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# Comparative studies on the effects of a yucca extract and acibenzolar-*S*-methyl (ASM) on inhibition of *Venturia inaequalis* in apple leaves

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**Abstract** The effect of an extract of *Yucca schidigera* on the control and infection process of the apple scab pathogen, *Venturia inaequalis*, was examined and compared with the chemical resistance inducer, acibenzolar-*S*-methyl (ASM). In seedling assays, both materials significantly reduced apple scab symptoms and pathogen sporulation on leaves and both showed similar control efficacies as the reference treatment, sulphur. Whereas yucca extract and sulphur gave significant inhibition of conidial germination *in vitro*, ASM did not inhibit germination. Histopathological studies of the infection process of *V. inaequalis* in apple leaves showed that the yucca extract primarily acted by inhibiting pre-penetration events and penetration itself. In contrast, the ASM treatment significantly inhibited more stages of the infection process (pre-penetration, penetration and post-penetration events). These observations suggest that the yucca extract acted mainly by a direct fungitoxic effect whereas ASM, as expected, acted as a resistance inducer. However, expression studies of two genes encoding the PR proteins, PR1 and PR8, in apple seedlings indicated that yucca extract may also affect plant defence as expression of both genes was up-regulated following yucca treatment, to a level similar to that observed after treatment with ASM. The fungitoxic effect of sulphur on *V. inaequalis* was also confirmed in this study.

**Keywords** Apple scab · Botanicals · Induced resistance · *Malus domestica* · Real-time RT-PCR · *Yucca schidigera*

## Introduction

Apple scab, caused by *Venturia inaequalis* is one of the most serious diseases of apples (*Malus domestica*) world-wide (MacHardy 1996). In organic fruit production, the use of resistant cultivars and cultural practices can reduce infections but many growers control the disease by spraying with copper-based fungicides. Since 2006, however, the amount of copper fungicide permitted for use in organic farming in the European Union has been reduced (Council Regulation (EEC) No 2092/91). Lime sulphur is often used as a fungicide for apple scab control, but even

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though it shows an efficacy as high as copper fungicides (Holb and Heijne 2001), phytotoxicity may also be a problem with this material (Holb et al. 2003). In some countries, e.g. Denmark and the Netherlands, organic growers are only allowed to use elemental sulphur, and especially when the timing of application is not optimal, sulphur is not very effective against scab. Alternatives to copper-based fungicides and sulphur are thus urgently needed for apple scab control in organic growing. Such materials may include salts (Schulze and Schönherr 2003), compost extracts (Cronin et al. 1996), plant oils (Northover and Schneider 1993) and plant extracts (Bosshard 1992). Plant extracts are potential sources of alternative control agents and a large part of the plant kingdom still remains unexplored for possible materials. With the purpose of identifying promising new materials for apple scab control in organic growing in Denmark, a national research project was initiated and continued as a part of the European project REPCO (Bengtsson et al. 2006b), where the main purpose was to find replacements for copper in apple and grape production. Several promising organically-based materials were found that consistently gave effective control of apple scab in seedling assays (Bengtsson et al. 2006a, b), and some of these were also tested in organic orchard trials (Heijne et al. 2007). Several plant extracts were found to be effective and especially one, prepared from *Yucca schidigera* was found to be consistently effective in controlling apple scab in seedling assays (Bengtsson et al. 2006b) and in field trials conducted in Denmark and the Netherlands (Bengtsson et al. 2007; Heijne et al. 2007). Yucca extract, made by crushing stems of *Y. schidigera* and other *Yucca* species, is reported to have a high content of steroid saponins (Cheeke 2001) and to contain polyphenolic compounds (Cheeke and Otero 2005). Both groups of compounds are involved in plant defence responses (Oleszek et al. 2001; Osbourn 2003). Amongst others, extracts from yucca are used as additives to foods and cosmetics, as adjuvants in vaccines and in animal feed to control intestinal protozoa and nematodes, and for the reduction of atmospheric ammonia and other odours in livestock buildings (Cheeke 2001; Cheeke and Otero 2005). Due to good surfactant properties at low concentrations, yucca extracts are also used as additives in spray formulations of pesticides and fertilisers.

The mode of action of potential disease-suppressing plant extracts remains unknown in many cases. In addition to the direct fungitoxic effects of plant extracts on a fungal pathogen, their potential as inducers of resistance against plant pathogens deserves investigation. A few plant extracts have been found to induce resistance in horticultural crops (Rocha and Hammersmidt 2005), including an extract of *Hedera helix* used against fire blight (*Erwinia amylovora*) in apple (Baysal and Zeller 2005). The use of induced resistance to control apple scab in the orchard may be a useful approach in the future, especially when integrated with other disease management practices (Rocha and Hammersmidt 2005).

In apple, chemically-induced resistance has been demonstrated with acibenzolar-*S*-methyl (ASM) against the fire blight pathogen, *Erwinia amylovora* (Brisset et al. 2000; Maxson-Stein et al. 2002) and against scab, caused by *Venturia nashicola* in Japanese pear (*Pyrus pyrifolia* var. *culta*) (Faize et al. 2004). In these studies, protection was accompanied by enhanced accumulation of several PR-proteins (PR1, PR2, PR8 and PR10). Furthermore, Ortega et al. (1998), who studied the development of *V. inaequalis* in apple leaves, suggested that resistance was induced in apple seedling leaves following treatment with methyl 2,6-dichlorosonicate (DCINA).

