Effect of Timing of Application of the Biological Control Agent *Microsphaeropsis ochracea* on the Production and Ejection Pattern of Ascospores by *Venturia inaequalis*

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Accepted for publication 28 July 2004.

ABSTRACT

Carisse, O., and Rolland, D. 2004. Effect of timing of application of the biological control agent *Microsphaeropsis ochracea* on the production and ejection pattern of ascospores by *Venturia inaequalis*. *Phytopathology* 94:1305-1314.

Field and in vitro trials were conducted to establish the influence of the biological control agent *Microsphaeropsis ochracea* on the ejection pattern of ascospores by *Venturia inaequalis* and on apple scab development, and to establish the best timing of application. The ejection pattern of ascospores was similar on leaves sprayed with *M. ochracea* and on untreated leaves. Fall application of *M. ochracea* combined with a delayed-fungicide program was evaluated in orchards with intermediate and high scab risk. For both orchards, it was possible to delay the first three and two infection periods in 1998 and 1999, respectively, without causing significant increase or unacceptable leaf and fruit scab incidence. To evaluate the best timing of application, sterile leaf disks were inoculated with *V. inaequalis* and then with *M. ochracea* 0, 2, 4, 6, 8, 10, 12, 14, and 16 weeks later. After incubation under optimal conditions for pseudothecia development, the number of ascospores was counted. Similarly,

M. ochracea was sprayed on scabbed leaves on seven occasions from August to November 1999 and 2000. Leaves were overwintered on the orchard floor and ascospore production was evaluated the following spring. Ascospore production was reduced by 97 to 100% on leaf disks inoculated with *M. ochracea* less than 6 weeks after inoculation with *V. inaequalis*, but ascospore production increased with increasing period of time when *M. ochracea* was applied 8 to 16 weeks after the inoculation with *V. inaequalis*. In the orchard, the greatest reduction in production of ascospores (94 to 96% in 2000 and 99% in 2001) occurred on leaves sprayed with *M. ochracea* in August. The production of ascospores was reduced by 61 to 84% in 2000 and 93% in 2001 on leaves sprayed with *M. ochracea* in September, reduced by 64 to 86% in 2000 and 74 to 89% in 2001 on leaves sprayed in October, and reduced by 54 and 67% in 2000 and 2001, respectively, on leaves sprayed in November. It was concluded that *M. ochracea* should be applied in August or September and that ascospore maturation models and delayed-fungicide program could be used in orchards treated with this biological control agent.

Additional keywords: pattern of ascospore ejection, sanitation.

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Wint., is of major economic importance in the north-eastern part of North America (38). If not managed, the disease can cause extensive losses following humid and cool weather conditions during the spring months. Direct losses result from fruit infections and indirect losses from defoliation, which can reduce tree vigor, winter hardiness, and subsequent yield (38).

The introduction of organic fungicides in the 1940s has had a major impact on the management of apple scab (35,36); gradually, fungicides became the most important tool for the control of the disease. Fungicidal control generally is considered to be the sole economically feasible control measure against apple scab. However, this may change due to the high costs of new fungicides, increased fungicide resistance in populations of *V. inaequalis*, and increasing concerns of environmental costs and consumers' negative perceptions of fungicide use (3). Over the years, *V. inaequalis* has become less sensitive to fungicides such as dodine, benomyl, the DMI fungicides (sterol demethylation inhibitors), and the newly developed strobilurin-based fungicides (9,16,32–34,44,51,52). Furthermore, cross resistance and partial cross resistance has been confirmed in populations of *V. inaequalis* from the United States (32). In addition, repeated use of

fungicides can interfere with pest and predator balance and have adverse effect on predacious mites (6–8). There are also increasing health concerns for both farmers and consumers (49). For all of these reasons, interest in the development of alternative strategies to manage apple scab and to reduce reliance on fungicides is increasing (14,54).

Although research in New York has shown that the fungus can overwinter as conidia on bud scales, *V. inaequalis* mostly overwinters in apple leaf litter (1). The main source of primary inoculum consists of ascospores that are produced within pseudothecia that develop in fallen leaves during the winter months. In the spring, at about the time of bud break, mature ascospores are forcibly ejected in response to rain (38). Ascospores continue to mature and are ejected over a period of 5 to 9 weeks, with a peak during the pink to petal fall apple phenological stages. Once ejected, ascospores are dispersed by wind. Few of the ascospores will reach the new growth of apple trees. The length of time required for infection depends on the number of hours of continuous wetness and the temperature during the wet period (40,43).

In northeastern North America, most fungicide programs aim at controlling the primary infections caused by the ascospores, thus avoiding secondary cycles. Removal or destruction of the leaves on the orchard floor greatly reduces the amount of primary inoculum and, as a result, reduces the risk of infection. Apple leaf litter can be destroyed by shredding the leaves (54), application of urea or biological control agents, and by combining leaf shredding with treatments such as urea or fungal antagonists (14,17,55).

