Toward an Integrated Use of Biological Control by *Cladosporium cladosporioides* H39 in Apple Scab (*Venturia inaequalis*) Management

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Abstract

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Apple scab, caused by *Venturia inaequalis*, is the most important disease in apple production, reducing yield and quality of fruit. Control of apple scab in commercial orchards currently depends on multiple applications of fungicides. The potential of the antagonistic isolate *Cladosporium cladosporioides* H39, originating from a sporulating colony of *V. inaequalis*, to control apple scab development was tested in eight trials during 2 years in orchards in Eperjeske (Hungary), Dabrowice (Poland), and Bavendorf (Germany) planted with different cultivars. Treatments were conducted as calendar sprays or after infection periods. Additional trials in an orchard in Randwijk (The Netherlands) focused on the effect of timing of antagonist application before or after infection periods. The overall results of the field trials consistently showed—for the first time—that

stand-alone applications of the antagonist *C. cladosporioides* H39 can reduce apple scab in leaves and fruit. This was demonstrated in an organic growing system as well as in conventional orchards by spray schedules applied during the primary or the summer season. In both systems, the same control levels could be reached as with common fungicide schedules. Efficacies reached 42 to 98% on leaf scab incidence and 41 to 94% on fruit scab. The antagonist was also effective if applied one or even several days (equivalent to approximately 300 to 2,000 degree h) after infection events in several field trials and a trial conducted in Randwijk with single-spray applications at different intervals before or after infection events. Better understanding of the biology of the antagonist will help to further exploit its use in apple scab control.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter, is the most important disease in apple production worldwide, reducing yield and quality of fruit (23,24). *V. inaequalis* overwinters mainly in fallen leaves but survival of conidia on twigs has also been reported (18). The saprophytological colonization of leaf litter leads to the formation of pseudothecia in which ascospores are produced. Such ascospores are released during the primary season in spring and may infect young apple leaves and young fruit. After this initiation of scab epidemics by primary ascospores, the further multicyclic development of the epidemic depends on the multiple development of the secondary conidia produced on leaves; this means that leaves and fruit can be infected throughout the whole growing season, although ontogenic resistance reduces the number of infections.

The development of preventive measures to limit the damage by apple scab focuses on the reduction of primary inoculum during overwintering in leaf litter. Removal or shredding of fallen leaves (13,28) or the application of antagonists (1,6,7) has been investigated. Mechanical measures can be part of a disease management strategy in commercial orchards, especially in organic farming (17). Despite the tremendous amount of research and number of publications on biological control of plant pathogens, biological control options for apple scab control are currently not available for use in practice (7). Another option is the use of resistant cultivars. However, limited acceptance by consumers as well as the breakdown of the major scab resistance gene *Vf* by *V. inaequalis* populations (14) limit the use of resistant cultivars.

Control of apple scab in commercial orchards currently depends on multiple applications of fungicides (25). Spray schedules with alternating use of active ingredients from several fungicidal groups are

common during the primary season as well as the summer season. Several weather-based decision support systems are available for growers to support their decisions on the timing of specific fungicide applications. Timing of applications during the primary season, guided by information on ascospore discharges, is critical to prevent the build up of scab epidemics because abundant amounts of young leaf and fruit tissue, highly susceptible to *V. inaequalis* infections, are present in spring. Often, failures in scab control during spring cannot be repaired by fungicide measures later in the season. Fungicide applications during the summer season aim at the protection of newly formed susceptible young leaf tissue to prevent further build up of epidemics. Fungicides are also sprayed to prevent late infections on old leaves, often leading to latent infections, to lower the primary inoculum load for the following season (22). Summer applications of fungicides also reduce the late infection of apple fruit which may result in scab lesions on stored fruit. *V. inaequalis* populations developed resistance to an increasing number of fungicides in major apple-growing areas during recent years (2,10). Furthermore, governmental regulations (e.g., in the European Union) restrict the use of fungicides (9). The increasing demand for fruit without or with low pesticide residues is another reason why alternatives for synthetic fungicides are needed.

Biological control of apple scab has been investigated for over 50 years (6), with most of the research focusing on overwintering *V. inaequalis*. The antagonists *Athelia bombacina* (15) and *Microphaeropsis* sp. (8) showed a high potential to reduce ascospore formation in leaf litter. Field application of *Microphaeropsis* sp. resulted in an ascospore reduction of 75 to 80%. Reports on biological control of apple scab in the canopy are rare (3,5,11,12). Recently, the antagonistic isolate *Cladosporium cladosporioides* H39 has been isolated from a sporulating colony of *V. inaequalis* on an apple leaf collected from a Dutch orchard (20,21). This isolate reduced the production of *V. inaequalis* conidia on apple seedlings when applied 1 to 3 days after inoculation with the pathogen.

The objective of our study was to assess the effect of applications of *C. cladosporioides* H39 on apple scab development under orchard

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conditions and to test the hypothesis that timing of antagonist applications may affect treatment efficacy. Treatments were conducted as calendar sprays during the primary season and the summer season. Another strategy was to apply the antagonist after predicted infection periods similar to the situation created during the screening bioassays (21). Additional experiments focused on the effect of timing of antagonist application before or after infection periods and its compatibility with agrochemicals commonly used in apple production.