Real-time reverse transcription-PCR (RT-PCR) has been used to study the response of plants to pathogen attack, where, for example, the expression of PR-proteins is used as an indication of systemic acquired resistance (Bonasera et al. 2006). Real-time RT-PCR has, however, only recently been used to study expression of defence genes in apples (Maxson-Stein et al. 2002; Kürkcüoglu et al. 2007).

The purpose of the present work was to study the effect and mechanisms by which a yucca extract inhibits infection by *V. inaequalis* in artificially inoculated apple leaves. This was done by carrying out a detailed microscopical study using a previously identified inducer of resistance, ASM, for comparison. In addition, the effect of yucca extract, ASM and sulphur on conidial germination was studied *in vitro*, and the expression of two PR-proteins, PR1 and PR8, in inoculated apple leaves treated with the three materials, was investigated.

## Materials and methods

### Plant material

Seeds of *M. domestica* cv. Golden Delicious (Eichenberg & Co, Miltenberg, Germany) were stored at 2°C. For stratification, seeds were primed under running tap water for 24 h, surface-sterilised for 1 min in 70% ethanol, 5 min in 2–3% sodium hypochlorite and rinsed three times in tap water. Seeds were placed in moist sand and incubated in a refrigerator (approximately 4°C) for 3–6 weeks. Germinating seeds were sown in small pots (5.5 cm diam) containing a peat-based substrate (Pindstrup Færdigblanding 2, Pindstrup Mosebrug A/S, Denmark) mixed with sand (4:1 *v/v*) in trays under plastic cover and grown in a growth chamber with cycles of 12 h light and 12 h darkness. Light was supplied by fluorescent tubes (Osram L36W/11-860 Lumilux plus Eco Daylight, Osram GmbH, Augsburg, Germany, 200 μE $m^{-2}$ $s^{-1}$). The temperature was 15–16°C while the relative humidity (RH) ranged from 50–60%. Four to 5 week-old plants with four to six developing leaves were used in the experiments.

### Preparation of fungal inoculum and treatments

Conidia from a single-spore isolate of *V. inaequalis* (MB363, isolated in 1998 from cv. Jonagold, Aarslev, Denmark) were produced on gauze by the 'bottle wick method' modified from Williams (1978) using 300-ml flat glass bottles containing 20 ml potato dextrose broth (PDB; Difco, Becton, Dickinson and Company, Sparkes, USA). Conidia were harvested by centrifugation and washed in sterile distilled water followed by re-suspension in sterile water. The concentration was adjusted to $1.5\times10^5$ conidia $ml^{-1}$ and inoculum was stored in centrifuge tubes at −18°C until use. In the plant assays, yucca extract, Norponin® BS Liquid (pure extract of *Y. schidigera*; Nor-Natur Aps, Denmark), was used at 5% (*v/v*) (this concentration was chosen due to its efficacy: no conidial germination in the *in vitro* assays) in distilled water, and acibenzolar-*S*-methyl, ASM (Bion WG 50 ™, Syngenta Crop Protection, Switzerland) was used at 200 ppm (*w/v*). Distilled water was used as the control treatment and elemental sulphur (0.27% (*w/v*) Kumulus S, BASF, Denmark), corresponding to a field dosage of 4 kg $ha^{-1}$ based on 1,500 l, as the reference treatment (Bengtsson et al. 2006a).

### Plant inoculation and disease assessment

Treatments were applied to plants 2 days before inoculation with the pathogen. Each treatment was applied to the adaxial surfaces of all leaves on each of eight replications (plants) with a plastic hand sprayer until run-off. Plants were arranged randomly in plastic trays and incubation took place in the growth chamber described above under a polyethylene bag to provide 100% RH and without light for the first 24 h. The temperature was 15–16°C. Light was provided for the next 24 h. Plants were then removed from the polyethylene cover, inoculated by spraying until run-off with a conidial suspension of *V. inaequalis* using a plastic hand sprayer and immediately returned to the polyethylene cover for 48 h without light and under the same conditions as described above. Light was again provided and holes were made in the polyethylene cover to allow air exchange. Symptoms on seedling leaves normally began to appear 7–8 days after inoculation (dai) and disease severity was assessed 14 dai on all individual inoculated leaves using the scale 0–7 derived from Croxall et al. (1952) and Parisi et al. (1993): 0=no visible symptoms; 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=sls>75%. The mean disease severity index was determined from the average score obtained from all inoculated leaves of each plant in each treatment. Experiments were repeated three times and the representative data from two experiments are presented.

### Determination of conidial production on apple leaves

In one selected experiment, conidial production on the scored leaves was quantified 14 dai. Treated leaves from each plant were sampled and pooled in 50 ml centrifuge tubes. After washing for 10 s on a Whirlmixer in 20 ml water, the number of conidia in the washing fluid was counted with a Fuchs Rosenthal haemocytometer (three replications) and the spore production per plant for each treatment calculated.

