Effect of Fall Application of Fungal Antagonists on Spring Ascospore Production of the Apple Scab Pathogen, *Venturia inaequalis*

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ABSTRACT

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The influences of *Microsphaeropsis* sp., *M. arundinis*, *Ophiostoma* sp., *Diplodia* sp., and *Trichoderma* sp., all antagonists of *Venturia inaequalis*, on ascospore production were evaluated under natural conditions and compared with urea and *Athelia bombacina*, a known antagonist. In the autumn, the fungi were applied to leaf disks artificially inoculated with *V. inaequalis* and to scabbed apple (*Malus domestica*) leaves incubated under controlled and natural conditions. In addition, large-scale trials were conducted with *Microsphaeropsis* sp. applied either as a foliar postharvest spray or as a ground application at 90% leaf fall. All fungal isolates, except *Ophiostoma* sp., were recovered from the leaf material that overwintered in the orchard. All treatments, except those with *Ophiostoma* sp.,

resulted in a significant reduction in *V. inaequalis* ascospore production on the leaf disks incubated under controlled conditions or in the orchard. In 1997, leaves with apple scab lesions treated with urea or *Microsphaeropsis* sp. produced significantly fewer ascospores of *V. inaequalis* than did nontreated leaves, with a reduction of 73.0 and 76.3%, respectively. In 1998, leaves treated with *Microsphaeropsis* sp., urea, *Trichoderma* sp., *A. bombacina*, and *M. arundinis* reduced ascospore production by 84.3, 96.6, 75.2, 96.6, and 52.2%, respectively. Based on all tests combined, the most efficient isolate was *Microsphaeropsis* sp. Postharvest applications of *Microsphaeropsis* sp. reduced the total amount of airborne ascospores trapped by 70.7 and 79.8% as compared with the nontreated plots in 1997 and 1998, respectively. *Microsphaeropsis* sp. provided a significant and consistent reduction in ascospore production in all tests.

Additional keywords: apple scab management.

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Wint., is considered to be the single most important disease of apples (*Malus domestica* Borkh.) in several production areas (28). Failure to control apple scab not only results in severe yield losses but also greatly reduces fruit market value. Currently, the strategy for apple scab control relies on multiple applications of fungicides (18,30). These sprays represent an appreciable cost to growers. Also, there are concerns about the development of resistance to fungicides (7,11,42).

Most research on apple scab has been focused on the control of primary infections and has resulted in spray schedules that essentially are based upon weather conditions that influence ascospore maturation (20) and infection (29), regardless of the inoculum potential. MacHardy et al. (30) showed that it is possible to delay the first fungicide applications up to the pink stage, when the inoculum potential is very low. The relation between the reduction of primary inoculum and scab severity has been clearly demonstrated by several authors (1,9,20,25,35). In cold temperate regions, *V. inaequalis* overwinters as immature pseudothecia in fallen apple leaves, and the ascospores produced in the spring are considered to be the principle source of primary inoculum.

Many attempts have been made to reduce the overwintering inoculum of *V. inaequalis*, including mechanical removal or destruction of the fallen leaves (27,32,44). The effect of various chemicals, including fungicides (benomyl, thiophanate-methyl, and carben-dazime) and other chemicals (dinitro-*o*-cresol [DNOC], mercury,

and lead arsenites) and urea (8,9,17,25,26) were investigated. Most of these chemicals have shown good efficacy, but their use pose several problems such as environmental concerns, possible detrimental effects on tree growth, phytotoxicity, and potential to enhance resistance in the *V. inaequalis* populations.

The first occurrence in the literature suggesting biological control against the saprophytic phase of *V. inaequalis* was by Simard et al. in 1957 (41). More recently, the possibility of using a fungal antagonist to reduce ascospore production has been investigated in vitro (23) and under natural conditions (12,24,31). From these studies, the fungus *Athelia bombacina* Pers. was identified as a potential biological control agent. However, complete pseudothecia inhibition was obtained in field trials only when very high antagonist inoculum doses were used (21). When a lower rate was used, pseudothecial inhibition was reduced by only 60 to 70% (31).