Material and Methods

Orchards and experimental design. Eight trials were carried out in orchards at three locations to assess the effect of applications of *C. cladosporioides* H39 on apple scab development. The effect of timing of single applications of *C. cladosporioides* H39 on sporulation of *V. inaequalis* was tested in additional trials in Randwijk. All trials were conducted in a fully randomized block design. Orchard characteristics and experimental designs are shown in Tables 1 and 2. During the trials, meteorological data were collected using a METOS agrometeorological station (Pessl Instrument GmbH, Weiz, Austria) in Eperjeske and Dabrowice, a Campbell CR-800 station (Campbell Scientific, Logan, UT) in Bavendorf, and a Mety station (Bodata, Dordrecht, The Netherlands) in Randwijk. Risks of infection periods by *V. inaequalis* were forecasted using RIMpro (Bio Fruit Advies, Zoelmond, The Netherlands) in Eperjeske, Dabrowice, and Randwijk and SCHORF (version 5.07; Farm Software GmbH, Bermatingen, Germany) in Bavendorf.

Treatments and assessments. Conidia of *C. cladosporioides* strain H39 (20) were produced on a cereal-based solid growth medium. Conidia were pilot formulated using a detergent to achieve optimum applicability and spread on the target. In an additional treatment in Eperjeske, Hungary, nonformulated spores were applied. Suspensions applied at the different locations contained 2×10^6 conidia/ml. In an additional treatment in Bavendorf in 2013, suspensions containing 6×10^6 conidia/ml were also applied for comparison.

The basic treatments in the trials were (i) untreated control; (ii) a reference fungicide schedule; (iii) *C. cladosporioides* H39 applications at regular intervals, usually 7 days but with extended intervals during periods of dry weather when no progress of scab epidemics was expected; (iv) *C. cladosporioides* H39 applications 1 day after predicted infection events by *V. inaequalis* on dry leaves, which was slightly modified in the trial in Eperjeske in 2013, where applications were applied 500 to 600 degree h after infection events on dry leaves; and (v) alternating applications of fungicides and *C. cladosporioides* H39. Treatments iii to v were not conducted in all trials. Fungicides used were copper oxychloride (Funguran, 500 g a.i./kg, applied at 1 kg/ha) and calcium polysulfide (lime sulfur, 290 g a.i./liter, applied at 1.5 l/ha) in the organically managed orchards in Eperjeske; and dithianon (Delan, containing 700 g a.i. kg⁻¹), applied at 0.65 kg/ha in Dabrowice and 0.25 kg/ha in Bavendorf, or dodine (Syllit, containing 400 g a.i./liter) applied at 0.625 liter/ha in the other orchards. Details on application frequencies and techniques are shown in Tables 1 to 3. Disease incidence (%) and phytotoxicity were assessed at the end of the primary or summer season (Table 2) on randomly chosen leaves and fruit. Infestation was assessed on 200 leaves and 50 fruit per plot in Eperjeske, on 200 leaves and 100 fruit in Dabrowice (where disease severity was also assessed using a six-point scale of evaluation from 0 = no symptoms to 5 = more than 75% of the area covered by scab in the case of leaves and more than 30% for fruit), and on 200 leaves and 200 fruit per plot in Bavendorf, except for the trial in 2013, where all leaves of 20 randomly chosen extension shoots per plot (ranging from 195 to 308 leaves) were assessed.

In most trials, fungicide schedules had been applied on all experimental plots before the trials started: 10 treatments with copper oxychloride or elementary sulfur in trial Eperjeske 2012; 2 treatments with copper oxychloride or dithianon in trial 1 in Dabrowice; 10 treatments with captan, copper oxychloride, difenoconazole, dithianon, pyrimethanil, thiram, or trifloxystrobin in trial 2 in Dabrowice; and 14 treatments with captan, copper oxychloride, dithianon, dodine, myclobutanil, pyraclostrobin, or trifloxystrobin in trial 2 in 2012 and

Table 1. Orchard characteristics and application techniques

Location	Cultivar and rootstock	Planting year	Planting distance (m)		Canopy height (m)	Spray applications	
			Within rows	Between rows		Equipment	Volume (liters/ha)
Eperjeske (48°21'30"N, 22°13'10"E) ^z	Idared on M26	1996	2.0	5.0	3.2	Handheld spray gun	600
Dabrowice (52°18'22"N, 19°4'44"E)							
Trial 1	Cortland on M26	2003	1.8	3.5	2.6	Air blast sprayer	1,300
Trial 2	McIntosh on M26	2003	1.8	3.5	2.6	Air blast sprayer	1,300
Bavendorf (47°45'37"N, 9°32'31"E)							
2012, Trials 1 and 2	Golden Delicious on M9	2001	0.8	3.2	2.25	Air blast sprayer	500
2013, Trials 1 and 2	Golden Delicious on M9	2008	1.0	3.5	2.0	Air blast sprayer	500
Randwijk (51°56'18"N, 5°42'23"E)	Jonagold on M9	1998	1.0	3.0	2.5	Knapsack sprayer	Until run-off

^z Hungarian organic production guidelines applied.