### In vitro conidial germination tests

Different concentrations of the compounds (2.5%, 5% and 10% for yucca extract; 200, 400, and 800 ppm for ASM; 0.2%, 0.4% and 0.8% for sulphur) were tested for their inhibitory effect on conidial germination of *V. inaequalis*. On single-well glass slides (Menzel, VWR International Aps, Denmark), 35 µl of conidial inoculum of *V. inaequalis* was mixed with 35 µl of test solution and incubated at 18°C in a moist chamber. After 24 and 48 h, the percentage of germinated conidia (among 100 randomly selected) was assessed using a light microscope and the treatments were compared to a water control, which was set to 100% germination. Conidia with visible germ tubes were considered to have germinated. The tests were repeated three times per test solution.

### Light microscopy of the early infection process of *V. inaequalis* in yucca extract and ASM-treated leaves

Four week-old apple seedlings (with four unfolded leaves) were arranged randomly in plastic trays and treated with water, yucca or ASM and incubated as above. Three days later, the adaxial leaf surfaces were inoculated with a conidial suspension of *V. inaequalis* ($1.5\times10^5$ conidia $ml^{-1}$) using a glass sprayer and incubated as above. The experiment was conducted with 20 plants per treatment. One, 3 and 5 dai, the two youngest leaves that were unfolded at the time of inoculation, from each of four plants, were sampled (eight leaves per treatment and time-point). The remaining eight plants per treatment were incubated until disease assessment as described above. At each sampling time, leaves were cleared on paper napkins saturated with a fixative (96% ethanol, 100% acetic acid; 24:1 *v*/*v*) in closed plastic boxes. The samples were incubated at 37°C for 2 days during which time the paper napkins and fixative were renewed, until no further chlorophyll could be extracted. Leaf samples were then treated for 2 h in a 1:1 solution of ethanol (96%) and lactic acid (90%) and stored in lactic acid (90%). Leaves were mounted on glass slides for light microscopy. At each sampling time, a number of events in the infection process were recorded for 50 germinated spores on each of the four examined leaves per treatment and time-point. First, each conidium was examined to see whether or not it had germinated. For 50 germinated conidia on each of the four examined leaves per treatment and time-point, the following parameters were recorded: number of germinated conidia, whether germinated conidia formed appressoria, primary stroma, runner hyphae and/or secondary stromata; whether germinated conidia caused penetration; whether appressoria caused penetration; whether primary stromata were produced; whether primary stromata formed runner hyphae and/or secondary stromata. The average length of germ tubes and the average number of runner hyphae per primary stroma-forming runner hyphae were also recorded.

### Data analysis

Data on disease severity, average length of germ tubes and average number of runner hyphae per primary stroma forming runner hyphae represent continuous variables. These parameters were analysed by analysis of variance assuming a normal distribution. Variances were stabilised by appropriate transformation of data if necessary and means separated by LSD-values. The remaining parameters in the histopathological study represent discrete variables since it was recorded whether or not a certain event had taken place (e.g. whether or not a conidium had germinated). These results were, therefore, analysed by logistic regression, assuming a binomial distribution (corrected for over-dispersion when present) (Collett 1991). For comparison of discrete variables (percentages), odds ratios were calculated, using the water-treated control plants as references (odds ratio=1.00). For example, odds ratio for percent germinated conidia is 0.55 for ASM (Table 1). This means that odds (that is, $p/(1-p)$, where $p$ is the probability of a conidium germinating) in ASM-treated plants is approximately half as big as odds for control plants. Data for conidial production also represents a discrete variable and were analysed by logistic regression, assuming a Poisson distribution. For both continuous and discrete variables, hypotheses were rejected at $P\leq0.05$. All data were analysed by PC-SAS (release 8.2; SAS Institute, Cary, NC).

### Quantification of gene expression of PR-proteins by real-time RT-PCR

Plants were treated with yucca, sulphur and ASM 2 days before inoculation with the pathogen, as described above. Water-treated non-inoculated plants

**Table 1** Incidence of pre-penetration and penetration events in the infection process of *Venturia inaequalis* 1, 3 and 5 dai of apple leaves, following treatment with yucca extract, acibenzolar-*S*-methyl (ASM; Bion WG 50™, Syngenta Crop Protection, Switzerland) or water (control)

| Infection events | Time after inoculation with *V. inaequalis* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 day | | | | | 3 days | | | | | 5 days | | | | |
| | Odds ratio[a] | | | | | Odds ratio | | | | | Odds ratio | | | | |
| | Yucca | ASM | Water | Yucca | ASM | Yucca | ASM | Water | Yucca | ASM | Yucca | ASM | Water | Yucca | ASM |
| Percent germinated conidia | 28.5 | 45.0 | 60.0 | 0.27*** | 0.55 *** | 27.6 | 46.0 | 64.0 | 0.24*** | 0.54*** | 26.9 | 50.5 | 58.0 | 0.26*** | 0.74* |
| Percent germinated conidia forming appressoria | 58.1 | 46.1 | 80.0 | 0.36*** | 0.21*** | 75.5 | 86.5 | 93.0 | 0.23*** | 0.48* | 79.5 | 86.0 | 94.5 | 0.22*** | 0.36** |
| Average length of germ tubes (μm) | 13.6 | 29.5 | 23.1 | $LSD_{95}$=5.0*, [b] | | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Percent germinated conidia causing penetration | 34.4 | 19.8 | 35.3 | 0.97 NS | 0.45** | 54.5 | 49.5 | 80.5 | 0.29*** | 0.24*** | 63.5 | 62.5 | 87.5 | 0.25*** | 0.24*** |
| Percent appressoria causing penetration | 58.5 | 43.2 | 47.2 | 1.64 NS | 0.83 NS | 70.4 | 53.4 | 81.2 | 0.55* | 0.27*** | 76.1 | 67.3 | 86.5 | 0.49** | 0.32*** |

