Project title: Role of ethylene and Actigard in defense against the gray mold pathogen *Botrytis cinerea*

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1. RESEARCH OBJECTIVES

Our main ***objective*** was to determine whether Actigard, an inducer of systemic acquired resistance (SAR), or ethylene can be used to manage postharvest *Botrytis cinerea* decay. Specifically, pears were treated with the ethylene agonist propylene, the ethylene action inhibitor 1-methylcyclopropene (1-MCP), and/or the 1-aminocycolopropane-1-carboxylic acid (ACC) synthase inhibitor aminoethoxyvinyl glycine (AVG) to alter ethylene biosynthesis and fruit ripening. Alternatively, Actigard containing 1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester (BTH) as the active ingredient was used to trigger SAR. As a result of these treatments, we expected differences in *Botrytis* susceptibility. Our ***rationale*** was that, once the effects of these treatments on gray mold decay were known, they could be implemented to control postharvest decay.

We used different pear (*Pyrus communis*) cultivars with and emphasis on 'd'Anjou', a winter pear, and 'Bartlett', a pear that does not require a chilling period for fruit ripening. We used wound inoculation because mycelial inoculation using agar plugs without wounding was highly variable and not effective. Fruits that were wounded and mock inoculated served as controls for all experiments. Robert Spotts and Joseph Postman (USDA/ARS National Clonal Germplasm Repository, Corvallis, OR) provided pear fruits for all of our studies.

2. SIGNIFICANT FINDINGS

- The *P. communis* varieties 'd'Anjou', 'Bartlett', 'Bosc', 'Comice', and *Pyrus pyrifolia* cv. 'Niitaka' differed in gray mold susceptibility and associated changes in ethylene production and fruit ripening.
- Treatments with propylene and 1-MCP accelerated and retarded disease-related ethylene biosynthesis, respectively, but had little influence on disease progression.
- Co-treatment of pears with 1-MCP and aminoethoxyvinyl glycine (AVG) blocked ethylene and 1-aminocycolopropane-1-carboxylic acid (ACC) biosynthesis for 4 d, but lesions started expanding on day 2 post inoculation.
- 1-MCP inhibited fruit softening in response to *Botrytis* infection but had little influence on the rate of lesion expansion.
- A mutant of *B. cinerea* with a defect in the polygalacturonase gene *Bcpg1* was less virulent on pear fruits, suggesting that pectin catabolism is important for invasion.
- Fruits became more susceptible to *Botrytis* as they matured during cold storage.
- 'Niitaka' and 'd'Anjou' were the least and most susceptible cultivars, respectively, whereas susceptibility of 'Bartlett' was intermediate.
- Actigard accelerated gray mold decay after cold storage of 'Bartlett' pear fruits and did not alter *Botrytis* susceptibility of 'd'Anjou' pears.

3. Methods

3.1. Biological Material

Fruits of the two pear (*P. communis*) cultivars, 'Bartlett' and 'd'Anjou', were collected on the commercial harvesting dates of August 9 (122 DAFB) in 2004 and August 12 in 2005 (124 DAFB), August 27 (142 DAFB) in 2004 and August 30 in 2005 (146 DAFB), respectively, at the Mid-Columbia Experiment Station, Hood River, OR. Fruits were divided in two batches for each cultivar, placed in the polyethylene bags, packed into cardboard boxes, and transferred into cold storage (-1^{o}C). 'Bartlett' was stored for 10 days and 'd'Anjou' was stored for 108 days. These disparate storage conditions have previously been reported to be adequate to elicit similar ripening behaviors in early and late ripening cultivars (1, 2). Pear fruits cv. d'Anjou' that were co-treated with AVG and 1-MCP after 60 d of cold storage and 'Niitaka' (*Pyrus pylifolia*) were harvested at USDA/ARS National Clonal Germplasm

Repository, Corvallis, OR, on September 20, 2004. 'Bosc' and 'Comice' fruits were harvested at USDA/ARS National Clonal Germplasm Repository in 2003.

B. cinerea strain B05.10, the mutants *ΔBcpg1* and *ΔBcpme1,* and the double mutant of *ΔBcpg1* and *ΔBcpme1* were obtained from Jan van Kan (Wageningen University, Netherlands) and cultured on potato dextrose agar.

3.2. Chemical Treatments

Freshly harvested or stored pears were transferred to room temperature 1 d prior to chemical treatments that altered ethylene biosynthesis. Pear fruits were exposed in jars to humidified propylene (500 ppm) supplied at a flow rate of 45-60 ml/min for 24 h prior to fungal infection (3). 1-MCP was supplied as SmartFresh^{TM} powder (0.14% active ingredient). According to the direction provided by AgroFresh, 16 mg of 1-MCP was mixed with 1 ml of water to generate 300 ppb of volatilized 1-MCP around pears in a sealed plastic container. Fruits were exposed for 24 h. Control fruits were kept in a sealed container for 24 h without any chemical additions. Pears were soaked in Retain (Valent Biosciences) solution containing 500 ppm AVG with 0.01% Tween 20 for 3 min and then dried. Retain was administrated first and 1-MCP last when pears were treated with both 1-MCP and Retain.