Over 300 species of fungi have been isolated from orchards of several apple-producing regions of Québec, Canada (5). Some of these fungal isolates were tested for the inhibition of pseudothecia and ascospore production using in vitro leaf disk assays (37). From this evaluation, five fungal isolates (*Microsphaeropsis* sp., *M. arundinis*, *Ophiostoma* sp., *Diplodia* sp., and *Trichoderma* sp.) were selected based on their capacity to inhibit ascospore production. Recently, Benyagoub et al. (4) studied the interaction between *Microsphaeropsis* sp. (strain P130A) and *V. inaequalis* and showed that *Microsphaeropsis* sp. was able to penetrate the *V. inaequalis* cell wall, inducing reduced growth or cell death.

The objective of this study was to evaluate the potential of the five fungal isolates to reduce the inoculum production of *V. inaequalis* under orchard conditions and to compare them with *A. bombacina* and urea, both known to inhibit pseudothecial production. Another objective was to evaluate the potential of *Microsphaeropsis* sp. to reduce airborne ascospore production.

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MATERIALS AND METHODS

Antagonist isolates and pathogen. Fungal antagonists were isolated from apple leaf litter in abandoned orchards of Quebec, Canada (5). Isolates P130A, previously identified as *Coniothyrium* sp., and P176A (6J-2), previously identified as *Phoma* sp., were reidentified as *Microsphaeropsis* sp. and *M. arundinis*, respectively. Isolate P11A (1H22) was identified as *Trichoderma* sp., isolate P164A as *Ophiostoma* sp., and isolate P138A as *Diplodia* sp. J. Andrews from the University of Wisconsin, Madison, kindly provided the culture of *A. bombacina*. All fungal cultures were stored on potato dextrose agar (PDA) (Difco Laboratories, Detroit) slants under mineral oil or in sterilized soil maintained at 2°C. Prior to inoculations, the fungal antagonists were cultured on PDA. When each fungus had covered at least two-thirds of the surface of a 9-cm petri dish, it was stored at 2°C for a maximum of 2 weeks until the day of inoculation. Mycelial slurries were prepared by homogenizing the fungal cultures in sterile distilled water at a ratio of 60 ml of water for each petri dish with a Stomaker laboratory blender (Seward Medical, London). For all inoculum preparations, the final concentration ranged from 1.87 to 3.56×10^4 , 1.78 to 3.41×10^4 , 2.28 to 4.78×10^4 , 7.84 to 9.86×10^3 , 1.37 to 2.47×10^4 , and 1.58 to 2.77×10^4 CFU/ml for *Microsphaeropsis* sp., *M. arundinis*, *Trichoderma* sp., *Ophiostoma* sp., *Diplodia* sp., and *A. bombacina*, respectively. Inoculum of *V. inaequalis* was prepared by culturing seven isolates (1An1.3, 9A1.1, 15A1.1, 18b2.2, 4b1.1, 26a3.3, and 15d2.1) obtained from our collection. These were grown on PDA at 15°C for at least 1 month. On the day of inoculation, one plate of each *V. inaequalis* isolate was inserted into a sterile plastic bag to which 100 ml of distilled water was added. The contents of the bags were then homogenized as described for the fungal antagonists. The final concentration of the preparations ranged from 1.26 to 1.97×10^6 CFU/ml.

Experiments with artificially inoculated leaf disks. The objective of the experiment was to determine the potential of the fungal antagonists to reduce ascospore production when applied under optimal conditions and to compare their efficacy under controlled and orchard conditions. Leaf disks of 2.7 cm in diameter were cut from apple leaves (cv. McIntosh) and sterilized by irradiation (10 h for a total of 40 kGy). In early October 1996 and 1997, leaf disks were inoculated with 50 µl of a mycelial suspension of a mixture of *V. inaequalis* isolates and placed in 500-ml glass jars as described by Philion et al. (37). The leaf disks were maintained at room temperature for 3 weeks to allow *V. inaequalis* to colonize most of the disk surface. Each disk was then inoculated with the fungal antagonists at a rate of 50 µl of mycelial slurry per leaf disk, 224 disks per treatment. After an incubation of 2 weeks at room temperature, a portion of the leaf disks (64 leaf disks) was incubated under controlled conditions in glass jars (four leaf disks per jar, 16 jars per treatment) as described by Philion et al. (37). The other leaf disks (160 disks) were incubated under orchard conditions in nylon mesh bags (18 × 40 cm) fixed to the ground. In early spring, the bags were shielded from the rain with wooden covers to avoid ascospore ejection. The experiment was conducted twice as a completely randomized design with eight treatments (six fungal isolates, urea, and a control with only *V. inaequalis*) and eight sampling units (bags of 20 disks). The 5% urea solution was applied at a rate of 1 ml per leaf (46% N, 5 g/100 ml of water) as described by Sutton (43).

