehyde present in pigment glands on the surface of the cotton plant including the seed. Approximately 0.5%-1% gossypol is found in most commercial cotton seeds (Calhoun et al., 2004). The currently planted cotton in Mississippi all produce gossypol. Pesticides are regularly used by cotton growers and the control of cotton insects still largely depends on insecticides. Insecticides are the most toxic class of pesticides, the top three insecticides spread to cotton fields are Acephate, Bifenthrin, and Imidacloprid. The top four varieties of Upland cotton (*Gossypium hirsutum*) grown in Mississippi are ST 5288 B2F, ST 5458 B2F, DP 0912 B2RF, and PHY 499 WRF. To protect the health of consumers, the gossypol and the three insecticides were detected and quantified in three cotton varieties (ST5288B2F, DP0912B2RF, and PHY499WRF) and a genetic standard line DES119 using HPLC/UV and HPLC/Mass and ELISA (Enzyme linked immunosorbent assay) methods. The gossypol concentration determined with LC/UV for ST5288, DP0912, PHY499 and DES119 was found to be 4937, 3696, 3726, and 4572 ppm, respectively. Using the LC/MS method, the gossypol concentration was determined to be 5256, 3723, 3735, and 4760 ppm, respectively, for ST5288, DP0912, PHY499 and DES119. In all LC/UV and LC/MS assays, extracts contained 0.1 mg seed sample per µl injected. The results indicates that the determined concentration values were fairly close using the two methods. The concentration of Bifenthrin was too low to be detected in all three varieties and DES119 using the LC/MS method. Acephate was also not detected in DP0912 and PHY499, and 0.044 ppm and 0.391 ppm of Acephate were detected in ST5288 and DES119, respectively. The concentration of imidacloprid was determined to be 6501, 3743, 6971, and 164 ppm for ST5288, DP0912, PHY499, and DES119, respectively. Using the competitive ELISA assay, the concentration of imidacloprid was found to be 4668±12.44, 1831±12.50, 764±11.47 ppm for PHY499, DP0912, and ST5288, respectively. DES119 has a concentration of imidacloprid less than 20±12.51 ppm, in the concentration range undetectable by the ELISA assay.

Project Results/Outcomes

In this box type 500—750 word summary of project results/outcomes.

The cottonseeds of the top three varieties grown in Mississippi (ST 5288 B2F, DP 0912 B2RF, and PHY 499 WRF) were obtained from a local cotton breeder, originally from Bayer CropScience (Stoneville, MS) and Phytogen (Corcoran, CA). One commercial oil product was also obtained from Delta Oil Mill (Jackson, MS). Gossypol and insecticides were extracted with a modified QuEChERs method. Briefly, cotton seeds were homogenized into fine powder with a coffee grinder. One gram powder was added into a 50 ml polypropylene test tube and extracted with 10 ml solution containing acetone/acetonitrile/HOAc (50/50/1) with a GenoGrinder. Ten mL of water and QuEChERs were subsequently added for further extraction. QuEChERs's salts were used to better separate the organic and inorganic layers. After centrifugation the gossypol or insecticides will be present in the top organic layer. The top layer will then be concentrated to approximately 2 mL in Roto-Vap and diluted to 10 mL with 1% HoAc/Acetronitrile. The samples after filtration were used for HPLC analysis. Samples were analyzed for Gossypol using both LC/UV at 254 nm and LC/MS and only using LC/MS for insecticide analysis. The HPLC analyses were performed by Mississippi State Chemical Laboratory at Mississippi State.

The highest gossypol concentration was detected in ST5288 with 4937 and 5256 ppm, respectively, by HPLC/UV and HPLC/MS. The concentration of gossypol in the genetic standard line DES119 were determined to be 4572 ppm and 4760 ppm, respectively, by HPLC/UV and HPLC/MS. In DP0912 and PHY499, the gossypol contents were much lower than that in DES119. For DP0912, the concentrations of gossypol were 3696 and 3723 ppm, respectively, by HPLC/UV and HPLC/MS. And for PHY499, the concentrations of gossypol were 3726 and 3735 ppm, respectively, by HPLC/UV and HPLC/MS (Extracts contained 0.1 mg sample per 1µL injected).