*ND* not determined, *NS* no significant difference

*$P$<0.05, **$P$<0.01, ***$P$<0.001

[a] Discrete variables: odds ratio for comparison of ASM- and Yucca-treated to water-treated (control) plants (water-treatment used as a reference, odds ratio=1.00)

[b] Continuous variables: analysed by analysis of variance

were used as controls. Four of the youngest leaves from each of six plants were harvested 1 day before and 2 and 5 dai, frozen in liquid nitrogen and kept at −80°C until use. RNA was extracted using the RNAqueous® Kit (Ambion, Applied Biosystems, CA, USA) with the addition of Plant RNA Isolation Aid (Ambion). The quantity and quality of RNA were checked in a spectrophotometer (NanoDrop Technologies, USA). Extracted RNA was further purified with the DNA-*free*™ Kit (Ambion) in order to avoid contamination with genomic DNA. RNA sub-samples (3.5 μg) were subjected to synthesis of cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) following the procedure described by the manufacturer. Genomic DNA contamination in RNA samples was detected by performing a 'no-reverse transcriptase' control in the cDNA synthesis procedure. Finally, 1 μl of each cDNA sample was used for real-time RT-PCR amplification. The cDNA synthesis for each RNA sample was conducted twice.

The design of the primers was carried out using the Primer3 programme (Rozen and Skaletsky 2000) based on PR1 and PR8 sequences from apples retrieved from GenBank with accession numbers AF507974, DQ318212 and AY548367, respectively. To confirm homology with the retrieved genes of PR1 and PR8 from GenBank, two degenerated primer pairs, aj264 and aj263 (PR1) and aj281A and aj282A (PR8) (Maxson-Stein et al. 2002), were tested using genomic DNA of apple (cv. Golden Delicious). The obtained PCR products were sequenced using the sequencing service from MWG-Biotech, Ebersberg, Germany, and homology was confirmed with the GenBank sequences. The sequence of the primers

for the real-time RT-PCR assays was PR1 RT-f, 5′ACTGCAATCTCGTGCACTCC; PR1 RT-r, 5′TAATGCCCACACACCTTTCC; PR8 RT-f, 5′ CGCCGGAAGTTACTCTCTCA and PR8 RT-r, 5′ AATCAACGCCGTCCAAAAC. Primers targeting 18S rDNA, originally designed for *Arabidopsis* (Shimada et al. 2003), but found in the present study to match 18S rDNA from apple 100%, were used as a constitutively-expressed control. The assays were carried out with the Mx3000P™ QRT-PCR System thermocycler including the associated software programme from Stratagene (La Jolla, CA USA). All reactions were set up in Stratagene 96-well reaction plates. Each 25 µl reaction was performed in Brilliant® SYBR® Green QPCR Master Mix Kit from Stratagene containing the SYBR Green I dye as fluorophor. A primer concentration of 150/150 nM (forward/reverse) was chosen for all genes based on optimisation of each primer set using standard curves. Negative control reactions replacing the cDNA template contained either sterile water or the no-reverse transcriptase control. Each reaction was repeated twice. PCR cycling parameters were 95°C for 10 min (denaturation), followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. After the 40 cycles, melting curves were acquired by heating to 95°C for 1 min cooling to 60°C for 30 s followed by ramping up the temperature to 95°C with 0.5°C $s^{-1}$ where the temperature was kept for 30 s. The fluorescence data was continuously collected during ramping from 60–95°C.

The $C_T$ value for each gene (18S, PR1 and PR8) was measured and the expression level of PR1 and PR8 in the different samples was calculated by the formula: $\text{Ratio} = (E_{\text{target}})^{\Delta\text{CT target (Mean control}-\text{Mean sample)}} / (E_{\text{ref}})^{\Delta\text{CT ref (Mean control}-\text{Mean sample)}}$, representing the *x*-fold difference from the calibrator (18S) (Pfaffl et al. 2002). The relative expression software tool REST© (Pfaffl et al. 2002) was used to test whether the expression differences were significant. Differences in expression between control and treated samples were assessed in group means for statistical significance by randomisation tests (Pair Wise Fixed Reallocation Randomisation Test©), according to Pfaffl et al. (2002). The experiment was conducted with similar results using two sets of cDNA, of which one set of data is presented.

