

# A Nylon Membrane Bag Assay for Determination of the Effect of Chemicals on Soilborne Plant Pathogens in Soil

**Youjian Lin**, Indian River Research and Education Center, University of Florida, Fort Pierce 34945 and Plant Protection College, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China; **Zhenli He**, Indian River Research and Education Center, University of Florida; **Erin N. Roskopf**, United States Horticultural Research Laboratory, Fort Pierce, FL 34945; **Kenneth L. Conn**, Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario N5V 4T3, Canada; **Charles A. Powell**, Indian River Research and Education Center, University of Florida; and **George Lazarovits**, Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada

## ABSTRACT

Lin, Y., He, Z., Roskopf, E. N., Conn, K. L., Powell, C. A., and Lazarovits, G. 2010. A nylon membrane bag assay for determination of the effect of chemicals on soilborne plant pathogens in soil. *Plant Dis.* 94:201-206.

A new nylon membrane bag (NMB) assay was developed for studies to determine the effect of chemicals added to soil on survival of soilborne plant pathogens. The rapid and effective assay can be used to study organisms for which there are no selective media or for which a selective medium is expensive or difficult to prepare. This assay consists of placing pathogens inside a bag made of small-pore (0.22- $\mu\text{m}$ ) nylon filtration membrane, which is placed in soil and later retrieved to determine survival of the pathogens on nonselective media. Chemicals but not other microorganisms can enter the bag from the soil. Using this assay, *Streptomyces scabies*, *Fusarium oxysporum* f. sp. *lycopersici* race 3, and *Ralstonia solanacearum* were successfully recovered from soil after 72 h as demonstrated by growth on a semiselective *Streptomyces* medium (*S. scabies*) or nonselective potato dextrose agar medium (*F. oxysporum* f. sp. *lycopersici* race 3 and *R. solanacearum*) with minimal microbial contamination. Addition of acetic acid (200 mM) to soil killed 100% of *S. scabies*. SPK (a mixture of organic chemicals) at a concentration of 1,500 mg kg<sup>-1</sup> of soil killed 83.3% of *F. oxysporum* f. sp. *lycopersici* race 3 culture plugs, 100% of *F. oxysporum* f. sp. *lycopersici* race 3 spores, and 97.2% of *R. solanacearum* cells. SPK at 1,000 mg kg<sup>-1</sup> of soil killed 50% of *F. oxysporum* f. sp. *lycopersici* race 3 culture plugs, 68.2% of *F. oxysporum* f. sp. *lycopersici* race 3 spores, and 12% of *R. solanacearum*. Benlate (500 to 1,500 mg kg<sup>-1</sup> of soil) did not kill the culture plugs of *F. oxysporum* f. sp. *lycopersici* race 3 but reduced the growth rate of *F. oxysporum* f. sp. *lycopersici* race 3. Benlate (500, 1,000, and 1,500 mg kg<sup>-1</sup> of soil) reduced *F. oxysporum* f. sp. *lycopersici* race 3 spore germination by 39.4, 49.3, and 50.4%, respectively. Streptomycin sulfate (1,500, 800, 400, and 200 mg kg<sup>-1</sup> of soil) caused 75.3, 21, 11.9, and 0.9% mortality, respectively, of *R. solanacearum*.

Screening chemicals, fungicides, bactericides, soil amendments, and biological control organisms for control of soilborne plant pathogens is one of the most active research areas of plant pathology (1,28,33,34). Methods developed for screening control agents for soilborne pathogens include in vitro and in vivo assays (34). In vitro assays include spore germination (11,16); paper disk diffusion, agar diffusion, and radial growth rate on agar plates (11,16,17); and shake-flask, broth microdilution, and microtiter plate assays (2,18,22,26,29). However, the results obtained by in vitro assays, mostly by agar plate assay procedures (16,34), some-

times correlate poorly with those of large-scale in vivo assays in the greenhouse and field (11,25).

In vivo assays take into account soil interactions with control agents and better reflect the eventual effect of control agents on soilborne diseases under field conditions. In vivo assays include soil-pathogen system assays (27,34) and soil-pathogen-plant system assays (17,27,34). Soil-pathogen-plant system assays are generally time consuming and laborious whereas soil-pathogen system assays are rapid and easier. Therefore, soil-pathogen system assays are more suitable for rapidly screening control agents for soilborne pathogens in laboratories (5,11,22,34,39,48). However, most of the soil-pathogen system assays employ autoclaved or sterile soil instead of using nonsterile or natural soil because of contamination problems that affect the analysis of test results.

Autoclaving destroys or changes the physical, chemical, and biological properties of soil that affect the efficacy of a

given control agent. Chemical control materials can undergo various changes in natural soil, including decomposition, which may enhance or reduce control of soilborne pathogens. In addition, control agents can be affected by numerous factors, including but not limited to soil temperature (6,8), aeration (24), microbial populations, pH (8,41,43,46), organic matter (21,24), clay content (40), cation exchange capacity (24), and moisture (24,39,44,45).

Much effort has been put into developing tools to determine the efficacy of control agents in natural soil (16,17,26). Target organisms can be detected in soil by use of selective media (14,16,23,42), burial and retrieval in nylon net bags (9), or polymerase chain reaction (PCR; 35,47) and immunoassays (1,2,12,19,20,31,32,38). However, there are limits to these assays. For example, PCR detects DNA from soil that may lead to the quantification of dead organisms, and selective media may not be available for target organisms or may be difficult or costly to prepare. Nylon net bag assays with selective media have been successfully used to determine the efficacy of chemicals and soil amendments on soilborne pathogens such as *Phytophthora* spp., *Sclerotium cepivorum*, and *Verticillium dahlia* in natural soil (4,7,8,42). This approach is most useful if selective media are available because microorganisms from soil can enter the bags through the pores. Thus, there is a need to develop assays that can rapidly and effectively determine the effect of chemicals added to soil on soilborne pathogens for which there are no selective media available.

