Progress Report (Research Proposal Jan. 2013) Project # 2013-12

Title: Assessment of Mycotoxin Contamination in Wines Produced from *Vitis vinifera* Grapes in the Southeastern U.S.

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Objectives:

1. To quantify the presence or absence of mycotoxins [ochratoxin A (OTA), fumonisin B1 (FB1), and fumonisin B2 (FB2)] through large-scale sampling of *Vitis vinifera* wines and winegrapes originating in the Southeast.

2. To isolate potentially mycotoxigenic fungi from winegrapes, identify these fungi to the species level, and determine if they produce mycotoxins in vitro.

3. To identify current beneficial and at-risk practices for mycotoxin contamination through a producer survey of viticultural and winemaking techniques.

Justification and Description:

Ochratoxin A (Figure 1), a mycotoxin produced by black-spored Aspergilli such as *A. niger* (Figure 2) and others, and present in cereals, coffee, and wine, has either been shown to be or is potentially nephrotoxic, immunosuppressive, teratogenic, genotoxic, embryotoxic, and cytotoxic (JECFA 2001). OTA is also a possible human carcinogen in Group 2B, with fellow members DDT, lead, and chloroform (IARC 1993). Although the European Union declared a maximum level of 2 µg per liter for ochratoxin A in wine in 2005 (Commission regulation [EC] No 123/2005, 26 Jan 2005), the United States has yet to conduct wide-scale sampling of wines (as suggested by the Joint FAO/WHO Expert Committee on Food Additives) (JECFA 2001) or to regulate the presence of OTA in wine. Several regions of the world have sampled their wines to assess exposure to this mycotoxin and ensure consumer safety, including Spain (Blesa et

al. 2004), South America (Chulze et al. 2006), Greece (Labrinea et al. 2011), Canada (Ng et al. 2004), Turkey (Var and Kabak 2007), Italy, and Hungary (Brera et al. 2005). Since the U.S. does have the potential in winegrapes for mycotoxin production, especially in the Southeastern region where the climate is conducive to the growth of *Aspergillus* species of molds on winegrapes, the levels must be evaluated to ensure public health safety.

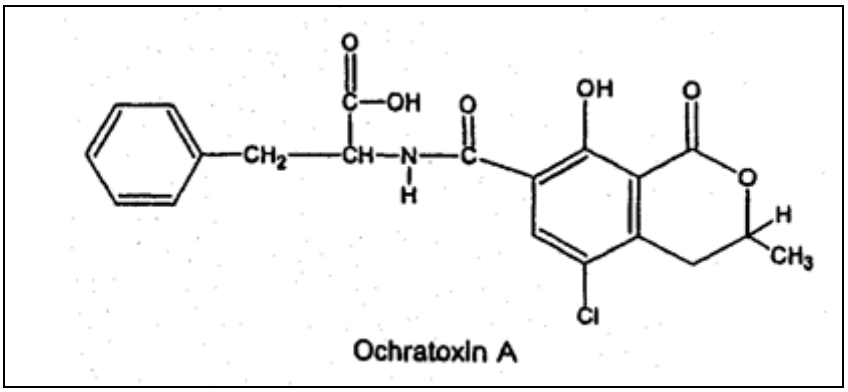

Figure 1. **Structure of Ochratoxin A**. Ochratoxin A is a nephrotoxic, immunosuppressive, teratogenic, genotoxic, embryotoxic, and cytotoxic compound produced by Aspergilli (http://www.bionano.re.kr/bbs/board.php?bo_table=menu05_03&wr_id=727).

