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Microassay for Biological and Chemical Control of Infection of Tobacco by *Phytophthora parasitica* var. *nicotianae*

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Abstract. We developed a simple, rapid, small-scale assay for infection of tobacco seedlings by *Phytophthora parasitica* var. *nicotianae*. One 7-day-old tobacco seedling was placed in each well of a 96-well microtiter plate and inoculated with 500 zoospores of *P. parasitica* var. *nicotianae*. After 72 h all of the inoculated seedlings of the susceptible cultivar, KY14, were infected, and the pathogen had produced sporangia that were visible on the surfaces of the seedlings. Sporangia did not develop on seedlings that were inoculated simultaneously with zoospores and either 1 μg/mL of the chemical fungicide metalaxyl or 5 μL of filtrate of a sporulated culture of the biocontrol agent, *Bacillus cereus* UW85. Seedlings of tobacco cultivar KY17 were infected by *P. parasitica* var. *nicotianae*, although mature plants of this variety are resistant to the pathogen. This microassay may facilitate the rapid screening of potential biological and chemical control agents and may be useful for studying mechanisms of infection and control of *Phytophthora* spp. under hydroponic conditions.

Phytophthora parasitica var. *nicotianae* (Breda de Haan) Tucker causes black-shank of tobacco (*Nicotianae tabacum* L.) in most tobacco-growing regions of the world. Although chemical and cultural control measures and cultivars with genetic resistance to black-shank are available, this disease still represents a major threat to the tobacco industry [5].

Water is a critical factor in *Phytophthora* diseases. Abundant water is required for release and chemotaxis of zoospores, which are regarded as a major infectious structure in the *Phytophthora* life cycle [1, 5, 7], and water facilitates spread of the inoculum [2]. Consequently, hydroponic plant culture provides ideal conditions for *Phytophthora* diseases. As hydroponic systems become increasingly important in culture of tobacco and other plants [7], new measures for control of black-shank and other diseases caused by zoosporic fungi will be needed in these systems [8].

To identify effective disease-control measures, including chemical and biological control agents or genetic resistance in the host, a rapid screen for infection of the host by the pathogen is desirable. The screen should be appropriate to the biology of the host and pathogen and to the environmental conditions that promote disease. In this paper we describe a rapid microassay for testing potential agents for control of infection of tobacco by *P. parasitica* var. *nicotianae* zoospores under hydroponic conditions.

Materials and Methods

Bacteria were grown in half-strength Trypticase soy broth. Isolation and characterization of *Bacillus cereus* UW85 and production of culture filtrates were described previously [4]. *P. parasitica* var. *nicotianae* Race O, isolate Ppn-O, was obtained from J. Helgeson (University of Wisconsin, Madison). The pathogen was grown for 5–7 days on oatmeal agar containing (per liter): 75 g oatmeal and 20 g agar. Tufts of aerial hyphae were transferred aseptically to sterile distilled water and incubated for 4–14 days to induce production of zoosporangia. The mycelia were transferred to fresh water and incubated at 4°C for 30–60 min to induce zoospore production. To induce zoospore release, the plate was placed at room temperature, and zoospores were collected after 1 h. The zoospore concentration was determined on a subsample with a hemacytometer after induction of the zoospores to encyst by vigorous agitation with a Vortex mixer.

Seeds of Burley tobacco cv. KY14 or cv. KY17 were surface-disinfected by soaking them in 20% Chlorox for 30 min. The seeds were washed with sterile distilled water, spread on moist, sterile filter paper, and incubated under a 40W cool white fluorescent light at 22°C for 5–7 days. One seedling was placed in each