Robert Spotts sprayed pear trees bearing 'd'Anjou' and 'Bartlett' fruits with Actigard one and two weeks prior to harvest. Four trees per treatment were used and the rates of application were 150 or 1,500 mg a.i./liter (4).

3.3. Plant Inoculations

For ethylene-related experiments, 10 and 5 fruits were inoculated per treatment in 2004 and 2005, respectively. For Actigard experiments, 10 fruits per treatment were used. Fruits were surface sterilized by dipping in 0.01 % sodium hypochlorite for 2 min and rinsing with sterilized water. Fruits were treated with chemicals as mentioned above and subsequently inoculated. Fruits were punctured along the equator with the tip of a syringe to generate a 4 mm deep and 4 mm wide hole and then inoculated with a conidial suspension of *B. cinerea* (2.5×10^5 conidia/2 µl in 2004 and 1×10^3 conidia/2 µl in 2005) in water (5). The high concentration was used whenever fungal concentration was not mentioned in the text. The same amount of sterilized water was used for wounded controls. Unwounded control fruits were left intact and were not inoculated. Unwounded control, wounded control, and infected fruits were placed in same moist chambers containing wet a cheesecloth and kept at room temperature.

3.4. Measurement of Ethylene and Lesion Diameters

Ethylene was measured in 2004. Each pear was sealed in a 1-l container for 1 h before each measurement. A sample of 1 ml was withdrawn from the headspace of the container with a syringe. The ethylene concentration of this sample was analyzed using a gas chromatograph (GC) equipped with a 4 ft × 1/8 inch activated alumina column packed with 80/100 mesh and a flame-ionization detector (Gow-Max Instrument Co., Series 580). Flow rates were air = 300 cc/min, hydrogen = 30 cc/min, nitrogen = 30 cc/min, and N_2 was used as a carrier gas. Temperatures were injector 101^{o}C, column 86^{o}C, and detector 104^{o}C. The first measurement was recorded 6 h post inoculation, followed by daily measurements for one week.

Once lesions were visible on the pear surface, lesion diameters were measured daily using a caliper.

3.5. Firmness Measurement

Fruit skin was removed and firmness of fruit flesh was measured as penetration force using a U.S. firmness tester with 0.78 cm penetrometer tip (Western Industrial Supply, San Francisco, California). Measurements were recorded twice per fruit on the non-diseased equatorial area of the fruit 7 d post inoculation.

3.6. Quantification of ACC

ACC was measured as described by Lizada and Yang (6) using pear fruits 7 d post inoculation. Infected and non-infected areas of three pear fruits were cut, pooled, and skin and flesh were frozen in liquid nitrogen. ACC was extracted from 5 g of frozen pear tissue with 10 ml of 5% sulfosalicylic acid. The extraction solution was stabilized by adding 1 μmol $HgCl_2$, and extracts were placed in a test tube sealed with a rubber stopper. A volume of 100 μl of a mixture of 5% NaOCl and saturated NaOH (2:1, v/v), generated on ice, was injected into the test tube. After adding this alkaline hypochlorite solution, the test tube was shaken using a vortex mixer for 5 sec and incubated on ice for 2.5 min. The test tube was agitated for another 5 sec and a volume of 0.5 ml of gas was withdrawn through the rubber stopper using a syringe. Ethylene was measured using a gas chromatograph.

3.7. Statistical Analysis

Data are presented as means ±SE. F-tests were used to compare variances and two-sample t-tests of two-tailed distributions were used to determine significant differences in softening (Microsoft Excel). Differences in lesion expansion were analyzed using the repeated measures procedure of a general linear model (GLM; $\alpha = 0.05$). Contrasts were used to determine significant differences between treatment and control means (SAS Institute, Cary, NC). A significance threshold of $P = 0.05$ was used.

4. RESULTS AND DISCUSSION

Ethylene was always produced when ‘d’Anjou’ and ‘Bartlett’ pears were infected with *B. cinerea*. In the case of ‘Bartlett’ pear fruits, propylene accelerated and 1-MCP suppressed ethylene production (Fig. 1A) and softening (Fig. 3A), but these changes had a relatively small influence on susceptibility to *B. cinerea* (Fig. 1B and C). 1-MCP increased the susceptibility of ‘Bartlett’ pears at harvest maturity. This effect was observed in both 2004 and 2005 trials when low (Fig. 1B; repeated measured option of GLM, $P < 0.0001$) or high concentrations of *B. cinerea* (Fig 1C; $P = 0.0192$) were used, respectively. Propylene only increased susceptibility to *B. cinerea* at harvest maturity in 2004 (Fig. 1B; $P = 0.0011$). This effect of propylene was no longer observed when a lower concentration of *B. cinerea* conidia was used in 2005 (Fig. 1C). Neither propylene nor 1-MCP had any effect on lesion expansion when ‘Bartlett’ fruits were challenged with a low or high concentration of *B. cinerea* after cold storage (Fig. 1E and F). Propylene no longer accelerated ethylene biosynthesis in response to *B. cinerea* once ‘Bartlett’ pear fruits were subjected to cold storage (Fig. 1D). 1-MCP still inhibited ethylene biosynthesis when ‘Bartlett’ fruits were subjected to cold storage (Fig. 1D), but the level of inhibition was less than in mature fruits (Fig. 1A).

