Effects of Pathogen Polygalacturonase, Ethylene, and Firmness on Interactions Between Pear Fruits and *Botrytis cinerea*

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ABSTRACT

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The plant hormone ethylene regulates developmental processes as well as responses to abiotic stress and pathogens. Ethylene influences interactions between the gray mold pathogen, *Botrytis cinerea*, and its hosts. The primary objective of this study was to determine the effect of ethylene on gray mold susceptibility of pear fruits. *B. cinerea* induced ethylene emission from infected pear fruits. As expected, ethylene production and softening of pear fruits were accelerated by propylene and inhibited by 1-methylcyclopropene (1-MCP), but these chemical treatments had a relatively small effect on the rate of lesion expansion after wound inoculation with *B. cinerea*. Cotreatment of pear fruits with 1-MCP and aminoethoxyvinylglycine (AVG) delayed the onset of ethylene emission well beyond the onset of lesion expansion. The trace amount of ethylene produced after inhibition with AVG and 1-MCP was at least partially produced via 1-aminocyclopropane-1-carboxylic acid. Gray mold susceptibility was cultivar-dependent; d'Anjou pears were more susceptible than Bartlett fruits. Storage enhanced the susceptibility of pear fruits. Virulence of the *B. cinerea* strain B05.10 on pear fruits was dependent on the polygalacturonase gene *Bcpg1*, but not on the pectin methyltransferase gene *Bcpme1*. Thus, we conclude that, unlike exogenous manipulation of ethylene and associated changes in fruit softening, pectin catabolism plays a major role in gray mold susceptibility of pear.

Additional keywords: cell wall, host-microbe association, pathogenesis, phytohormone

The plant hormone ethylene regulates various developmental processes and stress responses (43). Plant ethylene is synthesized from methionine via *S*-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (1). The conversion of SAM to ACC is catalyzed by ACC synthase, which is considered to be rate-limiting (31). ACC oxidase catalyzes the conversion of ACC to ethylene. ACC synthase and ACC oxidase are encoded by multigene families. Two systems regulate ethylene biosynthesis in plants. Ethylene is autoinhibited during system 1, but increases autocatalytically during system 2 (23).

Ethylene triggers and promotes ripening of climacteric fruits, including pear (*Pyrus communis*) (18). Transcription of pear polygalacturonase genes, which encode enzymes that hydrolyze pectins in cell walls of ripening fruits, is dependent on ethylene (18). Pear fruits are generally harvested at the preclimacteric stage and stored at low temperatures to enhance ripening via induction of ethylene biosynthesis (15,25). Chilling treatments are

crucial for ripening of winter pears like d'Anjou (15) and Passe-Grassane but not for early cultivars like Old Home (12) and Bartlett. Ethylene biosynthesis changes from system 1 to system 2 during cold storage of pear fruits (12). Different ACC synthase genes are activated when pear fruits are exposed to cold temperatures (12). Chilling treatments also increase ACC oxidase activity (25) and expression of the ethylene receptor *Pc-ETR1a* gene in pear fruits (13).

In order to investigate ethylene effects, chemicals can be used to inhibit or accelerate ethylene biosynthesis or action. Aminoethoxyvinylglycine (AVG), which inhibits ACC synthase activity (1), is used commercially as a plant growth regulator in the form of Retain (Valent Biosciences, Walnut Creek, CA). Retain applications delay ripening and softening of Bartlett pear fruits (9). 1-Methylcyclopropene (1-MCP) binds to ethylene receptors and suppresses ethylene biosynthesis in Braeburn apples (32,40) and a variety of pear fruits (24,29), including d'Anjou (3) and Bartlett (41). SmartFresh, a commercial formulation of 1-MCP, has been approved to treat fruits after harvest in order to enhance shelf life during long-term cold storage (17). The effects of 1-MCP on storage duration of many fruits, vegetables, and floricultural crops have been studied (7). Kubo et al. (24) reported that 1-MCP can also inhibit ethylene production after the initiation of ripening. The ethylene

analog propylene can be used to activate and monitor ethylene biosynthesis (15,36).

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*), the causal agent of gray mold, produces ethylene via 2-keto-4-methylbutyric acid (KMBA), a methionine-dependent but ACC-independent intermediate (8,10). *B. cinerea* attacks more than 200 host species, including pear (20), causing fruit decay during postharvest storage (26). *B. cinerea*, a necrotrophic pathogen, colonizes dying plant tissues and produces endo-polygalacturonases to hydrolyze pectic components of plant cell walls. The polygalacturonase gene *Bcpg1* was found to be required for complete virulence on tomato and apple fruits (38).

