MISSISSIPPI SOYBEAN PROMOTION BOARD
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TITLE: Identification of mycotoxins used in soybean root infection by *Macrophomina phaseolina* and other fungi.

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SUMMARY OF THE YEAR'S ACTIVITIES.

Substantial progress has been made in the isolation and structure identification of mycotoxins that may be used by the charcoal rot disease fungus, *Macrophomina phaseolina* (Mp) to infect soybeans through the roots, presumably by releasing a mycotoxin that kills dividing cells of the meristematic tissue and creating a necrotic area that facilitates entry of hyphae into the vascular system of the plant. Better understanding of the types of mycotoxins used to facilitate root infection of Mississippi soybeans by *M. phaseolina* and other fungi that use this infection mechanism may allow the development of improved methods to prevent root infection.

The major areas of research achievement have been as follows:
(i) development of a general methodology for the isolation and structural identification of mycotoxins used by *M. phaseolina* to kill and degrade root tips;
(ii) the identification of a new, known mycotoxin, (R)-(-)-mellein, as being produced by *M. phaseolina* isolates from Mississippi soybean plants with charcoal rot disease;
(iii) isolation and structure identification studies on the mycotoxin from *M. phaseolina* isolate *Mp* 205;
(iv) identification of mycotoxin production by other fungal pathogens of Mississippi soybeans; and
(v) preliminary studies on a *M. phaseolina* mycotoxin abatement strategy.

The research conducted already is consistent with various *M. phaseolina* isolates utilizing different mycotoxins to facilitate their root infection, and thus there is considerable additional research progress to be made in the future in this area.

Research progress was negatively impacted by the partial federal government shutdown that included the USDA, which prevented research from being accomplished during part of the year by the Principal Investigator, Dr. Hamed Abbas and the graduate research assistant, Mr. Vivek Khambhati, both of whom were US government employees.

OBJECTIVE

The objective of this research is to better understand the mechanism of root infection of soybeans from the soil reservoir by *M. phaseolina* and other fungi that infect the roots of soybean plants from soil reservoirs. These fungi access the vascular system of soybean plants through the roots by using a mycotoxin to induce localized root cell killing, which creates necrotic or tissue-free zones through which

fungal hyphae can readily penetrate. This research was conducted to better understand the range of mycotoxin types used by *M. phaseolina* isolates from Mississippi soybean plants to facilitate root infection in soybeans. The responses will be compared to those of a limited selection of other fungal pathogens that cause losses in Mississippi soybean production by infecting plants from the soil through the roots.

REPORT OF PROGRESS/ACTIVITY

Methodology development.

Initial research on the isolation and structural identification of mycotoxins used by *M. phaseolina* to kill and degrade root tips as part of their mechanism of root infection of soybean plants from the soil reservoir has focused on identifying and putting in place the needed methodology. The overall research strategy has been (i) identification of *M. phaseolina* isolates for which culture filtrates block the lateral root production response to hypoxic conditions in hydroponic cultures of soybean seedlings in tubes of unstirred 10% Villagarcia medium; and (ii) bioassay-guided fractionation of the activity targeting the dividing cells of the meristematic tissue near soybean root tips.

Initial studies used two *M. phaseolina* isolates, *Mp*205 in particular, and *Mp*3, for which ethyl acetate extracts were shown to effectively kill dividing NIH3T3 mouse fibroblasts and proliferation of *Bacillus subtilis* 1a1 bacterial isolates. Of these two bioassays, *B. subtilis* toxicity is by far the fastest, easiest, and most flexible, and particularly allows use of bioautography with preparative thin layer chromatography (TLC). Initial attempts at doing bioautography used the classical approach of inverting TLC plates on seeded agar; it was ineffective for everything except the tetracycline positive control. However, excellent results were obtained using the technique of spraying diluted *B. subtilis* culture medium onto the silica gel of the TLC plate, incubating overnight in a humidified box at 37^{o}C, spraying with MTT dye solution, and incubating an additional 4 hours. TLC bands with antibiotic activity give a white zone against a purple background. Bioautography is used to identify the UV_{254}-absorbing band associated with the mycotoxin, which is then purified using preparative TLC on silica gel. The sample is then subjected to reverse phase chromatography in the process of LC/MS/MS structure analysis.

(R)-(-)-Mellein, a new mycotoxin discovered in *M. phaseolina*.

A major achievement was the identification of a new, known mycotoxin, (R)-(-)-mellein, as being produced by *M. phaseolina* isolates from Mississippi soybean plants with charcoal rot disease. Gas chromatography/mass spectrometry (GC/MS) methods were developed for detection of (-)-botryodiplodin and for mellein. This research was carried out in collaboration with Dr. Jian Chen, Biological Control of Pests Research, NBCL, USDA, Stoneville, MS.

The limits of detection for (-)-botryodiplodin and mellein in fungal cultures were 0.1 and 0.625 ppm, respectively. Analysis was performed on two different sets of culture extracts for the presence of (-)-botryodiplodin (15 samples) and mullein (17 samples). These toxins were detected in fungal extracts at levels ranging from 0.1 to 100 ppm, with averages of 0.625 ppm to 20 ppm, respectively. The GC/MS method confirmed the validity of the in-culture color bioassay for the presence of (-)-botryodiplodin (i.e., red pigment formation in the presence of glycine).