Propylene had little effect on infected ‘d’Anjou’ pear fruits. Ethylene production was similar whether or not these fruits were treated with propylene after harvest or cold storage (Fig. 2A and D). Similarly to the situation in ‘Bartlett’ fruits (Fig. 1B), 1-MCP caused a statistically significant increase (Fig. 2B; $P < 0.0001$ and Fig. 2C; $P = 0.0017$) in susceptibility of ‘d’Anjou’ pears at harvest maturity, but not after cold storage (Fig. 2E). Conversely, propylene caused a statistically significant increase in susceptibility of ‘d’Anjou’ fruits after cold storage (Fig. 2E; $P = 0.0117$), but not immediately after harvest (Fig. 2B).

Propylene accelerated and 1-MCP inhibited softening of ‘Bartlett’ (Fig. 3A) and ‘d’Anjou’ pear fruits (Fig. 3B). Softening occurred at a faster rate when pear fruits were infected with *B. cinerea* relative to wounded control fruits. Botrytis infection caused an unexpected increase in firmness of untreated ‘Bartlett’ pears relative to wounded control fruits, but this difference was statistically not significant. Despite decreased softening of 1-MCP-treated pear fruits, there was no significant change in susceptibility of stored pears to *B. cinerea* (Fig. 1E).

Co-treatments with 1-MCP and Retain (AVG) were used to suppress ethylene biosynthesis even further in an attempt alter susceptibility to *B. cinerea*. For this experiment, ‘d’Anjou’ pear fruits were moved to room temperature after 60 d of cold storage. None of the stored fruits produced any ethylene (data not shown), suggesting that the physiology of these pears was similar to fruits at harvest maturity.