Experiments with naturally infected leaves. The objective of this experiment was to evaluate and compare the efficacy of the fungal antagonists under natural conditions, including microbial competition. In the beginning of October 1996 and 1997, apple leaves (*Malus domestica* cv. McIntosh) infected with *V. inaequalis* were collected in an experimental orchard with a high level of scab. To reduce the variation among sampling units, only leaves showing between five and eight typical scab lesions were selected. The leaves were stored at 2°C in paper bags until processed. On 25 October

1996 and 23 October 1997, the scabbed leaves were inoculated with the fungal antagonists at a rate of 1 ml of mycelial slurry per leaf. The leaves were then overwintered in nylon mesh bags (50 leaves per bag) fixed to the orchard floor. The experiment was conducted twice as a completely randomized design with eight treatments (six fungal isolates, urea, and a control with only *V. inaequalis*) and nine repetitions. All field trials were conducted at the Agriculture and Agri-Food Canada Experimental Farm at Frelighsburg, Québec, Canada.

Recovery of fungal antagonists. Colonization of the leaf disks by the fungal isolates was evaluated based on visual observations and reisolation. For each isolate, a portion of the leaf disks (32 incubated in vitro and 64 incubated in the orchard) was placed on petri dishes containing water agar amended with 100 mg of tetracycline (Sigma Chemical Co., St. Louis) per liter. The plates with leaf disks were kept at room temperature and observed every 3 days for the presence of fungal structures for a period of 1 to 6 weeks. The same procedure was followed for the naturally infected leaves, except that 45 whole leaves were used instead of leaf disks. Preliminary tests showed that surface sterilization was not necessary and could, in fact, inhibit the growth of some of the fungi tested. Consequently, leaf disks or leaves were not surface-sterilized. *Trichoderma* sp. was identified based on spore morphology and typical pigmentation; *Microsphaeropsis* sp. and *M. arundinis* (isolates P130A and P176A) were identified based on presence of pycnidia and spore morphology (pale brown, aseptate, smooth, thin-walled, guttulate, cylindrical, and $5 \mu \times 2$ to 2.5μ). *A. bombacina* was identified based on growth habit as described by Heye and Andrews (24). *Ophiostoma* sp. was identified based on shape of the mycelial aggregates, and *Diplodia* sp. was identified based on brown pigmentation of mycelium, presence of brown ostiolate pycnidia, and brown, thick-walled, two-celled pycnidiospores. When presence of the fungus was noted, it was isolated and cultured on PDA. Colonization was recorded as the proportion of the leaf disks or leaves from which the isolate was reisolated.

Field experiments with *Microsphaeropsis* sp. The objective of this experiment was to evaluate the potential of *Microsphaeropsis* sp. isolate P130A, to reduce the airborne ascospore concentration, and to compare the efficiency of a postharvest, preleaf fall tree application at about 10% leaf fall with a ground application at 90% leaf fall. The experiment was conducted during two seasons, 1996–1997 and 1997–1998, at the Agriculture Canada Experimental Farm in Frelighsburg, Québec, Canada. The experiment was conducted in a mature orchard of 0.41 ha planted with cvs. McIntosh (58%) and Lobo (42%). The dwarf trees grafted on M26 rootstock were planted at a distance of 5×2.5 m. Because of expected variations in scab levels among the plots, the potential ascospore dose (PAD) was measured in each plot, based on a late fall scab assessment, following the method described by MacHardy et al. (30). In each plot of 0.14 ha (five rows of 22 trees), 20 shoots were evaluated on 10 'McIntosh' trees chosen randomly in the subplot, for a total of 200 shoots. Scab incidence was assessed within 2 weeks after harvest by counting the number of infected leaves per shoot and expressed as percent scabbed leaves.

