A Rapid Microbioassay for Discovery of Antagonistic Bacteria for *Phytophthora parasitica* var. *nicotianae*

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ABSTRACT

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A simple, rapid, small-scale microbioassay for infection of tobacco seedlings by *Phytophthora parasitica* var. *nicotianae* was developed here. This assay uses tobacco seedlings cultivated in petri dishes for a standardized method for quantitation of initial zoospore inocula and high-throughput screening of antagonistic bacteria. Zoospore inocula between 10^2 to 10^5 spores per petri dish were inoculated on 14-day-old tobacco seedlings for the susceptibility test. The optimum inocula was established to be ten thousand zoospores. One hundred and fifty pure culture bacteria

with different pigments, growth rates, and morphologies were isolated from rhizosphere soil of tobacco and screened for protective ability against tobacco black shank. Fifteen bacteria presented high activity against *P. parasitica* on tobacco seedlings. They were identified by Biolog GEN III MicroPlate and distributed as *Bacillus amyloliquefaciens*, *B. licheniformis*, *Paenibacillus pabuli*, *B. atrophaeus*, *B. subtilis*, *B. pumilus*, and *B. endophyticus*, respectively. Four antagonists chosen randomly from the 15 bacteria all exhibited the same 100% protective activity in planta as that in the petri dishes. This microassay proved to be a rapid, reproducible, and efficient method for screening of potential biological agents or microorganisms and may be useful for studying mechanisms of infection and control of *Phytophthora* spp. under hydroponic conditions.

Phytophthora parasitica var. *nicotianae* (*P. parasitica*) Breda de Haan causes black shank of tobacco (*Nicotiana tabacum* L.) in most tobacco-growing regions of the world. It has a host range of more than 72 genera of *Solanaceae* plants (7), particularly tobacco. The impact of this pathogen on tobacco is enormous (5,8,15,20). Common annual losses attributed to black shank approach 85% in China (24). Currently, black shank is managed through the use of resistant cultivars, crop rotation, and the fungicide metalaxyl (23). However, it is still a major problem for farmers and there are increasing concerns over environmental problems and metalaxyl-resistant strains of *P. parasitica* (4). The development and application of microbial agents is one of the useful alternative measures and has been used successfully on many crops (3,6,11,19).

Biological control of *Phytophthora* spp. has been researched for many years worldwide. However, most of the microorganisms are easily influenced by the environment and normally have poor stability. To the best of our knowledge, few microorganisms which are antagonistic in the field are available. Given the enormous economic impact of *Phytophthora* diseases worldwide, discovery of new and novel biological control agents to manage tobacco black shank is an important priority in disease management. Plenty of fungi, bacteria, and actinomycetes have been screened in petri dishes (9,14,17). However, the in vitro results normally are insufficient. Efficacy assessments in vivo are normally conducted on greenhouse-grown plants. This method is

time consuming, material and labor intensive, and requires relatively large quantities of test microorganisms. The in vivo assays often supplement in vitro studies; these are tedious and relatively large scale. A need exists for a rapid, quantitative, efficient, and reproducible microbioassay method to screen microorganisms for potential control of black shank and other *Phytophthora* diseases.

Therefore, the objectives of the current study were (i) to standardize initial inocula concentrations, (ii) to screen potential antagonistic bacteria for control of infection of tobacco by *P. parasitica* on tobacco seedlings under hydroponic conditions in petri dishes, (iii) to compare the microbioassay with the efficacy assay conducted on greenhouse-grown plants, and (iv) to identify antagonistic bacteria with Biolog GEN III MicroPlate.