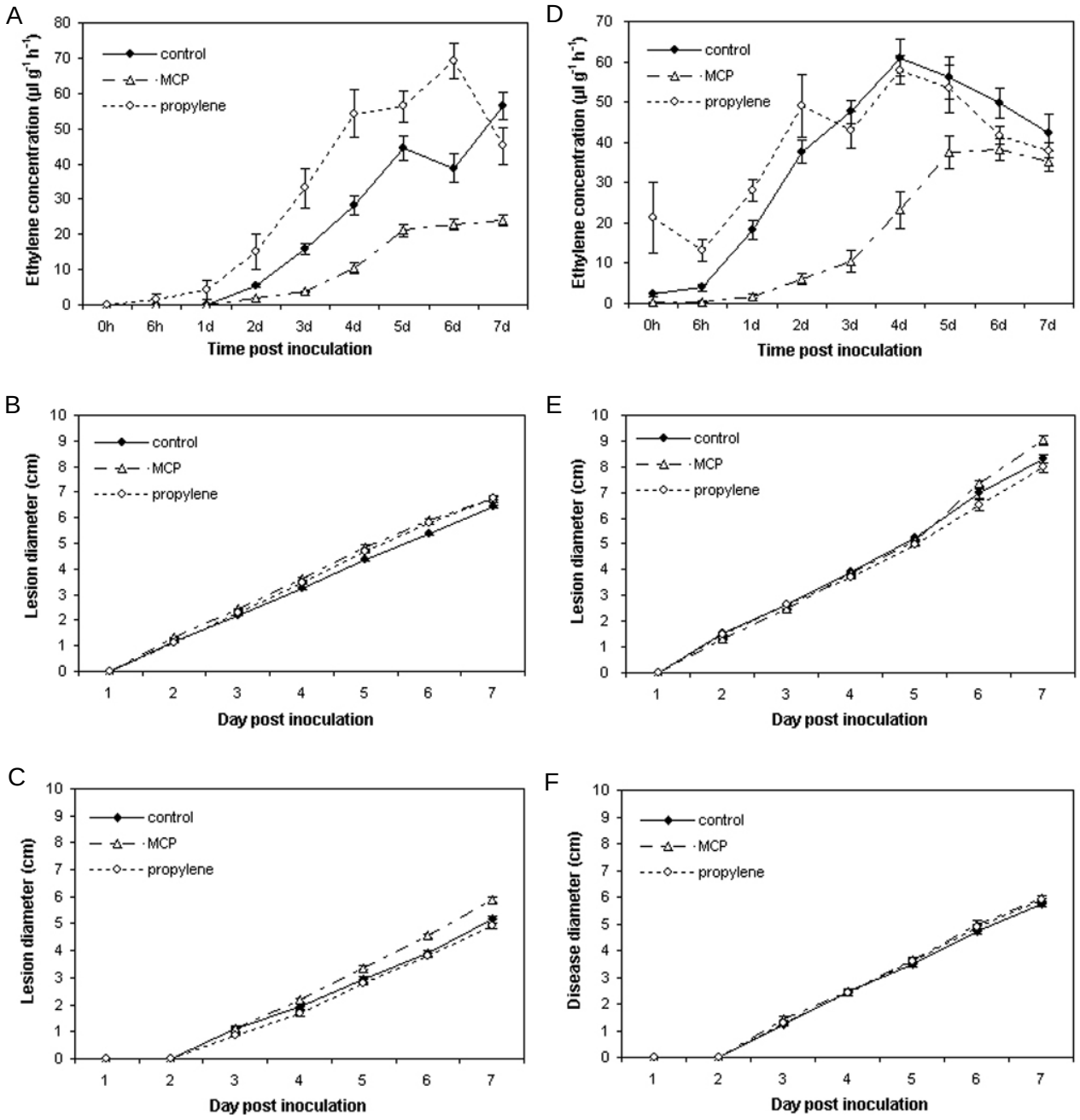

Fig. 1. Effects of 1-MCP and propylene on ethylene production and lesion expansion during infection of 'Bartlett' pear fruits by *B. cinerea*. Fruits were analyzed after harvest (A, B, and C) or 10 d after cold storage (D, E, and F). Results from 2004 (A, B, D, E) and 2005 (C, F) trials are shown. Ethylene production (A and D) and lesion expansion (B, C, E, and F) were measured in 'Bartlett' fruits that were untreated (control) or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were inoculated with 2.5×10^5 (B and E) or 1×10^3 (C and F) conidia per inoculum of *B. cinerea*. The repeated measures procedure of a GLM was used for statistical analysis. Contrasts were used to compare treated with control fruits.

Fruits of ‘d’Anjou’ pears started to produce ethylene 1 d post inoculation with *B. cinerea* (Fig. 4A). Despite pretreatment with Retain (AVG) and/or 1-MCP, ethylene production of infected ‘d’Anjou’ fruits increased significantly 5 d post inoculation (Fig. 4B and C). In addition to ethylene, ACC was detected 7 d post inoculation in ‘d’Anjou’ fruits that were pretreated with both 1- MCP and AVG (Fig. 4D). The presence of ACC suggests that ethylene was produced by the host via conversion of SAM. Despite large

