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Selection and orchard testing of antagonists suppressing conidial production by the apple scab pathogen *Venturia inaequalis*

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Abstract Apple scab caused by *Venturia inaequalis* is a major disease in apple production. Epidemics in spring are initiated by ascospores produced on overwintering leaves whereas epidemics during summer are driven by conidia produced on apple leaves by biotrophic mycelium. Fungal colonisers of sporulating colonies of *V. inaequalis* were isolated and their potential to reduce the production of conidia of *V. inaequalis* was evaluated on apple seedlings under controlled conditions. The four most effective isolates of the 63 screened isolates were tested subsequently under Dutch orchard conditions in 2006. Repeated applications of conidial suspensions of *Cladosporium cladosporioides* H39 resulted in an average reduction of conidial production by *V. inaequalis* of approximately 40%. In 2007, applications of conidial suspensions of *C. cladosporioides* H39 reduced conidial production by *V. inaequalis* by 69% on August 6 and by 51% on August 16, but no effect was found on August 20. However, viability of available conidia of *C. cladosporioides* H39 was low at the end of the experiment. Epiphytic and endophytic colonisation by *Cladosporium* spp. of leaves treated during the experiment with *C. cladosporioides* H39 was significantly higher than on control leaves sampled 6 weeks after the last application. It is concluded that *C. cladosporioides* H39 has promising potential as a biological control agent for apple scab control. More information is needed on the effect of *C. cladosporioides* H39 on apple scab epidemics as well as on mass production, formulation and shelf life of conidia of the antagonist.

Keywords Antagonist screening · Biological control · Sporulation

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Introduction

Apple scab, caused by *Venturia inaequalis* is a major disease in world-wide apple production (MacHardy 1996). Multiple fungicide applications in spring and summer are common to reduce yield and quality losses of fruits which are susceptible to the disease during the whole growing season. In organic farming scab control depends on fungicides based on copper or sulphur (Holb et al. 2003). Since the use of copper will be restricted in the European Union (European Commission 1991) and the use of sulphur and copper often leads to insufficient control and phytotoxicity, alternative control measures are needed. The use of microbial antagonists may be an alternative to fungicides for scab control.

Apple scab epidemics are initiated by ascospores as primary inoculum produced in overwintering leaves. Preventative measures such as mechanical shredding or removal of fallen leaves (Sutton et al.

2000; Gomez et al. 2007) and the application of antagonists (Andrews et al. 1983; Carisse et al. 2000) can reduce the primary inoculum load in orchards. After primary infections in spring, epidemics progress until autumn with multiple cycles. Epidemics during summer are driven by conidia produced by biotrophic mycelium developing under the cuticle of apple leaves (MacHardy et al. 2001; Holb et al. 2005). Although leaves develop an ontogenetic resistance as they age, newly formed young leaves are highly susceptible to infection by *V. inaequalis* and can produce large numbers of conidia. In this situation, antagonists present in the phyllosphere may interfere with conidia of the pathogen during sporulation or infection.

Research on biological control of *V. inaequalis* has until now mainly focused on reduction of ascospores in overwintering leaves as the primary inoculum of apple scab. Methods for reducing the spread of the disease during the season by suppressing conidial production or infection are needed especially in organic growing systems where sanitation measures are often used to minimise primary inoculum, but crop protection during scab epidemics depends on multiple spray applications of more or less effective products.

The use of antagonists to control apple scab by interfering with the pathogen in its biotrophic phase on leaves during summer epidemics first was investigated by Andrews et al. (1983) who assessed the antagonism of *Flavobacterium* sp., *Cryptococcus* sp., *Aureobasidium pullulans*, *Trichoderma viride*, *Chaetomium globosum*, *Microsphaeropsis olivacea* and two actinomycetes. They measured effects on mycelial growth, conidial germination and germ tube growth as well as on symptom development and conidial production on seedlings. Conidial production tended to correlate with disease indices in seedling tests in which antagonists had been applied before the pathogen. Since no separate seedling assays were conducted with antagonist applications after a homogeneous infection by *V. inaequalis*, a direct effect of antagonists on sporulation was not investigated. *Chaetomium globosum* was found to be the superior antagonist when results of the different assays were ranked. In subsequent experiments *C. globosum* showed poor epiphytic colonisation and the secreted antibiotics were not stable enough to achieve sufficient biocontrol levels on leaves (Boudreau and Andrews 1987). Amongst 931 bacteria and yeasts tested, an isolate of *Pseudomonas syringae* reduced infection by *V. inaequalis* in a series of bioassays on apple seedlings under controlled conditions (Burr et al. 1996). It was suggested that the antagonist produced an antibiotic suppressing conidial germination. Amongst more than 100 fungal isolates from apple leaves, Fiss et al. (2000) selected isolates of *Cladosporium* spp., *Aureobasidium* spp. and *Fusarium graminum* which reduced mycelial growth and conidial germination *in vitro* and tended to reduce disease incidence on seedlings. Also epiphytic yeasts have been evaluated but application of a selected isolate did not give reproducible results under orchard conditions (Fiss et al. 2003). Gessler et al. (2000) observed that *Acremonium strictum* can colonise old scab lesions. When applied to apple scab lesions under controlled conditions, sporulation of *V. inaequalis* was reduced by the antagonist. Applications in orchards had no effect on the pathogen.

The objective of our study was to evaluate whether fungi in the phyllosphere have a suppressive effect on *V. inaequalis* conidial production. Since the aim of the study was to contribute to the development of biocontrol products, only candidates were tested against *V. inaequalis* which fulfilled the following criteria to overcome major constraints in the development of biocontrol products. For applications in the phyllosphere, antagonists should be cold-tolerant and drought-tolerant (Köhl and Molhoek 2001). For production of inoculum, antagonists have to produce sufficient spores on inexpensive media. Considering registration procedures, it is an advantage to use antagonists not growing at temperatures >36°C and not belonging to genera that include also mycotoxin-producing species or human pathogens.

Fungal isolates were obtained from sporulating colonies of *V. inaequalis* collected at various locations and tested on apple seedlings. Selected isolates were subsequently tested under orchard conditions in two seasons. Some of the results presented here have been reported briefly at a conference (Köhl et al. 2008).