MATERIALS AND METHODS

Fungal strain and culture conditions. One isolate of *P. parasitica*, with wild-type sensitivity and pathogenicity to tobacco, was selected as the modal specie for assay development. The isolate was grown and maintained on lima bean agar medium (60.0 g of lima beans per liter boiled for 1 h and 16.0 g of agar per liter), in a controlled climate cabinet at 25°C in darkness. For zoospore production, agar plugs were removed from the edge of an actively growing culture and placed in petri dishes containing 8 ml of 10% V8 liquid medium (10% V8, 100 ml of Campbell's V8 juice to 900 ml of sterile water, and 0.2 g of CaCO₃). Following a 96 h incubation period at 25°C in light, the suspension was filtered through a double layer of sterile cheesecloth (Grade #40: 24 × 20 threads per inch) to remove mycelia fragments, and the resulting sporangial suspension was maintained at 4°C for 30 min and then at 25°C for 2 h in the dark to release the zoospores (23). For long-term storage, 5-mm agar plugs from the

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* The e-Xtra logo stands for "electronic extra" and indicates that Figures 1, 2, and 3 appear in color online.

leading edge of individual colonies were transferred into several sterile 1.5-ml microcentrifuge tubes containing 1 ml of sterile distilled water, and tubes were stored in the dark at 15°C (1,2).

The susceptible tobacco (*N. tabacum*) cultivar Honghuadajinyuan was used as host of black shank in this study. For the microbioassay, tobacco plants were cultivated (25°C) in petri dishes containing filter papers moistened with 5 ml of Hoagland's solution and were grown from sterile seeds at 12 plants per petri dish (90 mm in diameter). When two leaves were fully developed 3 weeks later, the plants were ready.

Standardization of inoculum concentration. Various inoculum concentrations were determined here for use in the screening of antagonistic bacteria. Zoospores of *P. parasitica* were collected as mentioned above. Inoculum concentrations were quantified by the use of a hemacytometer, and dilutions were made with sterile distilled H₂O to obtain 1 × 10², 1 × 10³, 1 × 10⁴, and 1 × 10⁵ zoospores/ml, respectively. One ml of various inocula was added to the tobacco seedlings. After inoculation, the petri dishes were incubated at 25°C, 100 to 120 μEm⁻² S⁻¹, relative humidity of >80%, and 16 h light per day for disease development. Number of diseased seedlings was checked 7 days after inoculation. The test was repeated twice, with three replications of each treatment.

Soil sampling and isolation of antagonistic bacteria. During the harvesting time of tobacco in 2010, rhizospheric soil about 15 cm away from healthy tobacco stems was collected from Fuquan and Libo tobacco commercial fields in Guizhou Province, southwest China. Several samples were selected randomly using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (13). Soil samples were taken from a depth of 10 to 20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded. Samples were air-dried at room temperature for 7 to 10 days and then passed through a 0.8-mm mesh sieve and were preserved in polyethylene bags at room temperature before use. Five grams of air-dried soil was mixed with 45 ml of sterile distilled water. The mixtures were shaken vigorously for 1 h and then allowed to settle for 1 h. Portions (0.5 ml) of soil suspensions (diluted 10⁻¹) were transferred to 4.5 ml of sterile distilled water and subsequently diluted to 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶. Aliquots of 100 μl of 10⁻³ to 10⁻⁶ soil dilutions were inoculated onto the surface of NA (beef extract [0.3%], peptone [0.5%], and agar [1.5%] in water [wt/vol]) medium plates (18). After inoculation, the petri dishes were placed at 30°C in darkness for 24 h. Bacteria with different pigments, growth rates, and morphologies were isolated from NA plates and refrigerated as pure cultures before use.

Screening of antagonistic bacteria. Pure bacterial solutions were incubated in NB (beef extract [0.3%] and peptone [0.5%] in water [wt/vol]) for 12 h, and 1 ml of each bacterial solution (1 ×

10⁸ CFU/ml) was added to the tobacco plants incubated in the petri dishes mentioned above. Afterwards, the petri dishes were incubated in the dark for 12 h. An additional 1 ml of a zoospore suspension adjusted to a concentration of 1 × 10⁴ spores/ml was added to each petri dish for inoculated treatments. The control was treated with just 1 ml of zoospore suspension. After inoculation, the petri dishes were incubated under the same conditions mentioned above. All incubation conditions were maintained similarly throughout the experiments. Results were obtained 7 days after inoculation based on a 0 to 5 scale: 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear on stems; 2, 30 to 50% of entire plant diseased; 3, 50 to 70% of entire plant diseased; 4, 70 to 90% of entire plant diseased; and 5, plant dead (21). The test was repeated twice, with three replications of each treatment.