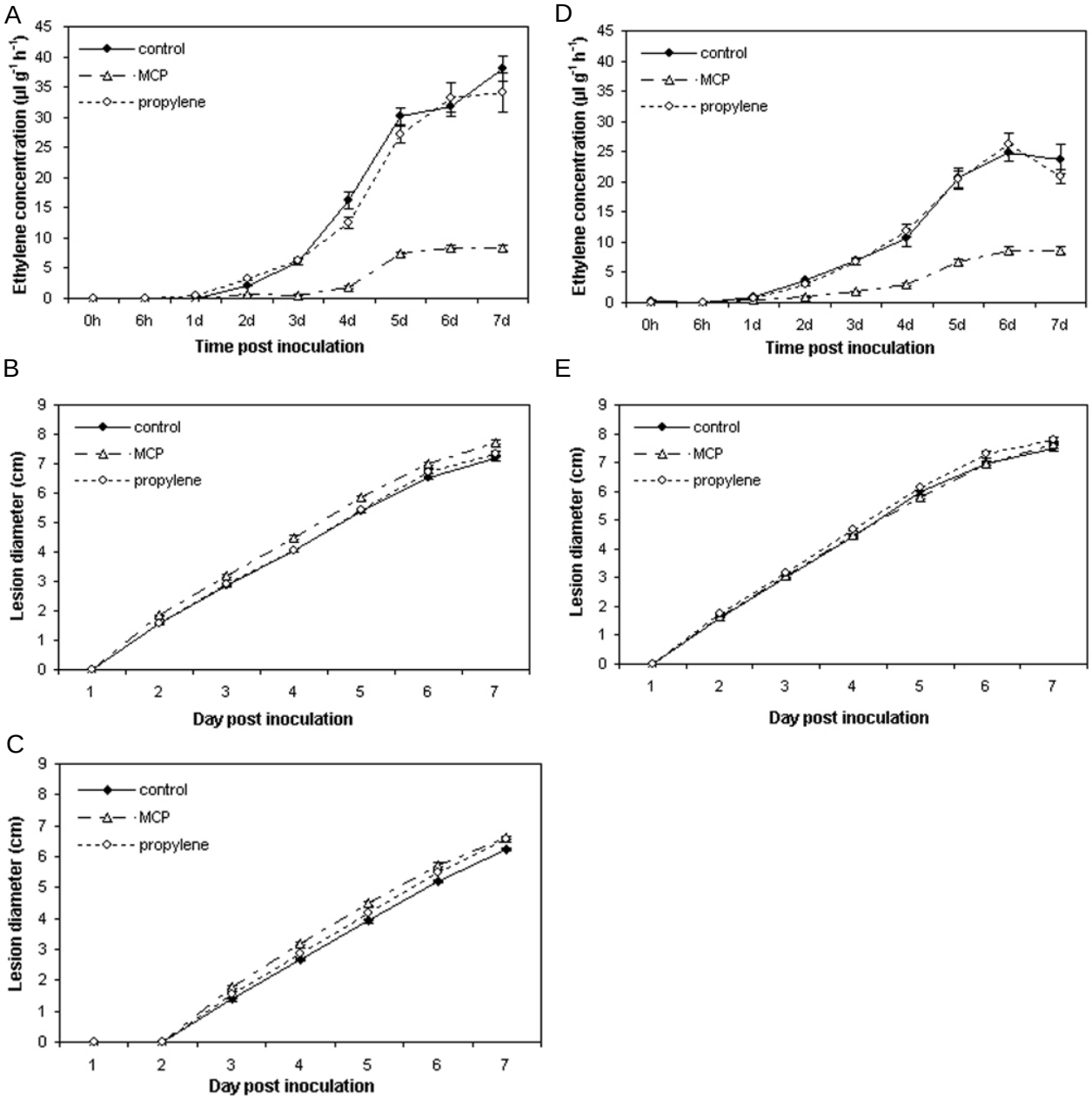

Fig. 2. Effects of 1-MCP and propylene on ethylene production and lesion expansion during infection of 'd'Anjou' pear fruits by *B. cinerea*. Fruits were analyzed after harvest (A, B, and C) or 108 d of cold storage (D and E). Results from 2004 (A, B, D, E) and 2005 (C) trials are shown. Ethylene production (A and D) and lesion expansion (B, C, and E) were measured in 'd'Anjou' fruits that were untreated (control) or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were inoculated with 2.5×10^5 (B and E) or 1×10^3 (C) conidia per inoculum of *B. cinerea*. The repeated measures procedure of a GLM was used for statistical analysis. Contrasts were used to compare treated with control fruits.

changes in ethylene production in response to ethylene inhibitors (Fig. 4A to C), differences in lesion expansion were relatively minor (Fig. 4E). 1-MCP caused a statistically significant increase in susceptibility of pears to *B. cinerea* ($P = 0.0238$). Conversely, AVG caused a statistically significant enhancement of resistance of 'd'Anjou' pears to this fungal pathogen (Fig. 4E; $P = 0.0003$). Gray mold susceptibility of pears that were co-treated with 1-MCP and AVG was not significantly different from

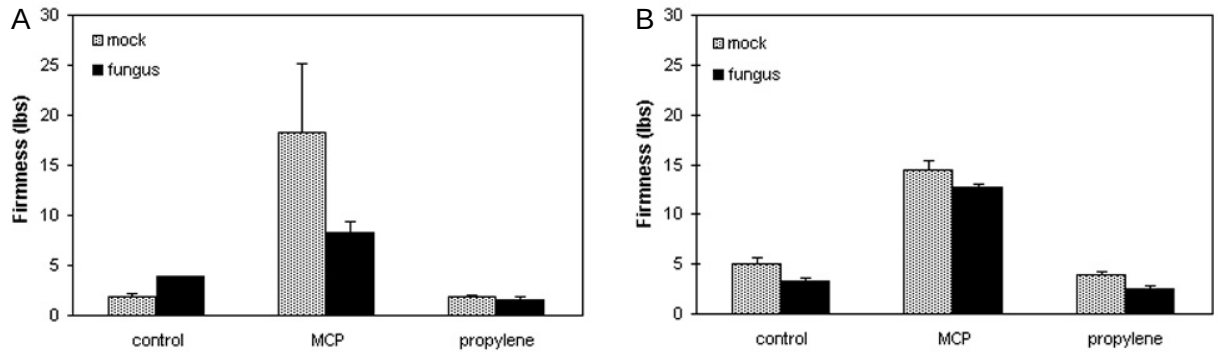

Fig. 3. Effects of gray mold infection and treatments with 1-MCP or propylene on softening of pear fruits. 'Bartlett' (A) and 'd'Anjou' fruits (B) were used after 10 d and 108 d of cold storage, respectively. Firmness of the healthy half of fruits exposed to *B. cinerea* for 7 d was compared to wounded control fruits. Results from the 2004 trial are shown.

untreated fruits, suggesting that the effects of these treatments canceled each other. After cotreatment with 1-MCP and AVG, lesions started to expand 2 d before the onset of ethylene production (Fig. 4C and E), suggesting that the onset of lesion expansion is independently of ethylene biosynthesis.

A mutant of *B. cinerea* with a deletion in the polygalacturonase gene *Bcpg1* exhibited reduced virulence when 'd'Anjou' pear fruits were wound-inoculated compared to wild type *B. cinerea* (Fig. 5A). In contrast, a *B. cinerea* mutant with a deletion in the pectin methylesterase gene *Bcpme1* did not alter lesion expansion in 'd'Anjou' pears (Fig. 5B). The reduced virulence of the *ΔBcpg1 ΔBcpme1* double mutant was likely caused by the deletion in the polygalacturonase gene because the deletion in the pectin methylesterase gene did not alter lesion expansion by itself (Fig. 5B). Thus, the endo-polygalacturonase gene *Bcpg1* increases the rate of gray mold infection in pear fruits, but the pectin methylesterase gene *Bcpme1* appears to be dispensable.