## Results

### Effect of yucca extract, ASM and sulphur on apple scab severity, sporulation and conidial germination

Spraying apple leaves with yucca extract, ASM or sulphur 2 days before inoculation with *V. inaequalis* significantly reduced the scab severity score compared to the control treatment (Fig. 1a). While the apple scab severity index for control plants was around 3.0, the score for sulphur, ASM and Yucca treatment was 0.7, 1.1 and 0.9, respectively (Fig. 1a). The consistency of the present data was confirmed in another experiment, where scab severity indexes of

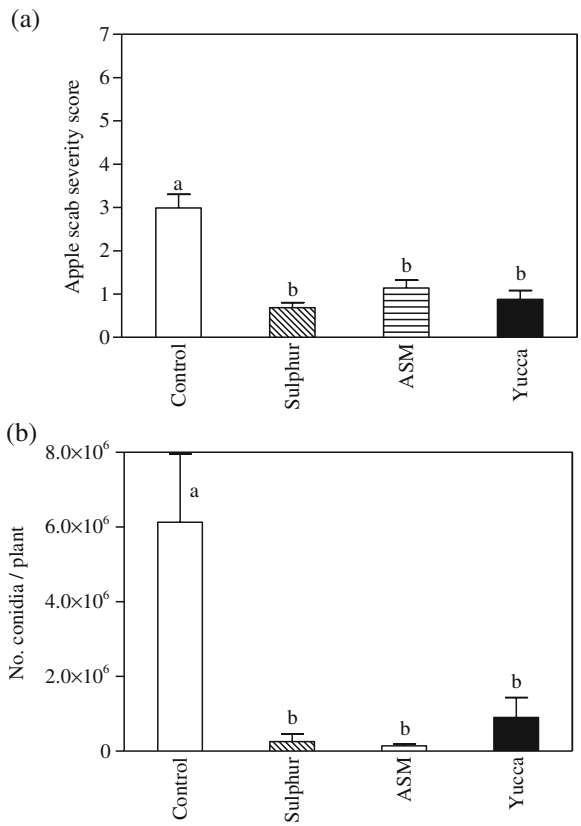


**Fig. 1** Effect of sulphur, acibenzolar-*S*-methyl (ASM) and yucca extract (**a**) on apple scab disease severity and (**b**) on conidial production in apple seedling assays. Treatments were applied 2 days before inoculation with conidia of *Venturia inaequalis*. Disease assessment (severity score 0–7) and conidial washings were performed 2 weeks after inoculation. Treatment means with the same letter are not significantly different (based on LSD test, $P \leq 0.05$). *Vertical bars* show standard deviation of the mean

3.2, 0.3, 0.4 and 0.3 were obtained for the control, sulphur, ASM and yucca treatments, respectively. Likewise, all treatments reduced the conidial production of *V. inaequalis* on leaves (Fig. 1b). While the number of conidia per plant in control plants was $6.1\times10^6$, the scores for sulphur, ASM and yucca were $0.2\times10^6$, $0.1\times10^6$ and $0.9\times10^6$ conidia per plant, respectively (Fig. 1b). Furthermore, yucca extract and sulphur significantly inhibited germination at all concentrations tested in the in vitro assay ($P<$ 0.0001) (Fig. 2) whereas ASM had no significant effect on conidial germination in any of the tested concentrations compared to the control at 24 and 48 h after treatment (Fig. 2). In another similar experiment, while the percentage of germinated conidia for control plants ranged from 75–95%, the germination scores obtained for sulphur, ASM and yucca ranged from 2–28%, 75–95% and 0–2%, respectively. In addition, conidia that germinated in sulphur solution had retarded germ tubes (data not shown).

## Comparison of the infection processes of *V. inaequalis* after different pre-treatments

On control plants, conidia of *V. inaequalis* most often germinated with one, occasionally two to three germ tubes on the leaf surface, and formed appressoria from which penetration of the cuticle took place within the first dai (data not shown). Three–5 dai, primary stromata developed under the cuticle at the point of primary penetration. From here runner hyphae (up to ten per stroma) developed and subsequently at day 5, secondary stromata also developed. Sporulation commenced 7–8 dai (data not shown).

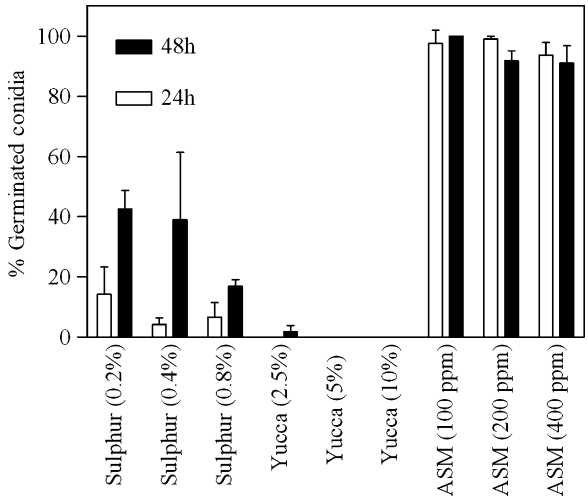


**Fig. 2** Effect of sulphur, yucca extract and acibenzolar-*S*-methyl (ASM) on conidial germination of *Venturia inaequalis* in vitro. Percentage of germinated conidia compared to water-control treatment, 24 and 48 h after incubation. *Vertical bars* show standard deviation of the mean