There is convincing genetic evidence for the involvement of ethylene in resistance of *Arabidopsis thaliana* to *B. cinerea*. Mutation in a central component of the ethylene signaling pathway, *ein2*, renders *Arabidopsis* plants hyper-susceptible to *B. cinerea* (2,39). Constitutive overexpression of the ethylene response factor *ERF1*, a transcriptional activator acting downstream of *EIN3* (34), activates expression of pathogenesis-related (PR) proteins in *A. thaliana* and enhances resistance to necrotrophic pathogens, including *B. cinerea* (6). Similarly rigorous genetic analysis of ethylene-dependent resistance to *B. cinerea* is lacking from other plant species. Instead, experiments have relied on chemical treatments, leading to conflicting results. Pretreatment of tomato (*Lycopersicon esculentum*) cv. Pearson and the ethylene receptor mutant *Never ripe* with ethylene induced expression of PR proteins and foliar resistance to *B. cinerea* strain B05.10 (11). However, Elad (14) reported that exogenously applied ethylene accelerated gray mold decay of VF198 tomato leaves while the ethylene biosynthesis inhibitors aminooxyacetic acid (AOA) and AVG and ethylene action inhibitor silver-thiosulfate retarded it. Ethylene also increased the susceptibility of mature green but not ripening tomato fruits cv. Faculta 38 to *B. cinerea* (4). Different tomato cultivars were used for these three publications. Elad (14) and Barkai-Golan and Lavy-Meir (4) used different *B. cinerea* isolates, but Diaz et al. (11) used the *B. cinerea* strain B05.10. It is, therefore, possible that the effect of ethylene on this host-pathogen interaction depends on specific combinations of *B. cinerea* strains and tomato cultivars. Alternatively, differences in treatment applications and inocu-

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lation methods may have contributed to these conflicting results in tomato (11). Treatment of Golden Delicious apples with 1-MCP prior to storage reduced the severity of *Botrytis* decay after 5 months of storage (30), but this may be a secondary effect on pectin metabolism because ethylene controls the accumulation of polygalacturonase.

It may be challenging to use ethylene for disease protection while maintaining fruit quality because this phytohormone promotes ripening. Cristescu et al. (10) proposed that softening fruit may provide a physically favorable condition for fungal invasion. The objective of this study was to evaluate the potential contributions of ethylene and fruit firmness to resistance of pear fruits against *B. cinerea*. We determined the influence of fruit ripening on *Botrytis* susceptibility by analyzing two developmental stages, preclimacteric and climacteric. We intentionally studied the short-term effects of 1-MCP because this would allow us to study ethylene-specific effects that are directly related to *Botrytis* interactions during two different developmental stages. Lastly, we analyzed the influence of pathogen polygalacturonase on disease progression.

MATERIALS AND METHODS

Biological material. Fruits of two pear cultivars, Bartlett and d'Anjou, were collected. Bartlett fruits were harvested 121 days after full bloom (DAFB) and 124 DAFB on the commercial harvesting dates of 9 August 2004 and 12 August 2005, respectively. D'Anjou pears were harvested on 27 August 2004 (143 DAFB) and 30 August 2005 (146 DAFB). All fruits were harvested at the Mid-Columbia Experiment Station, Hood River, OR. Fruits were divided into two batches for each cultivar, placed in polyethylene bags, packed into cardboard boxes, and transferred into cold storage (-1°C). Bartlett was stored for 10 days and d'Anjou was stored for 108 days. These disparate storage conditions have previously been reported as adequate to elicit similar ripening behaviors in early and late ripening cultivars (12,15). In a separate experiment, pear fruits cv. d'Anjou, which were treated with AVG and/or 1-MCP after 60 days of cold storage, were harvested at USDA/ARS National Clonal Germplasm Repository, Corvallis, OR, on 20 September 2004.

B. cinerea strain B05.10, the mutants $\Delta Bcpg1$ and $\Delta Bcpme1$, and the double mutant of $\Delta Bcpg1$ and $\Delta Bcpme1$ were obtained from Jan van Kan (Wageningen University, Netherlands) and cultured on potato dextrose agar.