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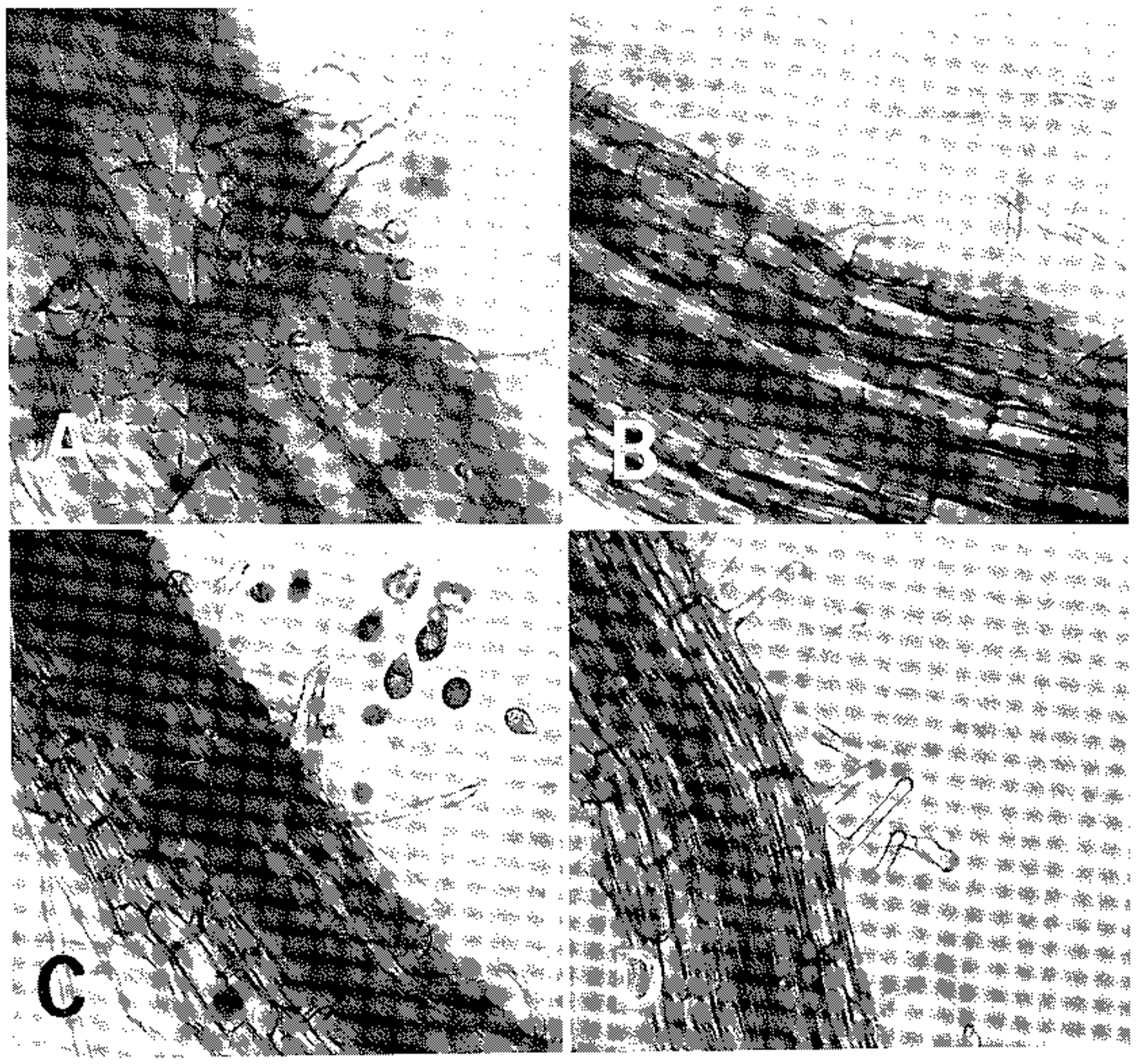

Fig. 1. Infection of tobacco seedlings by *Phytophthora parasitica* var. *nicotianae* in the presence or absence of UW85 culture filtrates. (A) 2 h after addition of 500 zoospores of *P. parasitica* var. *nicotianae*. Total magnification, 360×. (B) 2 h after addition of 500 zoospores and 5 μL of filtrate from a fully sporulated culture of UW85. Total magnification, 180×. (C) 48 h after addition of 500 zoospores to seedlings. Total magnification, 180×. (D) 48 h after addition of 500 zoospores and 5 μL of filtrate from a fully sporulated culture of UW85. Total magnification, 180×.