Greenhouse bioassay in planta. For efficacy assay, four bacteria (H11, H14, H19, and H113) with 100% protection were chosen randomly from the above microbioassay and were utilized here. Tobacco plants were cultivated in the greenhouse (20 to 30°C) and grown from seedlings at one plant per 0.2-liter pot with sandy loam soil. They were inoculated when six leaves were fully developed 4 weeks later. Thirty milliliters of pure bacterial solutions at a concentration of 1 × 10⁸ CFU/ml was added to the pot, and 24 h later, an additional 30 ml of a zoospore suspension (4 × 10⁴ spores/ml) was added to the pot for inoculated treatments. Twelve plants were treated per treatment, and the experiment was conducted twice. Results were obtained 30 days after inoculation according to the same method mentioned above.

Identification of antagonistic bacteria. All antagonistic bacteria were grown on Biolog BUG agar for identification by the

TABLE 1. Effect of different inoculum concentrations on the susceptibility of cultivar Hongda tobacco seedlings to infection by *Phytophthora parasitica*

Inocula ^x (spores/ml)	Infection ratio (%)		Disease incidence	
	Mean ^y	SE ^z	Mean ^y	SE ^z
0	0 c	0	0 d	0
1 × 10 ²	70.83 b	1.78	59.17 c	1.39
1 × 10 ³	87.50 b	1.31	77.50 b	1.11
1 × 10 ⁴	100 a	0	100 a	0
1 × 10 ⁵	100 a	0	100 a	0

^x Zoospore inocula of *P. parasitica* added to tobacco seedlings incubated on petri dishes.

^y Mean value from two independent assays, with 12 replicate tobacco seedlings per treatment in each assay. Means not followed by a common letter are significantly different ($P \leq 0.05$) according to least significant difference for each inoculum.

^z Standard error of the mean for two independent assays.

Fig. 1. Biolog GEN III MicroPlate composed of 71 carbon sources plus 23 chemical sensitivity assays.

Biolog system (Biolog Inc., Hayward, CA). In preparation for analysis, colonies were picked from pure cultures of bacteria and were plated as a lawn of bacteria onto a BUG agar plate. Separate plates were set up for each strain to be analyzed. The Biolog Microlog Bacterial Identification System consists of databases combined with specialized 96-well plates (test panels). A panel of 71 carbon source utilization assays and 23 chemical sensitivity assays (Fig. 1) provides a “Phenotypic Fingerprint” of the micro-organism that can be used to identify it at the species level (10). GEN III MicroPlate test panels were used for identification according to the manufacturer’s instructions throughout the experiment.

Data analysis. Results of laboratory and growth chamber experiments were reported. Data from repeated experiments were combined for analysis since variances between experiments were homogeneous. Inhibition on disease development was calculated as % protection = $100 \times (1 - x/y)$, where x and y were disease incidence in treated-challenged and control (untreated-challenged) tobacco seedlings, respectively (22).

RESULTS

Inoculum concentration effects. Inoculum of various zoospore concentrations had a significant effect on tobacco seedling infection by *P. parasitica*. Disease incidences and infection ratios increased greatly with the enhancement of inocula of the pathogen. Inoculum concentrations of 1×10^4 and 1×10^5 zoospore/ml

both provided a mean disease incidence of 100 over the course of the two repeated experiments. In contrast, inoculations of 1×10^2 and 1×10^3 zoospore/ml produced mean disease incidences of 59.17 and 77.50 and infection ratios of 70.83 and 87.50%, respectively (Table 1).

