

## Biological Control of Septoria Leaf Spot of Poplar by *Phaeotheca dimorphospora*

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### ABSTRACT

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*Phaeotheca dimorphospora*, a Deuteromycotina fungus found to be antagonistic in vitro against *Septoria musiva* and other tree pathogens, was tested for its ability to suppress Septoria leaf spot on poplar. In an in vitro leaf disk assay, *P. dimorphospora* significantly reduced the size of the necrotic area caused by *S. musiva* on *Populus tremuloides*, *P. grandidentata*, and *P. × berolinensis*. In the greenhouse, both pre- and posttreatment of cuttings of *P. × berolinensis* with *P. dimorphospora* resulted in a significant reduction in the severity and in the rate of development of Septoria leaf spot. *P. dimorphospora* was reisolated from poplar leaves 1 mo after inoculation. Application of *P. dimorphospora* culture filtrate and its medium extract on the foliage of poplar also reduced disease severity.

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Septoria leaf spot and stem canker, caused by *Septoria musiva* Peck, has devastated many young poplar plantations across North America (6,10). In Canada, this pathogen attacks many *Populus* species, especially hybrid poplars (11). Control of Septoria leaf spot

and canker by chemicals and cultural methods is expensive and efficiency is low (5). Planting clones with a high level of resistance to the pathogen was suggested as the best preventive management strategy (6). However, pathogens, insects, and abiotic factors need to be considered together, making it difficult and time-consuming to find a suitable clone. Introducing natural parasitic or saprophytic microorganisms that possess antagonistic characteristics to pathogens

and pests of poplars could be a valuable method for control of this disease.

A recently described fungal species, *Phaeotheca dimorphospora* DesRochers & Ouellette, isolated from elm wood, was shown to have antibiotic activity against the Dutch elm disease pathogen, *Ophiostoma ulmi* (Buisman) Nannf., in vitro (2,3). Further investigation of inhibition of a wide range of phytopathogenic fungi by *P. dimorphospora* showed that this fungus strongly inhibited most species tested, especially *S. musiva* (14). Therefore, *P. dimorphospora* appears to have potential as a biological control agent against Septoria leaf spot and stem canker.

In this study we examined the possibility of treating poplar foliage with *P. dimorphospora* or its metabolites to suppress leaf spots caused by *S. musiva* under laboratory and greenhouse conditions.

### MATERIALS AND METHODS

**Preparation of fungal spore suspensions.** Conidia of *S. musiva* (CLF-1326)

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were produced on a sporulation medium containing 180 ml of V8 juice, 2 g of CaCO<sub>3</sub>, 20 g of Bacto agar, and 800 ml of distilled water (7), under continuous light at 20 C. A conidial suspension was obtained from 10-day-old cultures by flooding them with sterile distilled water and gently agitating them with a glass rod. The conidial suspension was adjusted to a concentration of  $1 \times 10^6$  conidia per milliliter of water after filtering through four layers of cheesecloth. To estimate spore viability, 50  $\mu$ l of fresh conidial suspension was applied on a slide coated with 200  $\mu$ l of 0.5% water agar. Slides were incubated in a moisture chamber at 20 C with continuous light. Spore germination percentage was determined by observing at least 300 spores in each of three replications. A spore was considered germinated when the germ tube was approximately one-half the length of the spore.

Cultures of *P. dimorphospora* (CRBF-9166) were grown on potato-dextrose agar (PDA) and incubated at 23 C for 14 days in darkness. To induce the production of microconidia, cultures were transferred to an agar medium containing 1 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of KNO<sub>3</sub>, 0.5 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose, and 15 g of agar in 1 L of distilled water (4). The culture dishes were incubated at 20 C with continuous light for 10 days. Then, 2 ml of sterile distilled water was added to each dish, and microconidia were removed by gentle agitation with a glass rod. The mycelia were removed by filtering the suspension through four layers of cheesecloth. The spore concentration was determined with a hemacytometer and adjusted to  $1 \times 10^7$  microconidia per milliliter of water. Spore germination percentage was determined as described for *S. musiva*.

In another set of experiments, spores of the two fungi were mixed together (*S. musiva*  $1 \times 10^6$  per milliliter and *P. dimorphospora*  $1 \times 10^7$  per milliliter, 1:1, v/v) and 50  $\mu$ l of the suspension was placed on each of three slides coated with 0.5% water agar. The slides were maintained under the same conditions as described above. Controls were slides that contained either *S. musiva* spores or *P. dimorphospora* spores alone. The effect of *P. dimorphospora* microconidia on the germination of *S. musiva* conidia was observed under light microscopy daily for up to 5 days, and the experiment was repeated twice.