The top three insecticides Acephate, Bifenthrin, and Imidacloprid spread to cotton were also determined by LC/MS in this study. The contents of Bifenthrin were found to be too low to be quantified in all three varieties and DES119. Acephate could not be determined in DP0912 and PHY499; and 0.044 ppm and 0.391 ppm of Acephate were detected in ST5288 and DES119, respectively. Imidacloprid was detected in all the varieties and DES119. The concentration were determined to be 6501 ppm in ST5288, 3743 ppm in DP0912, 6971 ppm in PHY499, and 164 ppm in DES119, respectively (Extracts contained 0.1mg sample per 1 ul injected).

An ELISA kit obtained from ENVIROLOGIX was also used to quantify imidacloprid in cotton seed samples. In the assay, Imidacloprid pesticide residues in the seed sample compete with enzyme (horseradish peroxidase)-labeled imidacloprid for a limit number of antibody binding sites on the inside surface of the test wells. After several wash steps, the outcome of the competition was visualized with a color development step. The concentration of imidacloprid in cotton seeds was calculated based on a semi-log scaled standard curve ($y = -17.75 \ln(x) + 45.261$ $R^2 = 0.9942$). The ELISA results showed that the cultivar PHY499 had the highest imidacloprid contents in the seeds, which is 4668±12.44 ppm, followed by DP0912 with 1831±12.50 ppm, and ST5288 with 764±11.47 ppm. In DES119 seeds, the concentration of imidacloprid was too low to be quantified by the ELISA assay, lower than the detection limit of 20±12.51 ppm (Extracts contained 0.2mg sample per 1µL injected).

Project Impacts/Benefits

In this box type 250—300 words project Impacts/Benefits statement.

The detection and quantification of gossypol in Mississippi cotton seeds and their oil products will be useful for selection of cotton seeds with low levels of gossypol for oil extraction. The contents of gossypol in three most plant cotton varieties ST5288, DP0912 and PHY499 and a standard genetic line DES119 were determined in this project by Mississippi State Chemical Laboratory using LC/UV and LC/MS methods. The experimental results indicated that DP0912 and PHY499 have low contents of gossypol when comparing to the ST5288 which has a higher gossypol concentration. Therefore, the seeds from the two cotton cultivars DP0912 and PHY499 will be the better choice for cotton oil production, presumably less gossypol would be present in the crude oil product and less efforts are required for producing gossypol-free cotton seed oil. The residues of the three insecticides acephate, bifenthrin and imidacloprid in the cotton seeds were also determined by GC/MS and ELISA methods. There was none or very low contents of acephate and bifenthrin in the four cotton seeds. A high amount of imidacloprid, however, was found in ST5288, DP0912, and PHY499 seeds. The three cotton seeds of PHY499, DP0912, and ST5288 were probably pre-treated with imidacloprid for long-term storage. We have acquired a cotton seed oil sample from Delta Oil Mill but haven't quantified the gossypol and insecticides in the sample. An experimental protocol will have to be first developed to extract gossypol and insecticides from the oil sample for their analysis by HPLC/UV and HPLC/MS. This research will ultimately set up industrial standard in food safety to minimize health hazards via the consumption of cotton oil. During the last 15 years, my group has collaborated with two local Cotton Geneticists, Drs. Johnie Jenkins and Sukumar Saha (ARS/USDA at Mississippi State) in many cotton research projects. They have been also actively participating in our graduate program, serving on graduate committee for many of my graduate students.

Project Deliverables

In this box list complete citations for all publications, presentations, workshops, field days, and other deliverables that came out of this project. Please use the following style (J. of Food Sci):

Adachi N, Kinoshita H, Nishiguchi M, Takahashi M, Ouchi H, Minami T, Matsui K, Yamamura T, Motomura H, Ohtsu N, Yoshida S, and Hishida S. 2008. Simultaneous analysis of acephate and methamidophos in human serum by improved extraction and GC-MS. Forensic Toxicol 26: 76-79.

Alawi MA. 1983. HPLC-determination of acephate. Fresenius Journal of Analytical Chemistry **315**: 358-359.

Baskaran S, Kookana RS, and Naidu R. 1997. Determination of the insecticide imidacloprid in water and soil using high-performance liquid chromatography. Journal of Chromatography A **787**: 271-275.

Bell AA, and Stipanovic RD. 1978. Biochemistry of disease and pest resistance in cotton. Mycopathologia **65**: 91-106.