Materials and methods

Collection of candidate antagonists

Apple leaves infected by *V. inaequalis* were collected in September 2004 at 216 locations in The Nether-

lands (82 samples), Belgium (18 samples), northwestern Germany (11 samples), and central Germany (105 samples). At most locations, a high incidence of apple scab was observed on leaves and fruits. The majority of samples, consisting of 10–50 leaves, originated from old standard trees, e.g. planted along secondary roads, or abandoned orchards without any crop management. A few samples were also collected from organically managed orchards or orchards with integrated management. Leaves were stored in paper bags at 5°C and processed within 24 h. From each sample, green leaves with sporulating colonies of *V. inaequalis* were incubated for 3 days at 20°C in moist chambers. Thereafter, *V. inaequalis* colonies were visually inspected at ×10 to ×60 magnification for development of fungi different from *V. inaequalis*. Fungal mycelium or spores were isolated from the aerial parts of the colonies with a sterile needle and transferred onto oat meal agar (20 g oat meal, 15 g agar, 1,000 ml tap water) and V8 agar (200 ml V8 juice, 3 g $CaCO_3$, 20 g agar, 800 ml tap water), both containing 100 mg l^{-1} streptomycin and 15 mg l^{-1} tetracycline. Plates were incubated at 20°C in the dark and regularly inspected for developing fungal colonies. Pure cultures were prepared by transferring hyphal tips of developing colonies of fungi different from *V. inaequalis* onto sterile oat meal agar or V8 agar plates. Obtained isolates were stored at 4°C.

Pre-screening of candidate antagonists

Colonies of fungal isolates obtained from sporulating *V. inaequalis* colonies were inspected macroscopically and microscopically to exclude fungi belonging to the genera *Aspergillus*, *Penicillium* or *Fusarium*. The remaining isolates of hyphal fungi were cultured in Petri dishes on oat meal agar for 21 days at 18°C and 12 h per day NUV blacklight; yeasts were cultured on basal yeast agar (10 g bacteriological peptone, 1 g yeast extract, 20 g glucose, 20 g agar, 1,000 ml tap water) for 5 days at 18°C. For each isolate, suspensions of spores or yeast cells were prepared using sterile tap water containing 0.01% Tween 80 and the production of spores or yeast cells per plate was determined with the aid of a haemocytometer. Isolates producing $<1\times10^5$ spores or yeast cells per plate were discarded. Ten µl of the suspensions containing 1×10^5 to 1×10^6 spores or cells ml^{-1} were plated in duplicate in sterile wells (16 mm diam) containing 1.5 ml malt agar (1 g malt extract, 15 g agar, 1,000 ml tap water). Different plates with inoculated wells were incubated at 5°C, 18°C and 36°C in the dark for 14 days. An additional plate with wells containing malt agar adjusted to approximately −10 and −7 MPa by adding KCl (Campbell and Gardner 1971) were incubated at 18°C for 14 days. Wells were inspected for fungal growth and fungi producing colonies at 36°C and those not producing colonies at 5°C or at −10 or −7 MPa were discarded. Remaining isolates were grown on potato dextrose agar (Oxoid, 39 g, 1,000 ml tap water) and stored at 4°C until further use.

Cladosporium cladosporioides R407, which had been isolated from a fallen apple leaf sampled in the organically managed orchard of Applied Plant Research, Randwijk, was included in the 2007 orchard assay. This isolate strongly reduced ascospore production of *V. inaequalis* on overwintering leaves (Köhl et al. unpublished).

Isolates belonging to *Cladosporium* spp. were identified based on morphological characteristics according to Ellis (1990). Other isolates including a few randomly chosen *Cladosporium* spp. were identified by sequence analyses of the ITS1 region with primers ITS2 and ITS1-F (White et al. 1990). Sequencing reactions were performed using the DYEnamic ET Terminator cycle sequencing Kit (GE Healthcare, UK) on a 3730xL DNA Analyser (Applied Biosystems) and a 3100 Genetic Analyser (Applied Biosystems). The sequence alignment was produced with the aid of the SEQMAN Module of the LASERGENE System (DNASTAR, Inc.).

Production of fungal inocula

Conidia of the monospore isolate MB 363B of *V. inaequalis* obtained from M. Bengtsson, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark (currently renamed to Copenhagen University), were produced following the method of Williams (1978). Duran bottles (500 ml) with 40 ml potato dextrose broth (24 g l^{-1}) and a wick of cheese cloth sized 10×25 cm spread on the inner surface of the bottle, the lower part touching the medium, were autoclaved and inoculated with 250 µl of a suspension of mycelial fragments of *V. inaequalis*, prepared by flooding a culture of the fungus grown on potato dextrose agar with sterile water and gently rubbing with a sterile rubber spatula. Inoculated bottles were

incubated at 18°C in the dark. During the first 4 days, bottles were incubated in a horizontal position so that most of the wick was covered by the broth. Thereafter bottles were incubated in an upright position. Bottles were carefully rolled at days 2, 7 and 11 to distribute the broth with growing mycelium on the entire surface of the wick. After 14 days, the nutrient broth was decanted, 200 ml of sterile tap water added and bottles shaken thoroughly by hand for several minutes. The resulting conidial suspension was filtered through sterile nylon gauze with a mesh of 200 μm and centrifuged at 5,800×*g* for 30 min at 6°C. The pellet with conidia was re-suspended in tap water and the conidial concentration was determined with the aid of a haemocytometer and adjusted to 5×10^5 conidia ml^{-1} by adding sterile tap water. The yield was approximately 3×10^6 conidia per bottle. Conidia were stored in suspensions at −18°C until use.

For the screening experiments, candidate isolates of hyphal fungi were grown on oat meal agar for 28 days at 18°C and 12 h NUV blacklight per day; yeasts were grown on basal yeast agar for 5 days at 18°C. Suspensions of spores or yeast cells were prepared by flooding cultures with sterile tap water containing 0.01% Tween 80, gently rubbing with a sterile rubber spatula and filtration through sterile nylon gauze with a mesh of 200 μm. Concentrations of the suspensions were determined with the aid of a haemocytometer and concentrations were adjusted to 1×10^6 spores or cells ml^{-1} by adding sterile tap water containing 0.01% Tween 80.