Chemical treatments. Freshly harvested or stored pears were transferred to room temperature 1 day prior to chemical treatment. Fruits were surface-sterilized by dipping in 0.01% sodium hypochlorite for 2 min and rinsing with sterilized water.

Pear fruits were exposed in 19.8-liter jars to humidified propylene (500 ppm) supplied at a flow rate of 45 to 60 ml/min for 24 h prior to fungal infection (36). 1-MCP was supplied as SmartFresh (AgroFresh, Spring House, PA) powder (0.14% active ingredient). According to the direction provided by AgroFresh, 16 mg of 1-MCP powder was mixed with 1 ml of water to generate 300 ppb of volatilized 1-MCP around pears in a sealed 47.3-liter plastic container. Fruits were exposed for 24 h prior to fungal infection. Pears were soaked for 3 min in a Retain (Valent Biosciences) solution containing 500 ppm AVG and 0.01% Tween 20, and then dried. Control fruits were surface-sterilized but not soaked in 0.01% Tween 20. Retain was administered first and 1-MCP last when pears were treated with both 1-MCP and Retain. Fruits that were not treated with chemicals were kept in a sealed container for 24 h prior to fungal infection.

Fungal inoculation. Ten and five fruits per treatment were inoculated in 2004 and 2005, respectively. Fruits were first treated with chemicals, as mentioned above, and then subjected to the following inoculation methods. Fruits were punctured along the equator with the tip of a syringe to generate a 4-mm-deep and 4-mm-wide hole. Fruits were inoculated with 2 μl of a conidial suspension of *B. cinerea* (1.25×10^5 conidia/ μl in 2004 or 5×10^2 conidia/ μl in 2005) in water (35). The high concentration was used whenever fungal concentration was not mentioned in the text. The same amount of sterilized water was used for mock inoculations (wounded controls). Unwounded control fruits were left intact and placed in the same moist chamber, a closed container with wet cheesecloth on the bottom, as mock-inoculated and infected fruits.

Measurement of ethylene and lesion diameters. Ethylene was measured in 2004. Each pear was sealed in a 1-liter 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 evolution of this sample was analyzed using a gas chromatograph equipped with a 121.92×0.3175 cm activated alumina column packed with 80/100 mesh and a flame-ionization detector (Gow-Mac Instrument Co., Series 580, Bethlehem, PA). The first measurement was recorded 6 h postinoculation, followed by daily measurements for 1 week. Individual fruits were handled gently for repeated ethylene measurements, and no peel injury or bruising was observed.

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

Firmness measurement. The fruit skin was removed, and firmness of the fruit flesh was measured as penetration force using a U.S. firmness tester with a 0.78-cm penetrometer tip diameter (Western Indus-

trial Supply, San Francisco, CA). Measurements were recorded twice per fruit on the nondiseased equatorial area of the fruit 7 days postinoculation.

Quantification of ACC. ACC was measured as described by Lizada and Yang (27) using pear fruits 7 days postinoculation. Infected and uninfected parts 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.

Statistical analysis. Data are presented as means $\pm$ 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). In addition, a general linear model (GLM) was used to determine the influence of cultivar, chemical treatment, and infection on fruit firmness. Differences in lesion expansion were analyzed using the repeated measures procedure of a GLM ($\alpha = 0.05$). Contrasts were used to determine significant differences between treatment and control means (SAS Institute, Cary, NC). Least squares means (LSM) were used to determine significant differences among mean comparisons. The significance threshold was $P = 0.05$.

RESULTS

Figures 1 to 4 show interactions of time in cold storage with ethylene-promoting or ethylene-inhibiting chemicals with or without *Botrytis* infection for Bartlett and d'Anjou pear cultivars, respectively.

Ethylene production was monitored in mock-inoculated fruits to distinguish the effect of *B. cinerea* infection from wounding and ripening. Control experiments suggested no significant difference in ethylene production between wounded and intact pear fruits (data not shown). The onset of ripening-related ethylene biosynthesis was evident in Bartlett pears at harvest maturity (Fig. 1A) and d'Anjou fruits after 108 days of cold storage (Fig. 3D). A climacteric peak of ethylene production became apparent in Bartlett pears after a cold storage period of 10 days (Fig. 1D). Ethylene was not detected in wounded fruits of cv. d'Anjou immediately after harvest (Fig. 3A). This does not exclude the possibility that ethylene was produced below detection level, which could have been measured using a laser-based photoacoustic detection system (44).