Cai Y, Zhang H, Zeng Y, Mo J, Bao J, Miao C, Bai J, Yan F, and Chen F. 2004. An optimized gossypol high-performance liquid chromatography assay and its application in evaluation of different gland genotypes of cotton. Journal of Biosciences. **29**: 67-71.

Calhoun MC, Wan PJ, Kuhlmann SW, and Baldwin Jr, BC. 2004. Variation in the nutrient and gossypol content of whole and processed cottonseed. In: proceedings of the Mid-South Ruminant Nutrition Conference, 22 April, Arlington, TX.

Cotton Varieties Planted 2013 Crop. U.S. Department of Agriculture, Agricultural Marketing Service – Cotton Program, Memphis, Tennessee, Sept. 2013.

Dong H, Bi P, and Xi Y. 2008. Determination of pyrethroid pesticide residues in vegetables by solvent sublation followed by high-performance liquid chromatography. Journal of Chromatographic Science **46**: 622-626.

Hedin PA, Parrott WL, and Jenkins JN. 1992. Relationships of glands, cotton square terpenoid aldehydes, and other allelochemicals to larval growth of Heliothis virescens .Lepidoptera: Noctuidae.. Journal of Economic Entomology **85**: 359-364.

Lee JK, Ahn KC, Stoutamire DW, Gee SJ, and Hammock BD. 2003. Development of an enzyme-linked immunosorbent assay for the detection of the organophosphorus insecticide acephate. Journal of Agricultural and Food Chemistry **51**: 3695-3703.

Luo P, Wang YH, Wang GD, Essenberg M, and Chen XY. 2001. Molecular cloning and functional identification of .+.-δ-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase .CYP706B1. of cotton sesquiterpene biosynthesis. Plant Journal **28**: 95-104.

McMichael SC. 1959. Hopi cotton, a source of cottonseed free of gossypol pigments. Agronomy Journal **51**: 630.

McMichael SC. 1960. Combined effects of the glandless genes gl2 and gl3 on pigment glands in the cotton plant. Agronomy Journal **52**: 385-386.

National Agricultural Statistics Service. 2011. Mississippi Agricultural Overview .http://www.nass.usda.gov/Statistics_by_State/Ag_Overview/AgOverview_MS.pdf.

Nunes GS, Toscano IA, and Barcelo D. 1998. Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. Trends in Analytical Chemistry **17**: 79-87.

Palle SR, Campbell LM, Pandeya D, Puckhaber L, Tollack LK, Marcel S, Sundaram S, Stipanovic RD, Wedegaertner TC, Hinze L, and Rathore KS. 2013. RNAi-mediated ultra-low gossypol cottonseed trait: performance of transgenic lines under field conditions. Plant Biotechnology Journal **11**: 296-304.

Rathore KS, Sundaram S, Sunilkumar G, Campbell LM, Puckhaber L, Marcel S, Palle SR, Stipanovic RD, and Wedegaertner TC. 2012. Ultra-low gossypol cottonseed, generational stability of the seed-specific RNAi-mediated phenotype and resumption of terpenoid profile following seed germination. Plant Biotechnology Journal **10**: 174-183.

Risco CA and Chase Jr, CC. 1977. Gossypol. In: handbook of Plant and Fungal Toxicants .D'Mello, J.P.F., ed.., pp. 87-98. Boca Raton, FL: CRC Press.

Sun Q, Cai Y, Li S, Chen M, Mo J, He X, Jiang H, Liu J, and Lei K. 2013. Identification of the genes and pathways associated with pigment gland morphogenesis in cotton by transcriptome profiling of nesr-isogenic lines. Biologia **68**: 249-257.

Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD, and Rathore KS. 2006. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proceedings of the National Academy of Sciences USA, **103**: 18054-18059.

Sunilkumar G, Connell JP, Smith CW, Reddy AS, and Rathore KS. 2002. Cotton α-globulin promoter: isolation and functional characterization in transgenic cotton, Arabidopsis, and tobacco. Transgenic Research **11**: 347-359.

Vaissayre M and Hau B. 1985. New results on the susceptibility of glandless cotton varieties to phyllophagous insects. Coton et Fibres Tropicales **40**: 159-168.