For applications in the orchard, fungi were produced in the same way but concentrations were adjusted to 2×10^6 spores ml^{-1}. Conidia of isolates *C. cladosporioides* H39 and *Phoma pinodella* H3 were also produced in a Solid-State Fermentation (SSF) system. The harvested conidia were formulated as a water dispersible granule (WG) by PROPHYTA Biologischer Pflanzenschutz GmbH, Germany. For the preservation of product quality, the final product was stored at 4°C. Viability of dried conidia was determined on malt extract agar (1 g malt extract l^{-1}) before the beginning of the field experiments. Conidia incubated for 24 h at 20°C with germ tubes longer than half of the minimum diameter of a conidium were considered to be viable. The concentration of conidial suspensions was adjusted to 2×10^6 viable conidia ml^{-1}. Viability of spores in all suspensions applied to the field was always checked again at each application date by spraying suspension on malt extract agar amended with tetracycline at 15 mg l^{-1} and streptomycin at 100 mg l^{-1} in the orchard and assessing the percentage of germinated spores after incubation at 20°C for 24 h.

Seedling assay

Seeds of apple cv. Golden Delicious (G.J. Steingaesser & Comp. GmbH, Miltenberg, Germany) were seeded in moist sand and stratified at 4°C for 6 weeks in the dark. Thereafter, seeds were further grown in the moist sand at room temperature and daylight. After approximately 14 days, young seedlings were transplanted into potting soil, one seedling per pot (6 cm square, 8 cm height). Seedlings were grown for 28 days with cycles of 16 h light at 18°C and 8 h dark at 12°C. Plants used in experiments had at least four fully expanded leaves.

Seedlings were sprayed with conidial suspensions of *V. inaequalis* (1×10^5 ml^{-1}) until run-off and placed in moist chambers consisting of plastic trays closed by transparent plastic tops. After 2 days incubation at 15°C with diffuse light, tops were removed from the trays and seedlings further incubated for 3 or 4 days at 85% RH, 15°C and 16 h light per day. Thereafter, *V. inaequalis*-inoculated seedlings were sprayed with suspensions containing spores or yeast cells of candidate antagonists or water containing 0.01% Tween 80 as control. In the various experiments, seven to 10 candidate antagonists were tested per experiment. Two seedlings were used per replicate of each treatment. These sets of two inoculated seedlings were placed in a polyethylene tent in a block design with six blocks (replicates) and complete randomisation within blocks. Touching of leaves of neighbouring plants or the polyethylene was avoided. Seedlings were grown for 9 to 12 days at 15°C, with 16 h light per day at 138 μE s^{-1} m^{-2}.

From both seedlings of each replicate, the lowest five youngest leaves at inoculation time (experiments 1–5) or the youngest just unfolded leaf at inoculation time (labelled with a metal ring; 'young leaves') and the three next older leaves (experiments 6–14; 'old leaves') were carefully removed. Leaves of each replicate set of leaves were pooled, put into Duran bottles (100 ml) containing 15 ml of tap water with 0.01% Tween 80 for samples containing the two

youngest leaves or 30 ml for samples containing 10 (experiments 1–5) or six (experiments 6–14) leaves. Bottles were shaken with a flask shaker at 700 OCS min^{-1}. Concentration of conidia of *V. inaequalis* was determined for each suspension with the aid of a haemocytometer. Leaf surfaces of each replicate set of leaves were measured with an area meter (Li-COR Biosciences, model 3100, Lincoln, U.S.A.).

Orchard assays in 2006

Several rows of cv. Jonagold within an organically managed orchard at Applied Plant Research, Randwijk, The Netherlands, were pruned during spring and summer 2006 so that trees produced new shoots with young leaves highly susceptible to *V. inaequalis*. Depending on tree development and growth conditions for the trees, pruning was carried out approximately 3 to 5 weeks before a set of trees was used for an experiment. Also, the majority of young fruits were removed from the trees to stimulate the growth. Trees used for experiments in early summer (experiments 1 to 4) were not treated for scab control in 2006. Trees used in experiments 5 to 8 were treated with a sulphur schedule as is common for the organic orchard in the early season to slow down the scab epidemic but remained untreated from at least 3 weeks before and during the experiments.

A series of eight experiments was carried out, each on a different set of trees. For each experiment, two to six trees, depending on the number of newly produced shoots per tree, were chosen for each of six blocks (replicates). Within each block, seven treatments were carried out. For each treatment (per replicate) three shoots were used which were produced on the same branch. Shoots were labelled with coloured metal rings so that the two youngest leaves fully expanded on the day of the first treatment could later be distinguished from the other leaves of the shoot. Treatments consisted of spraying tap water containing 0.01% Tween 80 as control, or suspensions of freshly produced spores of the antagonists *Phoma pinodella* H3, *Coniothyrium cereale* H33, *Cladosporium sphaerospermum* H35 and *C. cladosporioides* H39. For *P. pinodella* H3 and *C. cladosporioides* H39 separate treatments were carried out with fermenter-produced spores formulated as water dispersible granules and re-suspended in tap water containing 0.01% Tween 80. Spray applications were done using a compressed air-driven knapsack sprayer (AZO, Edecon, Ede, The Netherlands) with one nozzle (Birchmeier helico saphir 1.2, 2F-0.6) at 250 kPa until run-off. The different experiments were carried out in the period between June 22 and September 28. Experiments started with the first treatment 1 to 3 days after an infection period for *V. inaequalis* had been predicted according to the Mills table based on leaf wetness duration and temperature. Starting dates for the eight experiments were June 22, June 29, July 6, July 31, August 3, August 7, August 21, and August 24. In experiment 8, the period between expected infection period for *V. inaequalis* and first application of antagonists was prolonged to 10 days, but leaves were labelled already 1 to 3 days after the expected infection period as in the other experiments. During all experiments, treatments were carried out at 3 to 4 day intervals resulting in five application dates in experiments 1 to 4, and six application dates in experiments 5 to 8. In the first three experiments, leaves were sampled 18 days after the first treatment (and thus 19 to 21 days after an expected infection period). In experiments 4 to 7, leaves were sampled 24 to 25 days after the first treatment (and thus 25 to 28 days after an expected infection period) to increase the time period for *V. inaequalis* sporulation and for possible antagonistic interactions. In experiment 8, leaves were sampled 24 days after the first application and thus 34 days after the expected infection period.