Ethylene production increased when fruits were challenged with *B. cinerea*. In the case of Bartlett fruits, propylene accelerated (Fig. 1A and B) and 1-MCP suppressed ethylene production (Fig. 1B and E). Compared with causing highly significant differences in ethylene biosynthesis, 1-MCP and propylene had no substantial effect on the rate of lesion expansion after wound inoculation of Bartlett pear fruits with *B. cinerea* (Figs. 1C and F, and 2A and B).

Fig. 1. Effects of 1-methylcyclopropene (1-MCP) and propylene on ethylene production and lesion expansion during infection of Bartlett pear fruits by *Botrytis cinerea* in 2004. Fruits were analyzed after harvest (A to C) or 10 days after cold storage (D to F). Ethylene production (A, B, D, and E) and lesion expansion (C and F) were measured in Bartlett fruits that were untreated or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were wounded and mock-inoculated with water (A and D) or inoculated with 2 μ l of a 1.25×10^5 conidia/ μ l suspension of *B. cinerea* (B, C, E, and F). Vertical bars show $\pm$ SE of 10 replications. The repeated measures option of a GLM was used for statistical analysis. Contrasts suggest significant differences in gray mold susceptibility between propylene-treated and untreated fruits ($P = 0.0011$ in C) and between 1-MCP-treated and untreated fruits at harvest maturity ($P < 0.0001$ in C).

Fig. 2. Effects of 1-methylcyclopropene (1-MCP) and propylene on lesion expansion during infection of Bartlett pear fruits by *Botrytis cinerea* in 2005. Fruits were analyzed after harvest (A) or 10 days after cold storage (B). Lesion expansion was measured in Bartlett fruits that were untreated or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were inoculated with 2 μ l of a 5×10^2 conidia/ μ l suspension of *B. cinerea*. Vertical bars show $\pm$ SE of 5 replications. The repeated measures option of a GLM was used for statistical analysis. Contrasts suggest significant differences in gray mold susceptibility between 1-MCP-treated and untreated fruits at harvest maturity ($P = 0.0192$ in A).

Inhibition of ethylene production by 1-MCP was stronger in pathogen-challenged d'Anjou pears (Fig. 3B and E) than in infected Bartlett fruits after harvest and cold storage (Fig. 1B and E). However, unlike Bartlett fruits, d'Anjou pears did not respond to propylene treatments with a change in ethylene production (Fig. 3B and E). Compared with the highly significant inhibition of ethylene biosynthesis by 1-MCP, changes in the rates of lesion expansion were relatively minor, suggesting that ethylene does not play an important role in controlling pathogen invasion of wounded pear fruits.

A comparison of decay progression illustrates that d'Anjou pears are more susceptible to *B. cinerea* (Figs. 3C and F, and 4) than Bartlett fruits (Figs. 1C and F, and 2). Moreover, susceptibility to *B. cinerea* of Bartlett (Figs. 1C and 2A versus Figs.

Fig. 4. Effects of 1-methylcyclopropene (1-MCP) and propylene on lesion expansion during infection of d'Anjou pear fruits by *Botrytis cinerea* in 2005. Fruits were analyzed after harvest. Lesion expansion was measured in d'Anjou fruits that were untreated or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were inoculated with 2 μ l of a 5×10^2 conidia/ μ l suspension of *B. cinerea*. Vertical bars show $\pm$ SE of 5 replications. The repeated measures option of a GLM was used for statistical analysis. Contrasts suggest significant differences between 1-MCP-treated and untreated fruits ($P = 0.0017$).

Fig. 3. Effects of 1-methylcyclopropene (1-MCP) and propylene on ethylene production and lesion expansion during infection of d'Anjou pear fruits by *Botrytis cinerea* in 2004. Fruits were analyzed after harvest (A to C) or 108 days of cold storage (D to F). Ethylene production (A, B, D, and E) and lesion expansion (C and F) were measured in d'Anjou fruits that were untreated or treated with 1-MCP or propylene prior to inoculation with *B. cinerea*. Fruits were wounded and mock-inoculated with water (A and D) or inoculated with 2 μ l of a 1.25×10^5 conidia/ μ l suspension of *B. cinerea* (B, C, E, and F). Vertical bars show $\pm$ SE of 10 replications. The repeated measures option of a GLM was used for statistical analysis. Contrasts suggest significant differences between 1-MCP-treated and untreated fruits after harvest ($P < 0.0001$ in C) and between propylene-treated and untreated fruits ($P = 0.0117$) after cold storage (F).