To produce large quantities of spores, *Microsphaeropsis* sp. mycelium was first produced in liquid cultures. Erlenmeyer flasks of 250-ml capacity containing 100 ml of sterilized malt extract (Sigma Chemical Co.) were inoculated with a 6-mm mycelial plug cut from the edge a 15-day-old culture of *Microsphaeropsis* sp. The flasks were maintained at room temperature in a rotary (240 rpm) shaker (Orbit Eviron-Shaker; Lab-Lie, Melrose Park, IL) for 15 days. Five hundred grams of barley grains was placed in 900-ml glass jars and soaked in distilled water for 24 h. The excess water was drained and the barley was autoclaved twice for 15 min at 121°C and 140 KPa. After cooling, each jar was inoculated with 500 µl of the liquid culture. The jars were then maintained at room temperature for 3 to 4 weeks and shaken every day for approximately 30 s. On the day of application, inoculum was prepared by mixing

two jars containing the barley grains covered with mature pycnidia with 2.5 liters of distilled water into 3-liter beakers. The mixture was agitated for 20 to 30 min and then filtered through two layers of cheesecloth. When the volume reached approximately 15 liters, the concentration was adjusted to 6.75×10^9 conidia per ml in order to obtain 4.5×10^5 conidia per ml once diluted in the tank mix.

The spore suspension was applied either to the tree canopy at less than 10% leaf fall or as a ground application at 90% leaf fall at a rate of 1,125 liters/ha, corresponding to approximately 1.2 liters per tree. The tree canopy application was done on 11 and 17 October in 1996 and 1998, respectively, and the ground application on 12 and 26 November in 1996 and 1998, respectively. The spore suspension was applied using a gasoline-powered Solo backpack sprayer (model 422; Solo, Sindelfingen, Germany) and a hand-held gun mounted with a single nozzle with an 80° flat spray tip operated at 620 KPa with a flow rate of approximately 2.2 liters/min. Five samples of 15 to 25 ml of the tank mix were collected while spraying each plot. Samples were kept on ice until the evaluation of spore concentration and germination. Within 24 h, the spore concentration was estimated using a hemacytometer. Spore germination was evaluated by pouring 1 µl of spore suspension onto water agar plates and incubating them at room temperature. After 24 h, 100 spores per plate were observed for germination. Spores were considered to have germinated when the germ tube was at least the length of the spore.

Quantification of ascospore production. Ascospore production on artificially inoculated leaf disks was evaluated when ascospores from nontreated leaf disks were mature as determined by squash mounts (19). To evaluate ascospore production per leaf disk, two leaf disks were placed in a 50-ml Falcon test tube (Fisher Scientific, Montreal, Canada) (16 tubes per treatments) with 20 ml of distilled water and exposed to air turbulence for 1 h (37). A few drops of Lugol's iodine solution were added to each ascospore suspension to stop germination, and the contents of the tubes were centrifuged at $2,000 \times g$ for 10 min (Accuspin FR, Beckman, CA). The liquid was carefully drawn out of the tubes so that the pellet and surrounding liquid were not disturbed in order to obtain 5 ml of ascospore suspension. Ascospore concentrations were determined using a hemacytometer and ascospore productivity reported for each treatment as the ascospore production per square centimeter of leaf tissue.

Ascospore production on naturally infected leaves was evaluated during the entire ascospore ejection period, from late April to the end of June. One leaf was selected arbitrarily from each sampling unit (nylon bag), for a total of nine leaves per treatment, and placed on the wire bottom of a wooden spore trap (14), the top surface facing up. Microscope slides, previously coated with petroleum jelly, were placed above the leaves at a height of 0.5 cm. After each rain, all the slides were collected and replaced by new ones, and the slides were stored at 10°C until examination. The number of ascospores present on 40% of the slide surface was counted under microscope at a magnification of $\times 250$, and ascospore production was expressed on a per slide basis.

