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An *in vitro* method for examining infection of soybean roots by *Macrophomina phaseolina*

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ABSTRACT

A general limitation when studying the early events in the infection process of a soil borne plant pathogen is the detection of the hypha arrival at the root surface. We describe a new method to infect plants with soil borne pathogens that may be applicable to examination of the prepenetration and the penetration phases of other soil borne fungi as well as the early responses of the host plant. Development of the *in vitro* method allowed us to obtain roots of soybean plants infected with *Macrophomina phaseolina* to characterize the infection process. Using two infection systems we provide evidence that *M. phaseolina* produces hyaline structures which are undetected by staining methods based on fungal chitin dyes and were identified exclusively by stained lipid vesicles produced by the pathogen within host tissues.

1. Introduction

Macrophomina phaseolina (*Mp*) is the causal agent of charcoal rot disease in soybean. Many economically important hosts, including cereals, legumes, vegetables, fruits and fiber crops, are attacked by this fungus [1]. Charcoal rot symptoms usually appear under high temperature conditions (28–35 °C) and low soil moisture, or when unfavourable environmental circumstances stress the plant [2,3]. Although initial infection occurs at the seedling stage, it usually remains latent until the soybean plant approaches maturity (growth stages R5–R7) [4]. Diseased plants may wilt and prematurely die with senesced leaves remaining attached to petioles. Seed yield is frequently reduced under these conditions. The diagnostic symptoms of charcoal rot on prematurely dying or dead plants are the sloughing of cortical tissues from the lower stem and taproot and the speckled grey appearance of these infected tissues due to abundant formation of microsclerotia in vascular, cortical, and pith tissues [5].

Light microscopy studies of plant fungal interfaces are routinely done by clearing and staining roots with dyes, such as trypan blue, acid fuchsine and chlorazol black, which specifically bind to chitin, a component of most fungal walls [6–8]. Alternatively, fungal lipids can be a detectable target molecule with Sudan IV as a dye. Interestingly, Barrow and Aaltonen [9], in their study on grasses, reported that fungi were not readily evident when chitin-targeted staining and traditional microscopic methods were used, probably because they could appear as protoplasts and hyaline structures. Therefore, they proposed the use of Sudan IV and a careful examination to follow hyphal growth through the plant tissues.

The first reports of *Mp* infection process in soybean were Ammon et al.'s [10,11], which were based on scanning electron microscopy analyses. They suggested that penetration through soybean cell walls occurred as a result of mechanical pressure and/or chemical softening. Ilyas and Sinclair [12] described the formation of intra-xylem sclerotia in wound-inoculated soybean plants, lacking the characterization of the initial penetration stages. To our knowledge, no other studies have tried to characterize the initial penetration stages of *Mp* in soybean plants since the 1970s.

As well as the scarce work describing *Mp* infection on soybean plants, a general limitation when studying the early colonization events of a soil borne pathogen is to actually observe the arrival of hyphae. Consequently, in the present work, we describe a new method to infect intact plants with soil borne pathogens and further characterize the infection process of *Mp* in soybean plants.

2. Materials and methods

2.1. Plant and fungal material

Uniform and healthy soybean seeds (*Glycine max* Don Mario 4800) were surface disinfested in 1% NaClO during 30 s and germinated in sterile *Petri* dishes with moist filter paper and cotton.

M. phaseolina (*Mp*), obtained from field infected soybean plants, was grown on potato dextrose agar (PDA) plates at 28 °C. The inoculum for pots assay was multiplied in potato dextrose broth under agitation for 48 h, until formation of microsclerotia.

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Microsclerotia were collected by filtration, rinsed and diluted in sterile distilled water. For inoculum for the *in vitro* method, *Mp* was also grown on PDA plates and a plug of fungal growth was placed in plates of solidified with Phytagel™ (SIGMA) Hoagland's solution.

2.2. Pot assay

Pregerminated disinfected seeds were placed in plastic pots with sterile Perlite, inoculated with 350 microsclerotia g^{-1} substrate, and maintained for 20 days in growth chamber under a 16 h light/8 h dark regime.

2.3. In vitro method

Sterile plastic Petri dishes (140 × 20 mm) were filled with 0.25% Phytagel™ solidified Hoagland's solution at 2×, 1× and 0.5× concentration.