**Preparation of culture filtrate of *P. dimorphospora*.** Liquid still cultures of *P. dimorphospora* were grown in 500-ml flasks containing 250 ml of 3% potato-dextrose broth at room temperature (approximately 20 C) for 6 mo (*P. DesRochers, personal communication*). The culture filtrate was obtained after removal of the hyphae by filtration through Whatman No. 1 filter paper and

centrifugation (5,000 g for 10 min at 4 C). This preparation was used immediately in the experiment.

**Preparation of a crude extract from *P. dimorphospora* culture medium.** A crude extract was prepared by growing *P. dimorphospora* from an inoculum disk placed over the surface of a dialysis membrane on PDA for 21 days at 23 C in darkness. The membrane with adhering fungus was then removed, and the medium under the membrane was collected from 10 culture dishes, ground in a Waring blender with an equal volume of sterile distilled water, and extracted with chloroform as described by Yang et al (14). The chloroform extract was evaporated to dryness in vacuo at 30 C. The residue condensed from 1 L of extract was solubilized in 5 ml of acetone. The final preparation was stored at 20 C and used in experiments as a 5% solution in water.

**In vitro screening.** Two *Populus* species and one hybrid were selected for the in vitro bioassay using the poplar leaf disk technique described by Spiers (12). *Populus tremuloides* Michx., *P. grandidentata* Michx., and *P. × berolinensis* Dippel are all considered susceptible to Septoria leaf spot and canker (1,6,10,13). Fully expanded leaves of *P. tremuloides* and *P. grandidentata* were collected from poplars on the Laval University campus, and leaves of *P. × berolinensis* were collected from rooted hardwood cuttings grown in the greenhouse. Leaves were rinsed thoroughly with sterile distilled water, and 18-mm-diameter leaf disks were cut with a cork borer and placed, abaxial surface up, into holes with the same diameter made in petri dishes containing 2% water agar. The *P. dimorphospora* spore suspension, culture filtrate, and medium extract were spread evenly over the surface of leaf disks (0.1 ml per disk). Immediately, 0.1 ml of conidial suspension of *S. musiva* was applied to the same leaf disk. Other treatments consisted of leaf disks inoculated with an equal volume of spore suspensions of *P. dimorphospora* or *S. musiva* alone or treated only with *P. dimorphospora* culture filtrate, its medium extract, or sterile distilled water. Dishes were incubated at 20 C under continuous light. After 14 and 28 days, a dot grid (25 dots per square centimeter) was used to measure the necrotic area on leaf disks. Ten leaf disks in two dishes (replications) were used in each treatment, and the experiment was done twice.

**Disease suppression in the greenhouse.** Hardwood cuttings of *P. × berolinensis* were propagated in 15-cm-diameter × 14-cm-deep plastic pots filled with a soil mix (500 g per pot, pH 4.8) containing 25% perlite and 75% peat and having a water content of 20%. The pots were placed in the greenhouse (18–25 C, 13-hr photoperiod), watered every 2 days, and

fertilized every 2 wk with a nutrient solution (Peters general purpose 20-20-20, 1:1,000). When the leaves had fully expanded, the following treatments were applied: 1) 10 ml of a spore suspension of *S. musiva* was sprayed on the foliage of each plant and 2 days later the same volume of *P. dimorphospora* spore suspension or culture filtrate or 5% medium extract was applied; 2) 10 ml of a spore suspension of *P. dimorphospora* was sprayed on each plant and 2 days later the same volume of *S. musiva* spore suspension was applied; 3) 10 ml of *P. dimorphospora* culture filtrate or its medium extract were sprayed on each plant and 24 hr later 10 ml of *S. musiva* spore suspension was applied; and 4) plants having received *S. musiva* or *P. dimorphospora* spore suspension alone, culture filtrate, crude extract, or distilled water served as controls. Each treatment contained five five-plant replications arranged in a randomized complete block design. After inoculation, pots were sealed with plastic bags for 2 days to maintain a high level of humidity. The development of the disease on foliage was observed weekly for 6 wk.