In each experiment, the two youngest fully expanded leaves at the beginning of the experiment together with the two next younger leaves (expanded during the course of the experiments) were pooled for the three shoots belonging to the same replicate so that a sample consisted of twelve leaves ('young leaves'). From the same shoot, the next older three to twelve leaves, depending on shoot size, were also sampled and pooled so that samples consisted of nine to 36 leaves ('older leaves'). The average number of older leaves sampled in the different experiments was 21.8. Samples of young leaves were put into 250-ml glass bottles. Within 2 h, 100–150 ml (depending on amount of leaves) of tap water with 0.01% Tween 80 was added and bottles were shaken with a flask shaker at 700 OCS min^{-1} for 10 min. Samples of older leaves were processed in the same way using 1,000-ml plastic bottles containing 150–350 ml

(depending on number of leaves) of tap water added before shaking. From the obtained suspensions, subsamples of 6 ml were stored at −18°C. The concentration of conidia of *V. inaequalis* was determined for each suspension with the aid of a haemocytometer. The leaf surface of all leaves per sample was measured with an area meter.

Orchard assay in 2007

The experiment was carried out in the organically managed orchard at Applied Plant Research, Randwijk, The Netherlands. The aim of the experiment was to control the summer epidemic of apple scab by antagonist applications. Therefore it was essential to allow an initiation of a mild to moderate epidemic in the orchards during the primary season. Unusual weather conditions during the primary season of 2007 with a period of 4 weeks without any rain were very unfavourable for *V. inaequalis* ascospore release and infection. Weather became more conducive after April and a mild apple scab epidemic had developed when the first antagonist suspension was applied. No fungicide treatments were carried out to reduce the progression of the epidemic before or during the experiment.

The experiment was carried out on 8 year-old trees cv. Jonagold in a design with six blocks, with two blocks in the same tree row. Each block consisted of three plots, each with four trees. Between plots, two untreated trees served as buffer. The three different treatments were randomly allocated to the plots. Trees were treated at a rate of 2 l per plot twice per week with the following treatments: (1) tap water amended with Tween 80 (0.01%) as control; and (2) conidial suspension of formulated H39 (2×10^6 conidia ml^{-1}). Applications were carried out twice per week at 16 dates between June 28 and August 20. (3) The third treatment consisted of multiple spray applications of conidial suspensions of *C. cladosporioides* R406. Conidia freshly produced at each application date were applied twice per week on the four trees of each replicate plot at twelve dates between July 12 and August 20.

Conidial production of *V. inaequalis* was assessed on susceptible young leaves developed during the course of the experiment at three sampling dates for leaves treated with Tween-water as control, or conidial suspensions of *C. cladosporioides* H39 and for two sampling dates for *C. cladosporioides* R406. Sampling dates were chosen so that sets of susceptible leaves present during a predicted infection period were sampled approximately 5 weeks after the infection period. The Mills table based on leaf wetness duration and temperature was used to predict infection periods. The second youngest just unfolded leaf was labelled 1 to 3 days after a predicted infection period on a set of three twigs belonging to the same tree in each plot. After 35 days, the two leaves just unfolded at the date of labelling and the next two younger leaves, unfolded after labelling, were sampled resulting in a sample consisting of twelve leaves per plot. Sampling dates were August 6 (of leaves labelled on July 2), August 16 (of leaves labelled on July 12), and August 20 (of leaves labelled on July 16). Leaves were further processed as described above.

Twenty labelled leaves of trees from the plots treated with Tween-water (control) or conidial suspensions of *C. cladosporioides* H39 or *C. cladosporioides* R406 were collected on October 4 from each plot. All sampled leaves had developed on the trees during the period between June 28 and August 20 when the series of spray applications had been carried out. It thus can be assumed that all leaves received one or several spore applications at young developmental stages.

From each leaf, one leaf disc (9 mm diam) was cut using a sterile cork borer. The resulting 20 leaf discs per sample were pooled in sterile 50-ml vials containing 10 ml of sterile Tween-water (0.01%) and vials were shaken using a flask shaker at high speed (700 OCS min^{-1}) for 10 min. From the resulting suspensions, serial dilutions (1:10) were prepared and 100 µl of undiluted and diluted suspensions were plated on each of two agar plates containing malt agar (10 g malt extract, 15 g agar, 1,000 ml tap water) amended with 100 mg l^{-1} streptomycin and 15 mg l^{-1} tetracycline. Plates were incubated at 20°C in the dark and colonies of *Cladosporium* spp., other hyphal fungi and yeasts were counted after 4, 7 and 11 days. From the colony counts, the number of colony forming units (CFU) cm^{-2} leaf surface was calculated for each sample.

After shaking the leaf disks to remove epiphytically present fungi, the pooled 20 leaf disks per sample were surface-sterilised by dipping in 96% ethanol followed by submersion for 1 min in 0.5% sodium hypochlorite

and subsequently rinsing three times with sterile water. Surface-sterilised leaf disks were subsequently homogenised in 5 ml sterile water using a sterile mortar. From the resulting homogenates serial dilutions (1:10) were prepared in sterile water and 100 µl of undiluted and diluted suspensions were plated on each of two malt agar plates containing 100 mg l^{-1} streptomycin and 15 mg l^{-1} tetracycline. Plates were incubated and colonies of endophytic fungi were assessed as described above.

Statistics

The number of *V. inaequalis* conidia produced cm^{-2} leaf surface of apple seedlings was calculated per replicate. If no conidia were detected, a detection limit was set at one conidium counted in the conidial suspension. This resulted in an average detection limit for the various experiments of approximately 100 conidia cm^{-2}, e.g. ranging between 74 and 212 cm^{-2}, depending on the leaf surfaces of the various replicates, for the first three experiments conducted under controlled conditions. Data obtained for different leaf ages were natural logarithmic-transformed and analysed separately per leaf age. Experiments conducted under controlled conditions were analysed separately by comparing the mean of the control treatment and the individual antagonist treatments using a LSD-test (α=0.05). Since *P*-values of ANOVAs often were >0.05, no further multiple comparisons between antagonist treatments were made (Ott and Longnecker, 2001). For the orchard assays in 2006, natural logarithmic-transformed data obtained for two different leaf ages were analysed separately per leaf age by ANOVA in a split-plot design with individual experiments considered as main blocks. Treatments were randomized within the six replicate blocks of each experiment. Each treatment was compared with the control treatment and statistically significant treatment effects (LSD-test; α=0.05) were indicated. For the orchard assay in 2007, natural logarithmic-transformed data on numbers of *V. inaequalis* conidia were analysed separately for each sampling date. Treatments were compared with the control using an one-sided LSD-test (α=0.05). Numbers of CFU of epiphytic and endophytic fungi were analysed by ANOVA and statistically significant differences were indicated (LSD-test; α=0.05).