Table 2. Experimental design of field trials

Trial	Number of			Period of applications		Assessment dates
	Treatments	Replicates	Trees per plot	Primary season	Summer season	
Eperjeske 2012	6	5	7	...	8 June–7 September	3 October
Eperjeske 2013	6	5	7	26 March–4 June	...	21 June
Dabrowice 2013, trial 1	4	4	5	29 April–29 May	12 June–22 August	25 June, 17 October (leaves); 26 July, 12 September (fruit)
Dabrowice 2013, trial 2	5	4	5	...	14 June–22 August	17 October (leaves), 10 September (fruit)
Bavendorf 2012 trial 1	3	4	12	29 March–16 May	...	10 May and 23 May
Bavendorf 2012 trial 2	3	4	12	...	11 June–18 September	6 June, 11 July (leaves); 8 August, 21 September (fruit)
Bavendorf 2013 trial 1	4	4	12	21 April–27 May	...	23 May, 5 June
Bavendorf 2013 trial 2	4	4	12	...	11 June–17 September	6 June, 18 July (leaves); 26 September (fruit)

trial 2 in 2013 in Bavendorf. These schedules were similar to common practice in commercial orchards in the growing areas, and application dates were chosen depending on predicted ascospore flights and infection periods.

Three additional small-scale trials were carried out in an orchard in Randwijk, The Netherlands, with single-spray applications of *C. cladosporioides* H39 on 'Jonagold'. These trials contained five treatments in a completely randomized block design with six blocks (replicates). Each plot consisted of one tree. The youngest just-unfolded leaves of 5 shoots/tree were labeled. Spray applications

were timed so that the antagonist was applied approximately 350 degree h before a predicted infection event. In the other treatments, *C. cladosporioides* H39 was applied approximately 1,000, 2,000, or 3,000 degree h after the predicted infection event. Untreated trees served as control. The labeled leaf, not yet unfolded at the beginning of the trial, and the next two elder leaves of each of the five labeled shoots per plot were sampled separately 20 days after the predicted infection event. The five leaves of similar age per plot were pooled and shaken in 30 ml of water containing 0.01% (vol/vol) Tween 80, and the number of *V. inaequalis* conidia per milliliter was

Table 3. Application dates of sprays of *Cladosporium cladosporioides* H39 and fungicides

Trial	Sprays	Applications dates															
Eperjeske 2012	Copper oxychloride	8 Jun	15 Jun	24 Jun	4 Jul	14 Jul	28 Jul	13 Aug	26 Aug	7 Sep	...	...	...	...	...	...	...
	H39, interval	8 Jun	15 Jun	22 Jun	29 Jun	5 Jul	13 Jul	20 Jul	27 Jul	2 Aug	9 Aug	16 Aug	23 Aug	30 Aug	6 Sep	...	...
	H39, after infection	9 Jun	14 Jun	20 Jun	27 Jun	4 Jul	11 Jul	22 Jul	2 Aug	14 Aug	18 Aug	26 Aug	5 Sep	...	...	...	...
Eperjeske 2013	Copper oxychloride	26 Mar	6 Apr	15 Apr	21 Apr	30 Apr	4 May	9 May	16 May	24 May	1 Jun	...	...	...	...	...	...
	H39, interval	26 Mar	2 Apr	9 Apr	16 Apr	23 Apr	30 Apr	7 May	14 May	21 May	28 May	4 Jun	...	...	...	...	...
	H39, after infection	28 Mar	9 Apr	17 Apr	24 Apr	30 Apr	7 May	12 May	19 May	25 May	4 Jun	...	...	...	...	...	...
Dabrowice 2013, trial 1	Lime sulfur	27 Mar	8 Apr	17 Apr	23 Apr	29 Apr	6 May	11 May	18 May	24 May	3 Jun	...	...	...	...	...	...
	Dithianon	29 Apr	6 May	13 May	21 May	29 May	5 Jun	12 Jun	24 Jun	15 Jul	...	...	...	...	...	...	...
	H39, interval	29 Apr	6 May	13 May	21 May	29 May	12 Jun	19 Jun	24 Jun	2 Jul	12 Jul	24 Jul	1 Aug	12 Aug	22 Aug	...	...
Dabrowice 2013, trial 2	Dithianon	14 Jun	24 Jun	15 Jul	23 Aug	...	...	...	...	...	...	...	...	...	...	...	...
	H39, interval	14 Jun	19 Jun	24 Jun	2 Jul	12 Jul	24 Jul	1 Aug	12 Aug	22 Aug	...	...	...	...	...	...	...
	H39, after infection	14 Jun	24 Jun	15 Jul	23 Aug	...	...	...	...	...	...	...	...	...	...	...	...
Bavendorf 2012 trial 1	Dithianon	29 Mar	2 Apr	10 Apr	17 Apr	25 Apr	2 May	9 May	16 May	...	...	...	...	...	...	...	...
	H39, interval	29 Mar	2 Apr	10 Apr	17 Apr	25 Apr	2 May	9 May	16 May	...	...	...	...	...	...	...	...
	Dithianon	11 Jun	18 Jun	26 Jun	3 Jul	10 Jul	17 Jul	24 Jul	31 Jul	7 Aug	13 Aug	21 Aug	28 Aug	5 Sep	11 Sep	18 Sep	...
Bavendorf 2012 trial 2	H39, interval	11 Jun	18 Jun	26 Jun	3 Jul	10 Jul	17 Jul	24 Jul	31 Jul	7 Aug	13 Aug	21 Aug	28 Aug	5 Sep	11 Sep	18 Sep	...
	Dithianon	11 Jun	18 Jun	26 Jun	3 Jul	10 Jul	17 Jul	24 Jul	31 Jul	7 Aug	13 Aug	21 Aug	28 Aug	5 Sep	11 Sep	18 Sep	...
	H39, interval	11 Jun	18 Jun	26 Jun	3 Jul	10 Jul	17 Jul	24 Jul	31 Jul	7 Aug	13 Aug	21 Aug	28 Aug	5 Sep	11 Sep	18 Sep	...
Bavendorf 2013 trial 1	Dodine	21 Apr	29 Apr	4 May	6 May	11 May	13 May	21 May	24 May	27 May	...	...	...	...	...	...	...
	H39, after infection	21 Apr	29 Apr	4 May	6 May	11 May	13 May	21 May	24 May	27 May	...	...	...	...	...	...	...
	Dodine	11 Jun	21 Jun	26 Jun	4 Jul	25 Jul	7 Aug	28 Aug	9 Sep	17 Sep	...	...	...	...	...	...	...
Bavendorf 2013 trial 2	H39, after infection	11 Jun	21 Jun	26 Jun	4 Jul	25 Jul	7 Aug	28 Aug	9 Sep	17 Sep	...	...	...	...	...	...	...
	Dodine	11 Jun	21 Jun	26 Jun	4 Jul	25 Jul	7 Aug	28 Aug	9 Sep	17 Sep	...	...	...	...	...	...	...
	H39, after infection	11 Jun	21 Jun	26 Jun	4 Jul	25 Jul	7 Aug	28 Aug	9 Sep	17 Sep	...	...	...	...	...	...	...

