MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 34-2015 (YEAR 1)
2015 Annual Report

TITLE: Mechanism of soybean root infection by *Macrophomina phaseolina*

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BACKGROUND AND OBJECTIVES

The fungus *Macrophomina phaseolina* causes charcoal rot disease by infecting soybeans from the soil reservoir. It is not understood (i) how the fungus locates soybean roots in the soil, or (ii) penetrates soybean roots to enter the plant's vascular system.

The objectives of the proposed research are to explore hypotheses (i) that *M. phaseolina* locates soybean roots in soil by chemotaxis toward one or more possibly volatile substances released from roots, and (ii) that when *M. phaseolina* propagates close to a root tip, it responds to the presence of either a different released substance or a surface component of the root by producing a mycotoxin, (-)-botryodiplodin, which kills rapidly dividing meristematic tissue in the root tip, thus generating necrotic tissue through which the fungus can readily propagate to enter the plant's vascular system. If agent(s) can be identified that trigger (-)-botryodiplodin release or any other step in the root infection process can be identified, plant breeders may be able to develop soybean cultivars that lack the response and would be expected to be charcoal rot disease resistant.

Objective 1: To examine the mechanism by which *M. phaseolina* locates soybean root tips in soil by screening, individually or as mixtures, as many as possible of the 80+ substances reported to be secreted from roots for ability to (i) induce chemotaxis by *M. phaseolina* isolates from Mississippi soybean fields, and (ii) stimulate synthesis and release of (-)-botryodiplodin. Both screening systems are usee in culture assay systems.

Objective 2: To examine the mechanism of entry of *M. phaseolina* hyphae into soybean seedling roots in hydroponic co-culture with fungal isolates from Mississippi soybean fields using TEM and ultrastructural analysis. The analysis will focus on meristematic tissue at root tips and compare necrotic effects on tissue by solutions of (-)-botryodiplodin with those of *M. phaseolina* in co-culture.

REPORT OF PROGRESS/ACTIVITY

Objective 1.

(i) How do *M. phaseolina* isolates from Mississippi soybean fields growing from plant debris in the soil find soybean roots to infect? Culture assay systems have been developed to search for evidence of chemotaxis exhibited by *M. phaseolina* isolates growing from a nutrient source toward a substance reported to be released from plant roots (Fig. 1). Chemotaxis is identified by the formation of a fungal colony that is not perfectly round due to preferential growth towards or away from the test substance.

Long range detection of the presence of roots in soil could be most efficiently achieved by sensing a volatile released substance, whereas shorter range detection would be expected to involve diffusible non-volatile substances. More than 80 substances have been reported to be released from the roots of various species of plants, including plant hormones, chelators of nutrient minerals, and a large number of other plant components for which there is no clear rationale for non-pathological release.

No chemotaxis was observed toward or away from any root-released substance that was tested. This is consistent with chemotaxis playing no role in soybean root location by *M. phaseolina*, although it does not exclude the possibility that an untested substance(s) might play this role. *M. phaseolina* in plant debris in soil appears to seek soybean root tips by extending hyphae in every direction. Limitations imposed by the soil surface and the need for oxygen by the strict aerobe result in a discoid-shaped search pattern with an infected piece of plant debris or soybean root at the center.

(ii) *M. phaseolina h*yphae propagating at random in every direction from a piece of infected plant debris or an infected root must have a signal that they have stumbled onto a soybean root tip and the rapidly advancing hyphae should stop advancing and begin secreting a phytotoxin such as (-)-botryodiplodin. What is that signal?

We have developed an in-culture assay system to detect the secretion of (-)-botryodiplodin that has proven to be an extraordinarily powerful tool for understanding the signals received and the responses of *M. phaseolina* to them in this part of the soybean root infection process. It has been known since the 1960's that (-)-botryodiplodin reacts with tissues to produce a red pigment. We have shown that the reaction is with amines, with glycine and β-alanine giving strong coloration. Incorporating either of them into culture medium such as Czapek-Dox agar provides a method for rapid visualization of (-)-botryodiplodin release from actively growing *M. phaseolina* cultures.