Results

Pre-screening

In total, 148 isolates representing various colony types were tested in the pre-screening trial. Seventeen isolates produced $<1\times10^5$ spores or yeast cells per plate and were discarded. All remaining 131 isolates were able to grow at 5°C. Sixteen isolates did not grow at −10 MPa and 14 isolates grew at 36°C. Since many of the isolates belonged to *Cladosporium* spp., only a sub-set of randomly chosen isolates of this genus was tested on seedlings. In total, 63 out of the 102 isolates were further tested in bioassays on apple seedlings. Isolates were identified based on their ITS sequences as *Aureobasidium pullulans* (three isolates), *Cladosporium cladosporioides* (10 isolates), *Cladosporium herbarum* (three isolates), *Coniothyrium cereale* (three isolates), *Cryptococcus victoriae* (two isolates), *Nectria galligena* (one isolate), *Phoma pinodella* (13 isolates), *Septoria passerini/triticii* (one isolate), *Stagonospora* sp. (three isolates), and *Trichothecium roseum* (one isolate). The remaining 23 isolates belonging to *Cladosporium* spp. were identified as *C. cladosporioides*, *C. herbarum* or *C. sphaerospermum* based on morphological characteristics.

Seedling tests

Fourteen experiments on seedlings were carried out to evaluate possible antagonistic effects of fungal isolates against *V. inaequalis* under controlled conditions. In the control treatments of the first five experiments, the conidial production on the five sampled leaves of the seedling was on average 3,596 conidia cm^{-2} and ranged for the different experiments between 1,339 and 10,509 conidia cm^{-2} (backtransformed values). Since conidial production was high on the youngest leaf and much less on the older leaves it was decided to sample such leaves separately in the subsequent experiments. In experiment 6–14, average conidial production on the young leaf was 2,896 conidia cm^{-2} and ranged for the different experiments between 728 and 6,186 conidia cm^{-2} (backtransformed values). For older leaves, average conidial production was 453 conidia cm^{-2} and ranged for the different experiments between 144 and 1,313 conidia cm^{-2} (backtransformed values).

Variation in conidial production was not only high between experiments but also within experiments between replicated sets of seedlings.

Most of the 63 candidate isolates tested on seedlings did not reduce conidial production of *V. inaequalis* statistically significantly (data not shown). Four isolates, *P. pinodella* H3, *C. cereale* H33, *C. sphaerospermum* H35, and *C. cladosporioides* H39 caused a significant reduction of *V. inaequalis* sporulation on the young or older leaves in at least two independent experiments. However, efficacies (calculated on base of backtransformed values for young or older leaves) of these antagonists varied between 18% and 80% for *P. pinodella* H3, 50% and 75% for *C. cereale* H33, 32% and 84% for *C. sphaerospermum* H35, and 19% to 79% for *C. cladosporioides* H39 in subsequent experiments and reduction of conidial production of *V. inaequalis* was not statistically significant in all replicated experiments (Table 1). A few more isolates showed a strong statistically significant antagonistic effect in one experiment but such effects could not be repeated in independent replicated experiments. Such isolates were *A. pullulans* H2, *C. cladosporioides* H8, H23, H26, and H40, *C. herbarum* H13, H27, and H28, and *P. pinodella* H10. In no case significant enhancement of conidial production of *V. inaequalis* after application of candidate antagonists was observed.

Orchard assays 2006

The apple scab epidemic developed moderately in the orchard before the first experiment started on June 22. In June and first half of July, dry conditions accompanied with day temperatures often >30°C were not favourable for further apple scab development. Thereafter, rainy and cold weather was strongly favourable for apple scab until the end of the experiments.

During the first three experiments conducted under dry and warm conditions, 2.1 to 12.8×10^3 conidia cm^{-2} (backtransformed values) were found on young leaves and 9.5 to 40.3×10^3 conidia cm^{-2} on older leaves on water-treated control plots (Tables 2 and 3). After weather had changed to moist and cold conditions during half of July, 46.3×10^3 conidia cm^{-2} on young leaves and 9.8×10^3 conidia cm^{-2} were found on leaves from control treatments in

Table 1 Effect of *Phoma pinodella* H3, *Coniothyrium cereale* H33, *Cladosporium sphaerospermum* H35 and *Cladosporium cladosporioides* H39 on conidial production by *Venturia inaequalis* on apple seedlings under controlled conditions

Treatment	Number of conidia cm^{-2}[a]						
	Exp 1 (nt=8)[b]	Exp 3 (nt=9)	Exp 9 (nt=10)	Exp 10 (nt=10)	Exp 11 (nt=10)	Exp 13 (nt=10)	Exp 14 (nt=11)
Youngest leaves							
Control	–[c]	–	1,960	2,393	4,188	728	1,480
P. pinodella H3	–	–	797[d] (59)	–	–	596 (18)	742 (50)
C. cereale H33	–	–	–	964 (60)	–	–	735 (50)
C. sphaerospermum H35	–	–	–	–	679[d] (84)	424 (42)	1,002 (32)
C. cladosporioides H39	–	–	–	–	863[d] (79)	330 (55)	572 (61)
Older leaves							
Control	1,339	4,915	331	359	1,313	144	614
P. pinodella H3	262[d] (80)	–	229 (31)	–	–	97 (33)	153[d] (75)
C. cereale H33	–	1,212[d] (75)	–	170 (53)	–	–	204[d] (67)
C. sphaerospermum H35	–	1,790 (64)	–	–	384[d] (71)	110 (24)	196[d] (68)
C. cladosporioides H39	–	–	–	–	354[d] (73)	116 (19)	162[d] (74)

In total, 63 fungal isolates were tested in 14 experiments. Only results of isolates significantly reducing *V. inaequalis* sporulation in at least two experiments are presented. In experiments (Exp) 1 and 3, the five youngest leaves at inoculation time per seedling were pooled; in the other experiments, the youngest leaf at inoculation time was sampled separately from the three next eldest leaves

[a] Backtransformed values; efficacy relative to control treatment in brackets

[b] Experiment number; total number of treatments in brackets

[c] Not tested

[d] Statistically different from the control treatment (LSD-test; $\alpha=0.05$)

Table 2 Effect of applications of *Phoma pinodella* H3, *Coniothyrium cereale* H33, *Cladosporium sphaerospermum* H35 and *Cladosporium cladosporioides* H39 on conidial production by *Venturia inaequalis* under orchard conditions