Table 4. Agrochemicals tested for their effects on *Cladosporium cladosporioides* H39

Product trade name	Active ingredient	Producer	Concentration of a.i.	Maximum rate of product tested ^z
Admire	Imacloprid	Bayer Crop Science	700 g/kg	0.5 g/liter
Armcarb	Potassium bicarbonate	Agrochemicals	850 g/kg	5.88 g/liter
Bellis	Pyraclostrobin	BASF	128 g/kg	4 g/liter
	Boscalid	BASF	252 g/kg	4 g/liter
Calypso	Thiacloprid	Bayer CropScience	480 g/liter	1.25 ml/liter
Delan WG	Dithianon	Cropcare	700 g/kg	2.125 g/liter
DiPel	<i>Bacillus thuringiensis</i>	Valent Biosciences	450 g/kg	5 g/liter
Exact	Triadimenol	Bayer CropScience	50 g/liter	2.5 ml/liter
Flint	Trifloxystrobin	Bayer CropScience	500 g/kg	0.5 g/liter
Folicur	Tebuconazole	Bayer CropScience	250 ml/liter	1 ml/liter
Funguran	Copper oxychloride	Spiess Urania	500 g/kg	1.25 g/liter
Gazelle	Acetamiprid	Certis	200 g/kg	1.25 g/liter
Insegar	Fenoxicarb	Syngenta	250 g/kg	1.5 g/liter
Kumulus	Sulfur	BASF	800 g/kg	10 g/liter
Yates lime sulfur	Calcium polysulfides	Yates Australia	200 g/liter	50 ml/liter
MaxCel	6-Benzyladenine	Valent Biosciences	19 g/liter	25 ml/liter
Merpan WG	Captan	Farmoz	800 g/kg	6.5 g/liter
Spoiler	Mineral oil	Van Wesemael	844 g/liter	30 ml/liter
Nimrod	Bupirimate	Farmoz	250 g/liter	2.5 g/liter
Pirimor	Pirimicarb	Syngenta	500 g/kg	2.5 g/liter
Runner	Methoxyfenozide	Bayer CropScience	240 g/liter	2 ml/liter
Scala	Pyrimethanil	Bayer CropScience	400 g/liter	3.75 ml/liter
Score	Difenoconazole	Syngenta	100 g/kg	1.875 g/liter
Steward	Inoxacarb	DuPont	158 g/liter	0.850 g/liter
Stroby	Kresoxim-methyl	BASF	500 g/kg	1 g/liter
Switch	Fludioxonil	Syngenta	250 g/kg	4 g/liter
	Cyprodinyl	Syngenta	375 g/kg	4 g/liter
Syllit	Dodine	Campbell Chemicals	400 g/liter	6.5 ml/liter
Teppeki	Flonicamid	Belchim	500 g/kg	0.7 g/liter
Topaz	Penconazole	Syngenta	100 g/kg	1.25 ml/liter
Tracer	Spinozad	Dow Agro Science	442 g/kg	0.75 ml/liter
Vertimec	Abamectine	Syngenta	18 g/liter	3.75 ml/liter

^z Based on a spray volume of 1,000 liters/ha for mineral oil and Armcarb and 200 liters/ha for all other agrochemicals.

counted using a hemocytometer. The leaf area of the sets of five leaves was measured (LI3100 area meter, LI-COR Nebraska) and the number of *V. inaequalis* conidia per square centimeter was calculated.

Compatibility of *C. cladosporioides* H39 with plant protection products. The effects of agrochemicals used in apple production in integrated pest management and organic systems (Table 4) on conidial germination and on mycelial growth of *C. cladosporioides* H39 were assessed in vitro.