The effect of *Microsphaeropsis* sp. on ascospore production in the orchard was evaluated by measuring the airborne concentration of ascospores of *V. inaequalis* during each period conducive to both ascospore ejection and infection. Homemade volumetric spore samplers (rotary impaction type) similar to the Rotorod device were used for this purpose (36). The onset of rain or dew, which triggers ascospore liberation, was determined using electrical grid leaf wetness sensors installed at the ground level. The signal was transmitted to a datalogger (CR-10; Campbell Scientific Inc., Logan, UT) that in turn activated the spore samplers. Two spore samplers were installed in each of the three plots and ran consecutively for 4 h, until the end of the rain, or for a maximum of 8 h. Since ascospore infection is not possible once the leaves are dry, the end of the rain event was determined with the drying of the wetness sensors installed within the tree canopy. The sampling heads were set

at 40 cm above the ground and shielded from the rain with a plastic cover 10 cm above the heads. For each rain conducive to infection, the number of ascospores on the entire surface of the exposed area of the rods was counted and ascospore concentration was expressed as the number of ascospores per cubic meter of air sampled. Airborne ascospores were monitored from 11 May to 20 June 1997 and from 24 April to 11 June 1998, for a total of ten and nine rain events in 1997 and 1998, respectively.

Statistical analysis. Tests of homogeneity of variances (*F* tests) were conducted to determine if data from the two experimental runs could be pooled and to ensure the underlying assumptions of the statistical models. Analysis of variance was used to test the effect of fungal isolates on total ascospore production; multiple comparison tests (least significant difference), to detect significant differences among means; and orthogonal comparison, to compare different levels of treatments with the control (*V. inaequalis* alone) at the 0.05 confidence level. Repeated measures analysis was used to test the effect of fungal isolates on ascospore production for each sampling date, because data were collected over time from the same leaves and, therefore, were not independent (Manova procedure). Analysis of variance and orthogonal comparisons were also used to test the effect of *Microsphaeropsis* sp. (strain P130A) on airborne ascospore production and to compare the two application types (tree versus ground). Statistical analysis was conducted using the SAS software program (SAS Institute, Inc., Cary, NC).

RESULTS

Recovery of fungal antagonists. All fungal isolates were recovered in high proportions (84 to 100%) from the leaf disks incubated in vitro. However, colonization was lower on the leaf disks and detached leaves overwintered in the orchard (Fig. 1). *Ophiostoma* sp. was recovered from less than 10% of the samples when leaf disks or entire leaves were overwintered in the orchard. In contrast, *Trichoderma* sp. was recovered from almost all samples (86 to 90%). The other fungal isolates were recovered in about two-thirds of the samples.

Experiments with artificially inoculated leaf disks. Based on homogeneity of variance, the data from the two experimental runs were pooled. When leaf disks was not treated, an average of 1.28×10^3 and 1.59×10^3 ascospores per cm² were obtained from leaf disks incubated under controlled conditions and overwintered in the orchard, respectively (Fig. 2A and B). On leaf disks incubated

Fig. 1. Recovery of the fungal isolates inoculated on apple leaf disks incubated under controlled conditions, on leaf disks overwintered in the orchard, and from detached apple leaves overwintered in the orchard. The percentage of recovery was calculated from samples of 32 leaf disks incubated under controlled conditions, 64 leaf disks overwintered in the orchard, and 45 apple leaves overwintered in the orchard.

under controlled conditions, significant ascospore reduction ($P \leq 0.0001$) was observed for all treatments except those with *Ophiostoma* sp. (Fig. 2A). The trend was similar for leaf disks incubated in the orchard, with significant ascospore inhibition ($P \leq 0.0001$) for all treatments except those with *Ophiostoma* sp. (Fig. 2B). On leaf disks incubated under controlled conditions, the greatest ascospore inhibition was observed on leaf disks treated with *A. bombacina*, followed by *Microsphaeropsis* sp., *Trichoderma* sp., urea, *M. arundinis*, and *Diplodia* sp., reducing ascospore production by 96, 94, 89, 85, 77, and 65%, respectively. On leaf disks overwintered in the orchard, the same treatments resulted in an ascospore reduction of 80, 96, 94, 89, 81, and 57%, respectively.