Table 1. Infection of tobacco seedlings by *Phytophthora parasitica* var. *nicotianae* in microtiter wells

Treatment	Tobacco cultivar	Infected seedlings (%)[a]
None	KY14	0 A[b]
Ppn[c] zoospores	KY14	100 C
Ppn zoospores	KY17	97 C
Ppn zoospores + TSB	KY14	94 C
Ppn zoospores + UW85 (vegetative)[d]	KY14	69 B
Ppn zoospores + UW85 (sporulated)[e]	KY14	3 A
Ppn zoospores + metalaxyl[f]	KY14	0 A

[a] Each value represents the mean percentage of 32 seedlings that were infected 3 days after inoculation with 500 zoospores of *P. parasitica* var. *nicotianae*.
[b] Values followed by different letters differ significantly at $P \leq .0002$.
[c] Ppn = *P. parasitica* var. *nicotianae*.
[d] 5 μL of filtrate of a culture of UW85 in vegetative growth phase was added.
[e] 5 μL of filtrate of a fully sporulated culture of UW85 was added.
[f] Metalaxyl was added at a final concentration of 1 μg/mL.

well of a 96-well microtiter plate with 150 μL of sterile distilled water. An additional 25 μL of water was added to wells for control treatments, and 25 μL of a zoospore suspension adjusted to a concentration of 2×10^4 zoospores/mL was added to wells for inoculated treatments. For the biocontrol treatments, an additional 5 μL of filtrate of the bacterial cultures was added to the wells, and for the chemical control treatment 20 μL of a solution of metalaxyl was added. The metalaxyl solution was made by suspending 10 mg Apron (25% active ingredient wt/wt) in 1 mL water. The microtiter wells were incubated under a 40W cool white fluorescent light and examined under a Zeiss inverted microscope at 100× magnification.

Results and Discussion

By 72 h after addition of 500 zoospores of the pathogen, all inoculated seedlings of the susceptible cultivar KY14 were infected. The zoospores encysted

and germinated on the seedling surface within 2 h of inoculation (Fig. 1A) and infected and formed sporangia within 48 h of inoculation (Fig. 1C). Sporangia often released new zoospores within 48 h after inoculation, and infection was assessed by the presence of sporangia on the seedling surface 72 h after inoculation.

To determine whether the microassay would be useful for screening potential agents for control of black-shank disease, we tested a biological and a chemical agent. *B. cereus* strain UW85, previously shown to lyse zoospores of *Phytophthora* [3] and to protect alfalfa from infection by *P. megasperma* f. sp. *medicaginis* [4], was tested in the microassay. Two hours after inoculation in wells to which 5 μL of filtrate of the UW85 culture had been added, the zoospores appeared lysed, whereas zoospores in the control wells were still swimming or encysted. Addition of 5 μL of filtrate from a fully sporulated culture of UW85 significantly reduced infection (Fig. 1B, 1D). Whereas 100% of the seedlings inoculated with zoospores alone were infected, only 3% of those inoculated with zoospores and culture filtrate of a sporulated culture of UW85 were infected (Table 1). In contrast, filtrate of a vegetative culture only reduced infection to 69% (Table 1). This is consistent with previous work, which showed that fully sporulated cultures of UW85 have superior disease suppression activity compared with vegetative cultures [4].

Metalaxyl, which is commonly used to control black-shank in the field, completely suppressed infection of tobacco seedlings in the microassay at a final concentration of 1 μg/mL (Table 1). Cultivar KY17 was susceptible to infection in the microassay (Table 1), although it is moderately resistant to black-shank in the field. This suggests that black-shank resistance is not expressed in seedlings of KY17 under the conditions of the microassay.

The microassay presented here is a rapid, simple technique for screening potential agents for control of *P. parasitica* var. *nicotianae* on tobacco. It may provide a preliminary screen for agents that will be useful in the field for control of black-shank, although not all agents that control infection under hydroponic conditions can be expected to work in soil in the field as well.

More importantly, the results in the microassay may be applicable to hydroponic plant culture. Hydroponics are becoming increasingly important in the greenhouse industry for large-scale production of seedlings for transplanting [6, 8], and since hydroponics provide an ideal environment for host infection by zoospore-producing pathogens [8], their control is important to the successful use of hydroponics. The microassay may provide a preliminary screen for biological and chemical agents for the control of oomycete diseases in hydroponic conditions. In addition, the microassay is ideal for studying mechanisms of infection by, and control of, zoosporic pathogens, since the infection process is readily visible in the microtiter wells.

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