Identification of mycotoxins used in soybean root infection by *Macrophomina phaseolina* and other fungi. Annual report - 34-2019

Maria Tomaso-Peterson, MariaT@pss.msstate.edu 662.325.2593
Hamed K. Abbas, Hamed.Abbas@usda.gov 662.686.5313
W. Thomas Shier, shier001@umn.edu 612-624-9465

The fungus *Macrophomina phaseolina* (Mp) causes charcoal rot disease by infecting soybeans through the roots from other infected plants or decaying material in the soil. *M. phaseolina* appears to locate meristematic tissue near soybean root tips in soil by detecting unique polysaccharides on the surfaces of viable sloughed off root cap cells that persist in viable form in soil near the meristematic tissue synthesizing mucilage used as a lubricant for root tip movement through the soil. During the root infection mechanism *M. phaseolina* releases a mycotoxin that kills dividing cells of the meristematic tissue near the root tip resulting in loss of the root cap and exposure of the plants vascular system to fungal hyphae. In some, but not all *M. phaseolina* isolates, (-)-botryodiplodin is the mycotoxin. Better understanding of the types of mycotoxins used to facilitate root infection may allow the development of improved methods to prevent root infection of Mississippi soybeans by *M. phaseolina* and other fungi that use this infection mechanism.

Objective: The objective of the proposed research is to better understand the range of mycotoxin types used by isolates of *M. phaseolina* and other pathogenic fungi from soybean plants to facilitate root infection. Potential remediation strategies, including biochar binding of the mycotoxins in soil around root tips, will be explored.

Results of Studies (April, 2019-June, 2020):

Soybean pathogen isolates (>150) collected from various sources in Mississippi and elsewhere were cultured and examined for ability to produce (-)-botryodiplodin using an in culture assay developed in these laboratories, which detects the toxin as a red pigment formed by reaction of the toxin with glycine. In addition to *M. phaseolina* isolates, soybean fungal pathogens *Lasiodiplodia theobromae*, *Sclerotinia sclerotiorum*, *Fusarium virguliforme*, *Phyllosticta sojicola*, *Alfimbria verrucaria* and *Cercospora sojina* were examined.

Soybean pathogens were grown in liquid culture and cell-free culture medium filtrates were tested for root toxicity with soybean (Saline, DT97-4290) seedlings grown in the greenhouse and transferred to hydroponic culture for testing when they reached the VC stage. In the root toxicity test, filter-sterilized culture medium samples were mixed at 50%, 10%, and 5% vol/vol in hydroponic culture medium bathing the roots, and the seedlings were monitored for toxicity signs over a 96-hour culture period in continuous light, when a damage rating in percentage was assigned to each seedling based on overall symptoms, and total volume of media consumed by the seedling was recorded. At 50% culture filtrate in medium, all filtrates showed symptoms of chlorosis, necrosis, leaf curling, and stunted growth on the leaves and stems of each plant, even for fungi that did not produce (-)-botryodiplodin. At 10% concentration, the filtrates of *L. theobromae*, *F. virguliforme*, and *A. verrucaria* resulted in varying levels of chlorosis, necrosis, and stunting. At 5% concentration, the filtrates of *L. theobromae* and *A. verrucaria* resulted in little damage.

Chemical analysis of culture filtrates exhibiting root toxicity, even when there was no (-)-botryodiplodin production detected, were carried out to identify and quantitate known mycotoxins and other secondary metabolites produced by the various fungi using LC/MS or GC/MS in our laboratory or LC/MS/MS in a service laboratory with this type of capability operated by Dr. Michael Sulyok at the University of Natural Resources and Life Sciences, Vienna, Austria. Previous studies in our laboratories using an in culture assay we developed showed that (-)-botryodiplodin is produced in detectable amounts by about 75% of *M.*

phaseolina isolates from soybean plants with charcoal rot disease in Mississippi. In the first collection of 79 *M. phaseolina* isolates studied using LC/MS/MS in collaboration with Dr. Sulyok, 78% produced (-)-botryodiplodin. These studies (i) confirm the frequency of production of (-)-botryodiplodin by pathogenic *M. phaseolina* isolates from Mississippi soybeans, and (ii) establish the validity of an in-culture assay for (-)-botryodiplodin production by *M. phaseolina* isolates developed in our laboratories. The percentage of (-)-botryodiplodin producers was slightly higher than that obtained using only the in culture assay developed in these laboratories, reflecting the greater sensitivity of the LC/MS/MS assay system.