Microsphaeropsis ochracea (Carisse & Bernier), a coelomycete isolated from dead apple leaves, recently has been identified as a

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Publication no. P-2004-0924-01R

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biological control agent of apple scab (4,12,17,46). In in vitro studies, this antagonist inhibited 98.6 to 99.2% of the ascospores produced on leaf disks (46). In a similar study, application of *M. ochracea* on leaf disks incubated under controlled and orchard conditions resulted in 94 and 96% fewer ascospores (17). When *M. ochracea* was applied onto naturally infected leaves that were overwintered on the orchard floor, ascospore production the following spring was reduced by 76 to 84% (17). Fall applications of *M. ochracea* on the tree canopy (at 10% leaf fall) and on the ground (at 90% leaf fall) were compared, resulting in 75.5 and 62% fewer ascospores, respectively (17). From this study, it was concluded that the biological control agent was slightly more effective when applied to the tree canopy, but the best timing of the application was unknown.

The objectives of this study were to (i) study the influence of fall application of *M. ochracea* on the ejection pattern of ascospores, (ii) evaluate the efficacy of *M. ochracea* under orchard conditions; and (iii) optimize the timing of application of *M. ochracea*. The information obtained was used to determine the best timing of application of *M. ochracea* and how fungicide spray programs should be adjusted in orchards treated with this biological control agent.

MATERIALS AND METHODS

Effect of *M. ochracea* on ejection pattern of ascospores by *V. inaequalis*. A first set of experiments was conducted with detached apple leaves and repeated during four seasons (1995–96 to 1998–99). In early October 1994, 1995, 1996, and 1997, apple leaves (*Malus pumila* Mill. cv. McIntosh) that were naturally infected with *V. inaequalis* and showing typical scab lesions were collected at the Agriculture Canada Experimental Farm, in Frelighsburg, Québec, Canada. On 25 October 1995, 25 October 1996, 17 October 1997, and 8 October 1998, the scabbed leaves were inoculated with *Microsphaeropsis ochracea*, 1 ml of mycelial slurry per leaf. The leaves then were overwintered in nylon mesh bags (50 leaves per bag) fixed to the orchard floor.

A second set of experiments (1996–97 and 1997–98) was conducted in a mature orchard of 0.41 ha planted with the cvs. McIntosh (58%) and Lobo (42%). The dwarf trees grafted on M26 rootstock were planted at a distance of 5 by 2.5 m. *M. ochracea* was applied, either to the canopy of McIntosh trees at 10% leaf fall or as a ground application under McIntosh trees at 90% leaf fall, at a rate of 1,125 liters/ha of a spore suspension of 4.5×10^5 spores/ml. The tree canopy application was done on 11 October 1996 and 17 October 1997 and the ground application on 12 November 1996 and 26 November 1997. The spore suspension was applied using a gasoline-powered Solo backpack sprayer (model 422; Solo Sindelfingen, Germany) and a handheld gun. A single nozzle with a 80° flat spray tip was operated at 620 kPa with a flow rate of approximately 2.2 liters/min.

The effect of *M. ochracea* on the ejection of ascospores in the orchard was evaluated by measuring the airborne concentration of ascospores of *V. inaequalis* during each major rain event. Home-made impaction spore samplers (rotary impaction type) similar to the Rotorod device were used for this purpose (17,18). 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, Edmonton, Canada), which 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. Ascospore infection is not possible once the leaves are dry; therefore, 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. The samplers were operated to spin at

2,400 rpm and to sample 19.99 liters of air per minute. For each rain conducive to ascospore ejection, the number of ascospores on the entire surface of the exposed area of the rods was counted under a microscope at $\times 250$, and the number of spores was transformed to ascospores per cubic meter as follows: (number of spores per rod $\times 1,000$ liters/m³)/(19.99 liters/min rod $\times 60$ min/h $\times$ running time in h). Airborne ascospore concentration was monitored from 1 May to 13 June 1997 and from 22 April to 11 June 1998, for a total of 11 and 10 rain events in 1997 and 1998, respectively.

Effect of fall treatment with *M. ochracea* on scab development the following spring. Experimental plots were established at the Agriculture Canada Experimental Farm, in Frelighsburg, Québec, Canada. The plots were established in two orchards selected based on their “scab risk” as determined by the method proposed by MacHardy (39). In the first orchard, the dwarf trees grafted on M26 rootstock were planted at a distance of 5 by 2.5 m. Each row (except for border rows) was composed of a repeated motif of four trees of Summerland McIntosh separated by one or two trees of Lobo, and the border rows were planted with Lobo only. Both cultivars are considered susceptible to apple scab (14). This orchard was split into subplots of 0.14 ha. In the second orchard, the dwarf trees grafted on M26 rootstock were planted at a distance of 4.5 by 2.5 m. This orchard, composed of McIntosh only, was split into subplots of 0.15 ha. Scab risk was estimated in each subplot on 28 September 1997 and 8 October 1998 using an adaptation of the method described by MacHardy (39) which is based on a late fall scab assessment. In each subplot, both surfaces of all leaves of 10 extension shoots from 10 trees selected at random were examined for the presence of scab lesions. When the number of scabbed leaves was ≤ 5 , between 5 and 25, and >25 , the subplot was considered to be at low, intermediate, and high risk, respectively. Because some treatments may influence the scab risk (i.e., cause an increase in the number of scab leaves present in the fall), each year the treatments were allocated randomly to the subplots within the two orchards.