Isolating and screening of antagonistic bacteria. In isolating and screening for bacteria having antagonistic activity against *P. parasitica*, the causal agent of tobacco black shank, 150 isolates of soil bacteria from tobacco rhizosphere were isolated. When adding test bacteria solution 12 h before that of the pathogen, 15 of the tested bacteria exhibited 100% protective efficacy, with a disease incidence value of 0 (Table 2). The others had much less activity, with highest disease incidence value of 100 (data not shown). Additionally, during the 7-day-incubation period, all control treatments resulted in the same disease incidence value of 100, and all tobacco seedlings died (Fig. 2).

Greenhouse bioassay in planta. The in planta efficacy of selected antagonists for the control of *P. parasitica* in tobacco plants was evaluated under greenhouse conditions and there were significant differences 30 days after transplanting. At 45 days after transplanting, antagonist bacteria significantly reduced tobacco black shank incidence compared with that of the control (Fig. 3). Generally, a positive correlation (data not shown) was found between petri dish screening and in planta suppression of disease.

Identification of antagonistic bacteria. All 15 selected antagonistic bacteria were identified to species level by Biolog GEN III

TABLE 2. Identification of antagonistic bacteria against tobacco black shank with Biolog GEN III MicroPlate^x

Strain number	Disease incidence ^y	Species	Similarity	Distance	Organism type
HI1	0	<i>Bacillus amyloliquefaciens</i>	0.653	5.055	GP ^z
HI2	0	<i>Bacillus amyloliquefaciens</i>	0.660	4.854	GP
HI3	0	<i>Bacillus amyloliquefaciens</i>	0.638	5.252	GP
HI4	0	<i>Bacillus amyloliquefaciens</i>	0.582	6.166	GP
HI5	0	<i>Bacillus amyloliquefaciens</i>	0.555	5.611	GP
HI6	0	<i>Bacillus amyloliquefaciens</i>	0.555	5.470	GP
HI7	0	<i>Bacillus licheniformis</i>	0.533	4.751	GP
HI8	0	<i>Bacillus licheniformis</i>	0.501	5.164	GP
HI9	0	<i>Paenibacillus pabuli</i>	0.569	6.340	GP
HI10	0	<i>Paenibacillus pabuli</i>	0.603	5.798	GP
HI11	0	<i>Bacillus atrophaeus</i>	0.610	5.694	GP
HI12	0	<i>Bacillus atrophaeus</i>	0.607	4.246	GP
HI13	0	<i>Bacillus subtilis</i>	0.596	5.861	GP
HI14	0	<i>Bacillus pumilus</i>	0.596	5.273	GP
HI15	0	<i>Bacillus endophyticus</i>	0.535	6.838	GP

^x Identification values of antagonistic bacteria with Biolog GEN III MicroPlate after 16 to 24 h incubation.

^y Values obtained from antagonistic bacteria screening bioassay on petri dishes.

^z Gram-positive bacteria.

Fig. 2. Antagonistic bacteria screening on tobacco seedlings under hydroponic conditions on petri dishes for control of infection of tobacco by *Phytophthora parasitica*: **A**, bacterium with 100% inhibition activity and **B**, dead tobacco seedlings in the control treatment.

Fig. 3. In planta efficacy of a select antagonist for the control of *Phytophthora parasitica* in tobacco plants under greenhouse conditions 45 days after transplanting: **A**, control and **B**, antagonistic bacterium treatment.

MicroPlate. If the similarity value was >0.5 during a 16 to 24 h incubation period, the identification result was acceptable. They were all gram-positive bacteria with similarity value of >0.5 . The isolates identified included seven genera: five *Bacillus amyloliquefaciens* species, two *B. licheniformis* species, two *Paenibacillus pabuli* species, two *B. atrophaeus* species, one *B. subtilis* species, one *B. pumilus* species, and one *B. endophyticus* species (Table 2).