In this article, we describe a nylon membrane bag (NMB) assay procedure which is different from nylon net bag assays in that small-pore (0.22- $\mu\text{m}$ ) nylon filtration membranes were used instead of large-pore (5- to 20- $\mu\text{m}$ ) nylon net. Using this NMB assay, various combinations of four chemicals (acetic acid, benomyl, streptomycin sulfate, and SPK [a proprietary mixture of organic chemicals]), two soils, and three soilborne pathogens (*Streptomyces scabies*, *Ralstonia solanacearum*, and *Fusarium oxysporum* f. sp. *lycopersici*)

Corresponding author: C. A. Powell  
E-mail: capowell@ufl.edu

Accepted for publication 4 October 2009.

doi:10.1094/PDIS-94-2-0201  
© 2010 The American Phytopathological Society

sici) were tested. Assessment of pathogen survival from the NMB assay was performed using nonselective or semiselective media.

## MATERIALS AND METHODS

**Soils.** Soil was collected at 0 to 15 cm of depth from a commercial potato field in Ontario, Canada (site G) and from a vegetable field in St. Lucie County, FL (site F). Site G soil was a sandy loam, Luvisolic, with a pH of 7.1 and organic carbon content of 12 kg<sup>-1</sup> of soil. Site F soil was sandy, siliceous, hyperthermic, Arenic, Glossaqualf with a pH of 7.6 and organic carbon content of 9.06 g kg<sup>-1</sup> of soil. Soils were air dried, passed through a 2-mm sieve, and stored at room temperature (24°C) prior to use. The water content of the soils was gravimetrically adjusted to 10% by adding deionized water before use. Then, the soils were weighed and treated with chemicals in the tests.

**Preparation of *S. scabiei* inoculum.** An isolate of the soilborne plant-pathogenic bacterium, *S. scabiei* strain SP, isolated from soil in Ontario, Canada (9) was used in this study. Spores from 2-week-old cultures grown on yeast malt extract (YME) agar medium were scraped off of the plate into sterile deionized water. The spore suspension was passed through a glass-wool filter to remove any excess growth media and finally suspended in 30 ml of sterile deionized water to get a uniform concentration prior to use. The con-

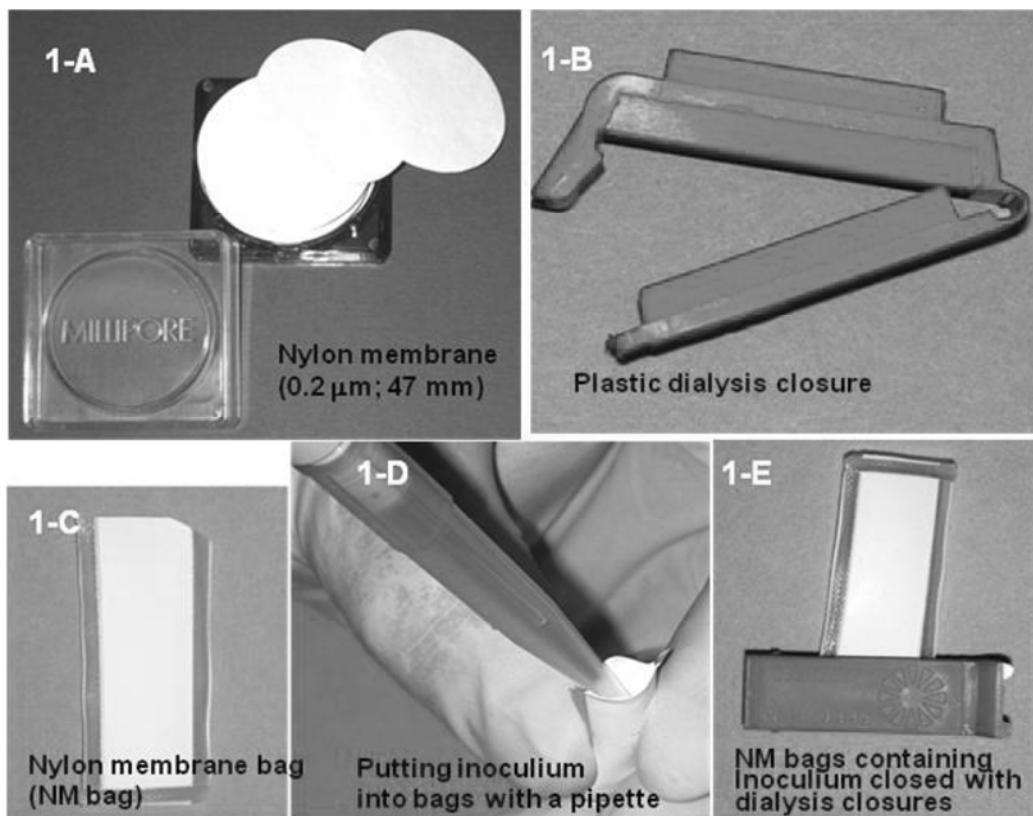
centration of spores was estimated by measuring absorbance at 590 nm. Viability of spores after exposure to chemicals in G site soil was determined by culturing on semiselective *Streptomyces* medium (STR medium) (3,9).

