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Screening of organically based fungicides for apple scab (*Venturia inaequalis*) control and a histopathological study of the mode of action of a resistance inducer

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Abstract: A range of possible substitutes for copper-based fungicides for control of apple scab (*Venturia inaequalis*) in organic growing were tested in laboratory and growth chamber experiments in the Danish project StopScab (2002-2004). Eighteen crude plant extracts, 19 commercial plant-based products and 6 miscellaneous compounds were tested for their ability to reduce scab symptoms on apple seedlings. Most of the compounds were also tested for their effect on conidium germination on glass slides. Fourteen of the crude plant extracts, 13 of the commercial plant products and 5 of the miscellaneous compounds showed promising control efficacies when used either preventively or curatively in the plant assay. A histopathological study was carried out on the mode of action of the resistance inducer, acibenzolar-S-methyl (ASM), which reduced scab severity and sporulation on apple seedlings in several plant assays when applied as preventive treatment. The effect of the inducer on key pre- and post-penetration events of *V. inaequalis* was studied and compared to these events in water-treated control leaves. The histopathological study showed that the inducer had its strongest effect on post-penetration events indicated by delayed infection and reduced stroma development. In addition, a small but significant inhibition of conidial germination and a stimulation of germ tube length were observed. This investigation provides new histopathological evidence for the mode of action of ASM against *V. inaequalis* and serves as a model for evaluation of the mechanisms by which the organically based fungicides reduce infection of *V. inaequalis*.

Key words: apple scab control, acibenzolar-S-methyl, botanical fungicides, induced resistance, organic growing, plant extracts, histopathological study, screening

Introduction

Apple scab (*Venturia inaequalis* (Cke.) Wint.) causes serious losses in quality and yield of organically as well as conventional grown apples. No effective eradicative or curative fungicides for apple scab control are presently available for use by organic growers, and while protective, copper-based fungicides and lime-sulphur are allowed in most European countries, only elemental sulphur is permitted in Denmark. Since sulphur is not very effective against apple scab, and the use of copper fungicides will be phased out in Europe within the next few years, alternative fungicides for apple scab control are increasingly needed.

As a part of the Danish research project StopScab (2002-2004) (Bengtsson & Hockenhull, *in press*; Bengtsson *et al*., 2004) we have been searching for and screening for organically based fungicides to control *V. inaequalis* in laboratory, growth chamber and green house experiments. Two routine screening systems were used, one testing the effect of materials on conidium germination on glass slides, and one testing the effect of compounds on disease severity on apple seedlings. As a model for evaluation of the mechanisms by which organically based fungicides reduce infection by *V. inaequalis*, a histopathological study was carried out on the mode of action of the resistance inducer, acibenzolar-S-methyl (ASM).

Materials and methods

Fungal inoculum

Conidia of a mono-conidial isolate of *V. inaequalis* were produced by the bottle wick method modified from Williams (1978). The concentration of harvested conidia was adjusted with sterile water to 1.5 x 10^5 conidia/ml, and the conidial inoculum was stored at –18°C until use.

Plant material

Apple seedlings were produced from seeds of *Malus* x *domestica* cv. 'Golden Delicious' (Eichenberg, Germany) and grown in small pots in a growth chamber at 15-16°C under cycles of 12 h light and 12 h darkness.

Preparation of test solutions

Water extracts and solutions (w/v or v/v) of test compounds, including crude plant extracts, commercial plant-based materials and miscellaneous commercial compounds were prepared according to protocols supplied by companies or based on literature studies. Water and elemental sulphur (0.27% (w/v); Kumulus S, BASF) were used as standard control treatments.

Glass slide test

Different concentrations of selected compounds were tested for effect on conidial germination. Inoculum of *V. inaequalis* was mixed with test solutions and incubated at 18°C. After 24 and 48 hours, the percentage of germinated conidia was assessed using a light microscope and compared to germination in the water control, which was set to 100 %.

Plant assay

Seedlings with four to six leaves were used and each treatment consisted of eight plants. Test solutions were applied to the adaxial leaf surface with a hand sprayer 1-3 days before inoculation (preventive treatment) or 1 day after inoculation with *V. inaequailis* (curative treatment). Plants were incubated in darkness under a clear plastic cover for 24 hours after the preventive / curative treatment and for a further 48 hours after inoculation. Disease severity was assessed 14 days after inoculation following a scale 0-7 (Croxall *et. al.*, 1952; Parisi *et al.*, 1993): 1 =: 0% < percentage of scabbed leaf surface (sls)< 1%; 2 = 1%<sls<5 %; 3 = 5%< sls< 10%; 4 = 10< sls< 25%; 5 = 25%<sls<50%; 6 = 50%<sls<75%; 7 = 75%<sls.

Histopathological study

Apple seedlings were sprayed with 200 ppm acibenzolar-S-methyl, ASM (Bion WG 50 ™, Syngenta Crop Protection) or water. Three days later, the adaxial leaf surfaces were inoculated and plants were incubated as described above. The 2 youngest leaves from each of 4 replications were harvested either 1 or 5 days after inoculation. Clearing was carried out by placing the leaves on absorbent paper saturated with a fixative (96% ethanol, 100 % acetic acid; 24:1 v/v) in closed plastic containers. The cleared leaves were mounted in lactic acid and examined using light microscopy. For each treatment the overall germination rate was determined and the following events were examined for 200 germinated conidia: formation of appressoria, cuticle penetration, formation of primary stroma, runner hypha and secondary stroma. In addition, the length of germ tubes and average length of runner hyphae per stroma were determined. All data were analyzed using SAS, version 8.2 (SAS Institute, Cary, NC). Continuous variables were analyzed by analysis of variance assuming a normal distribution. Discrete variables were analysed by logistic regression, assuming a binomial distribution.