1F and 2B) and d'Anjou pear fruits (Fig. 3C versus F) increased during cold storage, as expected (26). Cold storage had a larger effect on gray mold susceptibility of Bartlett than of d'Anjou pears.

Propylene accelerated ($P = 0.0224$) and 1-MCP inhibited fruit softening ($P < 0.0001$) after transfer of stored Bartlett (Fig. 5A) and d'Anjou pear fruits (Fig. 5B) to room temperature. Softening occurred at a faster rate when Bartlett and d'Anjou pear fruits were infected with *B. cinerea* relative to wounded (mock-inoculated) fruits ($P = 0.0036$). The overall significance of this data was not influenced by the unexpected firmness retention of inoculated Bartlett versus mock-inoculated control fruits (Fig. 5A). Although 1-MCP maintained the firmness of stored pear fruits (Fig. 5A and B), this ethylene action inhibitor had no effect on the susceptibility of stored pears to *B. cinerea* (Figs. 1F and 3F).

Cotreatments with 1-MCP and Retain (AVG) were used to further suppress ethylene biosynthesis. For this experiment, d'Anjou pear fruits were moved to room temperature after 60 days of cold storage. Unwounded control fruits did not produce any ethylene (data not shown), suggesting that the physiology of these pears was similar to that of fruits at harvest maturity. Fruits of d'Anjou pears started to produce ethylene 1 day postinoculation with *B. cinerea* (Fig. 6A). Despite pretreatment with Retain (AVG) and/or 1-MCP, ethylene production of infected d'Anjou fruits increased significantly 5 days postinoculation (Fig. 6B and C). In addition to ethylene, ACC was detected 7 days postinoculation of d'Anjou fruits that were pretreated with both 1-MCP and AVG (Fig. 6D). The presence of ACC suggests that ethylene was produced by the host via conversion of SAM. Despite large changes in ethylene production in response to ethylene inhibitors (Fig. 6A to C), differences in lesion expansion were relatively minor (Fig. 6E). After cotreatment with 1-MCP and AVG, lesions started to expand 2 days prior (Fig. 6E) to a rise in ethylene production (Fig. 6C), suggesting that the onset of lesion

expansion is independent of ethylene biosynthesis.

A mutant of *B. cinerea* with a deletion in the polygalacturonase gene *Bcpgl* exhibited reduced virulence when d'Anjou pear fruits were wound-inoculated compared to wild-type *B. cinerea* (Fig. 7A). 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. 7B). The reduced virulence of the $\Delta Bcpgl \Delta Bcpme1$ 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. 7B). Thus, the endo-polygalacturonase gene *Bcpgl* increases the rate of gray mold progression in pear fruits, but the pectin methylesterase gene *Bcpme1* appears to be dispensable.

Fruits of d'Anjou pears infected with the $\Delta Bcpgl$ mutant strain were slightly firmer than those infected with wild-type *B. cinerea* (Fig. 7C; t test, $P = 0.013$). Infection by the $\Delta Bcpgl$ mutant did not change the firmness of fruits treated with AVG and 1-MCP (Fig. 7C; t test, $P = 0.309$). Treatment with 1-MCP and AVG caused a three- to four-fold increase in firmness of pear fruits that were infected with the fungal mutant $\Delta Bcpgl$. Despite this major change in fruit softening, there was no change in the susceptibility of pear fruits to the $\Delta Bcpgl$ mutant (Fig. 7A). Conversely, the fungal polygalacturonase gene *Bcpgl* caused small changes in firmness compared to the wild-type strain B05.10, 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 $\Delta Bcpgl$ mutant nor wild-type *B. cinerea* (Figs. 5 and 7).

DISCUSSION

The relationship between ethylene biosynthesis and gray mold decay of pears. Our results demonstrate that, although ethylene production was drastically manipulated by 1-MCP, AVG, and propyl-

ene, ethylene had little effect on the disease progress of *B. cinerea* strain B05.10 in d'Anjou and Bartlett pear fruits. Lesions consistently started to expand 2 or 3 days after inoculation depending on the conidium concentrations, but the rise in ethylene production was delayed for another 2 days when both 1-MCP and AVG were used to inhibit ethylene biosynthesis. Thus, initiation of symptom development and ethylene production did not coincide.