**Preparation of *F. oxysporum* f. sp. *lycopersici* inoculum.** An isolate (no. 9602) of the soilborne plant-pathogenic fungus *F. oxysporum* f. sp. *lycopersici* race 3 (3), obtained from an infected tomato plant in a commercial production field in Saint Lucie County, FL, was used in this study. The culture of *F. oxysporum* f. sp. *lycopersici* race 3 was recovered from sterile soil tubes and grown on potato dextrose agar (PDA) medium (Difco Becton, Dickinson and Co., Sparks, MD) for 10 to 15 days. Agar plugs, consisting of a mixture of mycelia and spores of *F. oxysporum* f. sp. *lycopersici* race 3, were cut out of the cultures with a cork borer (1.0 cm in diameter). The culture plugs were completely dried by airflow in a Safearf class II safety cabinet for 12 to 24 h before use. The spore suspension, prepared by adding 15 ml of sterile deionized water to a culture plate and scraping off spores, was ready for use. The resulting spore suspension contained a mixture of micro- and macroconidia. The spore suspension was passed through a glass-wool filter to remove any excess growth medium and large pieces of mycelium. Uniform concentrations of spore suspension were used for all the treatments in each experiment. Viability of spores and

mycelia after exposure to chemicals in F site soil was determined by culturing on PDA and treatments were compared with an untreated control (4).

**Preparation of *R. solanacearum* inoculum.** An isolate of the soilborne plant-pathogenic bacterium, *R. solanacearum* (race 1, biovar 1; tomato strain Rs5), isolated in Quincy, FL (36,37) was used in this study. *R. solanacearum* was grown at 28°C either on casamino acid peptone glucose (CPG) agar medium (10 g of peptone, 1 g of casamino acids, 2.5 g of dextrose, 15 g of agar, and 1 liter of deionized water) for 48 hours or in CPG broth on a shaker (200 rpm) for 18 h (overnight; 36). Bacterial cells from 18-h CPG-broth culture were diluted with sterile deionized water prior to use. The concentration of the cells in the suspension was estimated by measuring absorbance at 590 nm. Viability of *R. solanacearum* after exposure to chemicals in F site soil was determined by culturing on PDA.

**Chemicals.** Chemicals added to the soils included glacial acetic acid (99.8%, Fisher Scientific), a broad-spectrum antimicrobial chemical that can kill soilborne plant pathogens (10,43); benomyl (Benlate 50% WP; DuPont, Wilmington, DE), a broad-spectrum systemic fungicide (13); streptomycin sulfate (Sigma-Aldrich, St. Louis), an aminoglycoside antibiotic for controlling bacterial diseases of crops (30); and SPK, a formulation of organic chemicals recently developed in our laboratory



**Fig. 1.** Illustration of materials and main steps for using a nylon membrane bag (NMB) assay. **A**, Nylon membrane; **B**, dialysis closure; **C**, prepared NMBs; **D**, placement of spore suspension into NMBs; and **E**, closure of NMBs with a dialysis closer.

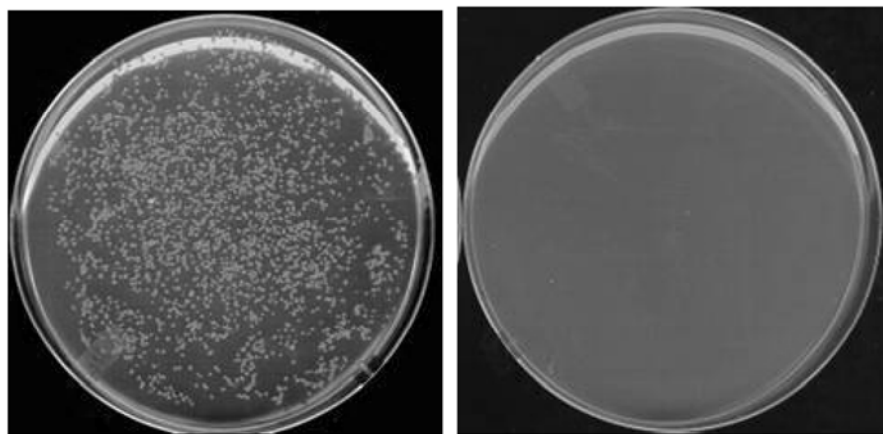
for controlling soilborne plant pathogens (Indian River Research and Education Center, University of Florida and United States Horticultural Research Laboratory, United States Department of Agriculture–Agricultural Research Service, patent pending; details of the composition can be made available to other investigators upon signing nondisclosure letter). The NMB assay was used to test toxicity of acetic acid against *S. scabies*, benomyl against *F. oxysporum* f. sp. *lycopersici* race 3, streptomycin against *R. solanacearum*, and SPK against both *F. oxysporum* f. sp. *lycopersici* race 3 and *R. solanacearum*. The concentration of acetic acid used was 200 mM in the water component of the soil. To achieve this, 0.5 ml of acetic acid (4.2 M) was added to 99.5 g of soil, giving a final moisture content of 10%. The concentrations used were benomyl at 500, 1,000, and 1,500 mg kg<sup>-1</sup> of soil; streptomycin sulfate at 200, 400, 800, and 1,500 mg kg<sup>-1</sup> of soil; and SPK at 500, 1,000, and 1,500 mg kg<sup>-1</sup>

of soil. Each trial contained an untreated check with no chemical treatment for comparison of viability or mortality.