M. ochracea was applied as described previously. To evaluate application efficacy, a sample of 15 to 25 ml of the tank mix was collected during the application in the middle of each half-row application. The samples were kept on ice until the evaluation of spore concentration and spore germination. Within 24 h, the spore concentration was estimated using a hemacytometer. Spore germination was evaluated by pouring 100 μ l of the solution onto water agar plates containing novobiocin (100 μ g ml⁻¹, Sigma-Aldrich, St. Louis), and observing the spores after 24 h of incubation at room temperature. Immediately after the application of the *M. ochracea*, five leaves per half row were collected and placed in plastic bags kept on ice. Within 24 h, the leaves were blotted on a water agar plate amended with novobiocin (100 μ g ml⁻¹). The plates were kept at room temperature (20 to 25°C) with a 12-h photoperiod and observed weekly for the presence of pycnidia during a period of up to 2 months. When pycnidia developed on the leaves, some of them were squashed and observed for the presence of spores typical of the *M. ochracea* (12).

To estimate the actual contribution of *M. ochracea*, the following four treatments were applied to four subplots within each orchard: no fall treatments and standard fungicide program; *M. ochracea* applied to the canopy at 10% leaf fall and delayed-fungicide program; *M. ochracea* applied as a ground application under the trees at 90% leaf fall and delayed-fungicide program; and no fall treatment and delayed-fungicide program. For the 10% leaf fall application, *M. ochracea* was applied on 16 October 1997 and 17 October 1998. For the 90% leaf fall application, *M. ochracea* was applied on 24 November 1997 and 13 November 1998. In the standard program, fungicides were applied to control all primary infection periods. In the delayed-fungicide program, fungicide application was delayed until after three infection periods or until the pink stage of bud development (which-

ever came first), unless the spore samplers indicated that a large amount of ascospores were ejected during an infection period. The concentration of airborne ascospores of *V. inaequalis* was evaluated during each major rain event as described previously. Leaf scab incidence was evaluated three times during the growing season, on 24 June, 15 July, and 13 August 1998 and 30 June, 21 July, and 17 August 1999. Fruit scab incidence was evaluated at harvest. Leaf scab incidence was evaluated based on a sample of 10 trees, 10 shoots per tree, for a total of 200 shoots. Fruit scab incidence was evaluated based on a sample of 200 fruit per subplot.

Timing of application of *M. ochracea*: laboratory tests. In the fall of 1999 and 2000, disease-free apple leaves were collected from *Malus domestica* Borkh. 'McIntosh' trees at the Agriculture and AgriFood Canada research station in Frelighsburg. Leaf disks, 2.7 cm in diameter, were cut and sterilized by irradiation (40 kGy [4 Mrad] of gamma radiation for 10 h). Petri dishes, 9 cm in diameter, were filled with 20 ml of autoclaved Perlite TM (Perlite Atlas Co. LTD., Beauport, Quebec, Canada) and 10 ml of sterilized distilled water. The irradiated leaf disks were placed on top of the perlite (two disks per dish).

A mycelial suspension of *V. inaequalis* was made from equal parts of a mixture of five isolates collected in orchards in Quebec, Canada. A mixture of isolates was used to increase the number of pairs of compatible mating types and, consequently, ensure the production of pseudothecia. All isolates were previously grown separately on potato dextrose agar (PDA; Difco Laboratories, Detroit) at 18°C. The growth rate of each isolate was different; therefore, when more than two-thirds of the surface of a 9-cm petri dish was covered with growth, the dish was stored at 2°C until inoculation of the leaf disks. To prepare the mixture of *V. inaequalis* isolates, one petri dish of each isolate was placed in a sterile plastic bag (Steward Medical, London) along with 200 ml of sterile water. The content of the bag was homogenized in a Stomacher laboratory blender (Seward Medical) for 1 min at normal speed. Sterile Eppendorf repeater-type Combitips TM syringes (Becton Dickinson, Canada Inc.) were used to draw the liquid directly from the bottom of the bag and to inoculate the disk surfaces. Each apple leaf disk was inoculated with 150 µl of *V. inaequalis* suspension. In order to estimate the concentration of the inoculum (mycelial slurry), serial dilutions were made and 100 µl of each dilution was spread onto petri dishes containing 10 ml of PDA (three plates per dilution). The number of CFU was counted after 21 days of incubation at 18°C.

Microspheeropsis ochracea was stored in silicagel at 4°C and cultured at room temperature (22 to 25°C) on Difco PDA medium (Difco Laboratories) amended with novobiocin (100 µg ml⁻¹; Sigma-Aldrich). When mature pycnidia were present, mycelial slurries were prepared by homogenizing the cultures in 60 ml of distilled sterile water with a Stomacher laboratory blender. For all inoculum preparations, the final concentration ranged from 1.21 to 3.04×10^2 CFU/ml. Each leaf disk previously inoculated with *V. inaequalis* was inoculated with 150 µl of this suspension. Sequential inoculation of the leaf disks with *M. ochracea* was done 0, 2, 4, 6, 8, 10, 12, 14, and 16 weeks after the inoculation of the leaf disks with *V. inaequalis*. For each inoculation, 10 dishes were inoculated along with a control consisting of 10 dishes inoculated only with *V. inaequalis*. The experiment was set up as a completely randomized design with 10 repetitions and the entire experiment was conducted twice. Several supplementary control dishes were prepared. These disks were used to follow the maturation of the pseudothecia and determine the developmental stage at the time of inoculation with *M. ochracea* (29) and to determine when the dishes should be placed under conditions that favor ascospore maturation and indicate when the ascospores were mature.

