New approaches in biological control of apple scab

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Abstract

Apple scab caused by Venturia inaequalis *is a major disease in apple production. In organic farming scab control depends on the use of fungicides based on copper or sulphur. Since the use of copper will be restricted in the European Union and the use of sulphur 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.*

Scab epidemics during summer are driven by conidia produced only on apple leaves. In this situation, antagonists present in the phyllosphere may interfere with conidia of the pathogen during sporulation or infection. Within the EU-funded project REPCO, more than hundred fungi were isolated from sporulating scab colonies and tested on apple seedlings for their potential to reduce sporulation of the pathogen. Since the aim of the study was to contribute to the development of a biocontrol product, only candidates were selected which fulfilled a range of additional criteria considering major constraints in development of biocontrol products. The best antagonists were applied under orchard conditions during two growing seasons. The antagonist H39 significantly reduced sporulation of V. inaequalis *after most applications. However, in a few cases no effect was found.*

Keywords: apple scab, biological control, antagonist, mass production, shelf life

Introduction

Apple scab caused by *Venturia inaequalis* is the major disease in European organic apple production. Control of the disease currently depends on frequent application of copper fungicides. Permitted amounts will be reduced stepwise during the following years (Council Regulation 2092/91, Annex II) to avoid environmental risks. The development of novel antagonists for biological control of apple scab may offer alternative options for disease control. Research on biological control of *V. inaequalis* mainly focussed on the overwintering stage of the pathogen in fallen leaves (Carisse *et al*., 2000). Only few reports described preliminary work on the possible use of antagonists during scab epidemics in summer (Fiss *et al*., 2000; Burr *et al*., 1996). Sporulating colonies of *V. inaequalis* may harbour antagonistic micro-organisms which may affect the sporulation capacity of the pathogen.

Objectives of our study were (1) to build-up a collection of micro-organisms obtained from *V. inaequalis* colonies on apple leaves, (2) to select possible antagonists suppressing sporulation of the pathogen, (3) to develop prototype production protocols and formulations for selected antagonists, and (4) to test selected antagonists under field conditions.

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Material and Methods

Sources of antagonists. Scab infected leaves were collected during late summer 2004 after a severe increase of the scab epidemics had been observed in orchards during September. In total, 216 leaf samples were collected in various parts of The Netherlands (82 samples), Belgium (18 samples), northwest Germany (11 samples) and central Germany (105 samples). Most samples originated from old standard trees (without any further cropping management), e.g. planted along secondary roads. Samples were also collected in organically managed orchards, abandoned orchards, or orchards with integrated management. Developing mycelium different from *V. inaequalis* mycelium was isolated from sporulating colonies of *V. inaequalis* on leaf parts not yet necrotic and pure cultures were obtained.

Pre-screening. A rapid throughput system was used for a first check of candidate antagonists regarding their potential risks and economical feasibility of the development of a biocontrol product. Fungi belonging to the genera *Aspergillus*, *Penicillium* or *Fusarium* were discarded because of the potential of various species within these genera to produce mycotoxins. The remaining isolates of fungi were cultured on nutrient media and the spore production was determined. Isolates producing less than 1×10^5 spores per plate were discarded because of a production of such isolates is considered not to be cost effective. Subsequently, isolate growing at 5°C and at low water activity were selected because they were considered to be cold tolerant and drought tolerant. These characteristics are pre-requisites for successful colonisation of the phyllosphere. Isolates growing at 36°C were discarded because such isolates may demand special risk studies during a registration procedure.

Screening on seedlings. The potential of candidate antagonists to suppress the conidia production of *V. inaequalis* on infected leaves was tested on young apple seedlings. Seedlings were sprayed with conidial suspensions of *V. inaequalis* (1×10^5 ml^{-1}) until run-off and placed in a moist chamber consisting of a plastic tray closed by transparent plastic top. After 2 days incubation at 15°C with diffuse light, the tops were removed from the trays and seedlings further incubated for 5 days at 85% RH, 15°C and 16 hrs light per day. Thereafter, *V. inaequalis*-inoculated seedlings were sprayed with antagonist suspensions (containing 1×10^6 spores or cells ml^{-1}) or water (containing 0.01% Tween 80) as control. Two seedlings were used for each replicate of each treatment. The sets of two inoculated seedlings were placed in a polyethylene tent in a block design with six blocks (replicates) and complete randomization 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 hrs light per day at 138 µE s^{-1} m^{-2}.