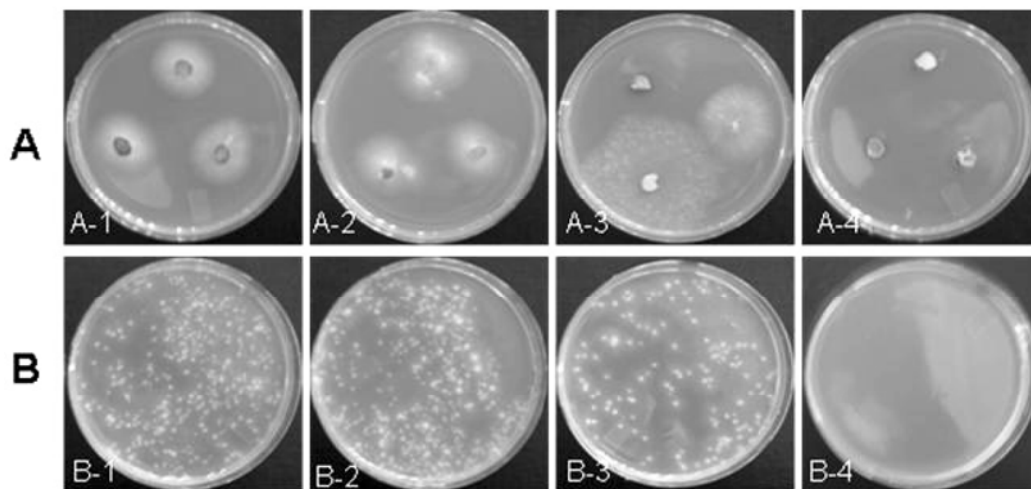
**Construction of NMBs.** NMBs (8 by 30 mm) were made of Millipore nylon hydrophilic membrane filter discs (0.22- $\mu$ m pore size and 47 mm in diameter, Millipore Corporation, Billerica, MA) and dialysis closures (23-mm width; Spectrum Laboratories, Inc.; Fig. 1A and B). An electron bag sealer (Daigger Lab Supplies, Vernon Hills, IL) was used to seal a section of the folded nylon-membrane disk in a rectangular shape for a final size of 4 by 0.8 cm. One of the two short-side edges was left unsealed (open) for placement of the pathogen suspensions or plugs (Fig. 1C).

**NMB assay procedure.** Effect of the various chemicals on *S. scabies*, *F. oxysporum* f. sp. *lycopersici* race 3, or *R. solanacearum* was determined by the NMB assay. Cell suspensions (200  $\mu$ l) of *S. scabies* or *R. solanacearum* or air-dried culture plugs (consisting of mycelia and

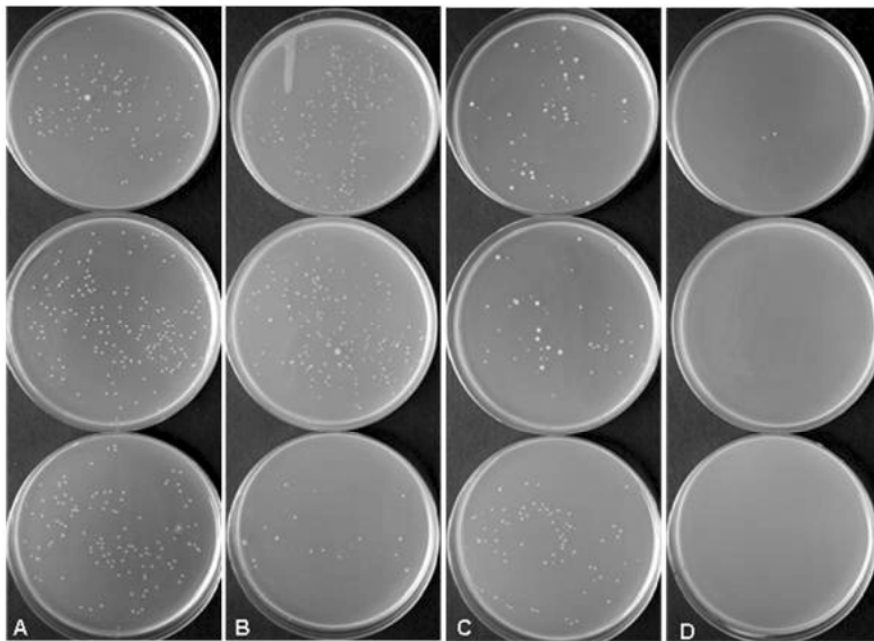
spores, 1 plug per bag) or spore suspensions (200  $\mu$ l) of *F. oxysporum* f. sp. *lycopersici* race 3 were placed into NMBs (Fig. 1D). Bags were completely sealed with dialysis closures (Fig. 1E) and stored less than 3 h in a refrigerator at 10°C prior to use. The water content of the soils was adjusted to 10% by weight by adding deionized water before the soils were weighed into 77-by-77-by-97-mm tissue culture vessels (150 g of soil in each vessel; Magenta GA-7 vessels; Carolina Biological Supply Company, Burlington, NC). The chemical solutions (6 ml each) with different concentrations were added to the soil samples in the vessels and mixed well using a stainless steel spatula. Then, the NMBs containing the pathogen propagules were buried into soil in the vessels. The treated vessels were incubated in the lab at room temperature (mean 24°C) for 72 h (5). The NMBs were removed from the soil in the vessels after 72 h and rinsed for 3 min with tap water followed by 3 min with sterile water. Any soil particles or other debris attached to the NMBs were removed by brushing the bags with a soft brush during the rinse. The culture plugs of *F. oxysporum* f. sp. *lycopersici* race 3 in the bags were transferred with sterile forceps onto PDA medium under a NuAire biological safety cabinet (class II, type A/B3, model Nu-425-600; NuAire Inc., Plymouth, MN). The spores of *S. scabies* and *F. oxysporum* f. sp. *lycopersici* race 3 and cells of *R. solanacearum* were recovered by cutting the washed NMB into small pieces and placing them in sterile glass test tubes under the safety cabinet, then adding sterile water to resuspend the spores or cells. After a serial dilution in sterile deionized water, the spore suspensions of *S. scabies* were spread on STR medium (6) and spores of *F. oxysporum* f. sp. *lycopersici* race 3 and cells of *R. solanacearum* were spread on PDA plates.



**Fig. 2.** Colonies of *Streptomyces scabies* growing on *Streptomyces* medium after a 72-h exposure in a nylon membrane bag to untreated natural soil (left) or soil amended with acetic acid (200 mM in water component of soil, right). The soil was a sandy-loam soil from a commercial potato field in Ontario, Canada.