The incubation conditions were described previously by Philion et al. (46). This set of conditions was chosen to optimize the pro-

duction and maturation of pseudothecia and ascospores. The leaf disks were maintained at room temperature for 3 weeks to allow *V. inaequalis* to colonize most of the disk surface. The dishes then were transferred to a cold room adjusted to 4°C with 90% relative humidity (RH) in complete darkness for a period of 4 weeks; then transferred to another cold room adjusted to 10°C with 90% RH and full darkness for an extra 10 to 12 weeks, depending on the degree of maturation of the pseudothecia in the controls. When most pseudothecia were mature, all the dishes were brought to the laboratory and kept at room temperature for another 2 to 3 weeks before ascospore extraction.

The production of ascospores on artificially inoculated leaf disks was evaluated at the end of the incubation period (19 to 21 weeks) determined by the presence of ascospores in pseudothecial squash mounts (23) prepared from control leaf disks inoculated only with *V. inaequalis*. To quantify ascospore production per leaf disk, two leaf disks were placed in a 50-ml Falcon test tube with 20 ml of distilled water and exposed to air turbulence for 6 h (46). A few drops of Lugol's iodine solution were added to each tube to stop germination, and the tubes were then centrifuged at 3,800 rpm for 10 min (Accuspin FR, Beckman, CA). The liquid was carefully drawn out of each tube so that the pellet and surrounding liquid were not disturbed, to obtain 5 ml of ascospore suspension. Ascospore concentration in each suspension was determined using a Fuchs Rosenthal R Haemocytometer (C.A. Hausser & Son, Max Levy Inc.). Ascospore productivity was reported for each treatment as ascospores per square centimeter of leaf disk. The surface of two disks was 11.45 cm² and the spore pellet was suspended in 5 ml, so the number of ascospores per square centimeter was calculated as (ascospore/ml $\times$ 5 ml)/11.45 cm².

Timing of application of *M. ochracea*: orchard trial with naturally infected leaves. The experiment was conducted during the 1999–2000 and 2000–01 seasons at the Agriculture and AgriFood Canada experimental farm in Frelighsburg, Québec, Canada. The experiment was conducted in a mature 0.41-ha orchard planted with cvs. McIntosh (58%) and Lobo (42%). The dwarf trees grafted on M26 rootstock were planted at a distance of 5 by 2.5 m. On seven occasions, from mid-August to early November 1999 and 2000, scabbed McIntosh apple leaves of the cultivar were inoculated with a spore suspension of *M. ochracea* at a rate of 0.5 ml per leaf of a suspension of 4.5×10^5 spores/ml. Inoculated leaves were enclosed in nylon mesh bags fixed to the branches. At the end of November, the bags were removed and fixed to the orchard ground for the overwintering period. In spring 2000 and 2001, the number of ascospores produced per leaf was quantified.

The production of ascospores on detached apple leaves was evaluated over the entire ascospore ejection period (i.e., from late April to the middle of June). One leaf was selected arbitrarily from each sampling unit (nylon bag) for a total of 10 leaves per treatment and placed on the wire bottom of a wooden spore trap (17) with the upper surface upward. Microscope slides, previously coated with petroleum jelly, were placed 0.5 cm above the leaves. After each major rain, all the slides were collected and replaced by new slides. Recovered slides were stored at 10°C until examination. The number of ascospores on 40% of the slide surface was counted under a microscope at a magnification of $\times 250$. Ascospore production then was expressed as the number of ascospores per centimeter of slide.

Statistical analysis. To test the effect of fall application of *M. ochracea* on the pattern of ascospores ejection, the number of ascospores trapped was transformed to seasonal proportion of ejected ascospores by dividing the number of ascospores trapped on a given date by the cumulative number of ascospores trapped for the entire season. Regression analysis was used to compare the seasonal proportion of ejected ascospores on leaves treated with *M. ochracea* in the fall and nontreated leaves. Analysis of

variance was used to test the effect of fall treatment and delayed-fungicide program the following spring on leaf and fruit scab and multiple comparison tests (least significant difference [LSD]) to detect significant differences among means. For the in vitro experiment, a test of homogeneity of variances (*F* test) was 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 the length of the *V. inaequalis* incubation period before the application of *M. ochracea* (in weeks) on the total ascospore production. Multiple comparison tests (LSD) were performed to detect significant differences among means, and orthogonal comparison was performed to compare different incubation periods with the control (*V. inaequalis* alone) at the 0.05 confidence level. Similarly, for the field trials, analysis of variance was used to test the effect of timing of application of *M. ochracea* on total production of ascospores and multiple comparison tests (LSD) to detect significant differences among means, and orthogonal comparison was performed to compare different timing of applications with the control (*V. inaequalis* alone) at the 0.05 confidence level. Because the production of ascospores was measured over time and from the same leaves, the observations were not independent. Therefore, repeated measures analysis was used to test the effect of timing of application of *M. ochracea* on ascospore production for each sampling date (MANOVA procedure). All analyses were done using the Statistical Analysis System (SAS) program (version 8; SAS Institute, Cary, NC).