The radicle of each disinfected pregerminated seed was carefully inserted through a small hole in the lid of a plastic *Petri* dish under sterile conditions, maintaining cotyledons outside to allow shoot growth. *Petri* dishes were maintained in a growth chamber under a 16 h light/8 h dark regime. When the first trifoliate leaf of each plant was completely expanded, they were inoculated with a plug of *Mp* as indicated above under a sterile flow chamber. The infection moment was monitored with a stereoscopic microscope through the *Petri* dish lid, and root samples of the infection zone were taken.

2.4. Processing of roots

Root samples were stained by the method described by Barrow [13]. Briefly, roots were cleared in 2.5% KOH before they were stained with Trypan blue (TB) or Sudan IV (SIV). Root segments were placed in several drops of glycerol on a microscope slide. A cover slip was placed over the root sections and pressed firmly to facilitate analysis at high magnification. Roots were examined with a Zeiss Axiophot microscope by conventional and DIC optics at 1000×. Specimens were photographed with a Sony Power HAD colour video camera.

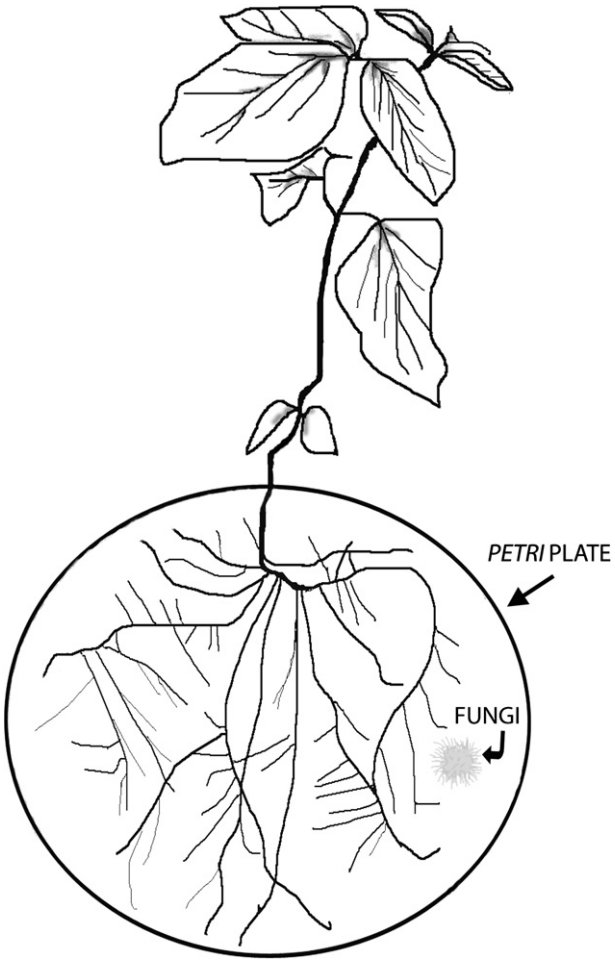

Fig. 1. Schematic representation of a soybean plant with a well developed root system growing in a *Petri* dish with Hoagland/Phytagel™ medium.

3. Results

3.1. In vitro method

Optimal concentration of Hoagland's solution for growing soybean plants *in vitro* was evaluated. Plants showed symptoms of nutritional deficiencies and toxicity when grown in the solutions of 0.5× and 2×, respectively. Plants grown in 1× Hoagland's solution remained healthy for one month. This concentration was chosen for the experiments here described. The diagram in Fig. 1 shows the experimental system.

Inoculation with *Mp* was carried out when plants were well established. Initially, an *Mp* colonized PDA plug was used, but the infection process was too fast, probably due to the PDA nutrients. Consequently, the fungus was grown in solidified with Phytagel™ Hoagland's solution (1×) and subsequently used as inoculum.

3.2. Infection process

The monitoring of the fungus arrival to roots was possible with the *in vitro* system by periodic observation through the *Petri* dish lid. Two days post-inoculation *Mp* hypha reached the surface of the roots (Fig. 2A).

The penetration process was characterized by staining roots of plants grown in pots as well as of plants grown in the *in vitro* assay. In both root samples, fungal structures with a swollen and pigmented cell wall were observed even without stain (Fig. 2B–D). In many cases, however, pigmented hyphae were continuous with hyaline hyphae (Fig. 2E and F) and could be distinguished by staining with Sudan IV. A pigmented swollen structure, with a hyaline hypha extending from it and contacting a soybean cell is observed in Fig. 2F.