Treatment	Ln-number of *V. inaequalis* conidia cm^{-2}[a]			
	Exp 1	Exp 5	Exp 6	Mean
Youngest leaves				
Control	9.5 (12.8)	10.1 (24.9)	9.8 (18.0)	9.8 (18.0)
P. pinodella H3	9.6 (14.2)	10.3 (29.1)	10.3 (30.4)	10.1 (24.3)
P. pinodella H3 formulated	9.7 (16.6)	10.0 (21.3)	10.6 (41.1)	10.1 (24.3)
C. cereale H33	–	9.6 (18.9)	10.9 (53.7)	10.3 (29.7)
C. sphaerospermum H35	9.2 (10.3)	10.4 (32.3)	9.8 (17.7)	9.8 (18.0)
C. cladosporioides H39	9.1 (8.6)	10.7 (46.1)	10.7 (42.5)	10.2 (26.9)
C. cladosporioides H39 formulated, low viability	–	10.4 (32.4)	10.4 (31.8)	10.4 (32.9)
Older leaves				
Control	(9.5)	9.2 (9.6)	9.3 (10.4)	9.2 (9.9)
P. pinodella H3	(6.6)	9.6 (15.0)	9.5 (13.8)	9.3 (10.9)
P. pinodella H3 formulated	19.6	9.5 (13.9)	9.5 (13.6)	9.6 (14.8)
C. cereale H33	–	9.6 (15.3)	10.3 (30.4)	9.9 (19.9)
C. sphaerospermum H35	16.1	9.3 (11.0)	9.0 (8.1)	9.3 (10.9)
C. cladosporioides H39	12.9	10.2 (26.1)	9.7 (16.1)	9.8 (18.0)
C. cladosporioides H39 formulated, low viability	–	9.3 (11.0)	9.7 (16.1)	9.5 (13.3)

Experiments (Exp) 1, 5, and 6; 2006

[a] Backtransformed values × 1,000 in brackets

Table 3 Effect of applications of *Phoma pinodella* H3, *Coniothyrium cereale* H33, *Cladosporium* sp. H35 and *Cladosporium cladosporioides* H39 on conidial production by *Venturia inaequalis* under orchard conditions

Treatment	Ln-number of *V. inaequalis* conidia cm^{-2}[a]					
	Exp 2	Exp 3	Exp 4	Exp 7	Exp 8	Mean
Youngest leaves						
Control	8.2 (3.6)	7.6 (2.1)	10.7 (46.3)	11.8 (132.1)	12.3 (227.3)	10.1 (25.3)
P. pinodella H3	8.4 (4.4)	8.4 (4.3)	10.7 (42.8)	11.8 (138.1)	12.5 (267.7)	10.4 (31.3)
P. pinodella H3 formulated	7.8 (2.3)	7.8 (2.3)	10.8 (50.3)	11.9 (146.8)	12.2 (196.8)	10.1 (24.0)
C. cereale H33	8.0 (3.1)	–	10.4 (34.1)	11.7 (126.0)	12.3 (230.6)	–
C. sphaerospermum H35	7.8 (2.4)	8.2 (3.6)	10.7 (42.8)	11.7 (122.2)	12.3 (218.7)	10.1 (25.2)
C. cladosporioides H39	8.9 (6.9)	7.9 (2.7)	10.2 (25.8)	12.4 (253.8)	12.1 (183.8)	10.3 (29.5)
C. cladosporioides H39 formulated	7.4 (1.6)	6.9 (0.9)	10.4 (31.7)	11.4 (90.4)	11.9 (147.2)	9.6[b] (14.6)
Older leaves						
Control	10.6 (40.3)	9.2 (10.3)	9.2 (9.8)	11.2 (72.8)	11.8 (132.1)	10.4 (33.0)
P. pinodella H3	10.3 (30.0)	9.3 (11.0)	8.2 (3.8)	11.2 (76.8)	11.7 (116.7)	10.2 (25.7)
P. pinodella H3 formulated	10.2 (27.2)	9.7 (15.9)	9.2 (10.2)	11.1 (63.3)	11.8 (137.1)	10.4 (32.8)
C. cereale H33	10.6 (41.3)	–	8.6 (5.5)	11.0 (62.8)	12.0 (167.3)	–
C. sphaerospermum H35	10.2 (27.3)	9.6 (15.4)	8.5 (4.8)	11.2 (75.4)	11.9 (150.0)	10.3 (29.6)
C. cladosporioides H39	10.5 (35.3)	8.9 (7.5)	8.7 (5.8)	11.4 (93.4)	11.9 (145.7)	10.3 (27.6)
C. cladosporioides H39 formulated	10.0 (21.3)	8.6 (5.5)	8.8 (6.6)	10.9 (52.3)	11.4 (90.7)	9.9[b] (20.5)

Experiments (Exp) 2, 3, 4, 7, and 8; 2006

[a] Backtransformed values × 1,000 in brackets

[b] Statistically different from the control treatment (LSD-test; $\alpha = 0.05$)

experiment 4. During the subsequent experiments conidial numbers increased to 227.3×10^3 conidia cm^{-2} on young leaves and 132.1×10^3 conidia cm^{-2} on older leaves in experiment 8.

Viability of spores applied at the 25 application dates during the course of the series of experiments was assessed for each suspension on one agar plate sprayed in the field. For spores of *P. pinodella* H3, freshly produced on agar plates for each application, germination on such plates was high with on average 94%, ranging between 79% and 99% for the different application dates. Formulated spores of *P. pinodella* H3 of the batch used during the entire series of field experiments had a viability of 62% at the beginning of the experiments. On plates sprayed in the orchard, on average 58% spores germinated. There was no trend of decreasing viability over time. Freshly produced spores of *C. cereale* H33 had an average viability of 73% (ranging from 44% to 97% on the different application dates). Viability of conidia of *C. sphaerospermum* H35 was, on average, 91% (ranging from 69% to 98%), and viability of freshly produced conidia of *C. cladosporioides* H39 was, on average, 92% (ranging from 82% to 99%). Formulated spores of *C. cladosporioides* H39 had a viability of approximately 47% at the beginning of the experiments. When conidial suspensions were prepared for field applications, formulated conidia of *C. cladosporioides* H39 produced following a first pilot protocol formed clusters consisting of approximately 5 to 50 conidia in the spraying suspensions so that a precise determination of spore germination was not possible. The majority of clusters in the suspensions sprayed on the first 10 application dates showed fungal growth after incubation on agar plates. From conidial clusters of suspensions applied between August 10 and August 29, only few or, on three occasions, no conidial clusters showed fungal growth on agar plates. It can be assumed that stored spores formulated as water-dispersible granules (WG) had been used which lost their vigour during storage for the last treatments during experiment 4 and all treatments during experiments 5 and 6. From the end of August onwards, a new batch of formulated conidia was used of which the majority of conidial clusters showed fungal growth.

