Development and Optimization of Diagnostic Protocols for *Macrophomina phaseolina* in soil and Microsclerotia in Strawberry Tissues

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Summary

Field soil artificially infested with *Macrophomina phaseolina* was used in a series of tests to evaluate the selectivity of four different media and two different plating techniques. In all tests, the rice bran (RB) medium was the most consistent for recovering and enumerating colony forming units (CFUs) of *M. phaseolina* in soil. Direct soil plating was compared with the Andersen sampler technique using a range of soil masses and incubation periods. An optimized Andersen sampler protocol was then used to quantify *M. phaseolina* in naturally infested soil collected from commercial fields in Oxnard and Santa Maria. Finally, a technique was developed to detect and visualize *M. phaseolina microsclerotia* in naturally infected strawberry tissue; microsclerotia were observed in all tissues tested, including crown, petiole and primary root samples.

Introduction

Crown rot, caused by *Macrophomina phaseolina*, is an emerging soilborne fungal disease in commercial strawberry production in California. *Macrophomina phaseolina* produces large numbers of melanized, multicellular structures referred to as microsclerotia. It is presumed that these structures serve as the primary source of inoculum in the subsequent strawberry crop in infested production fields. In other crops, the survival of *M. phaseolina* in soil and on host debris has been reported for up to fifteen years (Baird et al., 2003). Microsclerotia are produced on the outside of stems, collars, and/or root systems of other crops like soybean and corn, hence the name 'charcoal rot' or 'ashy stem blight'. Although microsclerotia are thought to play an important role in the disease cycle of strawberry crown rot, they have never been observed on or in infected strawberry tissue.

In addition, there are no culture-based or molecular assays that accurately detect and/or quantify *M. phaseolina* from soil. Therefore, researchers are limited in the ability to evaluate the efficacy of treatments (i.e., anaerobic soil disinfestation (ASD), low rates of fumigants, soil-applied fungicides, or steam) for reducing the population density of this pathogen. At current, the best ways to quantify the effects of such treatments are through plant assessments, greenhouse plant bioassays, and isolation from symptomatic plant samples collected systematically from treatment areas. Additionally, strawberry growers often want soil tested in advance of planting in new fields with unknown pathogen pressure. Information derived from this research will provide both researchers and growers a better understanding of the biology of this pathogen as well as the potential risks associated with planting in certain fields.

The objectives of this research are 1) to develop and optimize a culture-based assay to accurately detect and quantify *M. phaseolina* in infested field soil; and 2) to determine if microsclerotia are produced in infected strawberry tissue, and if so, in which specific tissues.

Materials and Methods

Evaluating the Selectivity of Different Media

Cornmeal sand inoculum of *M. phaseolina* was produced by using a method described by Mihail (1992). Field soil artificially infested with 5% and 10% v/v inoculum was used in a series of tests to evaluate the selectivity of specific media. Four media were evaluated, including: 1) RB medium (Cloud and Rupe, 1991); 2) a modified Mathur's mustard seed (MS) semi-selective medium (Zveibil et al., 2012); 3) Potato dextrose chloroneb streptomycin agar (Mihail and Alcorn, 1982); and 4) (Acidified Potato Dextrose) agar as a control.

Evaluating Plating Methods

Two different plating techniques were evaluated for their ability to recover and enumerate CFUs of *M. phaseolina* from artificially infested field soil (5% and 10% v/v). Direct soil plating was compared with the Andersen sampler technique (Butterfield and De Vay, 1977) using a range of different masses of soil (0.05, 0.1, and 0.5 grams) and incubation periods (5, 10, and 15 days).

Testing Naturally Infested Field Soil

An optimized plating protocol was used to quantify *M. phaseolina* in naturally infested soil from six commercial fields (in both Oxnard and Santa Maria) with confirmed cases of *Macrophomina*. Four to five soil cores were taken from the rhizosphere of plants with symptoms of stunting and chlorotic/necrotic foliage. These plants were also collected and processed on acidified potato dextrose agar to confirm the presence of the pathogen. The soil cores were composited and subsamples were air dried at room temperature for 14 days. After drying, all soils were ground individually with a mortar and pestle for 10 minutes before conducting the Andersen sampler assay. Samples of air dried soil were weighed out (0.1 grams) for plating onto RB medium. Soil was evenly dispersed onto the medium using the Stage 1 of the Andersen impactor and a Welch vacuum pump. Five plate replicates were used per sample composite. Plates were incubated in the dark for 15 days at 30 C. After incubation, the surface of each plate was rinsed under running tap water and excess soil was removed using a thumb pad. Plates were then visualized with the aid of a dissecting microscope at 16X magnification. Colonies with hyphal color and microsclerotia characteristic of *M. phaseolina* were then enumerated, and CFU per gram of soil were calculated.