Experiment with naturally infected leaves. On naturally infected apple leaves, more ascospores were observed on the control during the spring of 1998 than of 1997, with a seasonal total of 2.16×10^4 and 1.07×10^4 ascospores per leaf, respectively (Fig. 3A and B). In both years, *Diplodia* sp. failed to inhibit ascospore production. In 1997, leaves treated with urea or *Microsphaeropsis* sp. produced significantly fewer ascospores than did nontreated leaves ($P = 0.0109$ and 0.0254 , respectively), with a reduction of 73.0 and 76.3%, respectively (Fig. 3A). The other treatments did not result in a significant ascospore reduction (Fig. 3A). In 1998, leaves treated with *Microsphaeropsis* sp., urea, *Trichoderma* sp., *A. bombacina*, and *Ophiostoma* sp. produced significantly fewer ascospores ($P = 0.0048$, 0.0013 , 0.0116 , 0.0013 , and 0.0323 , respectively), with a percent reduction of 84.3, 96.6, 75.2, 96.6, and 52.2%, respectively (Fig. 3B). For the 2 years of the experiment, the pattern of ascospore ejection over time was similar on treated

and nontreated leaves, with maximum ejection occurring on 23 May 1997 and 5 May 1998 (Fig. 4A and B). Repeated measures analysis revealed that there was a significant effect of fungal isolates ($P < 0.0001$) and time ($P < 0.0001$) and that the effect of fungal isolates on ascospore production varied over time ($P = 0.05$). In general, the effect of fungal isolates on ascospore production was not significant when the amount of ascospores ejected was low. Overall, for all tests, the best four treatments in terms of reduction of ascospore production were those with *Microsphaeropsis* sp., urea, *A. bombacina*, and *Trichoderma* sp.

Field experiment with *Microsphaeropsis* sp. Percent germination of the *Microsphaeropsis* sp. spore suspension was high in all samples, ranging from 88 to 98%. In the fall of 1996, scab incidence in orchard plots prior to the treatments with *Microsphaeropsis* sp. was 2.56, 4.08, and 3.14% for the control, the canopy, and the ground applications, respectively. In 1997, for the same treatments, disease incidence was 9.0, 23.0, and 8.0%, respectively. Both ground and canopy application of *Microsphaeropsis* sp. significantly ($P = 0.0006$) reduced airborne ascospore concentration (Fig. 5). The application of *Microsphaeropsis* sp. to the tree canopy at less than 10% leaf fall resulted in a reduction in the total amount of ascospores trapped of 70.7 and 79.8% as compared with that of the untreated plot in 1997 and 1998, respectively (Fig. 5A and B). The ground application, at 90% leaf fall, resulted in a reduction in airborne ascospore concentration of 60.1 and 62.6% in 1997 and 1998, respectively. In both years, significantly fewer ascospores ($P = 0.0003$) were trapped when *Microsphaeropsis* sp. was applied on leaves still attached to the trees compared

Fig. 2. Ascospore production from leaf disks incubated under **A**, controlled conditions and **B**, orchard conditions. Bars with the same letters are not significantly different according to the least significant difference test (LSD, at the 0.05 level of confidence). Bars with * are significantly different from the control (nontreated leaves) based on orthogonal comparison ($P = 0.05$). Data from 1997 and 1998 were pooled.

Fig. 3. Total ascospore production during the spring of **A**, 1997 and **B**, 1998 from detached scabbed leaves treated with the fungal antagonists and urea. Bars with the same letters are not significantly different according to the least significant difference test (LSD, at the 0.05 level of confidence). Bars with * are significantly different from the control (nontreated leaves) based on orthogonal comparison ($P = 0.05$).

with that on fallen leaves. The canopy application was done in a plot with a ascospore potential of 1.5 and 2.5 times higher than that in the control in 1997 and 1998, respectively, which was conservatively established in the plot with the lowest level of scab.