No significant treatment effects were found when data were analysed for each experiment separately (Tables 2 and 3). For an overall analysis, experiments 1, 5 and 6 were excluded because formulated conidia of *C. cladosporioides* H39 (WG) were not available during experiment 1 and viability of formulated conidia was low during experiments 5 and 6. A statistically significant reduction of the number of conidia cm^{-2} was found for treatments with formulated conidia of *C. cladosporioides* H39 on both young and older leaves (Table 3). On average, conidial production was reduced by 43% on young and 38% on older leaves (based on backtransformed values). Such a trend was found during the first three experiments conducted under low disease pressure as well as during the last two experiments conducted under extremely high disease pressure. Applications of non-formulated conidia of the isolate did not reduce conidial production of *V. inaequalis*. When the formulation of *C. cladosporioides* H39 was applied containing only a few or even no living conidia as during experiments 5 and 6, no trend was found that the formulation itself had any reducing effect on conidial production of *V. inaequalis* (Table 2).

None of the other treatments with freshly produced spores of *P. pinodella* H3, *C. cereale* H33, or *C. sphaerospermum* H35 reduced conidial production of *V. inaequalis*. Also for spores of *P. pinodella* H3 formulated similar to those of *C. cladosporioides* H39, no treatment effects were found.

Orchard assay 2007

The primary season of the apple scab epidemic in the orchard started unusually late because no rainfall occurred during April. In the beginning of the experiments at the end of June only few scab lesions were present. Thereafter, weather conditions with frequent rainfalls and moderate temperatures were very favourable for apple scab during the period of the experiments. In the end of the experiments at the end of August, many scab lesions were observed on leaves within the experimental plots and in the adjacent plots.

Conidia of *C. cladosporioides* R406, produced freshly for each application date, had on average a viability of 93%. A batch of conidia of *C. cladosporioides* H39 was produced in spring 2007 and stored at 5°C. Conidia of *C. cladosporioides* H39 of this batch formulated as water-dispersible granules following the pilot protocol germinated at 20–30% during the

first weeks of the experiment but viability dropped to 4–11% for the last five treatments.

On leaves sampled on August 6, 16, and 20 from water-treated trees, 76,900 (13,900 to 139,800), 45,300 (35,200 to 55,200) and 32,200 (8,800 to 111,800) conidia of *V. inaequalis* cm^{-2} were produced on average, respectively (backtransformed means, range for six replicates in brackets) (Table 4). Variation between replicates was thus considerable. On leaves of trees treated with *C. cladosporioides* H39, the number of spores was statistically significantly lower with 24,100 conidia of *V. inaequalis* cm^{-2} (69% reduction based on backtransformed values) on the first sampling date and 22,000 conidia cm^{-2} (51% reduction) on the second sampling date. For the last sampling date, no treatment effect was observed for applications of *C. cladosporioides* H39. For the last two sampling dates when leaves treated with *C. cladosporioides* R406 were available, conidial counts for *V. inaequalis* on August 16 were 24,300 conidia cm^{-2} for leaves treated with *C. cladosporioides* R406 resulting in a statistically significant reduction of conidia by 46%. For August 20, only a reduction by 15% was observed and counts did not differ significantly from the control treatment. No treatment effects on leaf development or symptoms of phytotoxicity were observed.

Close to leaf fall, on October 4 apple leaves which had been sprayed as young developing leaves with *C. cladosporioides* H39 or R406 during summer were sampled from the different plots to assess the endo- and epiphytical colonisation by fungi. The epiphytic colonisation by *Cladosporium* spp. was significantly higher while hyphal fungi different from *Cladosporium* spp. were significantly reduced for leaves treated during the summer with conidial suspensions of *C. cladosporioides* H39 or R406 compared to leaves treated with water only (control) (Table 5). Endophytic colonisation by *Cladosporium* spp. was significantly higher and hyphal fungi different from *Cladosporium* spp. were significantly reduced for leaves treated with *C. cladosporioides* H39. No significant treatment effects were found for epiphytic or endophytic colonisation by yeasts and yeast-like fungi (data not shown).

Discussion

Amongst the 63 isolates fulfilling the requirements for the envisaged development of a commercial biocontrol product and tested on seedlings, 13 isolates significantly reduced conidial production by *V. inaequalis*, and finally one isolate, *C. cladosporioides* H39 showed reproducible effects under orchard conditions in two seasons. The efficacy of this isolate varied between 28% and 57% in 2006 unless conidia with low viability had been applied (Table 3). In 2007, control efficacy was 69% and 51% at the first two sampling dates but no effect was found at the last sampling date (Table 4). Interestingly, isolate *C. cladosporioides* R406 which had been selected in a parallel screening programme for selection of antagonists against ascospore production of *V. inaequalis* in overwintering leaves (Köhl et al. unpublished) and belonging to the same species, showed a similar antagonistic potential.

Table 4 Effect of treatments with conidial suspensions of *Cladosporium cladosporioides* H39 twice per week from June 28 until August 20 (2×10^6 conidia ml^{-1}; 16 applications in total) or of *Cladosporium cladosporioides* R406 twice per week from July 12 until August 20 (2×10^6 conidia ml^{-1}; 12 applications in total) on conidial production by *Venturia inaequalis* on apple leaves under orchard conditions

Treatment	Ln-number of *V. inaequalis* conidia cm^{-2} leaf surface (backtransformed×1,000)		
	August 6	August 16	August 20
Control	11.3 (76.9)	10.7 (45.3)	10.4 (32.2)
C. cladosporioides H39	10.1[a] (24.1)	10.0[a] (22.0)	10.5 (34.5)
C. cladosporioides R406	n.d.	10.1[a] (24.3)	10.2 (27.4)

Experiment 2007

n.d. not determined

[a] Statistically different from the control treatment (one-sided unprotected LSD-test; $\alpha=0.05$)

Various other isolates of *Cladosporium* spp. including *C. cladosporioides* were less effective when tested on seedlings.