DISCUSSION

In this investigation, we have developed a standardized micro-scale bioassay for screening potential antagonistic bacteria against *P. parasitica*. Whereas any one bioassay in vitro will likely be consistent with the in planta assay, the microbioassay provides a rapid, reproducible, simple technique for testing quantities of sample. It may provide a preliminary screen for agents that will be useful in the field for control of tobacco black shank, although not all bacteria that control infection on tobacco seedlings can be expected to work in soil and in the field as well.

Zoospores of *P. parasitica* were the primary infective propagule, and homogenized zoospore suspensions were considered to be a more appropriate assay target than mycelium (12). The optimum concentration of zoospores was quite important for standardization of inoculum concentration throughout the bioassay. Both 10^4 and 10^5 zoospores could generate the same disease incidence in tobacco seedlings during a short incubation period, while 10^4 zoospore treatment was chosen as the inoculum concentration for use in the assays because it combined technical expediency with consistency of results and it was easier to prepare the mounts of zoospores compared with that of 1×10^5 zoospores.

In this study, antagonistic bacteria were identified by Biolog GEN III MicroPlate. To the best of our knowledge, this is the first use of GEN III MicroPlate, the new microplate product of Biolog Corporation, in identification of antagonistic microorganisms. The test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of gram-negative and gram-positive bacteria. Biolog's advanced system of phenotypic testing is a natural progression from the traditional approaches to mycological identification. However, identification with the Biolog system is not enough for allocating an identity to the isolated strains. In contrast, bacterial identification based on polymerase chain reaction assays is always restricted by the sequencing information, database, and primers used. Phenotypic identification is quite interesting for precise genetic identification, however phenotype testing and genotype testing can complement one another (16).

In conclusion, the four bacteria have proven to be consistently efficient in the control of *P. parasitica* disease in an in planta biocontrol assay under greenhouse conditions. Field studies should be undertaken in the next step to confirm the effectiveness of the antagonistic strains under natural conditions and their modes of

action. In addition, this microbioassay provides an opportunity to screen useful microorganisms against other *Phytophthora* spp.

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LITERATURE CITED

- Ann, P. J., and Ko, W. H. 1990. Growth rate and colony morphology of progenies of zoospores and selfed oospores of *Phytophthora parasitica*. *Mycologia* 82:693-697.
- Boesewinkel, H. J. 1976. Storage of fungal cultures in water. *Trans. Br. Mycol. Soc.* 66:183-185.
- Boff, P., Köhl, J., Jansen, M., Horsten, P. J. F. M., Lombaers-van der Plas, C., and Gerlagh, M. 2002. Biological control of gray mold with *Ulocladium atrum* in annual strawberry crops. *Plant Dis.* 86:220-224.
- Cartwright, D. K., and Spurr, J. R. H. W. 1998. Biological control of *Phytophthora parasitica* var. *nicotianae* on tobacco seedlings with non-pathogenic binucleate *Rhizoctonia* fungi. *Soil Biol. Biochem.* 30:1879-1884.
- Dukes, P. D., and Apple, J. L. 1961. Chemotaxis of zoospores of *Phytophthora parasitica* var. *nicotianae* by plant roots and certain chemical solutions. *Phytopathology* 51:195-197.
- Han, J. G., Sun, L., Dong, X. Z., Cai, Z. Q., San, X. L., Yang, H. L., Wang, Y. S., and Song, W. 2005. Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as diazotroph and a potential biocontrol agent against various plant pathogens. *Syst. Appl. Microbiol.* 28:66-76.
- Hickmann, C. J. 1958. *Phytophthora* plant destroyer. *Trans. Br. Mycol. Soc.* 41:1-13.
- Jacobi, W. R., Main, C. E., and Powell, N. T. 1983. Influence of temperature and rainfall on the development of tobacco black shank. *Phytopathology* 73:139-143.
- Jin, F. L., Ding, Y. Q., Ding, W., Reddy, M. S., Dilantha Fernando, W. G., and Du, B. H. 2011. Genetic diversity and phylogeny of antagonistic bacteria against *Phytophthora nicotianae* isolated from tobacco rhizosphere. *Int. J. Mol. Sci.* 12:3055-3071.
- June, C. M., Roach, K., Levett, P. N., and Lavoie, M. C. 2006. Identification of *Streptococcus iniae* by commercial bacterial identification systems. *J. Microbiol. Methods* 67:20-26.
- Kloepper, J. W., Ryu, C. M., and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94:1259-1266.
- Kuhajek, J. M., Jeffers, S. N., Slattery, M., and Wedge, D. E. 2003. A rapid microbioassay for discovery of novel fungicides for *Phytophthora* spp. *Phytopathology* 93:46-53.
- Lee, J. Y., and Hwang, B. K. 2002. Diversity of antifungal actinomycetes in various vegetative soils of Korea. *Can. J. Microbiol.* 48:407-417.
- Lemessa, F., and Zeller, W. 2007. Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. *Biol. Control* 42:336-344.
- McCarter, S. M. 1967. Effect of soil moisture and soil temperature on black shank disease development in tobacco. *Phytopathology* 57:691-695.
- Morgan, M. C., Boyette, M., Goforth, C., Sperry, K. V., and Greene, S. R. 2009. Comparison of the Biolog OmniLog Identification System and 16S ribosomal RNA gene sequencing for accuracy in identification of atypical