An index described by Spielman et al (11) was modified to estimate the level of infection on each plant. A disease severity level was first described for each leaf according to the size of the necrotic area. The five levels of disease severity were 0 = healthy, 1 = <20%, 2 = 20–50%, 3 = 51–75%, and 4 = >75% of leaf surface necrotic. The index was then calculated on the basis of the summation of the proportion of leaves in each severity level multiplied by their severity category. For example, if a plant had 10 leaves, including five with a disease severity level of 1, three with a level of 2, and two with a level of 0, the index value would be  $[(5 \times 1) + (3 \times 2) + (2 \times 0)] / 10 = 1.1$ .

**Reisolation of *P. dimorphospora* and *S. musiva*.** One month after inoculation, 12 pieces each of dead and healthy leaf tissue (approximately 2 mm<sup>2</sup>) were arbitrarily selected and cut from leaves on cuttings in each treatment. The leaf tissues were washed with sterile distilled water, surface-sterilized in 70% alcohol for 30 sec, thoroughly washed twice with sterile distilled water, and placed on PDA dishes, four pieces per dish. The dishes were incubated at 23 C for 2 wk in darkness, and the fungal colonies formed were examined.

**Statistical analysis.** Data from all experiments were subjected to analysis of variance (8), and means were ranked by the cluster analysis method of Scott and Knott (9). Data from the greenhouse experiment were also subjected to an univariate repeated-measures analysis with repeated statement (8), with disease rating index as the dependent variable and elapsed time as the independent variable.

## RESULTS

Conidia of *S. musiva* germinated after 12 hr and 98% germination was achieved after 48 hr on 0.5% water agar. Germination of microconidia of *P. dimorphospora* was observed after 24 hr and germination increased gradually and reached 93% by 48 hr. When spores of *S. musiva* and *P. dimorphospora* were plated together, no significant change in the germination percentage or rate of either species was detected. However, germ tubes of *S. musiva* became abnormally branched and swollen after 48 hr, and their growth was stunted. After 72 hr in the presence of *P. dimorphospora*, hyphae of *S. musiva* had completely stopped growing and their cytoplasm had become coagulated. By contrast, the hyphae of *P. dimorphospora* kept developing normally in the presence of conidia of *S. musiva*.

In the in vitro leaf disk bioassay, disease symptoms were observed on the disks treated with *S. musiva* alone after 7 days, and the necrotic area continued to increase rapidly. By contrast, symptoms appeared 2 days later on leaf disks pretreated with *P. dimorphospora* spores, culture filtrate, or medium extract. In these disks, the size of the necrotic area increased very slowly compared with that of the control. In all leaf disks pretreated with either *P. dimorphospora* spores, culture filtrate, or medium extract, the size of the necrotic area was reduced significantly ( $P < 0.05$ ) (Fig. 1). No significant differences were observed among the means of these three treatments. On the leaf disks of *P. tremuloides*, *P. grandidentata*, and *P. X berolinensis* pretreated with *P. dimorphospora* spores, the size of the necrotic area caused by *S. musiva* was reduced by 98, 96.8, and 94.4% after 14 days and by 97.8, 72.3, and 92.3% after 28 days, respectively. Contamination by saprophytic fungi was observed on all other treatments at day 28.

In the greenhouse test, disease symptoms were observed on poplar leaves 14 days after inoculation with *S. musiva*. The disease developed rapidly and caused premature defoliation after 5 wk. On cuttings that were pretreated with *P. dimorphospora* spores, culture filtrate, or medium extract, the severity of the disease was significantly ( $P < 0.05$ ) lower than on those inoculated with *S. musiva* alone on every one of the dates when readings were taken. Univariate repeated-measures analysis indicated that the progression of the disease rating index was significantly ( $P < 0.01$ ) slower in these treatments and that linear effects accounted for over 97% of the progression of disease rating index curves over time (Fig. 2A). No significant differences were observed among cuttings treated with *P. dimorphospora* spores, culture filtrate, or medium extract. A similar trend was found in