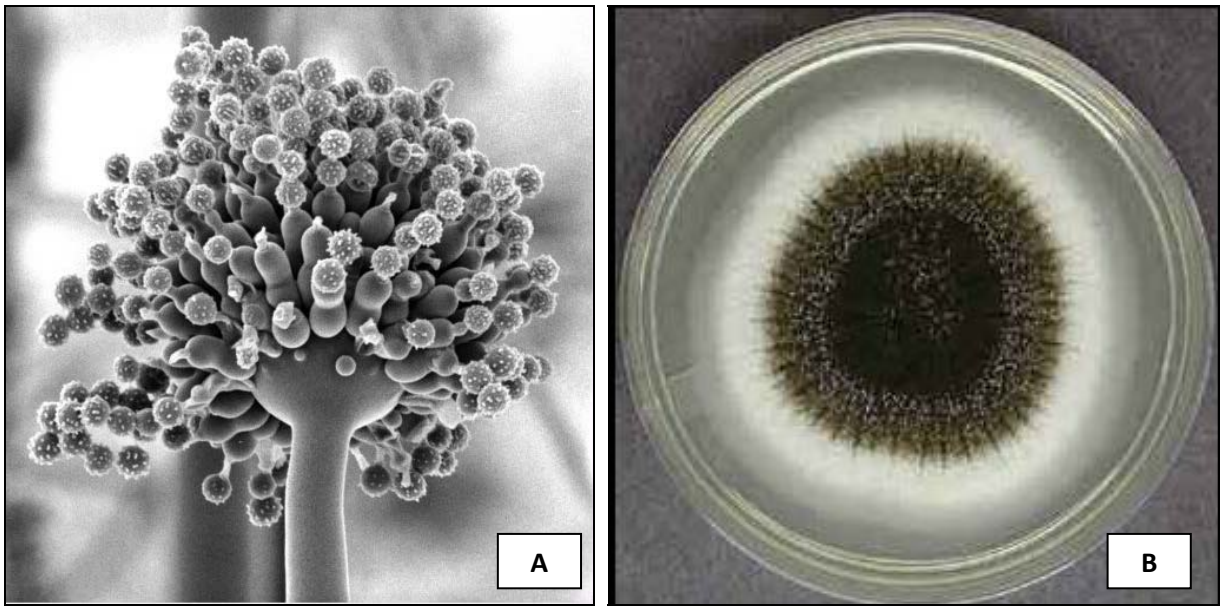

Figure 2. Black-spored Aspergilli, such as *Aspergillus niger*, are among those that produce toxins. (A) Scanning electron micrograph of *Aspergillus niger* (Read ND & Jeffree CE (1991) Low-temperature scanning electron microscopy in biology. *J Microsc* 161 :59-72); (B) *Aspergillus niger* colony in culture (http://labmed.ucsf.edu/education/residency/fung_morph/fungal_site/subpages/anigersdsp.html).

The fumonisin group of mycotoxins has recently been discovered in varying quantities in red wine (Mogensen et al. 2010). The most common fumonisin to date is FB1, which has been found to be neurotoxic, hepatotoxic, and nephrotoxic in animals, and joins OTA as a possible carcinogen (Group 2B) (IARC 2002) though further research is needed to prove a correlation. *Aspergillus niger* is capable of producing FB2, while some *Fusarium* species can produce both FB1 (Figure 3) and FB2. Since *Fusarium* was found in preliminary winegrape samples collected during this project, it was determined best to sample the winegrapes for both types of mycotoxigenic fungi – *Aspergillus* and *Fusarium*. One hypothesis in this stage of research is that

Fusarium infects vineyards in close proximity to cornfields, where the fungus is more common. In addition, the bottled wine samples will be tested for all three mycotoxins: OTA, FB1, and FB2.

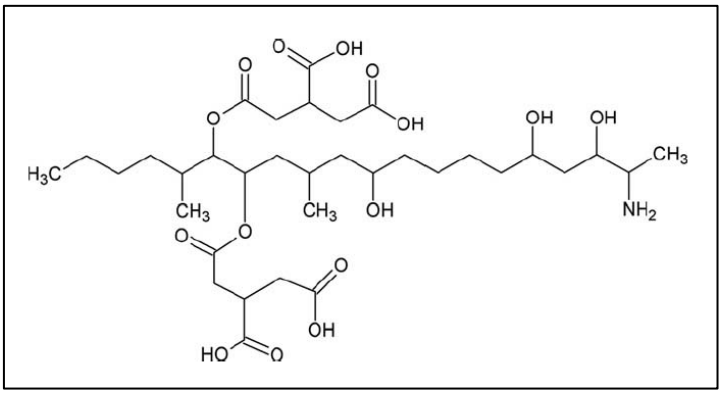

Figure 3. The molecular structure of FB1, a mycotoxin produced by some *Fusarium* fungi. (Stockmann-Juvala and Savolainen 2008).