Table 1. Overview of test results of different materials in the conidial germination test and plant assay screening for apple scab control.

	Glass slide test		Plant assay				
Type of material	No. tested	No. with moderate / strong effect[a]	No. tested	No. with no efficacy	No. with some efficacy[b]	No. with promising efficacy[c]	No. with high efficacy[d]
Crude plant extracts	9	6	18	3	1	14	0
Commercial botanical products	14	12	19	4	2	9	4
Other commercial compounds	3	2	6	1	0	3	2
Total	**26**	20	**43**	8	3	28	6

[a] Compounds able to reduce conidial germination or affect germ tube growth at different concentrations.
[b] Compounds showing a significant protective or curative effect in at least one plant assay.
[c] Compounds showing a highly significant protective or curative effect in at least one plant assay.
[d] Compounds repeatedly showing highly significant effect in several plant assays.

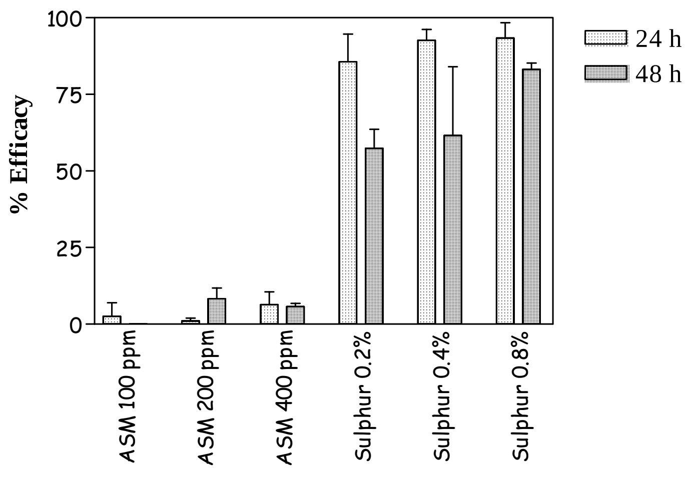

Figure 1. Efficacy (%) of ASM (100, 200 and 400 ppm) and sulphur (0.2, 0.4 and 0.8 %) in reducing germination of conidia in the glass slide test 24 and 48 hours after start of incubation. Bars represent standard deviations of means.

Results and discussion

Screening assays

Most of the materials tested in the glass slide test reduced conidial germination either moderately or strongly (Table 1). Twenty-eight materials, mainly crude plant extracts and commercial plant-based products, showed promising efficacy to control apple scab in the plant assay screenings and four showed high efficacy in this assay (Table 1). Two of the miscellaneous compounds, namely sulphur (Kumulus S) and acibenzolar-S-methyl, ASM, consistently showed repeatedly high efficacy in reducing apple scab severity when applied as protective treatments. Sulphur strongly reduced conidium germination in the glass slide test, while ASM did not (Figure 1).

Histopathological study

The histopathological observations of key infection events in treated leaves sampled one day after inoculation are presented in Table 2 and the observations made 5 days after inoculation are presented in Table 3.

Table 2. Effect of ASM on pre-penetration events of *Venturia inaequalis* on apple leaves, determined 1 day after inoculation.

Key events:	Control (water)	ASM
Percent conidia germinated	60.0	45.0***
Average length of germ tubes (µm)	23.1	29.5*

***=significant at P<0.001, and *=significant at P<0.05.

The ASM treatment showed a highly significant inhibition of conidial germination and a positive effect on germ tube length on leaves one day after inoculation (Table 2). Although less pronounced, the inhibitory effect on conidial germination was also found on the fifth day after inoculation (Table 3). This result is in sharp contrast to the results obtained in the conidial germination tests carried out on glass slides, where the inhibitory effect of ASM on spore germination (Figure 1) was very small. Strong effects of ASM were observed on penetration and post-penetration events as the infection process was delayed and development of primary and secondary stroma was reduced. Thus, inhibition of germination on leaves treated with ASM could be interpreted as an early expression of induced resistance. This, together with the observation that ASM did not inhibit conidial germination to any notable extent in the glass slide test, lead us to conclude that ASM mainly acts against the apple scab pathogen by the mechanism of induced resistance (Jørgensen *et al*., 2004). The developed system can serve as a model for evaluation of the mechanisms by which organically based fungicides reduce infection of *V. inaequalis*.

Table 3. Effects of ASM on pre- and post-penetration events of *Venturia inaequalis* conidia on apple leaves, determined 5 days after inoculation.

Key events:	Control (water)	ASM
Germinated conidia (%)	58.0	50.5*
Conidia forming appressoria (%)[a]	94.5	86.0**
Conidia attempting penetration (%)[a]	87.5	62.5***
Appressoria attempting penetration (%)	86.5	76.1***
Appressoria producing primary stromata (%)	59.2	36.2***
Conidia producing runner hyphae (%)[a]	50.5	27.0***
Number of runner hyphae formed per stroma	5.6	4.3*
Conidia forming secondary stromata (%)[a]	13.0	2.5**

***=significant at P<0.001, **=significant at P<0.01, and *=significant at P<0.05.

[a]: Events based on 200 germinated conidia

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