Careful examination of colonized root tissues at high magnification revealed internal colonization by hyaline hyphae that did not stain normally with trypan blue. By observing the stained vesicles, it was possible to detect *Mp* hyphae growing inter- and intracellularly in soybean roots. Intercellular growth was predominantly parallel to the root axis (Fig. 2H), whereas intracellular hyphae were perpendicular to it, with the production of lightly melanized swollen cells on the surface of each soybean cell wall (Fig. 2G).

In pot assays as well as in long term experiments (data not shown), cells filled with globular chitinized hypha were predominantly observed (Fig. 2I). Evidently, mycelium bound Trypan blue, a dye that specifically binds chitin. Conversely, in short term assays, *Mp* was present in host tissues as hyaline structures (Fig. 2J), which escape detection by fungal chitin staining but were visualized by their stained lipid vesicles.

4. Discussion

We present here a novel method to infect plant roots *in vitro* with a soil borne pathogen and to follow the progress of the infection. In the *in vitro* assay, it was necessary to adjust the inoculum growing conditions. Initially, *Mp* grown in PDA was used, but the infection

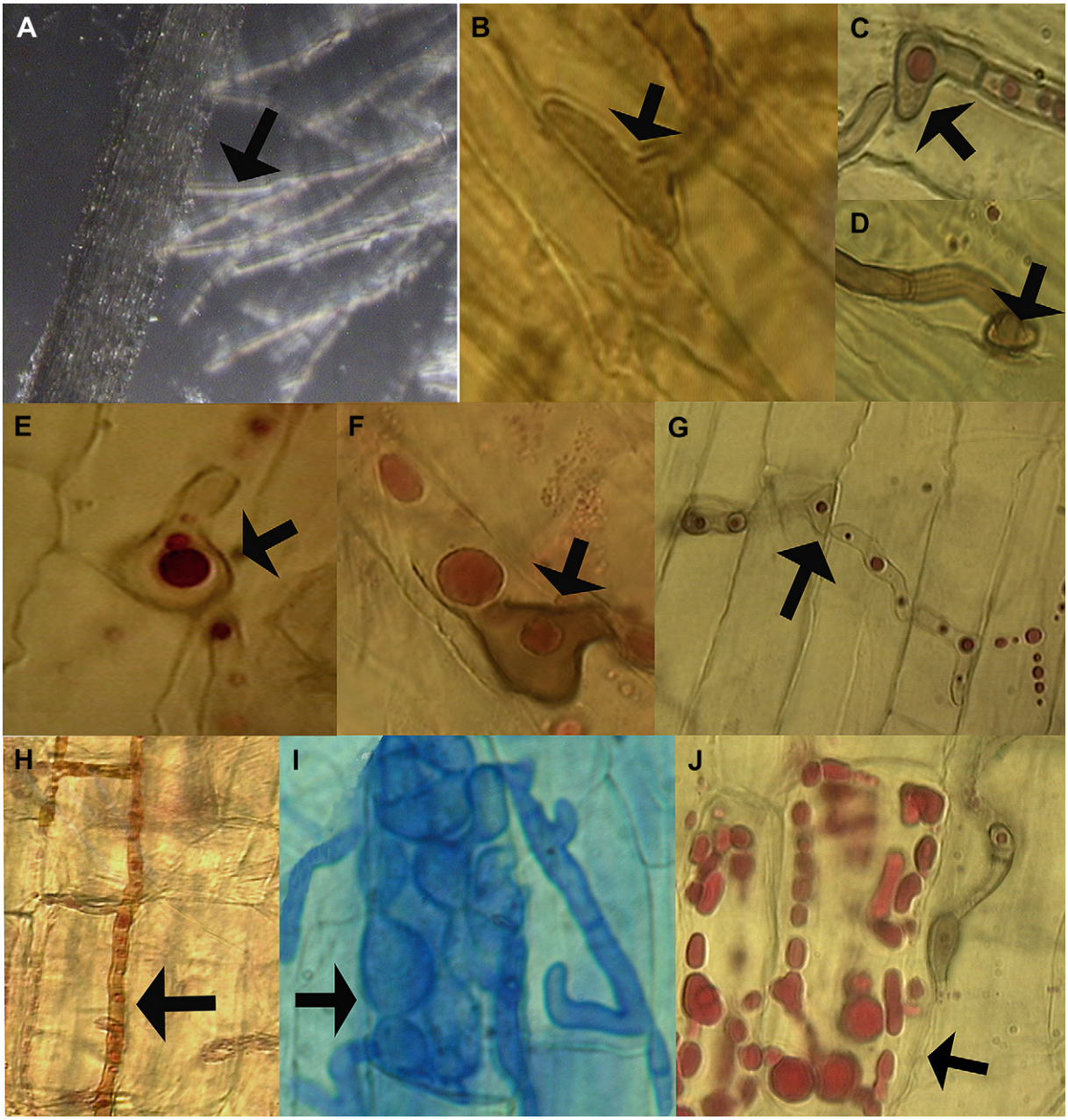

Fig. 2. Early colonization stages of soybean roots by *M. phaseolina*, *observed under* stereoscopic microscope (A) and microscope using DIC optics at 1000× (B–J). (A) Fungal hyphae contacting the host root surface. (B–D) Swollen and pigmented invasive structures. (E and F) Pigmented hyphae continuous with hyaline hyphae. (G) Intracellular growth of hyphae. (H) Intercellular growth of hyphae. (I) Soybean cells filled with chitinized globular structures. (J) Soybean cells filled with hyaline globular structures.

process was too aggressive and fast. The remaining PDA plug may have been supporting fungal growth. To obtain conditions similar to natural conditions, the fungus was grown in Hoagland's solution, the same medium as the plants. This improvement resulted in a slower growth of the fungus toward the roots and a slower infection development because the fungus derived substrates only from the soybean roots.

The ability to observe the colonization of root tissue from the very beginning allowed us to learn that *Mp* produced hyaline structures which were not detected by staining methods based specifically on fungal chitin and were only detected by lipid vesicles stain. In some cases, stained or melanized structures, formed a continuum with these hyaline hyphae. In accordance with this, several authors found these polymorphic structures studying the controversial subject of dark septate fungi [9,14–16]. Decreased visibility or lack of detection could be attributed to the absence of chitin, which prevented Trypan blue staining, and to the absence of melanin, which gives these fungi their characteristic dark colour [16]. Chitin and