## Pre-penetration events

Compared to water-treated leaves, pre-treatment with yucca extract or ASM significantly reduced conidial germination and percentage of conidia forming appressoria at 1, 3 and 5 dai (Table 1). Germination of *V. inaequalis* conidia on pre-treated leaves ranged from 26.9–28.5% for yucca, 45–50.5% for ASM and 58–64% on water-treated leaves during the experimental period (Table 1). The percentage of conidia forming appressoria in leaves pre-treated with yucca and ASM was also significantly reduced compared to the water control where the percentages were 58.1–79.5% for yucca, 46.1–86% for ASM and 80–94.5% in the water-treated control leaves (Table 1). At 1 dai, the average length of germ tubes was significantly reduced to 13.6 μm in leaves pre-treated with yucca compared to the control treatment (average=23.1 μm) (Table 1). By contrast, germ tubes on leaves pre-treated with ASM were significantly longer (average=29.5 μm) compared to the control (Table 1).

## Penetration events

At 1 dai, no significant difference was observed in the percentage of germinated conidia causing penetration of leaves pre-treated with yucca compared to control leaves (Table 1). However, the percentage of germinated conidia causing penetration in leaves pre-treated with ASM was reduced to 19.8%, which was significantly different to the control leaves (35.3%) (Table 1). At 3 and 5 dai, percentages of germinated conidia achieving penetration in pre-treated leaves were significantly reduced by the yucca treatment (54.5% and 63.5% respectively) and by the ASM treatment (49.5% and 62.5% respectively) compared to the control (80.5% and 87.5% respectively) (Table 1). The percentage of appressoria causing penetration was also significantly reduced by pre-treatment with both yucca (at day 3=70.4%; at day 5=76.1%) and ASM

(at day 3=53.4%; at day 5=67.3%) compared to water (Table 1).

Post-penetration events

Germinated conidia forming primary stromata, runner hyphae and secondary stromata were not observed on day 1 of the experimental period (data not shown). At 3 dai, these three parameters were not found to be significantly different in leaves treated with yucca extract to the control leaves (Table 2A). On day 5, with exception of the percentage of germinated conidia forming secondary stromata, the percentages of germinated conidia forming primary stroma (37.5%) and runner hyphae (28%) were significantly reduced in leaves pre-treated with yucca extracts compared to the control treatment (Table 2A). For the leaves pre-treated with ASM, the percentage of germinated conidia forming primary stromata was significantly reduced compared to the controls at 3 and 5 dai (on day 1, this event was not observed) (Table 2A). In the same treatment, germinated conidia forming runner hyphae were not observed on day 1 and 3 of the experimental period. By day 5, the percentages of germinated conidia forming runner hyphae (27%) and secondary stromata (2.5%) in leaves pre-treated with ASM were again significantly reduced compared to the control plants (Table 2A).

The percentage of penetrations giving primary stromata was significantly reduced by ASM (36.2%) 3 dai but not by yucca. By day 5, neither yucca nor ASM significantly reduced this parameter (Table 2B). While ASM significantly reduced the percentage of primary stromata forming runner hyphae on day 3 (23%), this was not seen 5 dai. At the same time, no significant effect on the percentage of runner hyphae forming secondary stromata was observed and yucca did not reduce either of these parameters significantly (Table 2B). Compared to the water treatment, the

**Table 2** Incidence of selected post-penetration events in the infection process of conidia of *Venturia inaequalis* 3 and 5 dai of apple leaves pre-treated with yucca extract, acibenzolar-*S*-methyl (ASM; Bion WG 50™, Syngenta Crop Protection, Switzerland) or water (control)

| *V. inaequalis* infection events relative to % germinated conidia (A) and a previous infection event (B) | Time after inoculation with *V. inaequalis* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 days | | | | | 5 days | | | | |
| | Odds ratio[a] | | | | | Odds ratio[a] | | | | |
| | Yucca | ASM | Water | Yucca | ASM | Yucca | ASM | Water | Yucca | ASM |
| A | | | | | | | | | | |
| Percent germinated conidia forming primary stroma | 26.0 | 7.0 | 33.5 | 0.15 NS | 0.69*** | 37.5 | 34.0 | 62.0 | 0.36** | 0.31** |
| Percent germinated conidia forming runner hyphae | 3.5 | 0.0 | 6.5 | 0.52 NS | 0.00*** | 28.0 | 27.0 | 50.5 | 0.38*** | 0.36*** |
| Percent germinated conidia forming secondary stromata | 0.0 | 0.0 | 0.0 | ENO | ENO | 7.5 | 2.5 | 13.0 | 0.54 NS | 0.17** |
| B | | | | | | | | | | |
| Percent penetrations resulting in primary stromata | 46.2 | 13.6 | 36.2 | 1.54 NS | 0.32*** | 58.5 | 53.7 | 68.2 | 0.53 NS | 0.39 NS |
| Percent primary stromata forming runner hyphae | 15.0 | 0.0 | 23.0 | 0.55 NS | 0.00* | 73.8 | 79.1 | 82.5 | 0.62 NS | 0.86 NS |
| Percent runner hyphae forming secondary stromata | 0.0 | 0.0 | 0.0 | ENO | ENO | 4.2 | 1.7 | 3.8 | 1.01 NS | 0.49 NS |
| Average number of runner hyphae per primary stroma forming runner hyphae | 1.0 | 0.0 | 1.2 | LSD=0.2***,[b] | | 5.5 | 4.3 | 5.6 | NS[b] | |