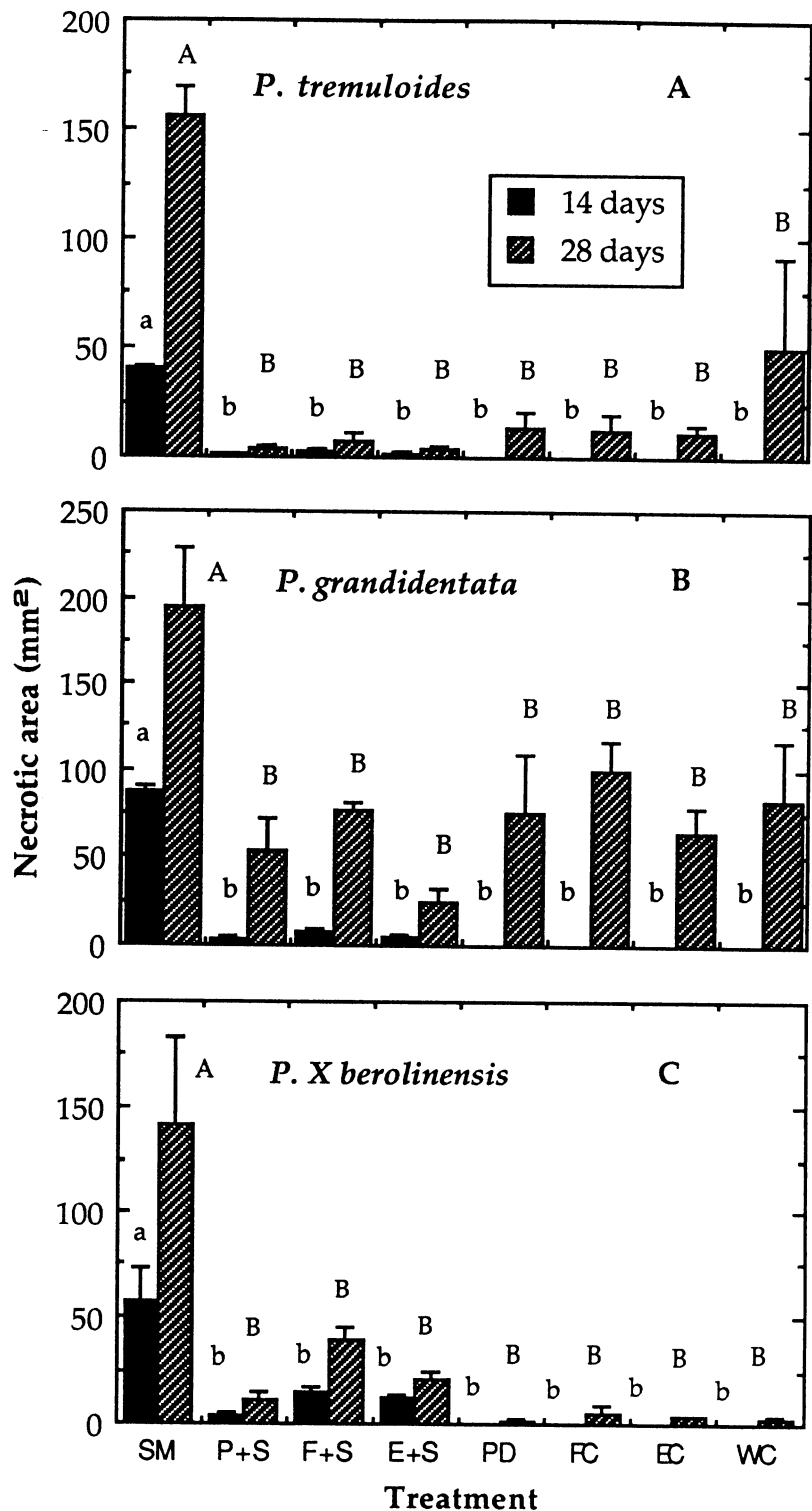


Fig. 1. Effects of *P. dimorphospora* and its culture filtrate and medium extract on control of Septoria leaf spot on poplar leaf disks. Values represent means of two five-leaf replicates, and vertical bars represent standard error of mean. For each poplar species, treatments followed by the same uppercase letter (28 days) or lowercase letter (14 days) are not significantly different ( $P < 0.05$ ) according to the cluster analysis test of Scott and Knott (9) following ANOVA. SM = *S. musiva* spores applied alone, P+S = *P. dimorphospora* and *S. musiva* spores applied together, F+S = *P. dimorphospora* culture filtrate and *S. musiva* spores applied together, E+S = *P. dimorphospora* medium extract and *S. musiva* spores applied together, PD = *P. dimorphospora* spores applied alone, FC = *P. dimorphospora* culture filtrate applied alone, EC = *P. dimorphospora* medium extract applied alone, and WC = distilled water applied alone.

posttreatment of cuttings with these biocontrol agents (Fig. 2B). No leaf necrosis was found on cuttings inoculated with *P. dimorphospora* spores alone. No toxic reaction was observed on cuttings treated with the *P. dimorphospora* culture filtrate or medium extract alone (Fig. 2).