Fruits of 'd'Anjou' pears infected with the *ΔBcpg1* mutant strain were slightly firmer than those infected with wild type *B. cinerea* (Fig. 5C; t-test, $P = 0.013$). Infection by the *ΔBcpg1* mutant did not change the firmness of fruits treated with AVG and 1-MCP (Fig. 5C; t-test, $P = 0.309$). Treatment with 1-MCP and AVG caused a 3 to 4 fold increase in firmness of pear fruits that were infected with the fungal mutant *ΔBcpg1*. Despite this major change in fruit softening, there was no change in the susceptibility of pear fruits to the *ΔBcpg1* mutant (Fig. 5A). Conversely, the fungal polygalacturonase gene *Bcpg1* caused small changes in firmness, but large differences in susceptibility of pear fruits to *B. cinerea*, suggesting that pectin degradation is a major factor in gray mold susceptibility. Thus, fruit firmness affected the virulence of neither the *ΔBcpg1* mutant nor wild type *B. cinerea* (Fig. 3 and 5).

Collectively, these data permit conclusions about the roles of ethylene, softening, and pectin hydrolysis in gray mold susceptibility. The 1-MCP data was consistent among cultivars and collection dates (Fig. 1, 2, and 4), suggesting that this ethylene action inhibitor increases the susceptibility of mature pear fruits (5 out of 5 datasets) but not stored fruits. The effects of propylene were variable; this ethylene agonist increased the susceptibility of mature 'Bartlett' pears and stored 'd'Anjou' fruits. AVG appeared to enhance resistance, but only one experiment was carried out using this ACC synthase inhibitor. In conclusion, manipulation of ethylene using exogenous treatments has limited influence on gray mold susceptibility of pear fruits. While 1-MCP did not alter gray mold susceptibility of stored fruits, this ethylene action inhibitor retarded softening of *Botrytis*-challenged fruits, thus demonstrating that fruit firmness is not related to gray mold infection. Lastly, deletion of a fungal polygalacturonase gene retards the rate of lesion expansion, implying that pectin catabolism promotes infection (7). It is, therefore, likely that pectin catabolism rather than softening increases gray mold susceptibility of pear fruits during storage.

Actigard sprays in the orchard one and two weeks prior to harvest did not affect gray mold susceptibility of mature pear fruits (Fig. 6A and B). When applied at a concentration of 1.5 g a.i./liter,