*ENO* event not observed, *NS* no significant difference

*$P<0.05$, **$P<0.01$, ***$P<0.001$

[a] Discrete variables: odds ratio for comparison of ASM- and Yucca-treated to water-treated (control) plants (water-treatment used as a reference, odds ratio=1.00)

[b] Continuous variables: analysed by analysis of variance

**Table 3** Real-time RT-PCR analysis of PR1 and PR8 gene expression after treatment with water, yucca extract, sulphur and acibenzolar-*S*-methyl (ASM) in apple seedlings inoculated with *Venturia inaequalis* (day 0)

| Time before and after inoculation (days) | Water | | Yucca | | Sulphur | | ASM | |
|---|---|---|---|---|---|---|---|---|
| | PR1 | PR8 | PR1 | PR8 | PR1 | PR8 | PR1 | PR8 |
| −1 | 0.4 | 0.2 | 5.4* | 6.8 | 2.6 | 0.6 | 2.9 | 2.2 |
| 2 | 0.9 | 3.9* | 3.9* | 3.3* | 2.0 | 0.4 | 0.6 | 8.1* |
| 5 | 0.1* | 0.5 | 6.4* | 7.2* | 0.3 | 0.6 | 2.3* | 8.0* |

*−1* 1 day before inoculation, *2* 2 dai, *5* 5 dai

*$P<0.05$ (Pair-Wise Fixed Reallocation Randomisation Test©; Pfaffl et al. 2002)

Values shown represent *x*-fold up-regulation compared with water-treated, non-inoculated seedlings, after normalisation to 18S rRNA

average number of runner hyphae produced per primary stroma (based on data from primary stromata that actually formed such hyphae) was significantly reduced for ASM 3 (average=0.0 μm) and 5 (average=4.3 μm) dai, but not for yucca (Table 2B).

Gene expression

The results from the relative quantification of expression of PR1 and PR8 are presented in Table 3. Significant ($P<0.05$) up-regulation of PR1 compared to water-treated, non-inoculated seedlings after normalisation to 18S rRNA was seen for yucca 1 day prior and 2 and 5 dai (Table 3). Treatment with ASM resulted in a significant up-regulation of this gene but only 5 dai and no significant up-regulation was seen following treatment with sulphur. In the case of the PR8 gene, significant up-regulation following yucca treatment was seen 1 day before inoculation and 2 and 5 dai. While for ASM significant up-regulations were seen both 2 and 5 dai, treatment with sulphur had no significant up-regulatory effect on PR8. Significant up-regulation of PR8 was recorded in the inoculated, water treatment 2 dai (Table 3). The tendency in up- and down-regulation was similar in both datasets.

## Discussion

As found in the present study and previous experiments (Bengtsson et al. 2006a, b), the treatment of apple seedling leaves with yucca extract, ASM or sulphur gives significant control of scab under controlled conditions. Promising results with different yucca extracts to control scab under field conditions, especially in combination with sulphur, are reported elsewhere (Heijne et al. 2007). Whereas sulphur seems to act only preventively, yucca extract has also shown promise as a curative treatment applied 1 dai with *V. inaequalis* (Bengtsson et al. 2006b).

The histopathological study of the mode of action of the yucca extract on *V. inaequalis* in apple leaves showed that it primarily acted by inhibiting conidial germination, which was also clear from the in vitro germination test. Germ tube elongation of conidia that survived the yucca treatment was significantly reduced compared to the water control. In addition, yucca extract had a significant inhibitory effect on several pre- and post-penetration stages of the infection process. Compared to the water control, fewer appressoria were formed by the surviving conidia and fewer penetrated successfully. Likewise, fewer appressoria formed primary stromata 5 dai but no influence on formation and number of runner hyphae was observed at this time. These observations indicate that yucca extract acts mainly by a direct fungitoxic effect, but it appears to have some effect on post-penetration events, which is supported by its ability to control scab as a curative treatment in seedling assays (Bengtsson et al. 2006a).

In contrast to the yucca treatment, the strongest inhibitory effect of ASM was on penetration and the stages thereafter. Penetration, primary stroma formation and the number of runner hyphae formed per stroma were all reduced. While the effect of ASM on primary stromata formation is in agreement with the observations of Ortega et al. (1998), who used DCINA (methyl 2,6-dichlorisonicotinate) as the inducer, these authors did not find any effects of DCINA on germination and appressorium formation (Ortega et al. 1998). Whereas in the in vitro test, ASM did not inhibit conidial germination, it did

inhibit germination in the histopathological study 1 and 3 dai when appressorium formation was also reduced. This inhibition could be interpreted as an early effect of induced resistance. Resistance can operate in grasses before penetration of various fungal pathogens (Lehnackers and Knogge 1990) and it could be speculated that induced resistance could also influence these processes. Future investigations are needed to examine this in more detail.