*P. dimorphospora* was reisolated from the foliage inoculated with this fungus alone in 41.7% of the attempts, whereas *S. musiva* was reisolated 90.5% of the time from inoculated leaf tissues after 1 mo (Table 1). In the case of cuttings inoculated with both fungi, *S. musiva* and *P. dimorphospora* were isolated

from both dead and healthy tissues. Recovery of *S. musiva* was as high as 76.2% from dead tissues, whereas recovery of *P. dimorphospora* was as high as 33.3% from healthy tissues.

## DISCUSSION

The inhibitory effect of *P. dimorphospora* against several fungal plant pathogens, including *S. musiva*, has been described previously (14). In tests conducted in vitro, *P. dimorphospora* prevented the growth of other fungi by causing abnormal elongation and lysis of their hyphae. Metabolites produced

by this fungus were fungicidal; the crude extract from the *P. dimorphospora* culture medium killed spores from several species, including *S. musiva* conidia, within 24 hr (14).

In the present study, it was observed that the germination of conidia of *S. musiva* was not affected when they were plated together with microconidia of *P. dimorphospora*. This is likely due to the fact that microconidia of *P. dimorphospora* germinated slightly slower than did those of *S. musiva*. Once microconidia of *P. dimorphospora* had germinated, however, they interfered with the normal growth of *S. musiva* germ tubes. The reaction of *S. musiva* was similar to the reaction observed in several other fungal species upon exposure to germinated spores of *P. dimorphospora* (14) and probably results from the production of antifungal compounds by the latter.

We chose a leaf disk bioassay (12) as a preliminary screening technique before greenhouse tests. Results showed that spraying *P. dimorphospora* spores, culture filtrate, or medium extract significantly reduced necrosis on the disks of the poplar species and hybrid tested. It seems most likely that the necrotic area observed after 14 days of incubation was due solely to *S. musiva*, since the water controls were not necrotic. After 28 days of incubation, however, necrosis was observed on water-treated leaf disks of *P. tremuloides* and *P. grandidentata*, suggesting that over a longer period, organisms other than *S. musiva* also contributed to symptom expression on detached leaves. Septoria leaf spot is not considered an important disease in natural stands of native poplars (10). We observed, however, that *S. musiva* caused extensive necrosis on detached leaves of *P. tremuloides* and *P. grandidentata*. As indicated by Ostry et al (7), the leaf disk bioassay provides optimum conditions for infection by *S. musiva* and may therefore overestimate the susceptibility of species or clones in the field.

Results from the greenhouse experiment showed that microconidia of *P. dimorphospora* reduced both the severity and the progression of the disease without causing any visible damage to poplar. Application of metabolites of *P. dimorphospora* resulted in similar effects and could be considered as an alternative to the use of a spore suspension.

Recovery of *P. dimorphospora* from surface-sterilized poplar leaves 1 mo after inoculation of a spore suspension suggests that the fungus was able to penetrate leaf tissues and produce viable structures within these tissues. Preliminary histological data indicate that microconidia of *P. dimorphospora* were able to germinate and form a loose hyphal network over the leaf surface. Some hyphae grew intercellularly, but not intracellularly, in the epidermal layer

