Identification of mycotoxins used in soybean root infection by *Macrophomina phaseolina* and other fungi. 34-2020

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Background and objectives

The fungus *Macrophomina phaseolina* causes charcoal rot disease by infecting soybeans through root tips it locates in soil. *M. phaseolina* then 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. Most, but not all, times the mycotoxin is (-)-botryodiplodin. Better understanding of the types of mycotoxins used to facilitate root infection may allow the development of novel methods to prevent root infection of Mississippi soybeans by *M. phaseolina* and other fungi that use this infection mechanism. Adding freshly-prepared biochar as a soil amendment to bind mycotoxins in the root zone is an example of novel root-protecting strategies under consideration.

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 in soybeans. Preliminary studies will be conducted on potential remediation strategies, including biochar binding of the mycotoxins in soil around root tips, as a possible approach to preventing fungal infection of soybean roots.

Progress Report

Soybean pathogen isolates (>150) collected from various sources in Mississippi and elsewhere were cultured and examined for ability to produce mycotoxins using the following three types of assays: (i) An in culture assay for (-)-botryodiplodin developed in these laboratories, which detects the toxin as a red pigment formed by reaction of the toxin with glycine; (ii) liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) instrumental-based assays allowing quantitative measurements of levels of known mycotoxins for which authentic standards are available; and (iii) a root toxicity assay using soybean seedlings in hydroponic culture to which known toxins at known concentrations can be added to the medium bathing the roots or mixed with filter-sterilized medium from cultures of fungi producing unidentified toxic secondary metabolites.

Mycotoxins produced by *M. phaseolina*:

A total of 140 *M. phaseolina* isolates from Mississippi, Tennessee, Illinois and Florida have been studied using the in culture assay, LC-MS and LC-MS/MS. The most information was obtained using analysis by LC-MS/MS carried out on samples prepared in our laboratories and analyzed by our collaborator, Dr. Michael Sulyok at the University of Natural Resources and Life Sciences, Vienna, Austria. The in-culture assay is rapid, simple, inexpensive and correlates well with LC-MS and LC-MS/MS results ($r_s = 0.70$, $p < 0.001$). The in-culture assay indicated that greater than two thirds of *M. phaseolina* isolates produced readily detectable levels of (-)-botryodiplodin. Use of LC-MS/MS has enabled the discovery that *M. phaseolina* isolates not only produce the known mycotoxins (-)-botryodiplodin and mellein, it has been shown for the first time in these studies that kojic acid was produced in 83.4% of isolates, (-)-botryodiplodin in 80.7%, moniliformin in 67.9%, orsellinic acid in 60.7%, cordycepin in 22.9%, mellein in 19.3%, cyclo[L-proline-L-tyrosine] in 16.4% and gliocladic acid in 15.7%. The following seven other secondary metabolites that were observed in <5% of isolates: as well as much less frequent production of emodin, endocrocin**,** citrinin, infectopyron, methylorsellinic acid, monocerin and N-benzoyl-phenylalanine.

Studies have been carried out to determine the root toxicity to soybean seedlings in hydroponic culture of mellein, kojic acid and orsellinic acid. Mellein, kojic acid and orsellinic acid all exhibited low toxicity

during 96-hr exposure to soybean seedling roots in the concentration range 0 to 100 µg/ml. In the range of 60 to 100 µg/ml, mellein induced symptoms of stunting and moderate wilting of the whole plant. At 0 to 40 µg/ml of mullein the only symptoms were wilting of the lower sets of leaves. In the 1.25 to 100 µg/ml range, kojic acid induced chlorosis on the entirety of the top set of leaves. The severity of chlorotic symptoms increased with kojic acid concentration. At all concentrations of orsellinic acid, there was no noticeable toxicity induced by the compound. However, some other plant pathogenic fungi that produce mycotoxins for which orsellinic acid is a biosynthetic precursor (e.g., 4-acetylantroquinonol produced by *Antrodia cinnamomea*) also release unconverted orsellinic acid. *M. phaseolina* may also be releasing as yet unidentified mycotoxins biosynthesized from orsellinic acid and methylorsellinic acid.

The observation that *M. phaseolina* produces moniliformin, which is usually associated with *Fusarium* spp. contamination, is a concern, because moniliformin 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. Moniliformin was evaluated for root toxicity in soybean seedlings in hydroponic culture. From 1.25 to 10 µg/mL, moniliformin did not induce any phytotoxic symptoms. At 20 µg/mL, only symptoms of chlorosis were observed on the top sets of leaves. At 40 µg/mL, chlorosis was observed on the top set of leaves as well as minor necrosis on the leaf edges. From 60-100 µg/mL, symptoms of chlorosis and necrosis were observed in increasing severity as the concentration was increased. No plants were killed by moniliformin at the concentrations tested.

Previous studies on (-)-botryodiplodin in soybean plants symptomatic with charcoal rot found the toxin only in root. Additional studies have also been conducted on soybean seeds from field trials with Asgrow 38x8 in collaboration with Dr. James R. Smith. Immature (R7) seeds were harvested by hand from six soybean plants symptomatic of charcoal rot, three with a high rating in the CRT severity scale and three with a low rating in the CRT severity scale, were extracted and processed for LC-MS/MS analysis. The six seed samples all contained abscisic acid, cordycepin and penicinoline, but no detectable (-)-botryodiplodin, kojic acid, moniliformin, mellein or other known mycotoxin. However, mature (R8) seeds machine-harvested from the same plots were found to contain brevianamide F, cercosporin, cyclo(L-Pro-L-Tyr), and moniliformin in four out of twelve samples, in addition to abscisic acid, cordycepin and penicinoline. One explanation for the appearance of fungal metabolites in harvested seed (R8 or later), but not immature (R7) seed in the plant is post-harvest production, like when corn is not dried rapidly enough.