From both seedlings of each replicate, the youngest five true leaves were carefully removed, put into Duran bottles (100 ml) containing 35 ml of tap water with 0.01% Tween 80. Bottles were shaken with a flask shaker and the concentration of conidia of *V. inaequalis* was determined for each suspension with the aid of a haemocytometer. Leaf surfaces were measured with an area meter.

Orchard experiments. Several rows of var. Jonagold within an organically managed orchard at Applied Plant Research, Randwijk, The Netherlands, were pruned during spring and summer 2006 and 2007 so that trees produced new shoots with young leaves highly susceptible for *V. inaequalis*. To stimulate the development of new shoots also the majority of young fruits was removed. A series of 8 experiments was carried out in 2006, each on a different set of trees. For each experiment 2 to 6 trees, depending on the number of newly produced shoots per tree, were chosen for each of 6 blocks (replicates). Within each block, 7 treatments were carried out.

Shoots were labelled with coloured metal rings so that the 2 youngest leaves fully expanded at 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 four different antagonists. Separate treatments were carried out with fermenter-produced spores of the antagonist H39 formulated as dry powder and resuspended in tap water containing 0.01 % TWEEN 80. Spray applications were done using a compressed air-driven knapsack sprayer at 250 kPa until run-off. The different experiments were carried out in the period between 22 June and 28 September 2006. 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. During all experiments, subsequent treatments were carried out at 3 to 4 day intervals. Leaves were sampled 3 to 5 weeks after the first treatment In each experiment, the 2 youngest leaves fully expanded at the beginning at the experiment together with the 2 next younger leaves (expanded during the course of the experiments) were pooled for 3 shoots belonging to the same replicate so that a sample consisted of 12 leaves. Leaves were shaken in bottles water containing 0.01% Tween 80 with a flask shaker. From the obtained suspensions, 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.
In 2007, a similar experiment was carried but the same trees were treated during a longer period of 8 weeks and leaves were repeatedly sampled from such trees.

Production and formulation. Promising isolates were selected and assessed in small scale Solid-State Fermentation (SSF) for their suitability for large scaled biotechnological production processes. Several fermentation conditions regarding media and incubation conditions were evaluated. Only antagonists which passed this screening step were tested in subsequent experiments in the orchard. Final experiments were carried out to develop protocols for mass production, down streaming and formulation for the two selected antagonists R406 and H39. Fermentation was based on the Prophyta laboratory scaled SSF system and formulated products were applied in the orchard experiments.

Results

Pre-screening and screening experiments. From 160 isolates tested, less than 50% of the tested isolates passed the first pre-screening steps. Details of preliminary results have been reported earlier (Köhl *et al*., 2000). Fourteen experiments on seedlings were carried out. Most of the 80 candidate isolates tested on seedlings did not statistically significantly reduce conidiation of *V. inaequalis*. Four isolates, H3, H33, H35, and H39 caused a significant reduction of *V. inaequalis* on the leaves which could be repeated in subsequent independent experiments. However, efficacies (calculated on base of backtransformed values) of these antagonists varied between 18 and 80% for H3, 50 and 75% for H33, 32 and 84% for H35, and 55 to 79% for H39 in the experiments in which they were tested and conidiation reduction was in some cases not statistically significant. A few more isolates showed a strong statistically significant antagonistic effect in one experiment but such effects could not be repeated. In no case significant enhancements of conidiation of *V. inaequalis* after application of candidate isolates was observed.

Orchard experiments. A series of 8 experiments was carried out in 2006, each on a different set of trees of the apple variety Jonagold within an organically managed orchard at Randwijk, The Netherlands. Trees were pruned during spring and summer so that trees produced new shoots with young leaves highly susceptible for *V. inaequalis*.

Four different candidate antagonists were applied to leaves and the production of conidia by *V. inaequalis* was quantified on treated leaves. From the four tested antagonists, only the first formulated spore product of the antagonist *Cladosporium cladosporioides* H39 reduced significantly pathogen sporulation by 35 to 55%. In 2007, apple trees were treated with formulated H39 with similar results. A significant reduction by 51 to 69% was found for two assessment dates (Figure 1). However, at a later assessment date no difference between treated and untreated trees was found. This may be due to a reduced quality of the available antagonist product during the field experiment which had an insufficient shelf life. A second antagonist, R406, which had shown high efficacy against ascospore production on overwintering leaves in the earlier experiment, had been included in the experiment and showed also partial antagonistic effect against conidia production of *V. inaequalis*.