Conidial germination. *C. cladosporioides* H39 was grown in petri dishes on potato dextrose agar (PDA; 39 g/liter; Oxoid) for 9 days at 20°C in the dark. To obtain conidial suspensions, cultures were flooded with sterile tap water containing 0.01% Tween 80. After gently rubbing with a rubber spatula, the resulting suspension was filtered through sterile 200- μ m nylon gauze. Concentrations of the conidial suspensions were determined by using a hemacytometer and

adjusted with sterile tap water containing 0.01% Tween 80 to 5×10^5 conidia/ml. Flasks containing the conidial suspensions were kept in ice water and suspensions were sprayed within 1 h on malt agar (1/10 strength) in petri dishes (60 mm in diameter) amended with various agrochemicals at different concentrations (Table 4; Figs. 1–3). Two petri dishes (60 mm in diameter) were used for each combination of agrochemical and concentration. Petri dishes were incubated for 24 h in the dark at 20°C. Thereafter, fungal growth in the dishes was stopped by adding 1 ml of household ammonia (4.8% [wt/wt] NH_4OH) onto a filter paper (40 mm) placed in the lid of the petri dish. Germination was assessed from 50 arbitrarily chosen conidia of *C. cladosporioides* H39. Conidia with germ tubes longer than half of the conidial length were considered as germinated. The experiment was conducted twice.

Mycelial growth. *C. cladosporioides* H39 was incubated for 14 days on malt agar (1/10 strength; malt extract [Oxoid] at 1 g/liter).

Fig. 1. Effect of fungicides used in apple orchards for **A** and **B**, scab control during the early season; **C** and **D**, powdery mildew control during the summer season; and **E** and **F**, scab control during the summer season on **A**, **C**, and **E**, conidial germination and **B**, **D**, and **F**, mycelial growth of *Cladosporium cladosporioides* H39 on agar in petri dishes. Bars indicate standard error of the mean.

Agar disks (4 mm in diameter) from the edges of colonies were transferred to malt agar amended with various agrochemicals at different concentrations (Table 4; Figs. 1–3). Two petri dishes (90 mm in diameter) were used for each combination of agrochemical and concentration. Petri dishes were incubated for 10 days in the dark at 20°C. Thereafter, the diameter of each *C. cladosporioides* H39 colony was measured twice on rectangular positions and the mean daily growth rate of the colony was calculated. The experiment was conducted twice.

Statistics. Data from each of the eight orchard trials in Eperjeske, Dabrowice, and Bavendorf were analyzed separately per assessment date by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) tests for multiple comparisons of the means of the different treatments (GENSTAT, 16th ed.; one- and two-way ANOVA). The numbers of *V. inaequalis* conidia produced on leaves in the trials in Randwijk were log₁₀ transformed before analysis to obtain normal distributions and the effects of antagonist treatments versus untreated control

were tested by one-sided LSD tests. Data on the efficacy of the agrochemicals compared with control treatments without agrochemicals are presented as overall mean and standard error of the mean if no differences were found between the repeated experiments.

Results

Spray schedule trials in Eperjeske, Dabrowice, and Bavendorf.

Apple scab epidemics occurred in the eight field trials carried out in Eperjeske, Dabrowice, and Bavendorf. Environmental conditions were more conducive in Eperjeske in 2012 and in Bavendorf in 2013.

In the trials in Eperjeske during the summer season 2012, applications of nonformulated or formulated conidia of *C. cladosporioides* H39 at weekly intervals reduced scab on leaves and fruit ($P < 0.001$) as effectively as the copper-based spray schedule commonly used in organic farming (Table 5, trial 12). Applications of the formulation without conidia of *C. cladosporioides* H39 did not reduce

Fig. 2. Effect of A–D, insecticides used in apple orchards and E, and F, a chemical thinner on A, C, and E, conidial germination and B, D, and F, mycelial growth of *Cladosporium cladosporioides* H39 on agar in petri dishes. Bars indicate standard error of the mean.

Fig. 3. Effect of fungicides and insecticides used in organic apple orchards on **A**, conidial germination and **B**, mycelial growth of *Cladosporium cladosporioides* H39 on agar in petri dishes. Effect on conidial germination was tested only for Armicarb and Tracer, and for lime sulfur at the lowest concentration. Bars indicate standard error of the mean.

Table 5. Effects of different application strategies of *Cladosporium cladosporioides* H39 on apple scab incidence on leaves and fruit in comparison with a copper spray schedule^w

Treatment ^x	Number of applications	Scab incidence (%) on	
		Leaves	Fruit
Trial 2012 ^y			
Untreated control	-	70.1 a	45.2 a
Copper oxychloride	9	35.8 c	20.2 d
H39, nonformulated conidia, interval	14	34.8 c	24.3 cd
H39, interval	14	39.7 c	26.0 cd
H39, after infection	12	52.6 b	30.5 bc
Pilot-formulation, interval	14	64.2 a	37.4 ab
Trial 2013 ^z			
Untreated control	-	46.7 a	32.5 a
Copper oxychloride	10	20.7 c	15.2 c
H39, interval	11	22.3 c	17.6 c
H39, after infection	10	30.5 b	24.8 b
H39, interval + lime sulfur if RIMpro > 200	11 + 10	16.4 c	13.5 c
Lime sulfur if RIMpro > 200	10	18.5 c	14.3 c

^w Orchards trials in Eperjeske, Hungary, on Idared.