Methodology:

This project has begun in-depth research into the levels and the potential precursors of ochratoxin A, fumonisin B1, and fumonisin B2 in Southeastern American wine and winegrapes. Wine and winegrapes from several states, especially wine produced in high-risk regions and during high-risk vintages, is being sampled and tested for the presence of OTA, FB1, and FB2 using LC/MS/MS and C-13 labeled standards, with method validation. LC/MS/MS analysis following sample extraction is favored over HPLC for mycotoxin analysis because it allows for detection and quantification of all three mycotoxins simultaneously. Winegrape production in 2013 was characterized by heavy rainfall, making fungal sampling ideal due to prime growth conditions. Wine bottle sampling focuses on red and sweet wines, where contamination potential is maximized due to prolonged contact with berries and prolonged pre-harvest time on the vine, respectively.

A survey of viticultural and winemaking practices will accompany wine samples (where possible) to assess at-risk or best-practice behaviors and conditions specific to American wines. The information gathered will be prepared in a clear and a respectful manner, keeping a strict anonymity of individual producers, for the education of the American winemaking community. Thus far, the winemaking community has been very receptive to the project, facilitating accessible sampling. All producers will be notified of the test results for both the wine and grape analysis. The current market value for the cost of a mycotoxin analysis (for OTA only) in finished wine is approximately $300.

Progress and Results:

Target wine samples – red *vinifera* wines made from 100% Southeastern grapes – have been collected by traveling to producers and purchasing wine on-site. Meetings with winemakers and vineyard owners provide detailed information about vintages/varieties more prone to disease, as well as production notes concerning varietal percentages, vintages, and sources for particular wines when the information does not appear on the bottle label. Southeast winegrowers face the added challenge of not only heavy pressure from Pierce's disease, but also berry damage from hurricanes and tropical storms. Wine produced during the year of Hurricane Katrina, 2005, is included in sampling whenever possible. A total of 60 bottles have been purchased from 25 producers in the three states of Georgia, North Carolina, and Virginia. More samples will be collected in Spring 2014 for a total of 200 representative wine samples. Once all 200 samples are collected, LC/MS/MS analysis on those samples will be conducted, in an effort to statistically reduce day to day variation in testing. In preparation, a baseline curve was created for LC/MS/MS analysis using Cabernet Sauvignon wine spiked with both C-13 labeled and unlabeled mycotoxins, along with unspiked wine.

All wine samples are given a randomly-assigned, unique 3-digit code. During harvest 2013, red winegrapes from 9 vineyard sites were collected within 10 days of harvest. Up to three different grape varieties were sampled per vineyard. Three samples, each consisting of two grape bunches (Figure 7, left), were collected per grape variety from different areas of the vineyard plot, following an approximate "X" shape when possible. The winegrape samples were transported in plastic Ziploc bags on ice to the laboratory for processing within 12 hours. The vineyard sites were located in Georgia, North Carolina, and Alabama. Upon return to the laboratory, winegrapes were crushed by hand and homogenized (Figure 7, right), then aliquoted and frozen to -80°C for further analysis. A total of nine different grape varieties (including one non-*vinifera* variety) were sampled: Cabernet Sauvignon, Cabernet franc, Tannat, Malbec, Merlot, Sangiovese, Touriga Nacional, Petit Verdot, and Norton. Vineyard elevations were recorded and vary from approximately 600-2300 ft. above sea level.

Figure 7. One wine grape sample (left). Two Tannat bunches in a Ziploc bag from Vineyard #20, sample #3. **Polytron homogenizer creates grape must in a sterilized beaker (right).**

Each sample was aliquoted into a portion for fungal analysis and a portion for direct mycotoxin analysis. The fungal analysis includes plating a small portion of each sample in duplicate onto both DRBC (Dichloran Rose Bengal Chlortetracycline agar) and BOA (2-benzoxazolinone) agar. DRBC selects for black-spored Aspergilli (Figure 8, left) whereas BOA plates select for *Fusarium*. Target fungal colonies chosen from these initial platings based on morphological characteristics are being isolated and purified (single spore isolation technique; Figure 8, right). Molecular techniques involving DNA extraction, PCR (polymerase chain reaction), gel electrophoresis, and sequencing are used to identify each isolated fungal colony to the species level. A phylogenetic tree will be produced from the results. This portion of the project will continue to take place over several months.