**Fig. 3.** Growth from culture plugs and micro- and macroconidia of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on potato dextrose agar after a 72-h exposure in a nylon membrane bag to natural soil amended with SPK (a novel mixture of organic chemicals). **A**, Growth of *F. oxysporum* f. sp. *lycopersici* race 3 from culture plugs in 1, untreated control or SPK at 2, 500; 3, 1,000; or 4, 1,500 mg kg<sup>-1</sup> of soil. **B**, Growth of *F. oxysporum* f. sp. *lycopersici* race 3 from spores in the treatments 1, untreated control or SPK at 2, 500; 3, 1,000; or 4, 1,500 mg kg<sup>-1</sup> of soil.



**Fig. 4.** Colonies of *Ralstonia solanacearum* (race 1, biovar 1; tomato strain Rs5) growing on potato dextrose agar after a 72-h exposure to soil amended with SPK (mixture of organic chemicals) using a nylon membrane bag assay. Soil was a sandy, siliceous, hyperthermic, Arenic, Glossaqualf from a vegetable field in Florida. **A**, Untreated control or SPK at **B**, 500; **C**, 1,000; or **D**, 1,500 mg kg<sup>-1</sup> of soil.

The plates were incubated at room temperature and examined after 3 and 7 days to determine the survival of the microbial propagules. The effectiveness of the chemicals on the pathogens was determined based on the viability of the pathogens. The experiments were conducted twice for the chemicals, pathogens, and soils. For *S. scabies*, three NMBs were placed into each of three vessels per treatment for each experiment. For *F. oxysporum* f. sp. *lycopersici* race 3 and *R. solanacearum*, there were three NMBs for each treatment in each experiment. The treatments, treated and untreated soil, and pathogen propagules in the experiment were randomized completely in the use of materials such as vessels and pathogen propagules prepared in nylon bags and the placement of vessel arrangement in incubation. The significant difference of the data was analyzed by a *t* test. Also, statistical regression analysis of data was conducted using SigmaPlot 10.0 (SPSS Inc., Richmond, CA).

## RESULTS

Very little microbial contamination resulted from the NMB assay for the pathogens tested (Figs. 2, 3, and 4). Even on a nonselective medium such as PDA, few nontarget organisms were observed (Fig. 3).

**Effect of acetic acid on *S. scabies* in soil.** A 72-h exposure of *S. scabies* to acetic acid in soil resulted in almost 100% mortality (Table 1; Fig. 2). Very little microbial contamination was observed on the agar medium (Fig. 2) (8). *S. scabies* colonies grew from the control treatment and

almost nothing grew from the acetic acid treatment.

### Effect of SPK on *F. oxysporum* f. sp. *lycopersici* and *R. solanacearum* in soil.

After a 72 h of incubation, SPK at the concentration of 1,500 mg kg<sup>-1</sup> of soil killed 83.3% of *F. oxysporum* f. sp. *lycopersici* race 3 culture plugs (Table 2), 100% of *F. oxysporum* f. sp. *lycopersici* race 3 spores (Table 2), and 97.2% of *R. solanacearum* cells (Table 3) in the NMBs placed in soil. SPK at 1,000 mg kg<sup>-1</sup> of soil killed 50% of *F. oxysporum* f. sp. *lycopersici* race 3 culture plugs (Table 2), 68.2% of *F. oxysporum* f. sp. *lycopersici* race 3 spores (Table 2), and 12% of *R. solanacearum* (Table 3). SPK at 500 mg kg<sup>-1</sup> did not kill *F. oxysporum* f. sp. *lycopersici* race 3 culture plugs or spores (Table 2) or *R. solanacearum* (Table 3). The effects of SPK concentrations on survival of *F. oxysporum* f. sp. *lycopersici* race 3 and *R. solanacearum* in soil were significant. The regression equation of *F. oxysporum* f. sp. *lycopersici* race 3 spore mortality (*y*) and the SPK concentration (*x*) was  $y = 3E - 05x^2 + 0.0282x - 4.975$  ( $R^2 = 0.9334$ ;  $P < 0.01$ ), and the regression equation of *R. solanacearum* mortality (*y*) and the SPK concentration (*x*) was  $y = 8E - 05x^2 - 0.066x + 3.1875$  ( $R^2 = 0.969$ ;  $P \leq 0.01$ ).

**Effect of benlate on *F. oxysporum* f. sp. *lycopersici* in soil.** After 72 h of incubation, benlate at the concentrations of 500 to 1,500 mg kg<sup>-1</sup> of soil reduced growth from but did not kill the culture plugs of *F. oxysporum* f. sp. *lycopersici* race 3 (Table 2). The average colony diameter of *F. oxysporum* f. sp. *lycopersici* race 3 in un-

**Table 1.** Survival of *Streptomyces scabies* in a nylon membrane bag (NMB) after burial in soil treated with acetic acid<sup>a</sup>

Treatments	No. of colonies	Mortality (%)
Trial I		
Untreated	11,000 ± 1,400	...
Acetic acid	27 ± 17	99.8
Trial II		
Untreated	33,000 ± 3,000	...
Acetic acid	0.0 ± 0.0	100

<sup>a</sup> NMBs containing *S. scabies* were placed into vessels containing untreated soil or soil treated with acetic acid (200 mM in water component of soil). Soil was a sandy-loam soil from a commercial potato field in Ontario, Canada. Bags were removed after 72 h and plated onto *Streptomyces* medium. Number of colonies are means ± standard error (*n* = 9).

treated controls was 3.3 ± 0.4 cm, while those treated with 500, 1,000, and 1,500 mg kg<sup>-1</sup> of soil were 2.3 ± 0.6, 2.3 ± 0.4, and 2.2 ± 0.4 cm, respectively (*n* = 6). Benlate killed spores of *F. oxysporum* f. sp. *lycopersici* race 3 in soil (Table 2). The mortality of spores was 50.4% at 1,500 mg kg<sup>-1</sup> of soil. Statistical analysis indicated that mycelium diameter and conidial survival differed between the control and benlate treatments (*t* test,  $P = 0.05$ ) but not among the different benlate concentrations. The regression equation of the *F. oxysporum* f. sp. *lycopersici* race 3 spore mortality (*y*) and the benlate concentration (*x*) was  $y = -4E - 05x^2 + 0.0897x + 1.035$  ( $R^2 = 0.9873$ ).