Visualizing Microsclerotia In Planta

Symptomatic plants were collected from specific areas of commercial fields with confirmed cases of Macrophomina crown rot. Each plant was split into different portions, including outer crown, inner crown, petiole, and primary roots. Tissue from each portion of the plant was sliced into thin sections with a sterile razor blade and soaked in a 5% sodium hydroxide solution for five days. Each sample was then visualized under dissecting and compound microscopes.

Results

Evaluating the Selectivity of Different Media

In all tests, the RB medium was the most consistent for recovering and enumerating CFUs of M. phaseolina from soil. The prolific growth of non-target fungal organisms made accurate quantification of M. phaseolina impossible on the other three media: modified Mathur's MS semi-selective agar, potato dextrose chloroneb streptomycin agar, and acidified potato dextrose agar (Figure 1).

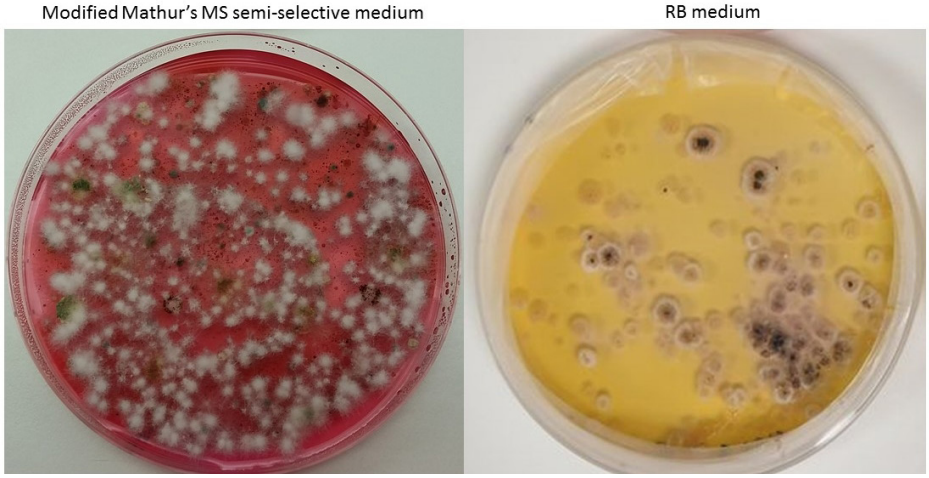

Figure 1. The rice brand medium was more selective than other media tested; Colonies of M. phaseolina were also easier to identify on RB medium due to the production of characteristic microsclerotia.

Evaluating Plating Methods

The Andersen sampler technique using 0.1 g to 0.05 g of soil per plate and incubation at 30 C for 15 days provided the most rapid and consistent results for identifying and enumerating colonies of *M. phaseolina* from artificially infested field soil.

Testing Naturally Infested Field Soil

Soil samples from the rhizosphere of plants naturally infected with *M. phaseolina* yielded a range of 1 to 2.5 CFUs per gram of soil.

Visualizing Microsclerotia *In Planta*

Microsclerotia were observed in all tissues tested, including crown, petiole and primary root samples (Figures 2, 3 and 4). Although microsclerotia were observed, they were impossible to quantify in any of the tissues using this method due to the consistency of the tissue after soaking in 5% sodium hydroxide.

Figure 2. Microsclerotia of *M. phaseolina* were observed in both inner and outer crown tissue of infected plants.