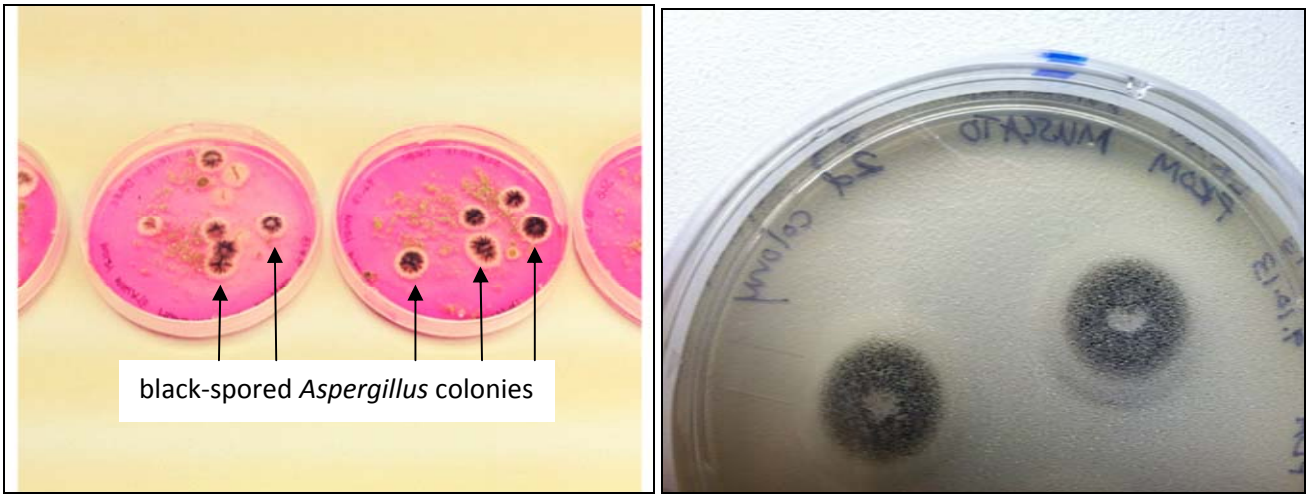

Figure 8. DRBC plates with black-spored *Aspergillus* colonies (left). An isolated colony growing in culture (right).

Additionally, isolated and purified colonies are grown in vitro and an agar plug will be taken from the colony to quantify the amount of mycotoxin produced using extraction followed by LC/MS/MS. It is important to know which species of *Aspergillus* and *Fusarium* present in vineyards produce specific mycotoxins.

The second aliquoted portion of the homogenized winegrape sample ("must") will be directly tested for mycotoxins using extraction and filtration followed by LC/MS/MS analysis. A base curve for the must samples was created using extra homogenized grape must and running it through the instrument with C-13 labeled mycotoxins, unlabeled mycotoxins, and no mycotoxins. Thus, the instrument is prepared and adjusted for must sample mycotoxin analysis.

Winegrape samples collected in 2012 during a preliminary study have now been isolated, purified, and sequenced, and a phylogenetic tree based on the ITS region (internal transcribed spacer) was created using Chromas, Sequencher v. 5.2.2, and Geneious v. 7 software (Figure 9). This phylogenetic tree, known as a neighbor-joining cladogram, was created from ten grape bunch samples from each of two Georgia vineyards with moldy fruit. A total of 29 black-spored Aspergilli isolates were found to be present in three clades: *Aspergillus niger*, *Aspergillus japonicus*, and *Aspergillus tubingensis*. To date, only *A. niger* has been proven to produce any of our mycotoxins of interest (OTA and FB2), but all isolates will be tested in vitro for mycotoxin production. Seven of the 29 isolates (24%) are identified as *A. niger*.