^x Values of the same assessment date followed by a common letter do not differ significantly (least significant difference test, $\alpha = 5\%$).

^y Treatments applied during the summer season from 8 June to 7 September 2012; assessment of scab incidence on 3 October 2012.

^z Treatments applied during the primary season from 26 March to 4 June 2013; assessment of scab incidence on 21 June 2013.

Table 6. Effects of multiple treatments of *Cladosporium cladosporioides* H39 and dithianon on apple scab disease incidence (DI) and severity (DS) on leaves and fruit^u

Treatment	N ^v	Scab on leaves				Scab on fruit					
		25 June		17 October		26 July		10 September		12 September	
		DI	DS	DI	DS	DI	DS	DI	DS	DI	DS
Trial on Cortland ^w											
Untreated control	...	62.3 c	1.4 b	100.0 d	4.9 c	65.6 c	1.6 c	...	...	83.8 c	4.7 c
Dithianon ^x	8	9.4 a	0.1 a	45.6 b	1.2 a	7.9 a	0.1 a	...	...	5.0 a	0.1 a
H39 interval	14	33.3 b	0.6 a	58.3 c	2.3 b	33.0 b	0.6 b	...	...	49.5 b	1.2 b
H39 interval + dithianon ^x	14 + 8	7.8 a	0.1 a	31.1 a	0.7 a	5.9 a	0.1 a	...	...	15.3 a	0.3 a
Trial on McIntosh ^y											
Untreated control	...	...	...	37.5 b	0.9 b	...	...	26.0 d	0.5 c	...	...
H39, after infection ^z	4	...	...	37.6 b	1.0 b	...	...	12.5 c	0.2 b	...	...
Dithianon after infection ^z	4	...	...	15.8 a	0.3 a	...	...	5.5 ab	0.1 a	...	...
Dithianon after infection + H39 interval ^z	4 + 9	...	...	12.9 a	0.2 a	...	...	4.0 a	0.1 a	...	...
H39 interval	9	...	...	20.6 a	0.4 a	...	...	8.5 b	0.1 ab	...	...

^u Orchard trials in Dabrowice in 2013. Values of the same assessment date followed by a common letter do not differ significantly (least significant difference test, $\alpha = 5\%$).

^v Number of applications

^w Treatments applied during the whole growing season from 29 April to 22 August on Cortland.

^x If RIMpro > 200.

^y Treatments applied during the summer season from 14 June to 23 August on McIntosh.

^z One day after RIMpro indicated infection.

apple scab. A first attempt to develop a rationale for an improved timing of spray applications focusing on scab development rather than fixed intervals as calendar sprays was made by spraying 1 day after predicted infection events. This strategy was slightly less effective than calendar sprays. Also, in the trial in 2013 at the same location during the primary season, calendar sprays of *C. cladosporioides* H39 were as effective as a copper-based spray schedule (Table 5, trial 2013). Sprays after predicted ascospore infections significantly reduced scab incidence on leaves and fruit ($P < 0.001$). However, such a schedule was less effective than calendar sprays. Combining weekly calendar sprays of *C. cladosporioides* H39 with lime sulfur application when high infection risks were predicted (RIMpro > 200) gave the best control effects but the additional effect of antagonist applications compared with the lime sulfur treatments without antagonist was not significant.

A scab epidemic throughout the whole season trial in Dabrowice on 'Cortland' apple was severe, with a leaf scab incidence of >60% already in July and 100% in October (Table 6, Cortland). Under these conditions, the weekly calendar application of *C. cladosporioides* H39 significantly reduced scab incidence and severity on leaves and fruit by approximately 50% ($P < 0.001$). Treatments with dithianon were significantly more effective. A significant additional effect of weekly sprays of *C. cladosporioides* H39 in combination with dithianon sprays (if RIMpro > 200) could be found for leaf scab incidence at the end of the season in October. In the trial at the same location carried out during the

summer epidemic on 'McIntosh', four applications of *C. cladosporioides* H39, applied after prediction of *V. inaequalis* infections by RIMpro, did not control leaf scab but significantly reduced fruit scab incidence and severity (Table 6, McIntosh) ($P < 0.001$). Nine weekly applications of the antagonist were more effective on fruit and also reduced leaf scab. Applications of dithianon after infection events tended to be more effective than *C. cladosporioides* H39 but differences were never significant. Combination of the dithianon spray schedule with the weekly spray schedule with *C. cladosporioides* H39 resulted in the highest control levels on both leaves and fruit.

In the trials in Bavendorf during the primary season, *C. cladosporioides* H39 was applied before predicted infection events in 2012 and after predicted infection events in 2013. Both spray schedules resulted in significant reductions of leaf scab (Table 7) ($P = 0.002$ for assessment on 10 May 2012 and $P < 0.001$ for other assessment dates). However, with 71 and 55% (two assessment dates), efficacy (calculated as relative reduction with untreated control = 0%) was higher when the antagonist was sprayed after infection events than when sprays were applied before infection events when values were 44 and 22% (two assessment dates). Application of a threefold dose rate of the antagonist did not enhance the effect. In the trial in Bavendorf during the summer epidemic 2012, scab incidence on leaves and fruit was low (Table 8, trial 2012). Weekly applications of *C. cladosporioides* H39 and of dithianon significantly reduced fruit scab ($P = 0.032$). A high fruit scab incidence of almost 71% was observed in the untreated control at the end of the 2013 season (Table 8, trial 2013). *C. cladosporioides* H39 applications after infection events significantly reduced scab incidence ($P < 0.001$) by more than 90%, not different from the effect of a dodine spray schedule.