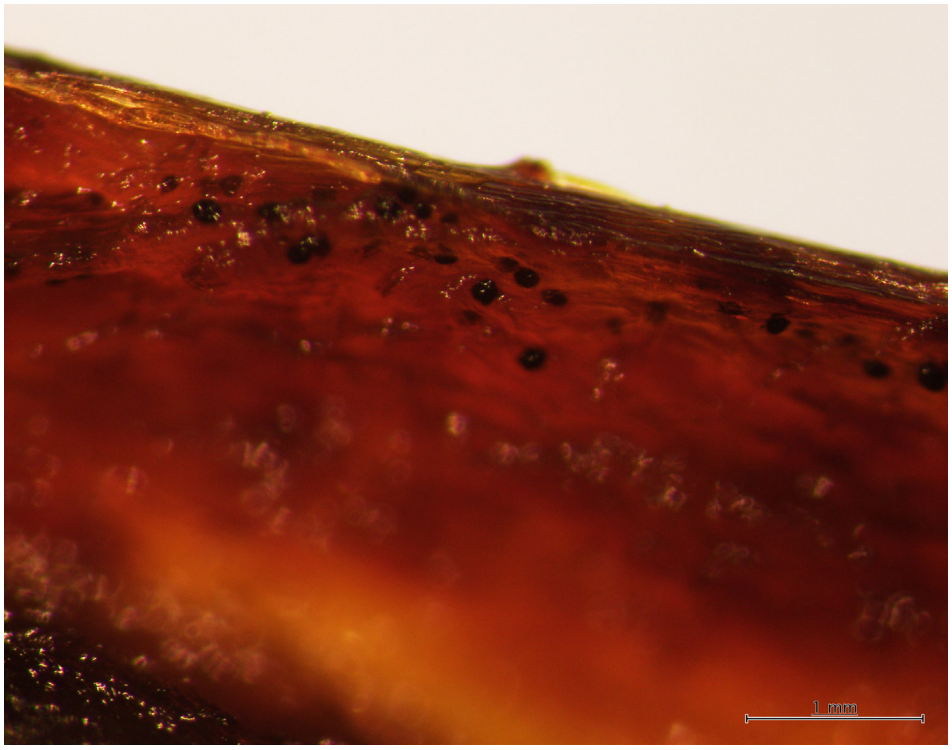

Figure 3. Microsclerotia of *M. phaseolina* were observed in petioles of infected plants.

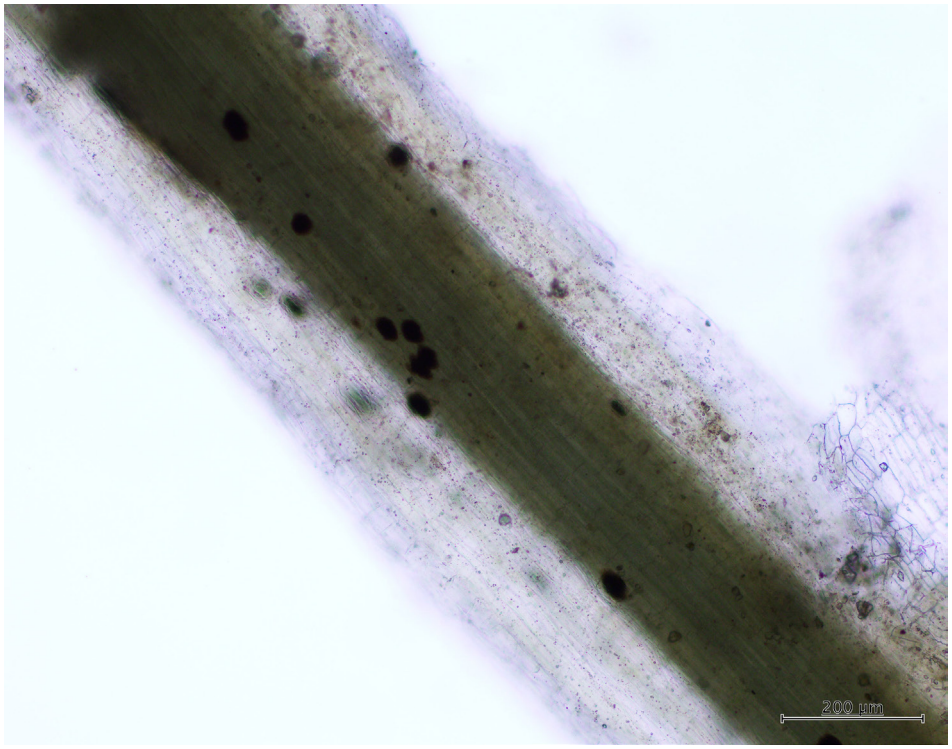

Figure 4. Microsclerotia of *M. phaseolina* were observed in primary roots of infected plants.

Discussion

Our lab has developed and optimized a culture-based assay with the resolution and sensitivity to detect and quantify *M. phaseolina* in naturally infested field soils. However, we feel this protocol could be further improved given the low level of colony forming units (1 to 2.5 CFUs per gram of soil) of the pathogen identified in the rhizosphere of infected plants. It is possible that the addition of a soil sieving step would increase the chances of finding this pathogen in bulk field soil. *Macrophomina phaseolina* is capable of producing microsclerotia in naturally infected plants, as large numbers of microsclerotia were visualized in inner and outer strawberry crown tissues, as well as petioles and primary root samples.

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