The in-culture model system has *M. phaseolina* growing out from a plug of Czapek-Dox agar containing sucrose across a plate of Czapek-Dox agar without sucrose toward a well containing a root-released substance being tested for ability to induce (-)-botryodiplodin release (Fig. 1). Two types of (-)-botryodiplodin-released inducers have been identified in these studies. The first are high concentrations of the simple sugars, sucrose and to a lesser extent glucose, which are presumed to represent an exit mechanism for *M. phaseolina* leaving root tips of infected soybean plants to spread to plants with interdigitating roots. The second are polysaccharides that may be similar or identical to ones associated with root tips.

The discovery of polysaccharide inducers of (-)-botryodiplodin release was made as a result of the serendipitous observation that yeast extract was a potent inducer of (-)-botryodiplodin release when it was tested as a convenient source of multiple B vitamins. Subsequent bioassay-guided fractionation of yeast extract by reverse phase chromatography showed that any vitamins present were not (-)-botryodiplodin-released inducers, whereas the activity was present in high molecular weight polysaccharide fractions.

A screen of 23 polysaccharides (Table 3) identified two strong activators: locust bean gum and guar gum, both manno-galactans. These polysaccharides are structurally similar to a unique

polysaccharide reported to be found on the surface of plant cells that get sloughed off and grow in suspension culture. Sloughable cells are released from root caps on root tips, where they are shed into the soil and live independently for 3 to 5 days, synthesizing mucilage that acts as a lubricant enabling the soft root tip to penetrate soil. Both the sloughed cell surface and the secreted mucilage are found in soil in exactly the right place to signal the presence of meristematic tissue nearby. The polysaccharide specific to sloughable cell has been purified from pea hull by a literature method and shown to be a potent inducer of hyperbranching, but a weak inducer of (-)-botryodiplodin release from *M. phaseolina*. The mucilage secreted by the roots of ~100 soybean (Saline) seedlings growing hydroponically is currently being collected for testing as an inducer of *M. phaseolina* hyperbranching and (-)-botryodiplodin release.

Studies are underway to understand the signaling role(s) of polysaccharides in the mechanism of root infection by *M. phaseolina*. The polysaccharide activators stimulate the following three readily-defined responses by *M. phaseolina*, all of which appear to be involved in either root infection or in the establishment of an endophyte relationship in soybean seeds: (i) hyperbranching; (ii) (-)-botryodiplodin release and (iii) microschlerotia formation.

In the in-culture model of *M. phaseolina* migrating out from a nutrient source, hyphae spread rapidly across the surface of the agar with occasional branching to yield a loose net across the agar surface in which anything as large as a soybean root would experience either a collision or a close encounter. In the presence of a (-)-botryodiplodin release-inducing polysaccharide, the rapid spread of hyphae across the agar surface stops when the occasional branching is replaced by intense branching, in which the resources that had gone into spreading became focused on complete coverage of the polysaccharide-rich zone. (-)-Botryodiplodin release occurs at the same time and would transform any nearby meristematic tissue into an easily-penetrated necrotic zone, which would inevitably be invaded by the hyperbranching hyphae. The third response to the polysaccharide inducer plays no apparent role in soybean root tip penetration by *M. phaseolina*, but would be expected to enable the fungus to identify the inner surface of the seed hull, which is where it should form microschlerotia, if it is to be an effective endophyte.

Objective 2.

Soybean seedlings have been shown to grow well in dilute hydroponic culture medium, including in individual glass tubes where an active lateral root production response occurs, presumably due to hypoxia resulting from lack of stirring. This experimental system has been used to investigate the anatomical location of (-)-botryodiplodin attack and hence *M. phaseolina* entry into the soybean root--specifically the hypothesis that the target is the meristematic tissue located near the root tip immediately behind the root cap. In root growth, all cell proliferation takes place in the meristematic tissue, and root elongation results from elongation of the new cells. Addition of (±)-botryodiplodin to the hydroponic medium preferentially inhibited lateral root growth, consistent with it targeting the meristematic tissue, which is more abundant in lateral roots.

The site of toxin attack on roots was shown to be root tips as predicted (Figure 3). A control root tip not exposed for 72 hours to (±)-botryodiplodin exhibited the expected centrally-located dividing cells surrounded by mature elongated cells, whereas the root tip exposed to 15 µg/ml (±)-botryodiplodin exhibited disruption of surface tissues, unoriented centrally-located dividing cells, and an absence of elongated cells. Ultrastructural analysis of (±)-botryodiplodin-treated

soybean root tip tissue has yet to be completed due to difficulties in identifying the required expertise. However, the completed research clearly establishes that the root tip is the target site for *M. phaseolina* entry into soybean roots.