Mellein (3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyran-1-one, also known as ochracin) is a dihydroisocoumarin compound. It is structurally related to the mycotoxin ochratoxin, and is believed to have a parallel biosynthetic route. Mellein has been reported to have a variety of biological activities, including antibacterial, antimalarial, antifungal, and anticancer activities. Mellein has been reported to be produced by several species of fungi, including *Aspergillus ochraceus* Wilhelm, *Parastagonospora nodorum* and *Lasiodiplodia pseudotheobromae*. To our knowledge, and to that of Google, mellein has never before been reported to be produced by *M. phaseolina* from any source, although it was noted that the *M. phaseolina* genome contains genes with similarities to ochratoxin biosynthetic genes. We have shown in our studies that mellein has phytotoxic activity against soybean seedling root tips in hydroponic culture, although the level of activity is much lower than that of (-)-botryodiplodin and other as yet unidentified mycotoxins produced by *M. phaseolina*. The weak activity makes it unlikely that mellein plays a substantial role in root infection by most *M. phaseolina* isolates, particularly by isolates that produce other mycotoxins.

Isolation and structure identification studies on the mycotoxin from *M. phaseolina* isolate *Mp* 205.

Reverse phase HPLC analysis using UV detection showed that ethyl acetate extracts of *Mp*205 and *Mp*3 do not contain any of the known *M. phaseolina* mycotoxins (i.e., no (-)-botryodiplodin, phaseolinone, or patulin). The *Mp*205 mycotoxin was most studied because it has the strongest *B. subtilis* toxicity. Bioautography was used with preparative TLC on silica gel in the solvent system chloroform:methanol 9:1 to purify the *Mp*205 mycotoxin, and activity confirmed the presence of *B. subtilis* toxicity in the isolated sample. In a subsequent reverse phase minicolumn chromatography using a step gradient of aqueous methanol to elute from Amberlite XAD-2 resin, the activity was lost. A second larger batch has been prepared and was being purified by the same method except that the reverse phase chromatography step was incorporated into LC/MS/MS analysis. The data from the study are still under analysis, but they do confirm the absence of any known *M. phaseolina* mycotoxins.

Identification of mycotoxin production by other fungal pathogens of Mississippi soybeans.

Screening culture filtrates from Mississippi *M. phaseolina* isolates shown to cause root toxicity with soybean seedlings in hydroponic culture in the absence of detectable (-)-botryodiplodin production capability identified four Mississippi *M. phaseolina* isolates that do not produce (-)-botryodiplodin (*M. phaseolina* isolates *Mp*3, *Mp*4, *Mp*6, *Mp*12 and *Mp*15,) and characterized their soybean seedling root toxicity responses. Additional studies have been carried out on a collection of endophytic fungi from soybean cultivars grown in Mississippi. None of the fungi in the collection produced detectable (-)-botryodiplodin, although three did respond to a polysaccharide (locust bean gum) that induces (-)-botryodiplodin synthesis, enhanced hyphal branching, and microsclerotia formation in many *M. phaseolina* isolates.

The three endophytes (Saline-1 (*Setosphaeria pedicellata,* based on Tub2 and Ef1); Manokin-2 (*Phoma eupyrena,* based on ITS1) and Manokin-4 (*Setosphaeria rostrata,* based on ITS1) responded to locust bean gum with enhanced hyphal branching and microsclerotia formation, but no (-)-botryodiplodin. With soybean seedlings in hydroponic culture, culture filtrates of Saline-1 and Manokin-2 cause severe toxicity to roots and aerial parts, whereas two other Mississippi soybean endophytes (Pharoah-1

(*Aspergillus ruber*, based on ITS1 and Tub2) and Manokin-6 (*Sarocladium kiliense*, based on ITS1, Tub2 and Ef1)) cause selective root toxicity under identical conditions. Numerous new isolates of *M. phaseolina* from soybean plants with charcoal rot disease in the 2018 Mississippi growing season have been added to the USDA, Stoneville collection.

Preliminary studies on a *M. phaseolina* mycotoxin abatement strategy.

A major research advance was made with the demonstration that (-)-botryodiplodin is bound very effectively by biochar. (-)-Botryodiplodin binding studies were carried out with commercially-available biochar (Wakefield Biochar Soil Conditioner, made from soft pine wood). Biochar was so effective at binding (±)-botryodiplodin at concentrations that are effective at destroying root tip meristematic tissue to create an entry site for *M. phaseolina* hyphae (10 µg/mL) that small enough biochar samples could not be meaningfully weighed out for studies. As a result, studies going forward will use soil-biochar mixtures. These studies have created the need for a convenient, effective, inexpensive assay for (-)-botryodiplodin in solution in the 5 to 20 µg/mL concentration range. Studies have been carried out on developing a colorimetric 96-well tray