- bacteria of clinical origin. J. Microbiol. Methods 79:336-343.
17. Nguyen, M. T., and Ranamukhaarachchi, S. L. 2010. Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. J. Plant Pathol. 92:395-406.
 18. Shahidi, B. G. H., Barkhordar, B., Pakgohar, N., Aghighi, S., Biglary, S., Farrokhi, P. R., Aminaii, M., Mahdavi, M. J., and Aghelizadeh, A. 2006. Biological control of *Phytophthora drechsleri* tucker, the causal agent of pistachio gummosis, under greenhouse conditions by use of actinomycetes. Plant Pathol. J. 5:20-23.
 19. Someya, N., Kataoka, N., Komagata, T., Hirayae, K., Hibi, T., and Akutsu, K. 2000. Biological control of cyclamen soilborne diseases by *Serratia marcescens* strain B2. Plant Dis. 84:334-340.
 20. Stokes, G. W., and Litton, G. C. 1966. Source of back shank resistance in tobacco and host reaction to races 0 and 1 *Phytophthora parasitica* var. *nicotianae*. Phytopathology 56:678-680.
 21. Sun, H. Y., Wang, H. C., Stammler, G., Ma, J. X., and Zhou, M. G. 2010. Baseline sensitivity of populations of *Phytophthora capsici* from China to three carboxylic acid amide (CAA) fungicides and sequence analysis of cholinephosphotranferases from a CAA-sensitive isolate and CAA-resistant laboratory mutants. J. Phytopathol. 158:244-252.
 22. Sunwoo, J. Y., Lee, Y. K., and Hwang, B. K. 1996. Induced resistance against *Phytophthora capsici* in pepper plants in response to DL- β -amino-n-butyric acid. Eur. J. Plant Pathol. 102:663-670.
 23. Yuan, S. K., Liu, X. L., Gu, B. G., Dong, J., Jiang, H., and Si, N. G. 2006. Sensitivity of *Phytophthora infestans* to flumorph: *In vitro* determination of baseline sensitivity and the risk of resistance. Plant Pathol. 55:258-263.
 24. Zhang, X. G., Sun, W. X., and Chang, C. J. 2003. Genetic and pathogenic variation among tobacco black shank strains of *Phytophthora parasitica* var. *nicotianae* from the main tobacco growing in China. J. Phytopathol. 151:259-266.