RESULTS

Effect of *M. ochracea* on ejection pattern of ascospores by *V. inaequalis*. When the number of ascospores of *V. inaequalis*

trapped was transformed in proportion of ejected ascospores, the patterns of ascospore ejection over time for the leaves sprayed with *M. ochracea* was similar to that of untreated leaves (Fig. 1). For the 4 years of the experiment, 84, 76, 78, and 89% less ascospores were collected from apple leaves sprayed in the fall with *M. ochracea* compared with the untreated leaves. When the experiment was conducted in orchard plots and *M. ochracea* applied onto the leaf canopy at 10% leaf fall and onto the ground at 90% leaf fall, a similar pattern of proportion of airborne ascospore concentration over time were observed in treated and nontreated plots (Fig. 2). However, in spring 1998, the number of ascospores trapped in plots where *M. ochracea* was applied the previous fall onto the tree canopy was lower than in nontreated plots or in plots treated the previous fall with *M. ochracea* applied to leaves on the ground. Regression analysis showed that there was a linear relationship between the proportion of season's ascospore matured and concentration of ascospore from leaves treated with *M. ochracea* and on nontreated leaves. The coefficient of determination (R^2) was 0.91 and the intercept and rate parameters were not significantly different than 0 and 1, respectively (Fig. 3).

Effect of fall treatment with *M. ochracea* on scab development the following spring. The first orchard was considered at intermediate risk because the number of scabbed leaves varied from 15 to 21 and the second orchard was considered at high risk because the number of scabbed leaves varied from 107 to 135 (Table 1). When *M. ochracea* was applied, the final concentration and percent germination of the solutions applied in each subplot varied from 7.27×10^5 to 1.47×10^6 conidia/ml with 85 to 100% percent spore germination. *M. ochracea* was recovered from 83 to 100% of the leaves.

Fall treatment and fungicide program had a significant effect on both leaf scab ($P < 0.0002$ and 0.0001 for 1998 and 1999, re-

Fig. 1. Proportion of ascospore of *Venturia inaequalis* ejected from leaves sprayed the previous fall with *Microsphaeropsis ochracea* and from unsprayed leaves. The solid line represents the predicted proportion of the season's ascospore matured calculated based on the equation developed by Gadoury and MacHardy (24). The symbols represent treatment observations and are averages of ascospore trapped from 10 leaves.

spectively) and fruit scab ($P < 0.0001$). Apple scab was more severe in 1988 than in 1999 and, for both years, more scab developed in the high-risk orchard than in the intermediate-risk orchard (Fig. 4). For both years and both orchards, there was no significant difference between leaf scab incidence in the subplots sprayed the previous fall with *M. ochracea* either to the canopy at 10% leaf fall or as a ground application under trees at 90% leaf fall with delayed-fungicide program in the spring compared with the standard program without fall treatment and all infection periods controlled (Fig. 4). However, when no fall treatment was applied and the fungicide program delayed the following spring, leaf scab incidence was significantly higher than in the other subplots (Fig. 4). Fruit scab incidence was not significantly different in subplots sprayed in the fall with *M. ochracea* at 10% leaf fall with a delayed-fungicide program in the spring compared with the standard program without fall treatment and all infection periods controlled (Fig. 5). Fruit scab incidence was significantly higher in subplots sprayed in the fall with *M. ochracea* under trees at 90% leaf fall with a delayed-fungicide program in the spring and in subplots with a delayed-fungicide program in the spring compared with the standard program without fall treatment and all infection periods controlled (Fig. 5). The first three and two infection periods were not treated in the subplots sprayed according to the delayed-fungicide program in 1998 and 1999, respectively (Table 2). However, in the subplots where *M. ochracea* was applied the previous fall under trees at 90% leaf fall, the third and second infection periods in 1998 and 1999, respectively, were treated because a large amount of ascospores were trapped (Table 2).

Fig. 2. Proportion of airborne ascospore concentration of *Venturia inaequalis* in orchard plots treated the previous fall with *Microsphaeropsis ochracea* applied as a foliar spray at 10% leaf fall or as a ground application at 90% leaf fall compared with a nontreated plot. Airborne ascospore concentrations were measured for each major rain event in spring 1997 and 1998. The solid line represents the predicted proportion season's ascospore matured calculated based on the equation developed by Gadoury and MacHardy (24).

Timing of application of *M. ochracea*: laboratory tests. The data from the two experimental runs were pooled based on homogeneity of variance ($P > 0.05$). Regardless of the time between the inoculations of the leaf disks with *V. inaequalis* and *M. ochracea*, significantly fewer ascospores ($P < 0.0001$) were produced on apple leaf disks treated with *M. ochracea* than on the disks inoculated with *V. inaequalis* only (Fig. 6). Low numbers of ascospores were recorded for the leaf disks inoculated with *M. ochracea* 0, 2, 4, and 6 weeks after inoculation with *V. inaequalis*, with 100, 99, 97, and 97% fewer ascospores, respectively. When *M. ochracea* was applied 8 to 16 weeks after the inoculation of the disks with *V. inaequalis*, the ascospores produced per square centimeter of apple leaf disk gradually increased with increasing period of time (Fig. 6). Based on phenological stages of development of the pseudothecia of *V. inaequalis* described by James and Sutton (32), the maximum stage observed (based on a sample of 20 observations) was noted on the day of inoculation of the leaf disks with *M. ochracea*. On leaf disks inoculated 2, 4, and 6 weeks after inoculation with *V. inaequalis*, the maximum stages observed were simple coiling (stage 2), formation of ascogonium from the initial (stage 3), and pseudoparaphyses beginning to appear as the ascogonium disappears (stage 5), respectively. On the leaf disks inoculated with *M. ochracea*, the pseudothecia matured from distinct pseudothecium (stage 6) 8 weeks after the inoculation with *V. inaequalis* to asci formed but contents not differentiated (stage 9) 16 weeks after the inoculation with *V. inaequalis*.