No phytotoxicity symptoms were observed in plots treated with *C. cladosporioides* H39 in the eight orchard trials.

Trials with single applications in Randwijk. Three additional trials with different timing of single treatments of *C. cladosporioides* H39 before or after an expected infection by *V. inaequalis* were carried out in 2013. Trial 1 started after a period of 16 days without infection events. After treatments had been carried out before and after an infection event on 13 June, five more infection events were recorded before leaves were sampled after 20 days. Under these conditions, most sporulation of *V. inaequalis* was found on the youngest leaves (Fig. 4). Single applications of *C. cladosporioides* H39 tended to reduce the number of *V. inaequalis* conidia, especially when sprayed after the infection event. A significant reduction by 74% was found on the youngest leaves when *C. cladosporioides* H39 was sprayed on 15 June, 774 degree h after infection. Pooled samples of the three leaf ages showed significantly reduced sporulation when *C. cladosporioides* H39 had been sprayed on 15 June (774 degree h after infection) or on 21 June (3,070 degree h after infection). Treatments of trial 2 were carried out before and after an infection

Table 7. Effects of multiple treatments of *Cladosporium cladosporioides* H39, dithianon, or dodine during the primary season on apple scab incidence on leaves^x

Treatment	Number of applications	Scab incidence (%) on leaves		
		10 May	23 May	5 June
Trial 2012 ^y				
Untreated control	...	19.5 a	58.1 a	...
Dithianon	8	2.5 c	15.9 c	...
H39, 2 × 10 ⁶ /ml	8	11.0 b	45.5 b	...
Trial 2013 ^z				
Untreated control	...	...	16.6 a	49.2 a
Dodine	9	...	0.0 c	1.5 c
H39, 2 × 10 ⁶ /ml	9	...	4.8 b	22.3 b
H39, 6 × 10 ⁶ /ml	9	...	3.9 b	19.6 b

^x Orchard trials in Bavendorf, Germany, on Golden Delicious. Values of the same assessment date followed by a common letter do not differ significantly (least significant difference test, $\alpha = 5\%$).

^y Treatments applied before predicted infection periods from 29 March to 16 May 2012.

^z Treatments applied after infection periods from 21 April to 27 May 2013.

Table 8. Effects of multiple treatments of *Cladosporium cladosporioides* H39 and of dithianon or dodine during the summer season on apple scab incidence on leaves and fruit^w

Treatment	N ^x	Scab incidence (%) on leaves			Scab incidence (%) on fruit		
		6 June	11 July	18 July	8 August	21 September	26 September
Trial 2012 ^y							
Untreated control	...	0.1 n.s.	1.6 n.s.	...	1.5 n.s.	5.5 a	...
Dithianon	15	0.4	1.8	...	0.7	0.8 b	...
H39, 2 × 10 ⁶ /ml	15	0.3	1.9	...	0.3	1.3 b	...
Trial 2013 ^z							
Untreated control	...	0.0 n.s.	...	17.6 a	...	...	70.8 a
Dodine	10	0.3	...	1.1 b	...	...	0.6 b
H39, 2 × 10 ⁶ /ml	10	0.3	...	0.7 b	...	...	3.5 b
H39, 6 × 10 ⁶ /ml	10	0.0	...	0.3 b	...	...	4.6 b

^w Orchard trials in Bavendorf, Germany, on Golden Delicious. Values of the same assessment date followed by a common letter do not differ significantly (least significant difference test, $\alpha = 5\%$); n.s. = no significant differences.

^x Number of applications.

^y Treatments applied before predicted infection periods from 11 June to 18 September 2012.

^z Treatments applied after infection periods from 11 June to 17 September 2013.

event on 19 June, only 5 days after an earlier infection event. There were four more infection events before sampling. Treatments of trial 3 were carried out before and after an infection event on 3 July but earlier infection events had been recorded 4 and 9 days before the trial started. There were two more infection events before sampling. Under the conditions of trials 2 and 3, more conidia of *V. inaequalis* were found on the elder leaves (indicating infections before the start of the experiment) and no treatment effects by single applications of *C. cladosporioides* H39 were found (data not shown).

Effect of crop protection products on *C. cladosporioides* H39. The various fungicides and insecticides commonly used in integrated pest management schedules in commercial orchards had a different effect on in vitro conidial germination and mycelial growth of *C. cladosporioides* H39 (Figs. 1–3). Delan (dithianon) and Stroby (kresoxim-methyl), commonly used for scab control during the primary season; Nimrod (bupirimate), used for powdery mildew control; and Folicur (tebuconazole) used for scab control and the control of various other fruit diseases during the summer season, did not completely inhibit *C. cladosporioides* H39 even at the highest dose (Fig. 1), whereas several other fungicide products such as Score (difenoconazole), Topaz (penconazole), and Bellis (pyraclostrobin combined with boscalid) inhibited the antagonist even at lower concentrations. Insecticides and a chemical thinner were not strong inhibitors (Fig. 2). Among the crop protection products commonly used in organic farming, insecticides and sulfur only had a weak effect on *C. cladosporioides* H39 whereas other fungicides strongly inhibited the antagonist (Fig. 3).