To our knowledge, this is the first report on the use of antagonists for biological control of *V. inaequalis* during its biotrophic stage resulting in statistically significant reductions of pathogen sporulation under orchard conditions. During the field experiments, sporulation on young and older leaves was assessed separately. Young, just emerged leaves had most likely only one *V. inaequalis* infection period and, due to their high susceptibility, produced high amounts of conidia under conducive conditions. Older leaves with ontogenic resistance (MacHardy 1996) had most likely been infected earlier during their susceptible stage before experiments started but later infections cannot be excluded (Li and Xu 2002). Applications of conidia of *C. cladosporioides* H39 formulated following a first pilot protocol resulted in reduction of conidial production in both situations, on young leaves with high susceptibility and young *V. inaequalis* colonies and on older leaves with ontogenetic resistance and most probably old colonies of *V. inaequalis* present.

However, experiments were conducted at the leaf level and conidial production was assessed only once on treated leaves. The effect of reduced conidial production on treated leaves on the further spread and increase of the disease has not been tested in our experiments. A reduction of approximately 40% of produced inoculum per sporulation event may result in a significantly stronger reduction of the disease after multiple infection-sporulation cycles as is common in apple scab epidemics. Thus, larger orchard plots have to be treated with *C. cladosporioides* H39 or R406 to assess the entire potential of the antagonists for biocontrol of apple scab.

Cladosporium spp. have been described in a few reports as antagonists of *Botrytis cinerea* (Newhook 1957; Eden et al. 1996). Fiss et al. (2000) found antagonistic activity of the fungus against *V. inaequalis* in *in vitro* studies. *Cladosporium* spp. are common epiphytic colonisers of leaf surfaces, often found in high densities (Dickinson 1981). Epiphytic colonisation by *Cladosporium* spp. is also reported for apple leaves (Fiss et al. 2000) and fruits (Teixidó et al. 1999). Such epiphytic populations of *Cladosporium* spp. may contribute to naturally occurring intrinsic biological control. In this case, conservation biological control (Eilenberg et al. 2001) protecting such beneficial populations by avoiding fungicides with side effects on non-target populations (Teixidó et al. 1999; Walter et al. 2007) or even selectively stimulating such naturally occurring beneficial populations, e.g. by application of specific nutrients, may contribute to scab prevention. Whether antagonism of *C. cladosporioides* H39 and R406 against *V. inaequalis* is an isolate-specific or a more common phenomenon in *Cladosporium* populations naturally present in orchards has to be investigated in further research.

Conidia of *C. cladosporioides* H39 produced on oat meal agar were not effective under orchard conditions but those produced in Solid-State Fermentation on grain culture substrates showed moderate effectivity. Microscopical observations showed that conidia produced in the fermenter were larger than

Table 5 Epiphytic and endophytic colonisation of apple leaves sampled on October 4

Treatment	Number of CFU cm^{-2} leaf surface			
	Epiphytes		Endophytes	
	Cladosporium spp.	Hyphal fungi different from *Cladosporium* spp.	*Cladosporium* spp.	Hyphal fungi different from *Cladosporium* spp.
Control	1,068c	428a[a]	0.27b	15.3a
C. cladosporioides H39	2,269a	155b	1.01a	2.6b
C. cladosporioides R406	1,525b	221b	0.24b	4.4b

Leaves treated with tap water containing 0.01% Tween 80 water (control) or with conidial suspensions of *Cladosporium cladosporioides* H39 twice per week from June 28 until August 20 (2×10^6 conidia ml^{-1}; 16 applications in total) or with *Cladosporium cladosporioides* R406 twice per week from July 12 until August 20 (2×10^6 conidia ml^{-1}; 12 applications in total). Experiment 2007.

[a] Values of the same column with common letters do not differ statistically significantly (LSD-test; $\alpha=0.05$)

those produced on oat meal agar. When oat meal agar was inoculated with such larger spores developing colonies again produced smaller conidia. The effect of growth medium on the spore quality of antagonists resulting in a better performance under environmental stress conditions has been demonstrated for several antagonists, e.g. cells of *Candida sake* produced on low-a_w-modified media (Teixidó et al. 1998). For further improvement of control level of *C. cladosporioides* H39, research is ongoing on mass production and further improvements of formulation since a strong effect of production conditions on effectivity has been observed and shelf life of the used pilot-product was a major obstacle. An improved shelf life will be needed if the development of a commercial biocontrol product will be envisaged. Furthermore, the possible effect of the used formulation on *V. inaequalis* has to be investigated in more detail. However, there is evidence that the formulation materials have no effect on *V. inaequalis* since applications of formulated conidia of *C. cladosporioides* H39 with low viability or of formulated spores of *P. pinodella* H3 did not result in any suppression of the pathogen.

Colonisation of apple leaves by introduced fungi depends on the biology of the introduced species (Kinkel et al. 1989). In their case study, introduced populations of *C. globosum* significantly decreased within a few days in contrast to populations of *A. pullulans*. In our study, antagonists were applied at 3 to 4 day intervals to guarantee high population levels on the leaves. Further studies are needed to assess the colonisation of apple leaves by the antagonist populations and their dynamics after application. Such knowledge is needed for optimising application timing and intervals. First results showed that conidial suspensions of *C. cladosporioides* H39 or R406 applied to leaves under orchard conditions had an effect on the population density of *Cladosporium* spp. on and within the treated leaves lasting at least 6 weeks. As a consequence, less frequent spray applications may be needed during summer epidemics to maintain an antagonist population on leaves sufficiently dense to reduce conidial production by *V. inaequalis*. The presence of higher populations of *Cladosporium* spp. in autumn close to leaf fall on and within leaves which had been treated during summer with both *C. cladosporioides* H39 and R406 may result in antagonism against *V. inaequalis* during the onset of its saprophytical colonisation of senesced apple leaves. For *C. cladosporioides* R406, a high potential in suppressing ascospore production by *V. inaequalis* during winter has been demonstrated after applications in autumn to fallen leaves (Köhl et al. unpublished).

Further research is needed on the effect of applications of *C. cladosporioides* H39 or R406 on scab epidemics, mass production and formulation and the feasibility to develop novel biocontrol products based on the antagonistic isolates.

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