Timing of application of *M. ochracea*: orchard trial with naturally infected leaves. Overall, more ascospores were collected during spring 2000 than in 2001 (Fig. 7). Regardless of the timing of the fall applications, *M. ochracea* significantly ($P < 0.0001$ and $P < 0.0002$ for 2000 and 2001, respectively) reduced the production of ascospores compared with the untreated leaves (Fig. 7). The effect of timing of application of *M. ochracea* was similar for both seasons, with lowest production of ascospores when the scabbed apple leaves were sprayed with *M. ochracea* in August, followed by an increase in the production of ascospores when the leaves were treated in September and October (Fig. 7). When the apple leaves were sprayed in August, the production of ascospores the following spring was reduced by 94 to 96% in 2000 and 99% in 2001. When the leaves were sprayed in September, the production of ascospores was reduced by 61 to 84% in 2000 and 93% in 2001. When the apple leaves were sprayed in October, the production of ascospores was reduced by 64 to 86% in 2000 and 74 to 89% in 2001. The early November application of *M. ochracea* resulted in 54 and 67% fewer ascospores the following spring in 2000 and 2001, respectively.

Fig. 3. Relationship between proportion of ascospores of *Venturia inaequalis* ejected on leaves treated the previous fall with *Microsphaeropsis ochracea* and with that of leaves that were not treated.

For both years, the pattern of ascospore ejection over time was similar on treated and nontreated leaves (Fig. 8) regardless of when the scabbed leaves were sprayed with *M. ochracea* the previous fall. Repeated measures analysis revealed that there was a significant effect of timing of application ($P < 0.0001$) and time ($P < 0.0001$) and that the effect of timing of application on pro-

duction of ascospores varied significantly over time ($P < 0.05$). The effect of timing of application on productions of ascospore was not significant when the amount of ascospores ejected was low. All applications of *M. ochracea* were done before leaf fall, which occurred around 8 November 1999 and 15 November 2000.

TABLE 1. Assessment of apple scab made in late fall 1997, 1998, and 1999 in two different orchards and as affected by application of *Microsphaeropsis ochracea* and fungicide program^a

Orchard ^b	Treatment ^c	Scab leaves/100 shoots			
		1997	1998 ^d	1998 ^d	1999
Orchard I	10% LF-delayed	18	17	31	19
Orchard I	90% LF-delayed	15	24	23	58
Orchard I	Delayed	19	31	24	42
Orchard I	Standard	21	23	17	24
Orchard II	10% LF-delayed	135	102	149	114
Orchard II	90% LF-delayed	127	152	152	167
Orchard II	Delayed	132	169	131	189
Orchard II	Standard	107	129	113	107

^a Scab risk was estimated in each subplot on 28 September 1997, 8 October 1998, and 12 October 1999 using an adaptation of the method described by MacHardy (39) which is based on a late fall scab assessment. In each subplot, both surfaces of all leaves of 10 extension shoots from 10 trees selected at random were examined for the presence of scab lesions. When the number of scabbed leaves was ≤ 5 , between 5 and 25, and >25 , the subplot was considered at low, intermediate, and high risk, respectively.

^b In orchard I the dwarf trees grafted on M26 rootstock were planted at a distance of 5 by 2.5 m. This orchard was composed of Summerland McIntosh Lobo. This orchard was split into subplots of 0.14 ha. In orchard II, the dwarf trees grafted on M26 rootstock were planted at a distance of 4.5 by 2.5 m. This orchard composed of McIntosh only, was split into subplots of 0.15 ha.

^c The treatments applied to four subplots within each orchard were no fall treatments and standard fungicide program (standard), *M. ochracea* applied to the canopy at 10% leaf fall and delayed-fungicide program (10% LF-delayed), *M. ochracea* applied as a ground application under the trees at 90% leaf fall and delayed-fungicide program (90% LF-delayed), and no fall treatment and delayed-fungicide program (delayed).

^d In orchard I, the same subplots were used for the 2 years of the experiment and treatments were assigned randomly each year. In orchard II, different subplots were used each year.

Fig. 4. Proportion of leaves with scab in subplots sprayed in the fall with *Microsphaeropsis ochracea* onto tree canopy at 10% leaf fall (10% LF-delayed) and onto the ground at 90% leaf fall (90% LF-delayed) with delayed fungicide program in the spring, not sprayed in the fall with delayed fungicide program in the spring (Delayed), and not sprayed in the fall with all infection periods controlled next spring (Standard). Bars with the same letters are not significantly different according to the least significant difference test at the 0.05 level of confidence. Orchard I was considered at intermediate scab risk and orchard II at high scab risk (39).

Fig. 5. Proportion of fruit with apple scab in subplots sprayed in the fall with *Microsphaeropsis ochracea* onto the canopy at 10% leaf fall (10% LF-delayed) and onto the ground at 90% leaf fall (90% LF-delayed) with delayed fungicide program in the spring, not sprayed in the fall with delayed fungicide program in the spring (Delayed), and not sprayed in the fall with all infection periods controlled next spring (Standard). Bars with the same letters are not significantly different according to the least significant difference test at the 0.05 level of confidence. Orchard I was considered at intermediate scab risk and orchard II at high scab risk (39).