Discussion

Eight trials were conducted under a range of environmental conditions during 2 years in Eperjeske and Dabrowice, locations with a continental climate, usually moderately conducive to apple scab; and in Bavendorf, a location with conditions highly conducive to apple scab due to the local climate. Four different cultivars were used: 'Idared', moderately susceptible to scab; 'Golden Delicious', moderately to highly susceptible to scab; and Cortland and McIntosh, highly susceptible to scab. The overall results of the eight field trials consistently showed—that stand-alone applications of the antagonist *C. cladosporioides* H39 can reduce apple scab in leaves and fruit. This was demonstrated in an organic growing system as well as in conventional orchards by spray schedules applied

during the primary or the summer season. In both systems, disease level reductions were the same as those reached with common fungicide schedules in some trials.

These results are a first step toward the integrated use of the antagonist *C. cladosporioides* H39 in scab control strategies. Optimum spray schedules in fruit production have to combine different crop protection products for control of apples scab, other diseases, and pests. Schedules will always depend on the specific situation in the orchard. Important factors are local and seasonal climate, cultivar, specific orchard conditions, including its scab history, but also grower's preferences and the availability of fungicides, the reduced susceptibility of the local *V. inaequalis* populations to certain fungicides, and market demands for maximum levels of residues on harvested fruit. In this complex situation, knowledge is needed for the optimum positioning of *C. cladosporioides* H39 in crop protection schedules. The information gained in our study leads to the conclusion that the antagonist can potentially replace fungicide treatments in spray schedules used for scab control. Furthermore, in vitro studies indicated that the antagonist may be compatible with certain crop protection products, so that combined or alternating use can be an option.

Another finding is that the antagonist is also effective if applied one or even several days after infection events (equivalent to approximately 300 to 2,000 degree h under field conditions). This has been found in several field trials and has been confirmed by the trial conducted in Randwijk with single-spray applications at different intervals before or after a predicted infection event. In this trial, the antagonist significantly reduced *V. inaequalis* sporulation when sprayed 774 or 3,070 degree h after an infection event and tended to reduce sporulation also when sprayed after 1,986 degree h ($P > 0.05$ due to variation between replicates). The finding is also in line with the screening procedure used during the selection of *C. cladosporioides* H39 in seedling tests where candidate antagonists were sprayed 5 to 6 days after inoculation with *V. inaequalis* (21). During this period, *V. inaequalis* is producing stromata under the cuticle, followed by the formation of conidiophores breaking through the cuticle after approximately 2,800 degree h (W. M. L. Molhoek et al., unpublished). Biological control effects of applications after infection are quite uncommon. In many cases, head starts of antagonists are a prerequisite (e.g., in case their mode of action is based on competition [19] or induced resistance [29]). *V. inaequalis* colonizes its host in a very specific way, with its characteristic subcuticular growth on the epidermis cells (4). Several chemicals have been successfully applied to induce resistance in apple against *V. inaequalis* (27) under field conditions. Ortega et al. (26) used methyl 2,6-dichlorisonicotinate to induce resistance in apple leaves against *V. inaequalis* and investigated the effects of induced resistance on the infection cycle of *V. inaequalis*. There was no effect on germination and appressorium formation on the apple leaf. After penetration of the cuticle, however, stromata were formed by the pathogen later and only 10% of such stromata developed runner hyphae. Only a few secondary stromata were produced, so that the colonization of leaves by *V. inaequalis* as well as the formation of conidia was strongly reduced. Thus, induced resistance resulting in a reduced or even completely inhibited sporulation may delay or even stop scab epidemics. Microscopic observations showed no signs of visible harm to the pathogen developing between cuticle and epidermis cells. Hypersensitive reactions by the host were not found (26). Ortega et al. (26) hypothesized that the postpenetration development of *V. inaequalis* is reduced as result of a reduced nutrient supply in the leaves. In susceptible apple tissue, melanoproteins of *V. inaequalis* increase the permeability of host cell membranes, resulting in an increasing nutrient supply by the host (16). *C. cladosporioides* H39 may also interfere with the pathogen mainly during this specific developmental stage. Studies on the specific mode of action of *C. cladosporioides* H39 as well as population dynamics of the antagonist after field applications are ongoing (J. Köhl et al., unpublished). Better understanding of the biology of the antagonist will help to further exploit its use in apple scab control.

Fig. 4. Effect of the time interval (expressed as degree hours) between a predicted infection event by *Venturia inaequalis* and a spray application of conidia of *Cladosporium cladosporioides* H39 on the number of *V. inaequalis* conidia produced on apple leaves of different age. Leaf 1 was the youngest leaf, not yet unfolded at the beginning of the trial, and leaves 2 and 3 were the next youngest leaves. Significantly lower numbers of conidia on treated leaves compared with the corresponding control leaves are indicated by asterisks (one-sided least significant difference test of $\log_{10}$ -transformed values, $\alpha = 5\%$). Backtransformed means of six replicates, each consisting of five leaves.

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