melanin increase wall rigidity and reduce permeability, protecting the fungus as it invades harsh environments [17–19]. Inside host tissues, *Mp* develops thin hyaline walls, which are presumed to be more permeable with increased potential for resource exchange with the host [9,16]. Something similar occurs with arbuscular mycorrhizal fungi, in which extra-radical hypha have thick composite walls with high chitin content, and a progressive reduction in wall thickness is found in internal hypha and arbuscules [20].

Also, we could observe the development of *Mp* structures with swelled and pigmented walls, produced either inter- and intracellularly. These structures, similar to appressoria, were

previously described as hyphopodia by Howard [21]. He described appressoria as structures that develop from swellings at the tips of conidial germ tubes and hyphopodia as structures that arise from mature vegetative hyphae. Hyphopodia have been defined as structures that allow the spreading of the fungus after infection of the plant, and they might enhance penetration or survival [21,22]. Hyphopodia generally are melanized and deposition of melanin, for instance, in the fungal cell wall of appressoria is associated with the generation of intracellular turgor pressure that provides the necessary force for plant penetration [18].

The ratio between stained and non-stained fungal structures correlated with the age of the plant. In experiments with mature soybean plants, cells filled with highly chitinized spherical structures, able to bind Trypan blue, or even melanized, were predominantly observed. On the contrary, in short term assays, *Mp* was present in the host tissues as hyaline structures which escape detection by chitin staining methods, and was detected exclusively by its stained lipid vesicles. This polymorphic nature of fungal structures was previously reported [9,16] in cases where stained and melanized associations were increasingly evident while plants became dormant. More specifically, dominant grasses and shrubs of arid south western USA ecosystems are colonized by structurally variable fungi that exist primarily as protoplasts, as well as hyaline, non-staining and non-pigmented fungal structures in physiologically active roots. Stained and melanized hyphae and microsclerotia develop as plant metabolism decreases during periods of stress [16].

Development of the *in vitro* method enhanced our ability to obtain *Mp*-infected roots of young and healthy soybean plants. This is not a trivial challenge with soil borne pathogens, especially when the objective is to characterize the penetration phase.

The *in vitro* method described in here may be a good system to obtain samples during the initial stages of infection of other interactions of a plant and a soil borne pathogen, and to observe, for example, early induction of plant gene expression.

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