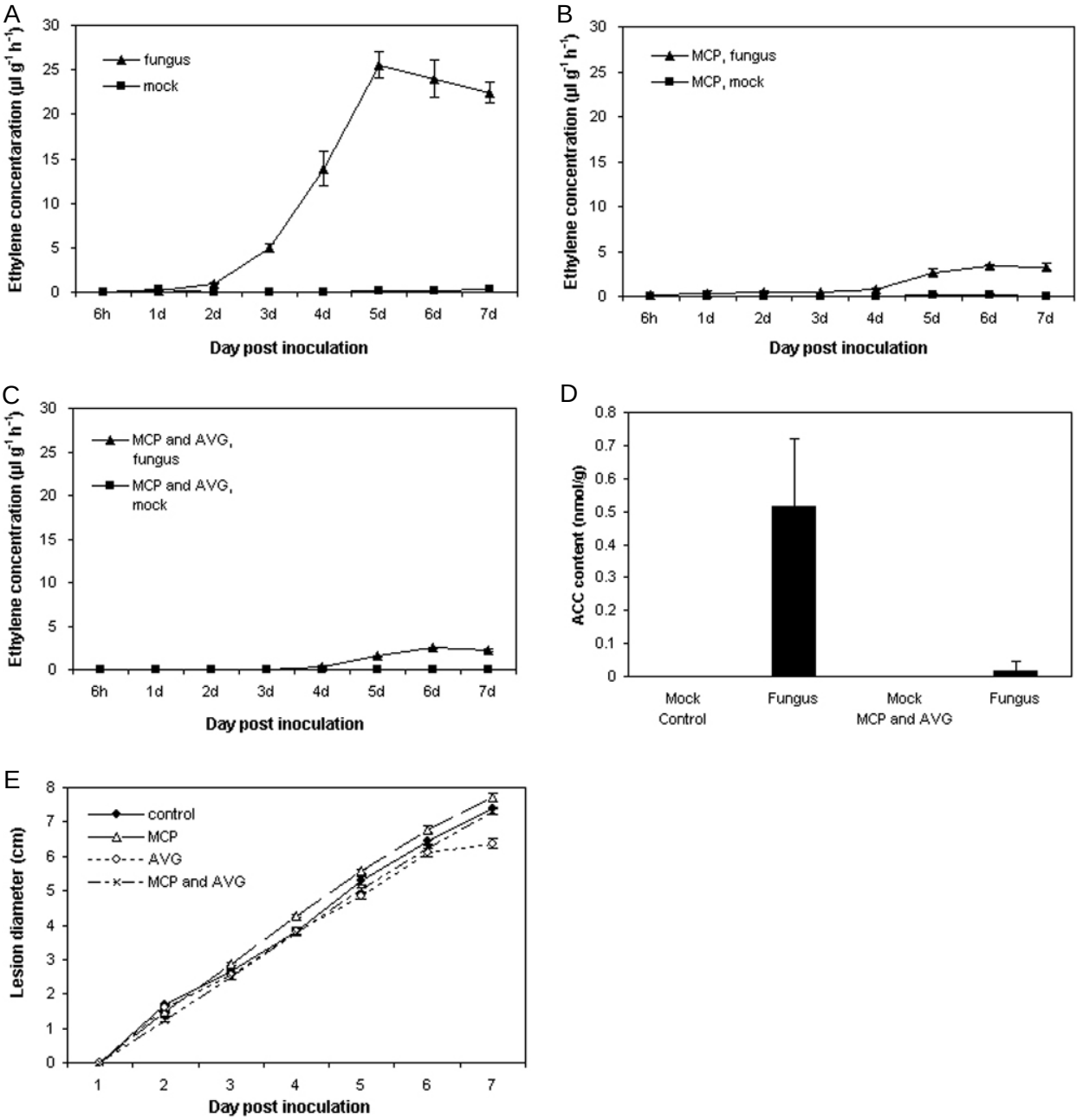

Fig. 4. Effects of 1-MCP and/or AVG on ethylene and ACC production after inoculation with *B. cinerea* of 'd'Anjou' pear fruits 60 d after cold storage. Ethylene production was measured in 'd'Anjou' fruits that were mock-inoculated or challenged with *B. cinerea* (A). Ethylene production in 'd'Anjou' fruits treated with 1-MCP prior to mock inoculation or gray mold infection (B). Ethylene production in 'd'Anjou' fruits treated with 1-MCP and AVG prior to mock inoculation or gray mold infection (C). ACC content was measured in 'd'Anjou' fruits that were not treated (control) or pretreated with 1-MCP and AVG and subsequently exposed to *B. cinerea* for 7 d (D). Lesion expansion in gray mold infected 'd'Anjou' fruits that were not treated (control) or treated with 1-MCP, AVG, 1-MCP and AVG before inoculation.

Actigard increased the rate of lesion expansion when stored 'Bartlett' pears were wound inoculated with *B. cinerea*. Collectively, these data demonstrate that the application of this inducer of SAR could not protect pear fruit after harvest when applied prior to harvest in the field. Analysis of PR gene expression has not yet been conducted because of the lack of a phenotypic effect. Actigard does protect grape fruits