DISCUSSION

Most of the fungal antagonists were recovered from the leaf material that overwintered in the orchard. *Ophiostoma* sp. was the exception and was recovered from less than 10% of the samples, which can be explained by the slow growth rate of this isolate and its poor leaf colonization ability. With the exception of *Ophiostoma* sp., all antagonists and urea significantly reduced ascospore production on the leaf disks artificially inoculated with *V. inaequalis*. These results are in complete agreement with those obtained by Phillion et al. (37), who selected these fungi based on a similar test. On the leaf disks, the fungal antagonist is in direct contact with *V. inaequalis*, which probably explains the high level of inhibition. The fungi had a similar effect on leaf disks incubated under controlled and orchard conditions. This indicates that microbial competition with other organisms may not play a major role in the interaction between the antagonists and *V. inaequalis*, except for *A. bombacina*. However, on the naturally infected apple leaves, the level of ascospore inhibition was lower for most of the fungal isolates. Scab on these leaves may have been more difficult to control than that on the artificially inoculated leaf disks, in part, because *V. inaequalis* was already established within leaves when the antagonists were applied, thus occupying a critical ecological niche partly shielding it from antagonism.

In a recent study by Ouimet et al. (34) on the effect of fungal antagonists on in vitro inhibition of vegetative growth of *V. inae-*

qualis, the isolate of *Ophiostoma* sp. completely prevented mycelial growth regardless of the temperature, pH, or light conditions. In the study by Phillion et al. (37), it reduced ascospore production by 88.7% in one trial; however, in another trial, ascospore inhibition was only 8.2%. In the current study, this isolate failed to inhibit ascospore production, with an average ascospore inhibition of 8.2% for all tests combined. A previous experiment (37) showed the potential of *M. arundinis* and *Diplodia* sp. for inhibiting ascospore production; but in our experiments, they did not provide a high and consistent level of inhibition, making them uninteresting for biological control.

In our study, the four best treatments were those with *Trichoderma* sp., *A. bombacina*, urea, and *Microsphaeropsis* sp., with an overall ascospore inhibition (all tests combined) of 83.7, 84.2, 87.7, and 90.4%, respectively. Our findings on the effect of *A. bombacina* are similar to those obtained by Heye and Andrews (24), Miedtke and Kennel (31), and Phillion et al. (37). Heye and Andrews (24) observed a complete ascospore inhibition on leaf disks incubated in the laboratory and on intact leaves incubated in the orchard. However, they used a large quantity of *A. bombacina* inoculum equivalent to approximately five times the amount used in the current study. In their study, Miedtke and Kennel (31) used lower amounts of inoculum, approximately double what was used here, and observed a reduction in ascospore production of only 60 to 70%. In our experiment, with relatively low amounts of inoculum, we observed an overall ascospore inhibition of 84.22%, although *Trichoderma* sp. and *Microsphaeropsis* sp. were as good as *A.*

Fig. 4. Ascospore production from detached scabbed leaves treated in the fall with the fungal antagonists and urea. Ascospore production was measured for each rain in **A**, 1997 and **B**, 1998.

Fig. 5. Airborne ascospore concentration in orchard plots treated in the fall with the fungal antagonist *Microsphaeropsis* sp. applied as a foliar spray at 10% leaf fall or as a ground application at 90% leaf fall as compared with a nontreated plot. Airborne ascospore concentration production was measured for each rain in **A**, 1997 and **B**, 1998.

bombacina in reducing ascospore production. *Trichoderma* sp. caused a reduction in ascospore production of 83.3 and 93.8% on leaf disks. On scabbed leaves, the reduction was only 68.1%. These results are similar to those observed by Ciotola (12), who reported the failure of *T. reesei* to reduce ascospore production under natural conditions.

Urea provided a high and consistent ascospore inhibition varying from 85.0 to 89.2%, depending on the experiment. The effect of autumn treatments with urea has been investigated by several authors (9,15,17,21,22,33,38). In most of these studies, urea provided complete eradication of pseudothecia or ascospore production and its effect is well documented. The application of urea stimulates the growth of microorganisms (13), which increases microbial competition. Among the microorganisms, some are antagonistic of *V. inaequalis* (22,38). Also, leaf softening induced by urea (8,13,23) promotes leaf removal by decomposing microorganisms (13). The application of urea causes an increased leaf nitrogen content and, consequently, a nitrogen-carbon imbalance that inhibits pseudothecia (13,23,38). Despite the obvious effect of urea application on ascospore production, very few experiments in large plots have been conducted. In such an experiment, Sutton (43) observed a reduction in relative ascospore dose of 74% following a spring urea application on leaf litter. However, the fall application of 5% urea failed to significantly reduce ascospore production. The effect of urea application on winter hardening of the apple trees is unclear. Shim et al. (40) showed that, following a postharvest urea application, most of the N content was translocated from leaves to storage tissues during leaf senescence; however, they did not observe winter injuries for the 2 years of the experiment.

