Selection of antagonists suppressing conidia production of *Venturia inaequalis*

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***Abstract* – Novel antagonists for biological control of *Venturia inaequalis* causing apples scab were screened. Several hundred fungal isolates were obtained from sporulating colonies of *V. inaequalis* on apple leaves collected at various locations. Candidate antagonists were pre-screened to exclude those isolates with potential risks or low economical feasibility. Remaining isolates were tested on apple seedlings for their ability to reduce conidiation of *V. inaequalis*. Several promising antagonists could be selected and will be tested under field conditions in summer 2006.[1]**

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. Sporulating colonies of *V. inaequalis* may harbour antagonistic micro-organisms which may affect the sporulation capacity of the pathogen. Objective of our study is to build-up a collection of micro-organisms obtained from *V. inaequalis* colonies on apple leaves and to select possible antagonists suppressing sporulation of the pathogen.

Materials and Methods.

Sampling

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.

Leaves with sporulating colonies of *V. inaequalis* on leaf parts not yet necrotic were placed in moist chambers and incubated for three days at 20 °C. Developing mycelium different from *V. inaequalis* mycelium was isolated on V8 agar and oat meal agar, both containing 100 mg/l streptomycine and 15 mg/l tetracycline. Agar plates were incubated at 15 °C for 3 to 5 days and mycelium from the edges of the developing fungal colonies (different from *V. inaequalis*) were subsequently transferred to agar plates to obtain pure cultures.

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Pre-screening of candidate antagonists

A rapid throughput system was used for a first check of candidate antagonists regarding potential risks and economical feasibility for the development of a biocontrol product. Pure cultures were first inspected for fungal genera. Those belonging to *Aspergillus*, *Penicillium* or *Fusarium* were discarded because of the potential of various species within these genera to produce mycotoxins. Other isolates of hyphal fungi were cultured in Petri dishes on oat meal agar for 21 days at 18 °C and 12 hrs per day blacklight; yeasts were cultured on basal yeast agar for 5 days at 18 °C. For each isolate, production of spores or yeast cells per plate was determined after preparation of suspensions using sterile tap water containing 0.01 % Tween 80. Isolates producing less than 1 x 10^5 spores or yeast cells per plate were discarded. Ten µl of the suspensions were plated in duplicate in sterile wells, 16 mm in diameter containing 1.5 ml malt agar. Different plates with inoculated wells were incubated at 5, 18 and 36 °C in the dark. An additional plate with wells containing malt agar adjusted to -10 and -7 MPa by adding KCl were incubated at 18 °C. All wells were inspected for fungal growth after an incubation period of 14 days.

Production of fungal suspensions

Conidia of *V. inaequalis* (of monospore isolate MB 363B obtained from M. Bengtsson, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark) were produced according to the bottle wick method (Williams, 1976, modified by M. Bengtsson) in Duran bottles containing potato dextrose broth. The obtained conidial suspension was stored at -18 °C until use.

Isolates of hyphal fungi were grown on oat meal agar for 28 days at 18 °C and 12 hrs 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 adding sterile tap water containing 0.01 % Tween 80.

Apple seedlings

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. Seedlings were grown for 28 days with cycles of 16 hrs light at 18 °C and 8 hrs dark at 12 °C. Plants used in experiments had at least four fully expanded leaves.

Seedling assay
Seedlings were sprayed with conidial suspensions of *V. inaequalis* (1 x 10^5/ml) 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 3 or 4 days at 85 % RH, 15 °C and 16 hrs light per day. Thereafter, *V. inaequalis*-inoculated seedlings were sprayed with antagonist suspensions (containing 1 x 10^6 spores or cells/ml) 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/m^2.

Assessments
From both seedlings of each replicate, the lowest 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

Statistics
The number of *V. inaequalis* conidia produced per cm^2 leaf was calculated per replicate. Natural logarithmic-transformed values were analyzed by ANOVA. If F-values indicated significant differences, means were separated by LSD-tests (α = 0.05).

RESULTS

Pre-screening
From the 159 fungal isolates, 20 isolates showed poor sporulation on agar. These isolates were excluded from further screening because spore production may not be economically feasible. From the remaining 139 isolates, spores of 13 isolates produced colonies at 36 °C. These isolates were excluded from further screening because of their potential risk for human health. All isolates tested produced colonies at 5 °C and can be considered as cold tolerant. Only 16 isolates out of 139 did not produce a colony at -10 MPa and were excluded from further screening because they may have low drought tolerance.
In summary, from 159 isolates tested, 49 were excluded from further screening for efficacy against *V. inaequalis* in bio-assays.

Bio-assay
A pilot experiment with ten replicates was carried out with *V. inaequalis*-inoculated seedlings. Based on the observed variation between replicates, and a test power of 90 %, it was estimated that with 6 replicates a reduction of sporulation by 80 % can be expected as statistically significant. A series of screening experiments has been carried out. Results of one experiment are given as an example in Table 1. In this experiment, four isolates out of seven reduced the sporulation of *V. inaequalis* statistically significant by more than 80%. In most other experiments, fewer antagonists with such a high efficacy were found.

Table 1. Effect of candidate antagonists on *V. inaequalis* conidiation on apple seedlings.

	Number of conidia/cm^2 leaf surface			
	Ln-transformed		Backtrans-formed	Relative to control*
Control	7.20	ab**	1339	100
Fungus 1	5.20	c	181	14
Fungus 2	5.57	c	262	20
Fungus 3	6.67	bc	788	59
Fungus 4	6.01	bc	407	30
Fungus 5	5.57	c	262	20
Fungus 6	8.32	a	4105	306
Fungus 7	5.55	c	257	19

* At backtransformed scale.
** Values with a common letter do not differ statistically significant ($LSD_{5\%}$ = 1.49).

DISCUSSION

Fungal colonisation of *V. inaequalis* colonies was frequently observed on scabbed apple leaves. Criteria for pre-screening of candidate antagonists were developed and a high throughput system was used to exclude isolates with possible risks or low economical feasibility. Approximately a third of the isolates did not fulfil the criteria and were excluded from further screening. First promising candidates were selected in the bio assays on apple seedlings under controlled conditions reducing sporulation of *V. inaequalis* for more than 80 %. Further screening experiments are ongoing. During summer 2006, a series of small scale experiments will be carried out under field conditions. Individual scabbed leaves will be treated with the most promising candidate antagonists and *V. inaequalis* conidiation will be quantified in comparison with untreated leaves.

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