IMPACTS AND BENEFITS TO MISSISSIPPI SOYBEAN PRODUCERS

The observation that certain polysaccharides can block the advance of fungal hyphae may result in the development of seed-coating components that reduce root damage. The identification of polysaccharides involved in the infection mechanism by *M. phaseolina* offers a strategy for developing resistance to charcoal rot disease in soybeans by developing cultivars that do not express the polysaccharide.

END PRODUCTS–COMPLETED OR FORTHCOMING

The studies conducted in this project have greatly advanced understanding of how root infection by *M. phaseolina* occurs. The results are expected to result in at least three publications in peer-reviewed research journals.

Graphics/Tables

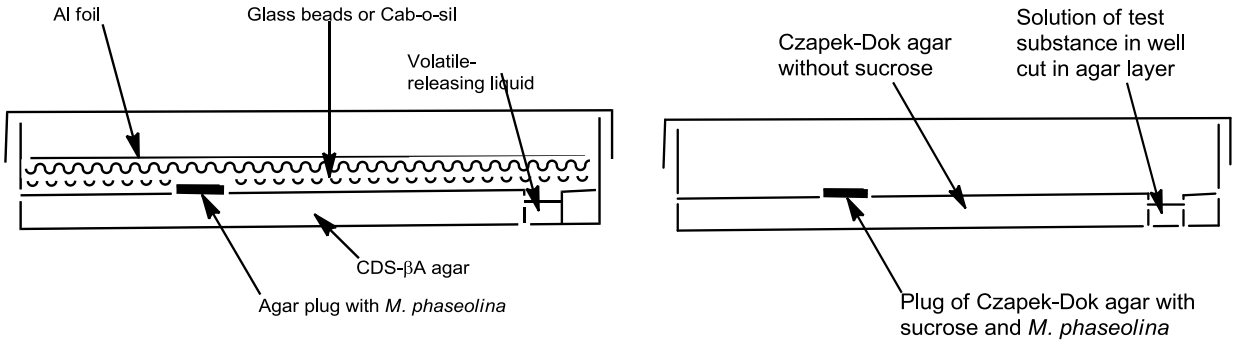


Figure 1. In culture assay systems for the detection of chemotaxis by *M. phaseolina* isolates from Mississippi soybean fields towards volatile (left) or diffusible (right) substances that have been reported to be released from plant roots.

Table 1. Substances reported to be released from plant roots that were shown to not induce any detectable chemotaxis by *M. phaseolina* isolates from Mississippi soybean fields.

A. Volatile Organic Compounds Tested: Isoprene, methyl salicylate, spermidine, spermine
B. Amino acids tested: Casein hydrolysate (Asp, Glu, Ser, Gly, His, Arg, The, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Try, Phe, Lys)
C. Organic acids: Citric, malic
D. Sugars: Glucose, sucrose, oligosaccharides
E. Vitamins: Biotin, thiamin, niacin, riboflavin, calcium pantothenate , pyridoxine, folic acid, biotin, α-lipoic acid, *myo*-inositol
F. Nucleosides: Cytidine, guanosine, thymidine, hypoxanthine, adenosine, guanine, uracil, uridine

Table 2. Substances reported to be released from plant roots that were shown to not induce any detectable (-)-botryodiplodin release by *M. phaseolina* isolates from Mississippi soybean fields.

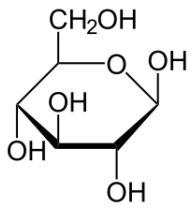
A. Volatile Organic Compounds Tested: Isoprene, methyl salicylate, spermidine, spermine
B. Amino acids tested: Casein hydrolysate (Asp, Glu, Ser, Gly, His, Arg, The, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Try, Phe, Lys)
C. Organic acids: Citric, malic
D. Vitamins: Biotin, thiamin, niacin, riboflavin, calcium pantothenate , pyridoxine, folic acid, biotin, α-lipoic acid, *myo*-inositol
E. Nucleosides: Cytidine, guanosine, thymidine, hypoxanthine, adenosine, guanine, uracil, uridine

Table 3. Polysaccharides that were shown to not induce any detectable (-)-botryodiplodin release by *M. phaseolina* isolates from Mississippi soybean fields.