Microsphaeropsis sp. was consistent in its effect on the production of ascospores by *V. inaequalis*, with ascospore inhibition varying from 81 to 96% in all the tests conducted with detached leaves. These results are similar to those obtained by Philion et al. (37), who observed an in vitro ascospore reduction of 98.6 and 99.2%. The effect of *Microsphaeropsis* sp. on ascospore reduction was higher on leaf disks (93.67 and 95.97%) than on naturally infected apple leaves (81.64%) and lowest when applied in large plots (75.27% for the canopy application). This diminution in efficacy can probably be explained by the different levels of contact between *Microsphaeropsis* sp. and *V. inaequalis* and by the conditions for leaf colonization by *Microsphaeropsis* sp. On the leaf disks, 2 weeks of incubation at room temperature were allowed for colonization. On the scabbed leaves, the antagonists were exposed to weather conditions immediately after their application on leaves, which may have had an effect on the ability of the antagonist to become established on or in the leaves. The tests in orchard plots were even more severe. For the canopy application, *Microsphaeropsis* sp. was exposed to adverse conditions due to the low level of humidity, fluctuating temperatures, and presence of wind. To our knowledge, it is the first evaluation of a microbial control agent for apple scab in large plots. This test was necessary because it is more similar to commercial conditions than to detached leaf tests. The mode of action of this isolate of *Microsphaeropsis* sp. is not fully established. However, Benyagoub et al. (4) reported that the interaction between *V. inaequalis* and *Microsphaeropsis* sp. proceeds via mycoparasitism and involves both enzymatic activity and antibiosis. This is in contrast to a report by Andrews et al. (3), who suggested that microbial antagonism of the vegetative stage of *V. inaequalis* by *M. olivacea* operated through nutrient competition.

The isolate of *Microsphaeropsis* sp. tested in this study produces large quantities of spores, making it a good candidate for commercialization. However, the effect of *Microsphaeropsis* sp. on scab development remains to be established under commercial conditions. Regardless of how the ascospore reduction is achieved, it will not, by itself, solve the problem of apple scab. Much more work is needed to integrate biological control in scab management. Research on biological control of apple scab has been oriented

toward interrupting the secondary cycles by preventing conidial infection (10,16) or toward reducing primary inoculum by interfering with the overwintering of pseudothecia (12,23,31,37). In general, biological control of primary and secondary infections using microorganisms either failed (6) or testing did not reach the field level (10). Chemical fungicides are generally efficient in controlling primary and secondary infections; consequently, biological control strategies aiming to control leaf infection have a limited commercial future. Since chemical fungicides should not be used in the fall because of detrimental environmental impact and spread of resistance, the reduction of the initial inoculum as a result of the application of a biological control agent can be part of integrated scab management. If the primary inoculum is substantially reduced, the fungicide program could be delayed (30) or ended earlier, resulting in fungicide savings. The ascospore threshold at which fungicide application is required remains to be established. Currently, the action threshold is determined by simply relating the PAD reduction to a theoretical delay in epidemic onset. Anagnostakis and Aylor (2) and Sanogo and Aylor (39) reported that the infection efficiency of ascospores was 5 to 14% on apple leaves and 6 to 21% on apple flower buds based on actual ascospore concentrations measured at the leaf level using microscope slides. Philion et al. (36) reported that leaf infection on potted trees occurred when more than 150 ascospores per spore trap were collected.

From the current study, it can be expected that *Microsphaeropsis* sp. could reduce overwintering inoculum by at least 75% and may serve as insurance for the elimination of these early season sprays. Nevertheless, results are encouraging if a similar effect could be obtained in a commercial orchard. For instance, by improving formulation and the application technique, we could envision a scab control program in which at least the first few fungicide applications could be avoided.

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