TABLE 2. Description of the fungicide programs used during spring 1998 and 1999^a

Year, fungicide	Rate (kg/ha)	Date applied	Standard	Delayed	10% LF-delayed	90% LF-delayed
1998						
Polyram 80DF	3.60	24 April	X	—	—	—
Dithane GD	4.80	1 May	X	—	—	—
Captan 80WP	1.69	5 May	X	—	—	X
Nova 40WP	0.153	7 May	X	X	X	X
Captan 80WP	1.69	13 May	X	X	X	X
Captan 80WP	1.69	20 May	X	X	X	X
Maestro 75DF	4.00	28 May	X	X	X	X
Captan 80WP	1.69	1 June	X	X	X	X
Captan 80WP	1.69	10 June	X	X	X	X
1999						
Polyram 80DF	6.00	5 May	X	—	—	—
Captan 80WP	1.69	7 May	X	—	—	X
Nova 40WP	0.153	11 May	X	X	X	X
Polyram 80DF	3.00	11 May	X	X	X	X
Nova 40WP	0.340	21 May	X	X	X	X
Captan 80WP	3.50	21 May	X	X	X	X
Captan 80WP	1.69	24 May	X	X	X	X
Nova 40WP	0.340	27 May	X	X	X	X
Captan 80WP	3.75	27 May	X	X	X	X
Equal 65WP	1.63	1 June	X	X	X	X
Captan 80WP	1.80	11 June	X	X	X	X
Nova 40WP	0.340	11 June	X	X	X	X

^a X indicates that fungicide was applied and — indicates that fungicide was not applied. The treatments applied were no fall treatments and standard fungicide program (standard), no fall treatment and delayed-fungicide program (delayed), *Microsphaeropsis ochracea* applied to the canopy at 10% leaf fall and delayed-fungicide program (10% LF-delayed), and *M. ochracea* applied as a ground application under the trees at 90% leaf fall and delayed-fungicide program (90% LF-delayed).

DISCUSSION

In most apple-production areas, frequent applications of fungicide have been and are still the main approach to manage apple scab (38). Over the years, researchers have developed several tools to effectively target the applications of fungicide during the primary stage of apple scab development by integrating models or techniques to estimate the potential ascospore dose or scab risk

Fig. 6. Production of ascospores of *Venturia inaequalis* on leaf disks inoculated with *Microsphaeropsis ochracea* 0, 2, 4, 6, 8, 10, 12, 14, and 16 weeks after inoculation with *V. inaequalis*. 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 (not inoculated with *M. ochracea*) based on orthogonal comparisons ($P = 0.05$). Data from the two experimental runs were pooled.

Fig. 7. Total production of ascospores by *Venturia inaequalis* from detached scabbed leaves sprayed with *Microsphaeropsis ochracea* on different dates in the fall. The production of ascospores was measured for eight major rain events in 2000 and 2001. 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 (non-treated leaves) based on orthogonal comparisons ($P = 0.05$).

(25,37,39,41), ascospore maturity in the spring (24,30,53), and risk of infection period (40,43). MacHardy et al. (41) showed that delaying the first sprays in orchards with a low potential ascospore dose does not result in significantly or unacceptable fruit scab at harvest. Furthermore, to aid in decision making on whether early spring fungicide application could be eliminated without significant increase in the incidence of fruit scab at harvest, action thresholds based on fall scab assessments have been developed (39). Thus, the reduction of primary inoculum is crucial for optimal management of apple scab.

Conidia and mycelium of *V. inaequalis* do not survive the low temperature and freezing of colder climates (1). Consequently, ascospores are the main source of primary inoculum. It has been known for more than a century that *V. inaequalis* overwinters in scabbed leaves (50), but the relationship between the amount of scab lesions on leaves present in the leaf litter and the amount of inoculum was only established by Curtis in 1924 (20). Methods for destroying *V. inaequalis* in leaves have been studied for more than 70 years (14). Early studies used materials such as lime sulfur or Bordeaux mixtures applied to the leaves either on the tree just prior to leaf fall or to leaves on the ground. More recent studies have examined the effects of applications of benzimidazole and sterol inhibiting fungicides just prior to leaf fall (21). These fungicides were very effective at reducing the primary inoculum; however their use has been discouraged because of the possibility of developing resistance (5,31,45).

Sanitation measures disappeared from the recommendations until 1990, when similar effects of leaf removal on reduction of initial inoculum were reported by Rosenberger in 1990 (47) and subsequently by Sutton et al. in 2000 (54). Sutton et al. (54) reported that, if all apple leaves were shredded in the fall or in the early spring, the primary inoculum was reduced by 80 to 90%. However, the authors also reported that, when incomplete shred-

Fig. 8. Production of ascospores by *Venturia inaequalis* over time from detached scabbed leaves sprayed with *Microsphaeropsis ochracea* on different dates in the fall. Each value is an average of ascospore production from 10 leaves.

ding of leaves was done, primary inoculum was reduced by only 50 to 65%. Leaf shredding or removal is weather dependant because it can be done only when most leaves are on the ground and depends on the region, because this may be at the time of first snowfall.