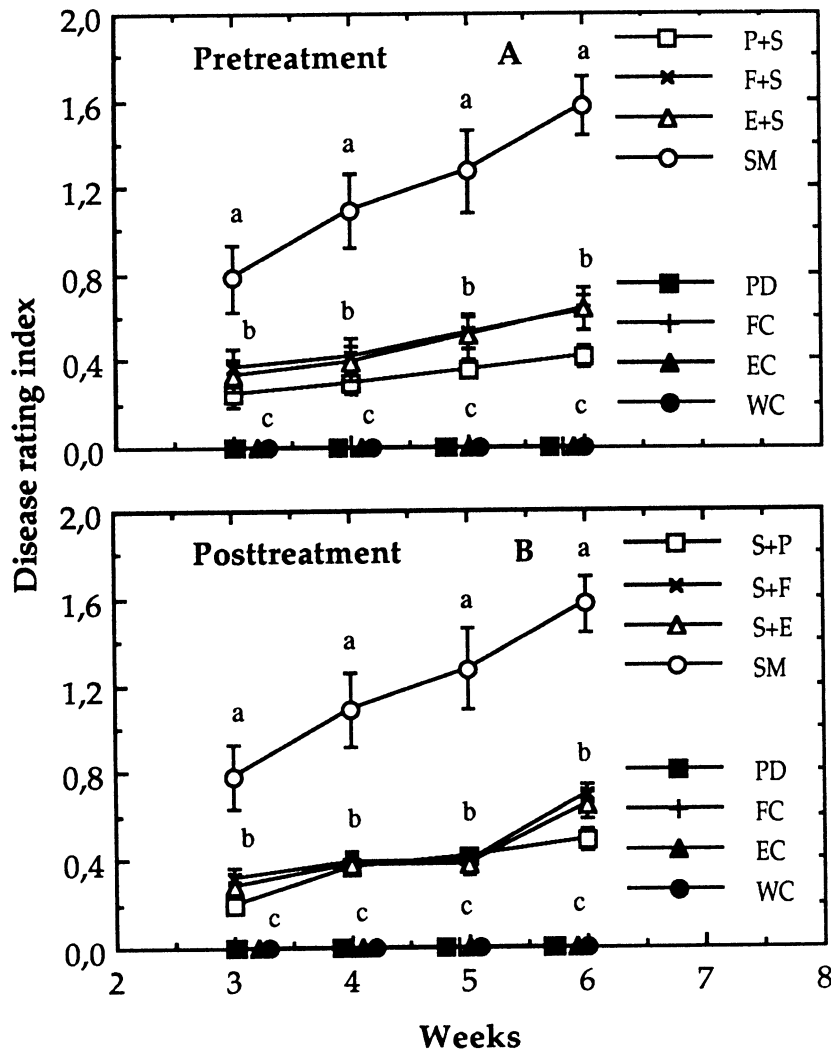


Fig. 2. Effects of *P. dimorphospora* spores, culture filtrate, and medium extract on control of Septoria leaf spot of poplar in the greenhouse. Values represent means of five replicates, and vertical bars represent standard error of mean. Within each week, treatments followed by the same letter are not significantly different ( $P < 0.05$ ) according to the cluster analysis test of Scott and Knott (9) following ANOVA. P+S = *P. dimorphospora* spores applied first and *S. musiva* spores applied 48 hr later, F+S = *P. dimorphospora* culture filtrate applied first and *S. musiva* spores applied 24 hr later, E+S = *P. dimorphospora* medium extract applied first and *S. musiva* spores applied 24 hr later, SM = *S. musiva* spores applied alone, PD = *P. dimorphospora* spores applied alone, FC = *P. dimorphospora* culture filtrate applied alone, EC = *P. dimorphospora* medium extract applied alone, WC = distilled water applied alone, S+P = *S. musiva* spores applied first and *P. dimorphospora* spores applied 48 hr later, S+F = *S. musiva* spores applied first and *P. dimorphospora* culture filtrate applied 48 hr later, and S+E = *S. musiva* spores applied first and *P. dimorphospora* medium extract applied 48 hr later. Univariate repeated-measures analysis indicated that the disease rating index of poplar cuttings inoculated with *S. musiva* alone increased significantly faster ( $P < 0.01$ ) than indices measured in all other treatments.

**Table 1.** Reisolation of *Phaeothea dimorphospora* and *Septoria musiva* from poplar foliage 1 mo after inoculation

Treatment <sup>a</sup>	Isolation frequency (%)		
	<i>P. dimorphospora</i>	<i>S. musiva</i>	Other microorganisms
<i>S. musiva</i> first <sup>b</sup>			
Dead tissue	4.8	76.2	19.0
Healthy tissue	33.3	8.3	58.4
<i>P. dimorphospora</i> first <sup>c</sup>			
Dead tissue	16.7	50.0	33.3
Healthy tissue	25.0	16.7	58.3
<i>S. musiva</i> alone			
Dead tissue	0.0	90.5	9.5
Healthy tissue	0.0	66.7	33.3
<i>P. dimorphospora</i> alone	41.7	0.0	58.3
Water only	0.0	0.0	100

<sup>a</sup>A 10-ml spore suspension of *S. musiva* ( $10^6$  spores per milliliter) or of *P. dimorphospora* ( $10^7$  spores per milliliter) was applied to each cutting.

<sup>b</sup>*P. dimorphospora* was applied 48 hr later.

<sup>c</sup>*S. musiva* was applied 48 hr later.

(D. Yang et al, unpublished). These results suggest that *P. dimorphospora* could possibly become established in poplar plantations after being applied as a spore suspension. Additional studies are necessary to quantify the surviving population and determine if it is sufficient to protect leaves from subsequent infection by *S. musiva*. Field trials under a wide range of conditions are also needed to fully assess the potential and limitations of *P. dimorphospora* as a biological control agent in nature.

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