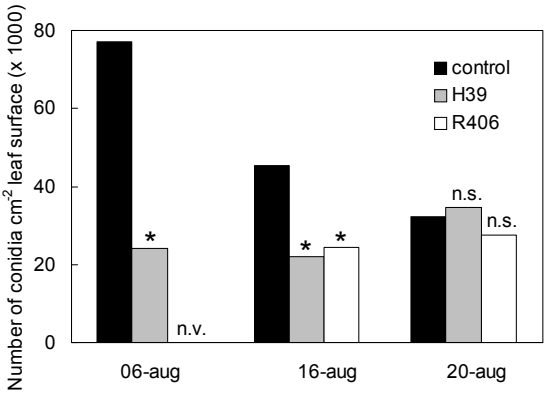

Figure 1. Conidia production of *V. inaequalis* on apple leaves treated twice per week with H39 or R406 (approximately $2x10^6$ viable spores ml^{-1}) in an organically managed orchard. Randwijk 2007. Statistically significant effects of individual treatments in comparison with the control treatment are indicated by '*' separately per sampling date; one-sided unprotected LSD-test (α = 0.05); n.s.: no significant difference; n.v.: no value.

Production and formulation. The spore production in Solid-State Fermentation was investigated cultivating isolates on substrate mixtures consisting of oats and rolled oats. For the strain H39 it was possible to harvest more than 1.5×10^9 conidia per gram culture substrate (dw input). For R406, spore production depended on substrate composition and duration of incubation. However, the overall quantities and qualities of produced spore were not satisfying.

Unfortunately, most samples of the produced final products of both isolates showed a limited shelf life. The only exception was a formulation of spores of isolate H39 as a wettable powder. Even after 8 months of storage at 4°C or -20°C, sufficient spores were viable and able to germinate on nutrient agar (Figure 2). Since spore products were developed for applications on leaf surfaces, UV light may affect spore survival after application. Spores produced and formulated under various conditions were tested for their susceptibility toward UV light. Spores of H39 formulated as wettable powder showed a lower susceptibility to exposure to UV light than spores produced and formulated under other conditions.

Unfortunately, the final formulated product, optimised in spore production, shelf life and UV stability, could not be tested for its potential in apple scab control in orchards during the project period.

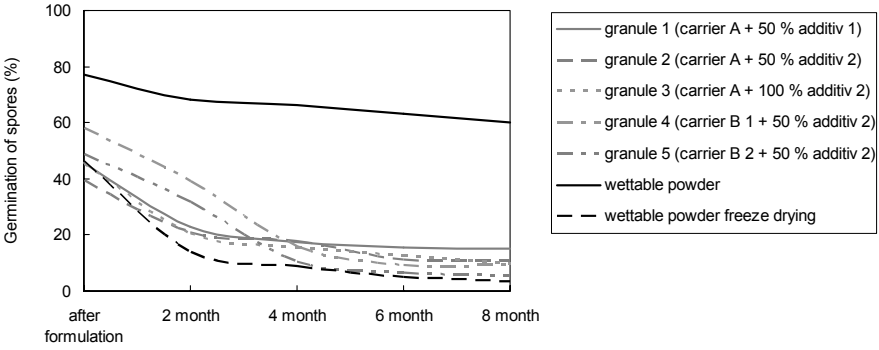

Figure 2. Effect of different formulation techniques on shelf life of spores of H39 mass produced in Solid-State Fermentation and stored at +4 °C.

Discussion

A surprisingly high number of very different fungi were found in sporulating lesions of *V. inaequalis*. The number of candidate isolates for the labour-intensive screening experiments could be reduced significantly by a pre-screening procedure. The protocol of the pre-screening had been developed considering important characteristics regarding economical feasibility (spore production on growth medium), important ecological characteristics for field applications (cold tolerance, drought tolerance) and possible risks for users. Screening experiments on seedlings under controlled conditions showed high variation in conidia counts for *V. inaequalis* between experiments but also within experiments between single seedlings and leaves of seedlings. However, it was possible to select a few antagonistic fungal isolates which suppressed sporulation of *V. inaequalis* under these conditions for more than 80%. Out of these isolates, those were chosen which showed a satisfying production in Solid-State Fermentation. In orchards experiments conducted in two different years, multiple applications of one formulated isolate resulted in a significant reduction of *V. inaequalis* conidia production by 31- 69%. Since epidemics of apple scab are polycyclic, such a level of reduction in each reproduction cycle may have a strong effect on the progression of the epidemic.

The spores of H39 in the formulated product used in the field experiments had a limited shelf life. This major problem was solved by developing new protocols for the product formulation.

Further research is now needed to investigate the effect of newly developed formulations of H39 on *V. inaequalis* under field conditions and to assess the effect of antagonist applications on the entire summer epidemics of apple scab.

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