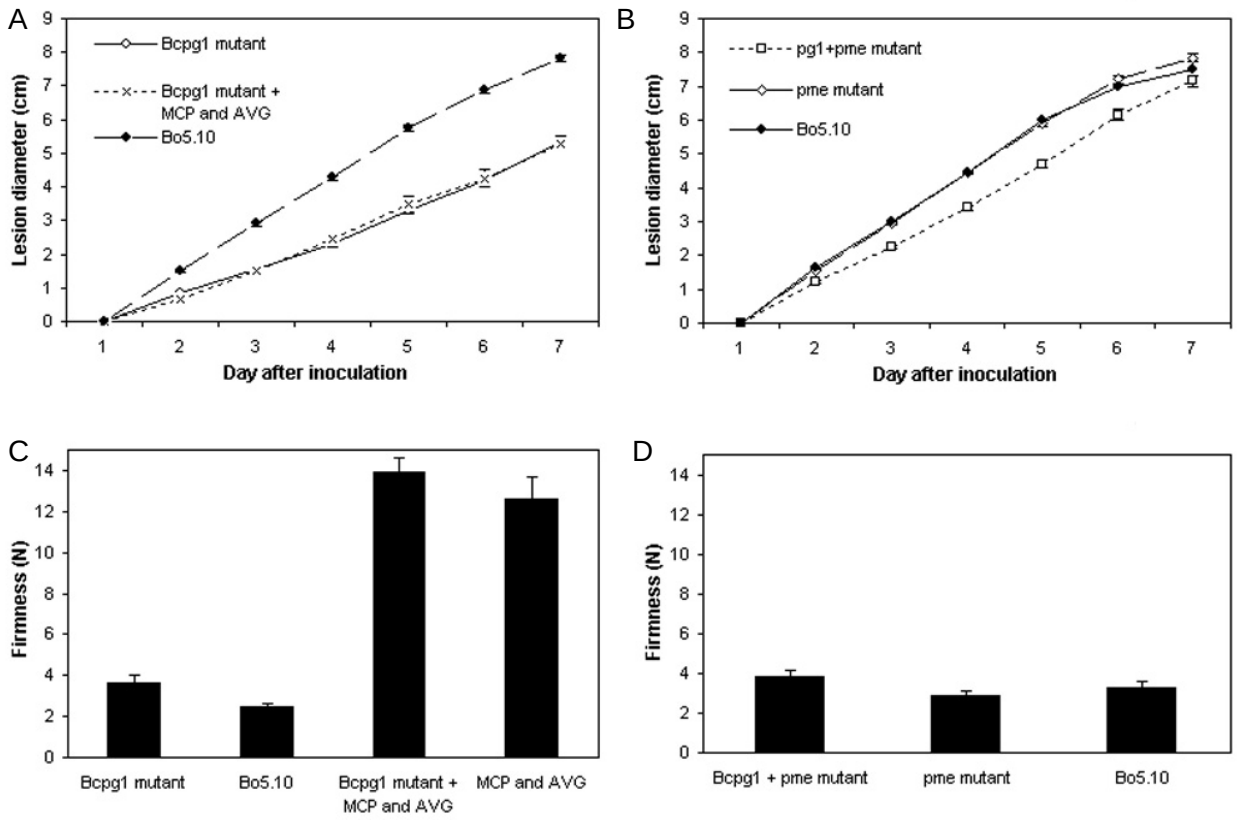

Fig. 5. Unlike pectin methylesterase *Bcpme1*, the polygalacturonase gene *Bcpg1* is required for virulence of *B. cinerea* in pear fruits. Fruits of 'd'Anjou' pears were used after 60 d (A and C) or 108 d of cold storage (B and D). Fruits were pretreated with 1-MCP and AVG and subsequently infected with a deletion strain of *B. cinerea*, *ΔBcpg1*, or the parental wild type B05.10 (A and C). Otherwise, fruits were infected with B05.10, *ΔBcpme1*, or the double mutant *ΔBcpg1 ΔBcpme1* (B and D). Lesion expansion (A and B) or firmness (C and D) was measured.

in the field (8), suggesting that the protective effect of this SAR inducer depends on the attachment of fruits to the plant. Actigard can induce PR gene expression in leaves of apple seedlings (4), but it is unknown whether this SAR inducer affects fruits. However, our preliminary indicate efficacy of Actigard in NahG mutants of tomato (9), which do not accumulate salicylic acid. In these plants Actigard increase resistance to *B. cinerea* in leaves and fruits. In conclusion, Actigard does not alter the susceptibility of pear fruits after harvest either because it is unable to trigger a systemic response in fruits or because SAR does not affect *B. cinerea* (10).

5. REFERENCES

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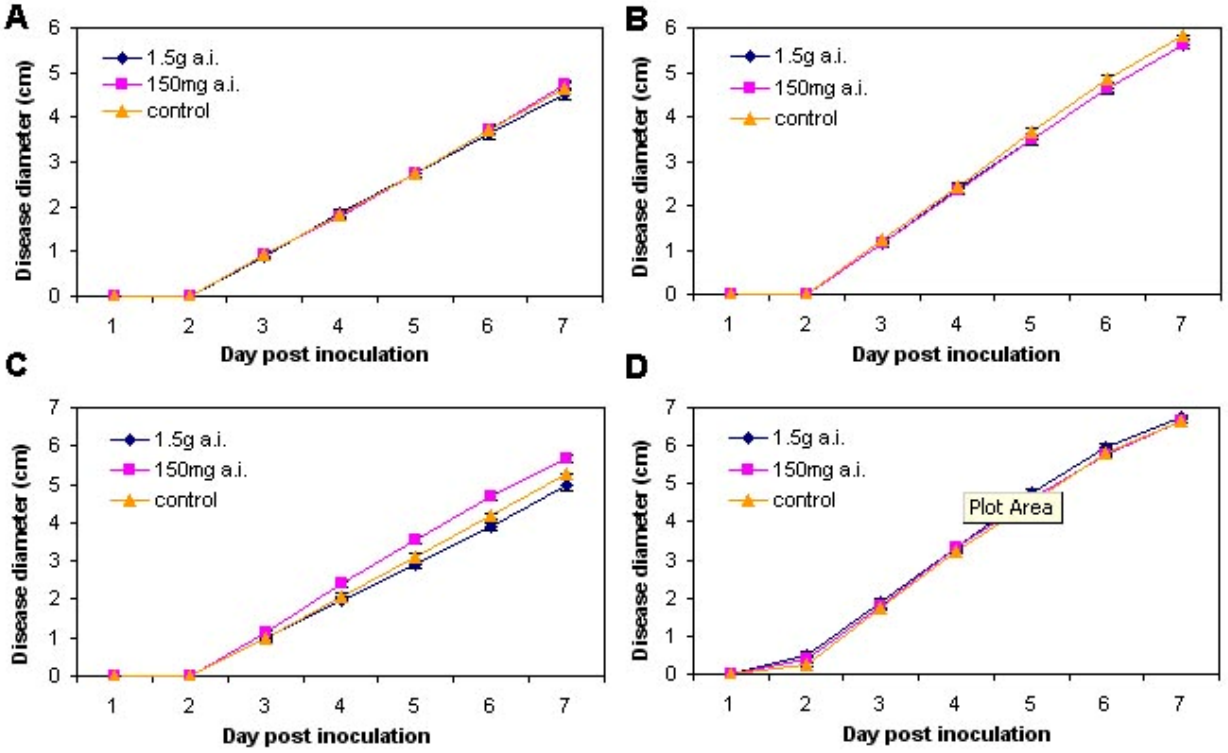

Fig. 6. Effect of Actigard on gray mold susceptibility of pear fruits. 'Bartlett' (A and C) and 'd'Anjou' pear fruits (B and D) were sprayed with 150 mg a.i. or 1,5 g a.i. per liter Actigard one and two weeks prior to harvest. Freshly harvested (A and B) or stored fruits (C and D) were wound inoculated with *B. cinerea*.

6. BUDGET

Project title:	Role of systemic resistance in defense against the gray mold pathogen *Botrytis cinerea*
PI:	Henrik Stotz
Project duration:	2003 to 2005
Final year:	2005
Project total (three years):	$77,020
Current year request:	$29,184