**Effect of streptomycin on *R. solanacearum* in soil.** The toxicity of streptomycin on *R. solanacearum* increased with its concentration in soil after a 72-h exposure (Table 3). The highest mortality, 75.3%, of *R. solanacearum* occurred in the soil treated with streptomycin at 1,500 mg kg<sup>-1</sup> of soil. The mortality of *R. solanacearum* among the control and treatments of streptomycin at concentrations ranging from 400 to 1,500 mg kg<sup>-1</sup> of soil was significantly different (*t* test,  $P = 0.01$ ) but not between the control and the treatment of streptomycin at the 200 mg kg<sup>-1</sup> of soil concentration ( $P = 0.05$ ). The regression equation of the *R. solanacearum* mortality (*y*) and the streptomycin concentration (*x*) in soil was  $y = 3E - 05x^2 + 0.0068x + 0.318$  ( $R^2 = 0.9927$ ).

## DISCUSSION

The new NMB assay can be used to rapidly and effectively determine the effect of chemicals added to soil on survival of soilborne plant pathogens. This assay consists of placing pathogens inside a bag made of small-pore (0.22-μm) nylon filtrate membrane which is placed in soil and later retrieved to determine survival of the pathogens on nonselective or semiselective media. A novel component of this assay is that chemicals but not microorganisms can enter the bag from the soil. *S. scabies*, *F.*

**Table 2.** Mortality of *Fusarium oxysporum* f. sp. *lycopersici* micro- and macroconidia pores and mycelia in nylon membrane bags (NMBs) after burial in soil treated with benlate and SPK (a novel mixture of organic chemicals)<sup>a</sup>

Treatments (mg kg <sup>-1</sup> of soil)	No. of viable mycelium plugs/no. of plugs tested	No. of colonies from spores	Spore mortality (%)
Untreated	6/6	245,000 ± 45,000	0.0
Benlate			
500	6/6	148,500 ± 15,000	39.4
1,000	5/6	124,000 ± 7,000	49.3
1,500	6/6	12,1500 ± 3,500	50.4
SPK			
500	6/6	240,800 ± 28,900	1.7
1,000	3/6	177,900 ± 11,500	68.2
1,500	1/6	0	100.0

<sup>a</sup> NMBs containing of *F. oxysporum* f. sp. *lycopersici* were placed into vessels containing untreated soil or soil treated with benlate or SPK. Soil was a sandy, siliceous, hyperthermic, Arenic, Glossaqualf soil from a vegetable field in Florida. Bags were removed after 72 h and plated onto potato dextrose agar. Number of colonies are means ± standard error from two experiments ( $n = 6$ ).

*oxysporum* f. sp. *lycopersici* race 3, and *R. solanacearum* were successfully recovered from soil after 72 h, as demonstrated by growth on a semiselective STR medium (*S. scabies*) or nonselective PDA medium with minimal microbial contamination. This assay was especially useful for testing the effect of chemicals on fungal mycelia in soil because the fungal mycelia (culture plugs) could be easily transferred onto nonselective media, such as the PDA used in this test, to determine viability.

In this study, only nonselective PDA medium or semiselective STR media were used to determine the viability of pathogens after they were exposed to chemicals in soil. However, we believe that, if the NMB assay is combined with selective media, it may become an even more powerful and useful assay for testing the effect of control agents on different kinds of soilborne pathogens. This assay provides a tool that can be used in native, unpasteurized soils; thus, the test results may more closely reflect the efficacy of the control agents in the field. However, there are some factors that may affect the assay results. The NMBs must be checked for leakage after organisms are added and the bags are sealed. Leaking may cause contamination and affect test results. The second is the rinsing of NMBs. We found that rinsing the NMBs with running tap water and with sterile deionized water and brushing the surface of the bags with a soft brush during the rinse, then immediately placing the clean NMBs into sterile containers until they are opened under a safety cabinet, can reduce the risk of contamination.

Some of the results obtained by the NMB assay were different from previous reports. For example, benlate at concentrations ranging from 500 to 1,500 mg kg<sup>-1</sup> of soil did not kill *F. oxysporum* f. sp. *lycopersici* race 3, and streptomycin at concentrations ranging from 200 to 1,500 mg kg<sup>-1</sup> of soil did not completely kill *R. solanacearum*. Previous work using agar plate assays or field tests suggested that benlate is effective for control of *Fusarium*

spp. (15) and streptomycin is deleterious to *Ralstonia* spp. (30). The results obtained by in vitro assays in previous reports may not show the direct or indirect effects of the chemicals on the pathogens when applied to natural soil. The NMB assay may include direct effects of the test chemical and effects mediated by factors present in natural soil. The lack of effect may also be strain specific, because the baseline sensitivity of these isolates to the test materials was not characterized. Second, the results in our tests were obtained after a 72-h interaction between the control agents and pathogens in soil, whereas previous field tests were conducted over several months. Additionally, the membrane may affect the exposure of the pathogens to the chemicals in soil due to the small size of pores. The suitability of the NMB assay for testing effects of longer interactions between the chemicals and pathogens in soil is unknown.

