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Ethylene Analog and 1-Methylcyclopropene Enhance Black Spot Disease Development in *Pyrus pyrifolia* Nakai

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***Abstract*. Black spot disease is one of the most serious diseases in Asian pear cultivation, with the commercial cultivar Nijisseiki being susceptible. Ethylene is known to play major roles in regulating plant defense responses against various pathogens. We investigated the relationship between ethylene synthesis and black spot disease in 'Nijisseiki' pear leaves by treatment with an analog of ethylene and 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action. Interestingly, both treatments enhanced black spot disease symptoms. Both treatments also increased ethylene production in accordance with disease symptoms through altered gene expression of ethylene biosynthetic enzymes, especially 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*PpACS3* and *4*). Chemical names used: 1-methylcyclopropene (1-MCP), 1-aminocyclopropane-1-carboxylate (ACC).**

Pears are the third most important temperate fruit species after grapes and apples (Itai, 2007). There are three major species of pear, *Pyrus communis* (European pear), *P. bretschneideri* Rehd. or *P. ussuriensis* Maxim. (Chinese pear), and *P. pyrifolia* Nakai (Japanese pear: Nashi), which are commercially cultivated in temperate zones (Bell, 1990). Pears are susceptible to a number of diseases, mostly caused by fungi. In European varieties, resistance against fungal diseases such as scab, powdery mildew, brown spot, and fire blight is an important breeding objective (Bell et al., 1996). In Asian pears on the other hand, resistance against fungal diseases such as scab, rust, and black spot is receiving attention with resistance to black spot disease being a major objective in Japan (Itai, 2007; Kajiura, 1994).

Black spot disease, which is caused by a Japanese pear pathotype of *Alternaria alternata* (Fr.) Keissler, is one of the most serious diseases in Japanese pear cultivation in Japan (Sanada et al., 1988) and the commercial cultivars Nijisseiki, Shinsui, and Nansui are susceptible (Kajiura, 1994). Successful fruit production is currently maintained by repeated fungicide application and by covering the fruits with paper bags during the growing season. The causal pathogen produces a host-specific toxin named AK-toxin (Nakashima et al., 1982; Tanaka, 1933), which causes necrosis on fruit skin and leaves resulting in decreased yield. AK-toxin is toxic to susceptible cultivars only and is harmless to resistant cultivars and nonhost plants. Susceptibility is controlled by a single dominant gene (A), the locus of which is heterozygous in most susceptible cultivars (Kozaki,1973; Terakami et al., 2007).

Ethylene is known to play major roles in regulating plant defense responses against various pathogens, pests, and abiotic stresses (Broekaert et al., 2006). Ethylene is known to increase either susceptibility or resistance in various plants, depending on the plant–pathogen interaction. For example, treatment of tomato with ethylene enhances resistance to the fungus *Botrytis cinerea* (Diaz et al., 2002), whereas it promotes susceptibility to *Xanthomonas campestris* pv. *vesicatoria* (Lund et al., 1998). A similar variety of effects has been observed in *Arabidopsis*, soybean, and tobacco (reviewed in Van Loon et al., 2006). The role of ethylene in plant defense responses is therefore versatile. Pear farmers have experiential knowledge that fruit infected with black spot disease result in early ripening. Fruit ripening is also related to ethylene production in Asian pears (Itai et al., 1999, 2003a). Thus, there seems to be a relationship between ethylene synthesis and black spot disease. However, despite this knowledge, no report has yet documented the correlation between ethylene production and black spot disease. We report the effects of propylene, an analog of ethylene, and 1-MCP, an inhibitor of ethylene action, on the development of black spot disease concomitant with gene expression of ethylene biosynthetic enzymes.

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Materials and Methods

Plant material. Japanese pear (*Pyrus pyrifolia* Nakai) 'Nijisseiki' was grown at the Tottori University orchard. Immature leaves were detached and transferred to the laboratory immediately and inoculated with conidia suspensions of nonpathogenic *A. alternata* strain O-94 (2×10^5 conidia/mL) and virulent *A. alternata* strain O-276 (2×10^5 conidia/mL) (Egusa et al., 2009). Water-sprayed leaves were used as controls. Leaves inoculated with O-276 were divided into three treatment groups (air, 1-MCP, and propylene), exposed to 2 $\mu L \cdot L^{-1}$ 1-MCP (EthylBloc; Rhome and Hass Co., Springhouse, PA) or 1000 $\mu L \cdot L^{-1}$ propylene in a moist plastic chamber, and then incubated at 25 °C for 24 h. Fifteen randomly selected leaves per treatment and per sampling time (0, 1, 6, 12, 24 h) were used in this experiment. Leaf lesions were determined by scanning for spots in five leaf pictures and the numbers of pixels were quantified using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health). The results were expressed as the percentage of spot area vs. total leaf area (spot area/total leaf area) × 100 to compensate for differences in leaf size. Data were converted to the arcsine for further statistical analysis.