Although papilla-like structures in apple leaves were observed by Ortega et al. (1998) in a few sites of unsuccessful penetration attempts by *V. inaequalis* following treatment with DCINA, histological indicators of defence reactions (e.g. hypersensitive responses and fluorescent papillae), which occur in many host–pathogen interactions including the barley–*Drechslera teres* pathosystem (Jørgensen et al. 1998), were not detected in the present work.

In the molecular study, compared with the water control, *V. inaequalis* temporarily induced PR8 expression 2 dai, while PR1 was slightly down-regulated at day 5. The temporary up-regulation of PR8 in the inoculated water-treated sample might reflect the plant's response to the pathogen. The effect of treatment with yucca or ASM resulted in more pronounced inductions of the two PR-proteins than after treatment with the pathogen alone. However, since the experiment was carried out with inoculation of *V. inaequalis*, the effect of yucca and ASM alone could only be assessed 1 day before inoculation.

In the case of sulphur, no significant regulation of either of the two PR-proteins was found, thus indicating that sulphur did not trigger the defense responses of the plant. This was in marked contrast to yucca and ASM, whose induction patterns were similar. While the results of both the histological study and the in vitro germination test firmly establish that yucca extract has a strong fungicidal effect on conidia of *V. inaequalis*, up-regulation of PR1 and PR8 following yucca treatment would also seem to indicate that an effect of induced resistance by some components in the extract also occurred. Plant extracts contain many different compounds and while several of these may effect conidial germination and infection, possibly by different mechanisms, the same or other compounds may effect infection by activating the plant's resistance mechanisms. The major constituents in yucca extract are steroid saponins (Hostettmann and Marston 1995). Saponins are found in many plant species, especially dicots, and they may have potent antifungal activity (Osbourn 2003). *Yucca schidigera* may also be a good source of polyphenols, e.g. resveratrol, methoxystilbene and yuccaols, which have been extracted from its bark (Oleszek et al. 2001). The content of such constituents in yucca products can, however, vary depending on the origin of plants and the extraction method (B. Nielsen, Nor-Natur ApS, Denmark, personal communication). It is currently not known which constituents of the yucca extract (Norponin® BS Liquid) elicit the up-regulation of PR-proteins in apple seedlings, found in the present study.

ASM has previously been reported to elicit systemic resistance in apple against fire blight (Brisset et al. 2000; Maxson-Stein et al. 2002; Baysal and Zeller 2005) and against scab in Japanese pear (Faize et al. 2004). In contrast to the present studies, which were carried out with seedlings, Bonasera et al. (2006) found no induction of PR-proteins (PR2, PR5, PR8 and PR1) in young shoots of 1 year-old apple plants following treatment with ASM. Although several authors have demonstrated induction of β-1,3 glucanase activity in different apple tissues upon treatments with ASM (Brisset et al. 2000) and several microorganisms, including *V. inaequalis* (Gau et al. 2004), *Pseudomonas fluorescens* (Kürkcüogler et al. 2007), and *Candida saitoana* (Ghatouth et al. 2003), we have not been able to find significant differences in activity of this enzyme in inoculated leaves of apple seedlings, treated with ASM, sulphur or yucca extract (Bengtsson et al. unpublished).

Our model system using ASM as a reference inducer treatment is a useful tool in work on the elucidation of the mode of action of plant extracts to distinguish materials working as inducers of resistance from those with direct fungitoxic activity. Thus, strong inhibition of spore germination and strong reduction in germ tube extension of surviving conidia are strong indicators of fungitoxic action by the materials tested. By contrast, weak inhibition of spore germination together with stimulation of germ tube length, appear to be indicators of induced resistance. These parameters could be used as morphological markers when screening materials for induced resistance, as they are relatively simple and quick to determine. Parallel, Real-Time RT-PCR for studying expression of genes involved in defence responses in apple, e.g. the PR-proteins PR1 and PR8, also seems to be a promising tool for future studies.

While the results from the present study demonstrate that yucca extract has an interesting potential as a control material for use against scab in organic apple production, whether or not this will materialise will depend on future developments. The yucca extracts used in the present experiments were commercially available products manufactured for purposes other than for disease control in plants. As named in the introduction, extracts from yucca plants are widely used as additives to animal and human food-stuffs as well as in non-food products. That yucca extract is already widely used for a number of other purposes means that its market price is relatively high. Cheapening the cost of a disease control material containing yucca extract might be attained by combining it with other, cheaper, control materials. The potential of this approach has already been demonstrated in the REPCO orchard trials, where sulphur and yucca extract were used in combination and with good efficacy. A further approach would be to utilise cheaper sources of yucca extract. One possibility might be extracts made from the wastes remaining after initial extraction and other steps in the manufacturing process. As it is already an accepted additive to food and non-food products used by humans as well as in animal feedstuffs, registration of disease control products containing yucca extract should, hopefully, be a less extensive process than for other, less well tried materials. The results presented here also indicate that it should be worthwhile to initiate further studies in order to learn more about the fungicidal and resistance-inducing fractions occurring in extracts from *Yucca* spp.

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