Prior to this study, toxicity of acetic acid added to soil to had not been demonstrated for *S. scabies*. A previous study demonstrated with a nylon net bag assay that acetic acid kills *V. dahliae* in soil (10). The results of the NMB assay confirmed that acetic acid can kill *S. scabies* in soil and may partially explain the fact that liquid swine manure, which contains acetic acid, can reduce potato scab caused by *S. scabies* (7)

Although this NMB assay allows the study of interactions between chemicals in soil and pathogens, it cannot take into account potential interactions between the chemicals, microorganisms in soil but outside the bags, and pathogens in the bags. Sublethal doses of chemicals may not kill the pathogens but may stimulate or suppress the growth of other organisms which, in turn, could affect pathogen growth, survival, or ability to cause disease.

In summary, the NMB assay is a rapid, reliable, and effective assay for evaluating the effect of chemicals on soilborne pathogens in natural soil. The NMB assay can be used to investigate the interactions be-

**Table 3.** Mortality of *Ralstonia solanacearum* (race 1, biovar 1; tomato strain Rs5) in nylon membrane bags (NMBs) after burial in soil treated with streptomycin sulfate and SPK (a novel mixture of organic chemicals)<sup>a</sup>

Treatments (mg kg <sup>-1</sup> of soil)	No. of colonies	Mortality (%)
Untreated	258,000 ± 32600	...
Streptomycin		
200	256,000 ± 16,500	0.9
400	227,000 ± 78,100	11.9
800	204,000 ± 82,900	21.6
1,500	63,800 ± 42,300	75.3
SPK		
500	236,200 ± 11,800	0.85
1,000	227,300 ± 12,800	12.0
1,500	7,200 ± 5,200	97.2

<sup>a</sup> NMBs containing *R. solanacearum* (Race 1, biovar 1; tomato strain Rs5) were placed into vessels containing untreated soil or soil treated with streptomycin sulfate or SPK. Soil was a sandy, siliceous, hyperthermic, Arenic, and Glossaqualf soil from a vegetable field in Florida. Bags were removed after 72 h and plated onto potato dextrose agar. Number of colonies are means ± standard error from two experiments ( $n = 6$ ).

tween soil properties and the efficacy of chemicals in the control of soilborne pathogens in soil. For example, the effect of moisture, temperature, pH, and concentrations of related specific elements on the efficacy of chemicals on soilborne pathogens can be examined using this assay. In addition, this assay can be used to determine the duration of chemical activity in soil in the laboratory.

#### ACKNOWLEDGMENTS

We thank T. M. Momol and P. Ji for providing the isolate of *R. solanacearum* (race 1, biovar 1; tomato strain Rs5) and M. A. Ritenour and C. Ohs for their critical comments with regard to the preparation of the manuscript.

#### LITERATURE CITED

- Boam, M., and Boland, G. J. 2000. Evaluation of polyclonal-antibody-based immunoassays for detection of *Sclerotinia sclerotiorum* on canola petals, and prediction of stem rot. *Can. J. Microbiol.* 46:723-729.
- Cahill, D. M., and Hardham, A. R. 1994. A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soil. *Phytopathology* 84:1284-1292.
- Chellemi, D. O., Dankers, H. A., and Crosier, B. 1992. First Report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on tomato in North-west Florida and Georgia. *Plant Dis.* 76:861.
- Chellemi, D. O., Olson, S. M., and Mitchell, D. J. 1994. Effect of soil solarization and fumigation on survival of soilborne pathogens of tomato in northern Florida. *Plant Dis.* 78:1167-1172.
- Chinn, S. H. F., and Ledingham, R. J. 1962. A laboratory method for testing the fungicidal effect of chemicals on fungal spores in soil. *Phytopathology* 52:1041-1044.
- Coley-Smith, J. R. 1990. White rot disease of *Allium*: problems of soil-borne diseases in microcosm. *Plant Pathol.* 39:214-222.
- Conn, K. L., and Lazarovits, G. 1999. Impact of animal manures on Verticillium wilt, potato scab, and soil microbial populations. *Can. J. Plant Pathol.* 21:81-92.