Mycotoxins produced by other fungal pathogens of soybean:

Soybean fungal pathogens *Lasiodiplodia theobromae, Sclerotinia sclerotiorum, Fusarium virguliforme, Phyllosticta sojicola, Alfimbria verrucaria, Cercospora sojina* and *Phoma* sp were examined by growing 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.

Research on potential remediation strategies:

(-)-Botryodiplodin is known to bind to activated charcoal, which was used in its initial purification. Freshly-prepared biochar, a soil amendment, is actually charcoal. The amount of biochar needed to remove

(±)-botryodiplodin from water solution at effective concentrations (~10 µg/ml) were too small to weigh. Culture filtrates of *M. phaseolina* TN 281 containing detectable (-)-botryodiplodin were treated with a range of biochar amounts (0-4% w/v) from hardwood and from coconut shell, filtered and residual (-)-botryodiplodin levels assessed using the colorimetric assay being developed in the laboratory. Efficient removal of (-)-botryodiplodin was observed. Biochar added to soil in the root zone of soybean plants as a control for biochar in seed coatings had no significant effect of soybean plant growth or general health.

Impacts and Benefits to Mississippi Soybean Producers

Understanding the role of mycotoxins in the mechanism of soybean root infection by *M. phaseolina* and other fungi enables the development of resistant soybean cultivars, protective agronomic approaches like biochar seed coatings, and strategies for preventing mycotoxin contamination of seeds. To know the mycotoxins in fungal infection is to know the enemy and that is the first step in defeating this enemy.

End Products

Publications:

1. Abbas H.K., 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.K., 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.
3. Khambhati V.H., Abbas, H.K., Sulyok, M., Tomaso-Peterson, M., & Shier, W.T. (2020). First report of the production of mycotoxins and other secondary metabolites by *Macrophomina phaseolina* (Tassi) Goid. isolates from soybeans (*Glycine max* L.) symptomatic with charcoal rot disease. *J. of Fungi*, 6:332, doi.org/10.3390/jof6040332.
4. Khambhati, V.H., Abbas, H.K., Sulyok, M., Chen, J., Tomaso-Peterson, M., & Shier, W.T. (2021). The bioactivity and production of mellein by cultures of *Macrophomina phaseolina*. In Preparation.
5. Khambhati, V.H., Abbas, H.K., Sulyok, M., Chen, J., Tomaso-Peterson, M., & Shier, W.T. (2021). Phytotoxicity and production of kojic acid *Macrophomina phaseolina*. In Preparation.

Presentations:

1. Khambhati, V.H., Abbas, H.K., Shier, W.T., Tomaso-Peterson, M., Chen, J., Kotowicz, J., Bellaloui, N., & Mengistu, A. (2019). Determination of secondary metabolites and their role in root infection by soybean fungi, *Macrophomina phaseolina* and other pathogens. *Phytopathology*, 109(10S), S2.37. (2019 APS Annual Meeting, Poster)
2. Khambhati, V.H. (2019). Identification of Secondary Metabolites Produced by *Macrophomina phaseolina* and Other Soybean Pathogens, and Their Role in Root Infection. *Journal of Mississippi Academy of Sciences*, 65(1), 32. (Mississippi Academy of Sciences – Eighty-Third Annual Meeting)
3. Khambhati, V.H., Abbas, H.K., Shier, W.T., Tomaso-Peterson, M., Chen, J., Kotowicz, J., Bellaloui, N., & Mengistu, A. (2019). Uncovering Secondary Metabolites Involved in the Pathogenesis of Charcoal Rot Disease Fungus, *Macrophomina phaseolina*. Proceedings in MEA/MAPPAN Joint Annual Meeting, Mississippi State, MS. October 21-22, 2019.
4. Khambhati, V.H., Abbas, H.K., Shier, W.T., Tomaso-Peterson, M., Chen, J., Kotowicz, J., Bellaloui, N., & Mengistu, A. (2019). Identifying Secondary Metabolites Produced by Charcoal Rot Disease Fungus, *Macrophomina phaseolina*, and Their Role in Pathogenesis. *Journal of Mississippi Academy of Sciences*, 65(1), 43. (Mississippi Academy of Sciences – Eighty-Fourth Annual Meeting, Poster)

5. Khambhati, V.H., Abbas, H.K., Shier, W.T., Tomaso-Peterson, M., Chen, J., Bellaloui, N., & Mengistu, A. (2020). Identification and evaluation of phytotoxic secondary metabolites produced by charcoal rot disease fungus, *Macrophomina phaseolina*. *Phytopathology*, 110(10S), S2.15 (2020 APS Annual Meeting, Poster)

Seminars:

1. Khambhati, V.H. (2019). Uncovering the role of secondary metabolites in the root infection process of *Macrophomina phaseolina* and other soybean pathogens. Mississippi State University, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, EPP 8111, Seminar, Caprio, M.A.
2. Khambhati, V.H. (2019). Identification of mycotoxins used in soybean root infection by *Macrophomina phaseolina* and other fungi. Mississippi State University, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, EPP 8111, Seminar, Caprio, M.A.
3. Khambhati, V.H. (2020). Identification, evaluation, and management of mycotoxins produced by *Macrophomina phaseolina*. Mississippi State University, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, EPP 8121, Seminar, Caprio, M.A.