Statistically significant differences in lesion expansion among treatments were revealed in several trials, but the magnitude of these differences was very small (Figs. 1 to 4 and 6). The suggested effects of propylene were inconsistent because, in one case, an acceleration of lesion expansion was observed (Fig. 3F) even though no impact on ethylene biosynthesis was measured throughout the experiment (Fig. 3E). Moreover, the susceptibility-enhancing effect of 1-MCP on mature pear fruits (Figs. 1 to 4) contradicted the effects of propylene (Fig. 1C) and AVG (Fig. 6E). For these two reasons, we conclude that these small treatment effects on gray mold susceptibility are stochastic in nature and not biologically relevant.

Variable results have previously been obtained using 1-MCP within the context of postharvest decay. Saftner et al. (30) observed that susceptibility of apples to *B. cinerea* was reduced by prestorage but not by poststorage application of 1-MCP. Conversely, Janisiewicz et al. (19) reported that 1-MCP treatment before storage increased the susceptibility of Golden Delicious apples to two other major postharvest pathogens, bitter rot caused by *Colletotrichum acutatum* and blue mold caused by *Penicillium expansum*. Our study differs from these published data because we evaluated the ripening-dependent short-term effects of this ethylene blocker.

Okumura et al. (28) hypothesized that ethylene produced in sweet potato (*Ipomoea batatas*) during infection by the black rot fungus *Ceratocystis fimbriata* is independent of the ethylene biosynthetic pathway of the host. Externally supplied

Fig. 5. Effects of gray mold infection and treatments with 1-methylcyclopropene (1-MCP) or propylene on softening of pear fruits. Bartlett (A) and d'Anjou fruits (B) were used after 10 and 108 days of cold storage, respectively. Firmness of the healthy area of fruits that were exposed to *Botrytis cinerea* for 7 days was compared to mock-inoculated fruits. Vertical bars show $\pm$ SE of 10 replications. A GLM in conjunction with LSM was used to determine the significance of cultivar, infection, and chemical treatment effects.

ACC did not increase ethylene generation, and methionine was not converted to ethylene effectively (28). In contrast, our data clearly show that ethylene was produced via the SAM-ACC pathway in pear fruits. We detected 0.018 nmol g⁻¹ fresh weight of the ethylene precursor ACC even when ethylene biosynthesis was strongly suppressed in d'Anjou pear fruits by 1-MCP and AVG. Thus, *B. cinerea* activated the ethylene biosynthetic pathway of the host during a developmental stage that did not support endogenous ethylene biosynthesis.

B. cinerea, including strain B05.10 (8), produces ethylene in vitro using the KMBA pathway (8,10), but our data demonstrate that pear fruits are the significant source of ethylene biosynthesis during *B. cinerea* infection. Nevertheless, we cannot exclude the contribution of fungal ethylene biosynthesis because 1-MCP and AVG decreased pathogen-induced ethylene production only 10-fold (Fig. 6A and C), whereas ACC content was reduced 29-fold (Fig. 6D). Tomato pericarp, treated with AOA prior to infection with *B. cinerea*, generated low levels of ethylene compared

to tomato pericarp treated with water prior to infection (10). Cristescu et al. (10) concluded that this low amount of ethylene is produced by *B. cinerea* itself, but they did not measure ACC. It is, therefore, possible that tomato pericarp produced ethylene despite treatment with AOA prior to inoculation. Activation of ACC synthases may be responsible for the production of ACC and ethylene that we observed in pear fruits after pretreatment with both AVG and 1-MCP and subsequent inoculation with *B. cinerea*. A multigene family encodes ACC synthases, and distinct ACC

Fig. 6. Effects of 1-methylcyclopropane (1-MCP) and/or aminoethoxyvinylglycine (AVG) on ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) production after inoculation with *Botrytis cinerea* of d'Anjou pear fruits 60 days 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 or pretreated with 1-MCP and AVG and subsequently exposed to *B. cinerea* for 7 days (D). Lesion expansion in gray mold infected d'Anjou fruits that were not treated or treated with 1-MCP, AVG, or 1-MCP and AVG before inoculation (E). Vertical bars show $\pm$ SE of ≥ 4 replications. The repeated measures option of a GLM was used for statistical analysis. Contrasts suggest significant differences between AVG-treated and untreated fruits ($P = 0.0003$) and between 1-MCP-treated ($P = 0.0238$) and untreated fruits (E).

synthase isoforms are activated during different stages of fruit ripening (12). Based on our ACC and ethylene measurements, we conclude that, during infection, both pear fruits and *B. cinerea* produce ethylene, even after treatment with AVG and 1-MCP.