8. Conn, K. L., and Lazarovits, G. 2000. Soil factors influencing the efficacy of liquid swine manure added to soil to kill *Verticillium dahliae*. *Can. J. Plant Pathol.* 22:400-406.
9. Conn, K. L., Leci, E., Kritzman, G., and Lazarovits, G. 1998. A quantitative method for determining soil populations of *Streptomyces* and differentiating potential potato scab-inducing strains. *Plant Dis.* 82:631-638.
10. Conn, K. L., Tenuta, M., and Lazarovits, G. 2005. Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acid, nitrous acid, and ammonia toxicity. *Phytopathology* 95:28-35.
11. Corden, M. E., and Young, R. A. 1961. Evaluation of eradicant soil fungicides in the laboratory. *Phytopathology* 52:503-509.
12. De Cal, A., Martinez-Treceno, A., Salto, T., Lopez-Aranda, J. M., and Melgarejo, P. 2005. Effect of chemical fumigation on soil fungal communities in Spanish strawberry nurseries. *Appl. Soil Ecol.* 28:47-56.
13. Edgington, L. V., Khew, K. L., and Barron, G. L. 1971. Fungi toxic spectrum of benzimidazole compounds. *Phytopathology* 61:42-48.
14. Engelbrecht, M. C. 1994. Modification of a semi-selective medium for the isolation and quantification of *Pseudomonas solanacearum*. *ACIAR Proc.* 10:3-5.
15. Evens, S. G. 1976. Evaluation of benomyl soil drenches for the control of *Verticillium* and *Fusarium* wilt of Carnations. *Plant Pathol.* 25:81-84.
16. Fang, Z. D. 1979. *Methods for Research on Plant Diseases*. Agriculture Press, Beijing.
17. Forster, H., Kanetis, L., and Adaskaveg, J. E. 2003. Spiral gradient dilution, a rapid method for determining growth response and 50% effective concentration values in fungus-fungicide interactions. *Phytopathology* 94:163-170.
18. Gadd, G. M. 1986. Toxicity screening using fungi and yeast. Pages 43-77 in: *Toxicity Testing Using Microorganisms*, Vol. II. B. J. Dutka and G. Bitton, eds. CRC Press, Boca Raton, FL.
19. Gan, Z., Marquardt, R. R., Abramson, D., and Clear, R. M. 1997. The characterization of chicken antibodies raised against *Fusarium* spp. by enzyme-linked immunosorbent assay and immunoblotting. *Int. J. Food Microbiol.* 38:191-200.
20. Gautam, Y., Cahill, D M., and Hardham, A. R. 1999. Development of a quantitative immunodipstick assay for *Phytophthora nicotianae*. *Food Agric. Immunol.* 11:229-242.
21. Gottlieb, D., Siminoff, P., and Martin, M. M. 1952. The production and role of antibiotics in soil. IV. Actidione and clavacin. *Phytopathology* 42:493-496.
22. Hadacek, F., and Gregr, H. 2000. Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochem. Anal.* 11:137-147.
23. Hawke, M. A., and Lazarovits, G. 1994. Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. *Phytopathology* 84:883-890.
24. Kaufman, D. D. 1977. Soil-fungicide interactions. Pages 1-49 in: *Antifungal Compounds*. Vol. II Interactions in Biological and Ecological Systems. M. R. Siegel and H. D. Sisler, eds. Marcel Dekker, Inc., New York.
25. Knight, S. C. A., Anthony, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schulz, M. A., Spinks, C. A., Worthington, P. A., and Youle, D. 1997. Rationale and perspectives on the development of fungicides. *Annu. Rev. Phytopathol.* 35:349-372.
26. 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* 94:46-53.
27. Latham, A. J., and Linn, M. B. 1968. A comparison of soil column and petri dish techniques for the evaluation of soil fungitoxicants. *Phytopathology* 58:460-463.
28. Lazarovits, G., Tenuta, M., and Conn, K. L. 2001. Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops. *Australas. Plant Pathol.* 30:111-117.
29. Major, J. S. 1995. Current screening practices in the pharmaceutical industry. *Proc. Briton Crop. Weeds* 1:89-96.
30. McManus, P. S., Stockwell, V. O., Sundin, G. W., and Jones, A. L. 2002. Antibiotic used in plant agriculture. *Annu. Rev. Phytopathol.* 40:443-465.
31. Miller, S. A., Bhat, R. G., and Schmitthenner, B. 1994. Detection of *Phytophthora capsici* in pepper and cucurbit crops in Ohio with two commercial immunoassay kits. *Plant Dis.* 78:1042-1046.
32. Miller, S. A., Madden, L. V., and Schmitthenner, B. 1997. Distribution of *Phytophthora* spp. in field soils determined by immunoassay. *Phytopathology* 87:101-107.
33. Noling, J. W. 2002. The practical realities of alternatives to methyl bromide: concluding remarks. *Phytopathology* 92:1373-1375.
34. Pelletier, E. N. 1977. Detecting potential protective and systemic antifungal compounds. Pages 51-68 in: *Antifungal Compound*. Vol. I. Discovery, Development, and Uses. M. R. Siegel and H. D. Sisler, eds. Marcel Dekker, Inc., New York.
35. Poussier, S., Cheron, J., Couteau, A., and Luisetti, J. 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. *J. Microbiol. Methods* 51:349-359.
36. Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., and Jones, J. B. 2005. Application of acibenzolar-s-methyl enhances host resistance in tomato. *Plant Dis.* 89:989-993.
37. Pradhanang, P. M., and Momol, M. T. 2001. Survival of *Ralstonia solanacearum* in soil under irrigated rice culture and aquatic weeds. *J. Phytopathol.* 149:707-711.
38. Robold, A. V., and Hardham, A. R. 1998. Production of species-specific monoclonal antibodies that react with surface components on zoospores and cysts of *Phytophthora nicotianae*. *Can. J. Microbiol.* 44:1161-1170.
39. Saeed, I. A. M., Rouse, D. I., Harkin, J. M., and Smith, K. P. 1997. Effects of soil water content and soil temperature on efficacy of metham-sodium against *Verticillium dahliae*. *Plant Dis.* 81:773-776.
40. Sanchez-Martin, M., Andrades, M. S., and Sanchez-Camazano, M. 2000. Soil properties influencing the adsorption and mobility of penconazole in vineyard soils. *Soil Sci.* 165:951-960.
41. Sheng, G., Yang, Y., Huang, M., and Yang, K. 2005. Influence of pH on pesticide sorption by soil containing wheat residue-derived char. *Environ. Pollut.* 134:457-463.
42. Smolinska, U. 2000. Survival of *Sclerotium cepivorum* sclerotia and *Fusarium oxysporum* chlamydospores in soil amended with cruciferous residues. *J. Phytopathol.* 148:343-349.
43. Tenuta, M., Conn, K. L., and Lazarovits, G. 2002. Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. *Phytopathology* 92:548-552.
44. Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soil—a review. *J. Plant Nutr. Soil Sci.* 166:145-167.
45. Walker, A. 1987. Further observation on the enhanced degradation of iprodione and vinclozolin in soil. *Pestic. Sci.* 21:219-231.
46. Warton, B., and Matthiessen, J. N. 2005. The crucial role of calcium interacting with soil pH in enhanced biodegradation of metam-sodium. *Pest Manage. Sci.* 61:856-862.
47. Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., and Stead, D. E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol.* 66:2853-2858.
48. Zentmyer, G. A. 1955. A laboratory method for testing soil fungicides with *Phytophthora cinnamomi* as test organism. *Phytopathology* 45:398-404.