Watanabe E, Eun H, Baba K, Arao T, Ishii Y, Endo S, Ueji M. 2004. Evaluation and validation of a commercially available enzyme-linked immunosorbent assay for the neonicotinoid insecticide imidacloprid in agricultural samples. Journal of Agricultural and Food Chemistry **52**:2756-2762.

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, and Waterhouse PM. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant Journal **27**: 581-590.

Graphics

Include one or two graphics (picture or figure, colored preferred) that illustrate project outcomes. Please make sure you provide labels and appropriate units for all dimensions, and a title with a brief explanation for each figure/graph.

Varieties	Imidacloprid (ppm, n=3)
DP0912	1831.56±12.50**

A

B

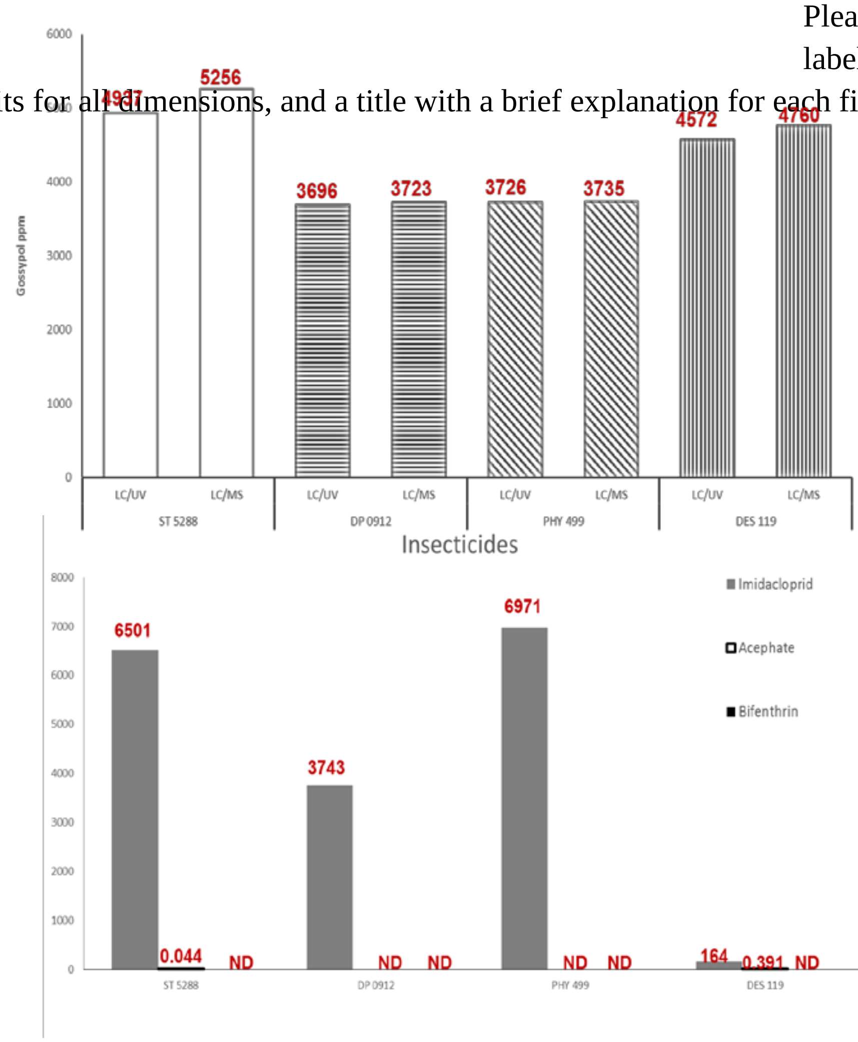

Figure 1. Quantification of gossypol and three insecticides in four cotton varieties by HPLC. A) Gossypol quantification by HPLC/UV and HPLC/MS; B) Quantification of three insecticides by HPLC/MS (Extracts contained 0.1mg sample per 1µL injected).

Table 1. Quantification of Imidacloprid by ELISA in four cotton varieties

DES119	<20±12.51**
ST5288	764.47±11.47**
PHY499	4668.42±12.44**

** Significant at p<0.01

Attached Refereed Journal Publications in Separate Files

Please attached published journal articles (in pdf format if available) for this project. Manuscripts accepted or in review process may be submitted in word files. Thank you very much for your cooperation.