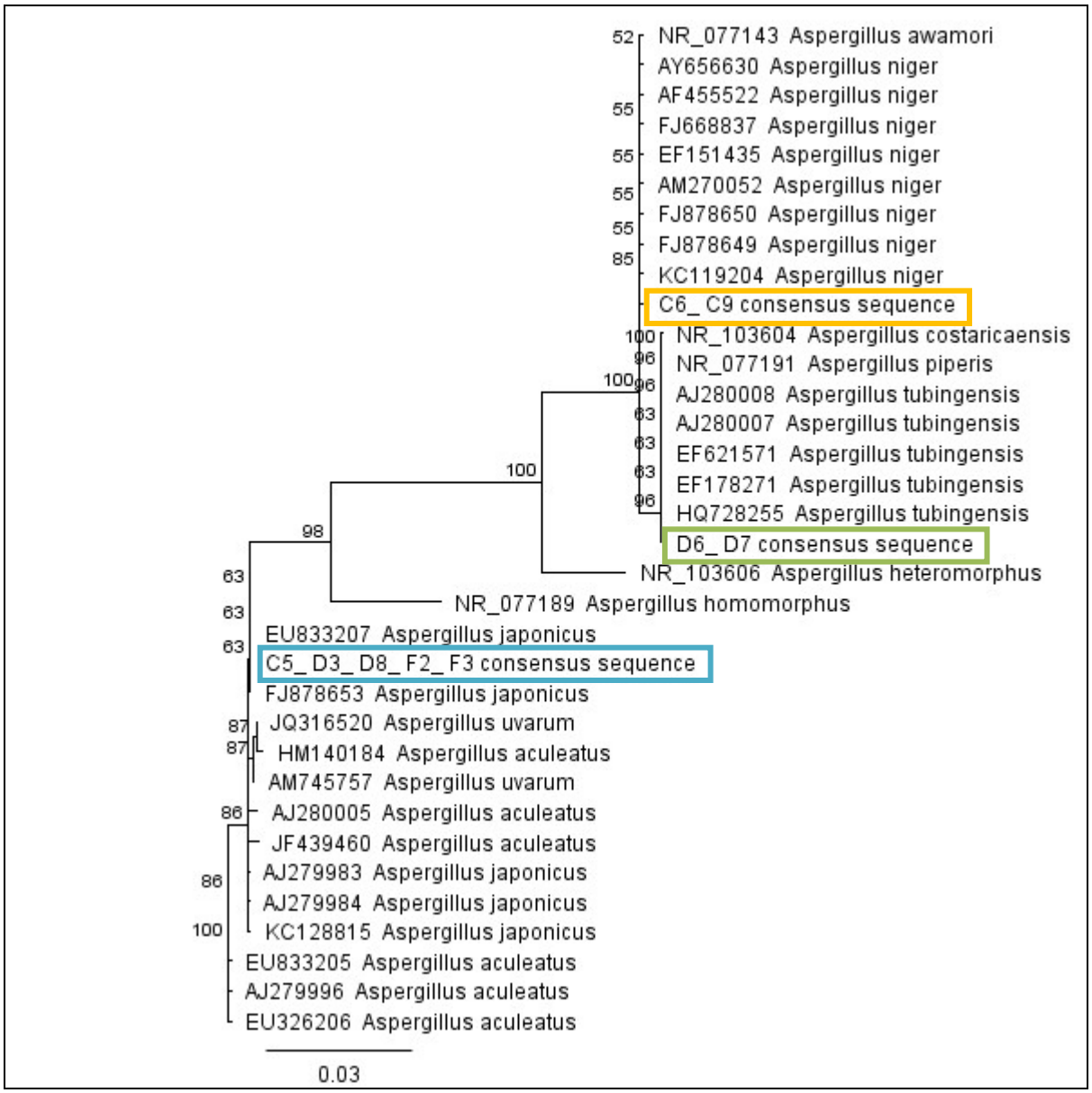

Figure 9. Neighbor-joining cladogram. Cladogram is based on nucleotide sequence of the ITS region of ribosomal DNA showing species identification of the black-spored *Aspergillus* isolates obtained from two vineyards sampled in fall of 2012. Numbers on branches indicate bootstrap support values (1000 replications). Highlighted consensus sequences show representative sample colonies, whereas all other listed isolates are from the NCBI BLAST library (http://blast.ncbi.nlm.nih.gov).

Impact Statement:

Where environmental conditions are conducive to fungal colonization, as in the Southeastern United States, it is imperative to determine whether or not there is a mycotoxin contamination risk in winegrape production. *Vinifera* winegrape growers deal with many challenges and damaged grapes are more prone to fungal invasion. If species of fungi which produce mycotoxins are discovered and/or mycotoxins are found in the finished wine product, corrective steps involving best viticultural and enological techniques can be learned from the surveys and put into practice to lower the contamination risk factors. By facing the mycotoxins in wine issue with careful consideration and planning, everyone involved will be better educated and public health maximized.

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