Current studies in our laboratories have detected production of the following mycotoxins and other secondary metabolites not previously known to be produced by *M. phaseolina* isolates from soybeans with charcoal rot disease: mellein, kojic acid, orsellinic acid and moniliformin. The discovery of mellein production by pathogenic *M. phaseolina* isolates from soybean plants with charcoal rot disease in Mississippi was made in our laboratory using gas chromatography-mass spectrometry (GC/MS). The extract from one culture (0.99%) produced mellein as the only identified secondary metabolite, while extracts from 25 cultures (24.75%) produced mellein and kojic acid. Extracts from an additional 48 cultures (47.52%) produced kojic acid, but no mellein. Kojic acid is a well-known secondary metabolite of fungi sometimes marketed in beauty products to lighten skin color (it inhibits tyrosinase) and in health products for treating skin conditions such as sun damage, scars and age spots. An additional secondary metabolite, orsellinic acid (specifically o-orsellininc acid), but no methylorsellinic acid, was identified as being produced by 49 out of 101 culture filtrates (49%) from *M. phaseolina* isolates from soybeans with charcoal rot disease in our completed study. Some isolates released relatively high levels of orsellinic acid (up to 44.2 µg/ml culture fluid). No reports of orsellinic acid toxicity could be found. The known mycotoxin moniliformin was found in very low (<0.15 µg/ml culture fluid) to trace levels in 60 out of 101 (59%) culture filtrates from *M. phaseolina* isolates from soybeans with charcoal rot disease in our completed study. This observation is a concern because moniliformin, which is usually associated with *Fusarium* spp. contamination, has been reported to cause toxicity in laboratory animal studies, although it is considered to have a low to negligible risk for feeding animals (swine, poultry, mink) at the levels found in naturally-contaminated grains. Additional studies are needed to establish that, as expected, no detectable moniliformin gets into harvested soybean seeds from fields contaminated by *M. phaseolina* isolates capable of producing the mycotoxin. An additional 90 *M. phaseolina* isolates from soybean plants with charcoal rot disease were cultured during, the culture media filter-sterilized and freeze-dried to prepare samples that were submitted for LC/MS/MS analysis.

In summary, extensive progress has been made in identifying previously unknown mycotoxins and other secondary metabolites as being produced by isolates of *M. phaseolina* from soybean plants exhibiting charcoal rot disease in Mississippi.

Studies have been carried out on the effectiveness of freshly-prepared biochar in soil at binding (-)-botryodiplodin and other mycotoxins, which fungi may use to facilitate entry into the soybean vascular system by damaging root tips or other places in roots. Studies on (-)-botryodiplodin binding in soil have as yet yielded only qualitative results due to lockouts associated with COVID-19 responses at all project research sites. Only the graduate student, Vivek Khambhati, was designated an essential employee and could carry out limited laboratory-based research activities during this period. Studies that have been completed used the colorimetric assay for (-)-botryodiplodin being developed in these laboratories. Unfortunately, humic and fulvic acids that leach from some soils interferes with this assay. Results are not yet available for studies using LC/MS/MS to quantitate reduction in (-)-botryodiplodin levels during exposure to biochar types.

Publications:

1. Abbas H., Bellaloui N., Accinelli C., Smith J.R., Shier W.T. (2019) Toxin production in soybean (*Glycine max* L.) plants with charcoal rot disease and by *Macrophomina phaseolina*, the fungus that causes the disease. *Toxins*, 11:645, doi:10.3390/toxins11110645.
2. Abbas H., Bellaloui N., Butler A., Nelson J., Abou-Karam M., Shier W.T. (2020) Phytotoxic responses of soybean (*Glycine max* L.) to botryodiplodin, a toxin produced by the charcoal rot disease fungus, *Macrophomina phaseolina*. *Toxins*, 12:25, doi:10.3390/toxins12010025.