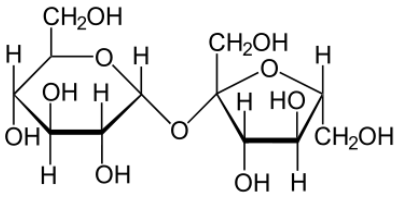
Carrageenan, Carboxymethylcellulose, Na Dextran sulfate, Corn starch, Dextrans, Na Polygalacturonate, Gum Ghatti, Yeast Mannan, Gum Arabic, Xanthum gum, Gum storax, Cellobiose, Chitosan hydrochloride, γ-Cyclodextrin.

Figure 2. Structures of saccharides shown to induce (i) hyperbranching, (ii) (-)-botryodiplodin release and (iii) microschlerotia formation by *M. phaseolina* isolates from Mississippi soybean fields.

a) Simple sugars that may play a role in the exit of fungal hyphae from infected roots.

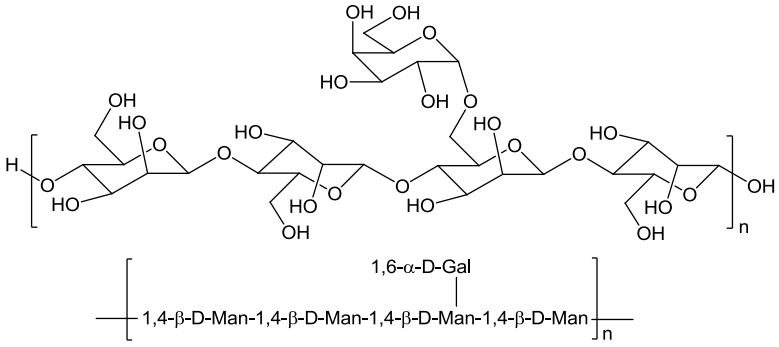


D-Glucose

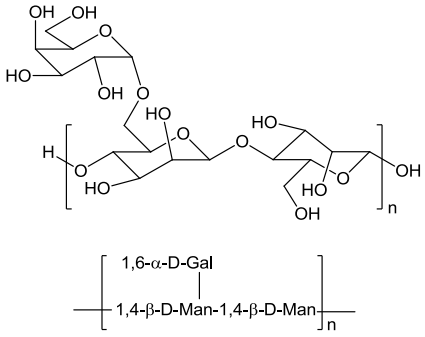


Sucrose

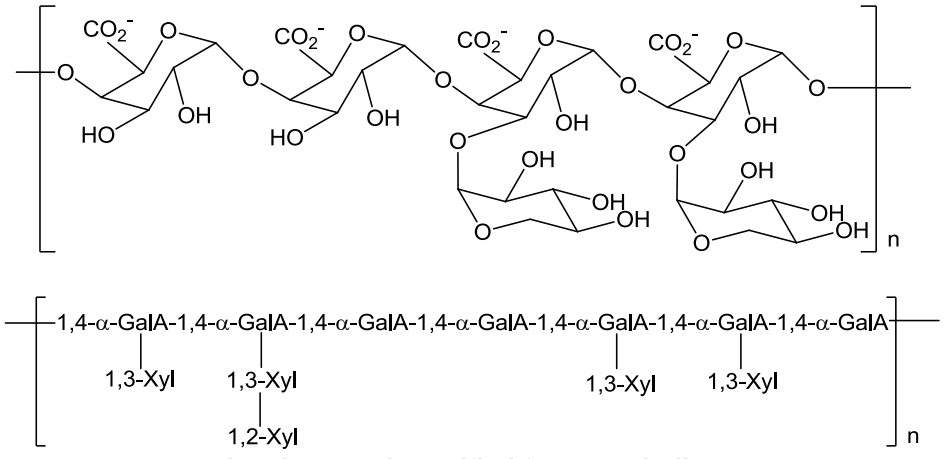
b) Polysaccharides that may signal a close encounter with a soybean root tip in soil



Locust (Carob) Bean Gum



Guar Gum



Xylogalacturonin purified from pea hulls

Figure 3. Light micrographs of soybean lateral root tips from hydroponically grown seedlings exposed to no (±)-botryodiplodin (left) or 15 µg/ml (±)-botryodiplodin (right) in the hydroponic medium.