Softening and disease progression. Cristescu et al. (10) proposed that ethylene leads to softening of plant tissue and provides a physically favorable environment for fungal invasion. However, our data showed that 1-MCP-dependent inhibition of fruit softening did not influence the susceptibility of pear fruits to *B. cinerea*.

Manipulation of polygalacturonase levels in transgenic tomatoes suggests that the activity of this cell wall hydrolase increases the susceptibility of fruits to pathogen attack (5). However, this enzyme is not necessarily required for tomato softening (16,33). In pears, a low level of polygalacturonase *PC-PGI* is transcribed during the preclimacteric stage, but ethylene induces high levels of *PC-PGI* gene expression (18). Correlated changes in polygalacturonase gene expression also occurred after inhibition and acceleration of ripening by 1-MCP and propylene, respectively (18). We investigated the effect of fungal polygalacturonase activity on softening using the *B. cinerea* mutant $\Delta Bcpg1$ because transgenic pears with

altered polygalacturonase expression do not exist. The reduction of firmness was significantly less when $\Delta Bcpg1$ infected pear fruits compared to the wild-type fungus. However, the change in firmness was relatively minor, and in the presence of AVG and 1-MCP, the $\Delta Bcpg1$ mutant had no effect on pear fruit softening. Thus, 1-MCP had a much larger impact on pear fruit softening than the presence or absence of a single polygalacturonase gene in *B. cinerea*.

B. cinerea produces various endopolygalacturonases during plant infection, and the expression of individual polygalacturonase genes depends on the host and the stage of infection (37). In the present study, polygalacturonase *Bcpg1* was necessary for full virulence of *B. cinerea* in pear fruit, but pectin methylesterase *Bcpme1* was dispensable. These results are consistent with previous reports (22,38). In contrast to *B. cinerea* strain B05.10, *Bcpme1* in a different genetic background, *B. cinerea* strain Bd90, has been shown to contribute to virulence on various hosts (42). Gene replacement of *Bcpme2* by itself or in combination with *Bcpme1* did not alter growth on 75% methylated pectin or virulence of the *B. cinerea* B05.10 strain on tomato or grapevine leaves (21). The full genome sequence of B05.10 strain suggests the existence of *Bcpme3*, but the only

enzyme activity that was detected in wild-type fungus was *Bcpme1* (21). It was therefore concluded that *B. cinerea* strain B05.10 can degrade pectin without prior demethylation.

In conclusion, our data suggest a major influence of pectin catabolism on lesion expansion when pear fruits were infected with *B. cinerea* B05.10. Increased susceptibility of pear fruits during cold storage may be a direct consequence of pectin breakdown. In contrast, changes in ethylene biosynthesis and fruit softening had no major effect on the susceptibility of d'Anjou and Bartlett pear fruits to *B. cinerea*. The previously reported increase in resistance of apples to *B. cinerea* by 1-MCP (30) may be based on a long-term inhibition of pectin metabolism because ethylene regulates the accumulation of pear polygalacturonase in fruits (18). It is possible that differences in gray mold susceptibility among cultivars are based on pectin composition and/or polymerization.

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Fig. 7. Unlike pectin methylesterase *Bcpme1*, the polygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea* in pear fruits. Fruits of d'Anjou pears were used after 60 days (A and C) or 108 days of cold storage (B and D). Fruits were pretreated with 1-methylcyclopropene (1-MCP) and aminoethoxyvinylglycine (AVG) and subsequently infected with a deletion strain of *B. cinerea*, $\Delta Bcpg1$, or the parental wild-type B05.10 (A and C). Fruits were infected with B05.10, $\Delta Bcpme1$, or the double mutant $\Delta Bcpg1 \Delta Bcpme1$ (B and D). Lesion expansion (A and B) or firmness (C and D) was measured. Vertical bars show $\pm$ SE of 10 replications.

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