Several researchers (10,11,19,26,48,54) studied the role of urea in the inhibition of *V. inaequalis* pseudothecia development. Fall application of urea induces modifications in chemical and microbial components of apple leaf surfaces and causes deterioration of the physical structure of the leaves, which contributes to the suppression of the production of ascospores (19). Despite the apparent effect of urea application on ascospore production, very few large-scale experiments have been conducted. In such an experiment, Sutton et al. (54) reported a decrease in relative ascospore dose of 74% following a spring application of urea on leaf litter. In the same study, the fall application of 5% urea failed to significantly reduce ascospore production. Biosanitation (i.e., the use of a biological control agent to prevent disease by the reduction of primary inoculum) is one avenue for management of primary inoculum.

Researchers have investigated the possibility of using fungal antagonists to interfere with pseudothecial development of *V. inaequalis* (14,27,28). *Athelia bombacina* was selected based on its ability to inhibit pseudothecial development. In a field trial, Young and Andrews (56) showed that *A. bombacina* inhibits hyphal growth and initiation of pseudothecia by *V. inaequalis*. However, complete pseudothecial inhibition was obtained in field trials only when very high doses of the antagonist inoculum were used (27,28). When a lower rate was used, pseudothecial inhibition was reduced to only 60 to 70% (42). Up to now, *A. bombacina* has not been developed as a commercial biological control agent.

In previous studies, *M. ochracea* was identified as a good antagonist of *V. inaequalis* (17,46); however, its effect on ejection pattern of ascospores and scab development has not been established. The results of the present study showed that, although a late-summer application of *M. ochracea* reduced the production of ascospores of *V. inaequalis* the following spring by $\approx 90\%$, the ejection pattern of ascospores over time was not modified. This is important because it means that maturation models developed for different apple-growing areas (24,30,53) could be used in orchards treated with *M. ochracea*. In this study, *M. ochracea* was evaluated as a sanitation practice in orchards with moderate and high scab risk. The first three and two infection periods, in 1998 and 1999, respectively, were not treated with fungicide in the plots sprayed the previous fall with *M. ochracea* at 10% leaf fall without causing significant increase in fruit scab incidence. When the same infection periods were not treated with fungicides in plots not sprayed the previous fall with *M. ochracea*, the fruit scab incidence was significantly higher and at a level unacceptable for growers. Furthermore, for both years and both orchards, the delayed-fungicide program in plots sprayed the previous fall with *M. ochracea* did not result in an increase in scab risk. Delaying fungicide program would reduce the total amount of fungicide applied per orchard and, consequently, reduce costs of labor, equipment, and material, as well as early season passes through the orchard under wet conditions. Reduction in amount of fungicide applied also may reduce pressure for the development of fungicide resistance in *V. inaequalis* population.

Previous studies showed that *M. ochracea* has the potential to reduce the production of ascospore by 81 to 96% (17,46). However, in large field trials, the reduction in ascospore production was only 75.27%, indicating the need for optimization, including timing of application. Because *M. ochracea* was isolated on leaf litter (4) and accelerated leaf decomposition (46), and because wetness of leaf litter usually is not a limiting factor in the fall, it was hypothesized that this antagonist should be applied on leaf litter at $\approx 90\%$ leaf fall. However, there are limitations to this approach. In a previous study, greater reduction in the production of

ascospores was observed when *M. ochracea* was applied onto the apple tree canopy at the time of 10% leaf fall (17). *M. ochracea* germinates and grows best at temperatures from 20 to 25°C (13), which is more likely to occur at 10% leaf fall stage than at the 90% leaf fall stage when the average daily temperature is low (below 20°C); and, if snow cover is present, ground applications will not be feasible.

Timing of application is critical for chemical as well as for biological fungicides. *M. ochracea*, for example, is capable of reducing initial inoculum of *V. inaequalis* by $\approx 80\%$ (17), but the best timing of application was unknown. In the present study, the in vitro and orchard experiments showed that *M. ochracea* was most effective when applied during late summer or early fall rather than during late fall. Under laboratory conditions, *M. ochracea* was more efficient at reducing the production of ascospores by *V. inaequalis* when it was applied before the formation of distinct pseudothecium. The exact mode of action of *M. ochracea* is not understood, but the higher efficiency of late-summer applications may be explained by the parasitic activity and the attraction of *M. ochracea* to melanized fungal structures, including mycelium of *V. inaequalis* (2,15,22). It could be that *M. ochracea*, when applied on apple leaves, is attracted by apple scab lesions (presence of tissues with necrosis and of fungal melanin) (22). Another possibility is that *M. ochracea* colonizes the lesion and parasitizes *V. inaequalis* mycelium, preventing mating and, later, the formation of pseudothecia.

The use of *M. ochracea* should be considered as a sanitation or orchard hygiene practice and not as a replacement for fungicides during the primary scab season. It probably could be best used in conjunction with a fall assessment of potential ascospore dose (PAD) (25,37,39,41). Knowing ahead of time how much primary inoculum is expected in the orchard can assist growers in developing control strategies the following spring. For example, in orchards with high inoculum (high PAD), *M. ochracea* could be used to reduce the inoculum potential and, consequently, bring the scab risk down to a manageable level. In orchards with low inoculum potential, *M. ochracea* could be used to secure delayed-fungicide spray strategies (39,41).

ACKNOWLEDGMENTS

This work was financially supported by a Matching Investment Initiative between Agriculture and Agri-Food Canada and PhilomBios.

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