Ethylene measurement. Three leaves per treatment with five replications were placed in sealed 0.5-L jars at 20 °C. After 2 h incubation, 2-mL headspace gas samples were withdrawn and analyzed on a gas chromatograph with a flame ionization detector and 60/80 mesh-activated alumina column as described previously (Itai et al., 2003b).

*RNA extraction and cloning of the 1-aminocyclopropane-1-carboxylate synthase gene (*PpACS4*).* To get ACC synthase genes related to pathogen infection, total RNA was extracted from leaves 0 h and 12 h after inoculation according to the hot borate method (Wan and Wilkins, 1994). First-strand cDNA was synthesized from 1 μg of total RNA from O-276-inoculated 'Nijisseiki' leaves using M-MLV reverse transcriptase (ReverTra Ace; Toyobo, Tokyo, Japan). The ACC synthase gene was amplified from cDNA by polymerase chain reaction (PCR) using the following degenerate oligonucleotide primers: sense 5′-ATICARATGGGIYTIGCIGARAAYCA -3′ and antisense 5′-GCRAARCAIACICKRAAC CAICCIGGYTC -3′ based on conserved regions of ACC synthases (Itai et al., 1999). PCR reactions for all clones were performed using 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) under the following conditions: hot start 5 min at 94 °C followed by 40 cycles at 94 °C (1 min), annealing at 55 °C (1 min), extension at 72 °C (2 min), and a final extension at 72 °C for 5 min. Amplified fragments were purified and cloned into a pGEM-T easy vector (Promega). Cloned cDNA fragments were used as probes. DNA sequencing was performed using a Big Dye terminator cycle

sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and analyzed using DNASIS pro software (Hitachi Software Engineering Co., Tokyo, Japan). Full-length cDNA was obtained using 3′ and 5′ RACE methods with 3′- and 5′ Full Race Core kits (Takara, Kyoto, Japan) according to the manufacturer's instructions.

Northern blot analysis. To examine gene expression of ACC synthase and ACC oxidase, Northern blot analysis was used in this experiment. Ten micrograms of total RNA were denatured and loaded onto 1.2% (w/v) agarose gel containing 2% formaldehyde in 1× MOPS running buffer [20 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)]. RNA was separated at 50 V for 2 h, transferred onto a Hybond N^+ nylon membrane (GE Healthcare Bio-science, Buckinghamshire, U.K.), and fixed by exposing the membrane to ultraviolet in a ultraviolet crosslinker (Hoefer Pharmacia Biotech., San Francisco, CA). The blots were hybridized overnight in modified Church and Gilbert buffer [0.5 M phosphate buffer, pH 7.2, 7% (w/v) sodium dodecyl sulfate (SDS), 10 mM EDTA, 100 μg/mL denatured and fragmented salmon sperm DNA] at 65 °C with gene-specific probes of *PpACS1* (Genbank Accession no. AB015624), *PpACS2* (No. AB007639), *PpACS3* (No. AB015625) (Itai et al., 2003b) and *PpACS4* (No. AB610771), and *PpACO1* (No. D67038), *PpACO2* (No. AB042105), and *PpACO4* (No. AB042111). The probes were labeled with ^{32}P-dCTP using a random prime labeling kit (GE Healthcare Bio-science). Partial cDNA fragments of specific 3′ ends of all cDNAs were used as probes (Itai et al., 2003b). After hybridization, the membranes were washed twice in 2× SSC/0.1% SDS solution at 65 °C for 15 min, three times in 0.2× SSC/0.1% SDS solution at 65 °C for 15 min, and then exposed to an imaging plate (Fuji Film, Tokyo, Japan). Signals were detected with an image analyzer (FLA5000; Fuji Film). The relative mRNA level was determined using Image Gauge software Version 4.0 (Fuji Film). Data were subjected to one-way analysis of variance (ANOVA) according to treatment. All ANOVAs were performed with JMP statistical software (Version 6.0; SAS Institute, Cary, NC) using treatments as a statistical parameter at a significance level of $P < 0.05$. Means were separated using Tukey's multiple range test.

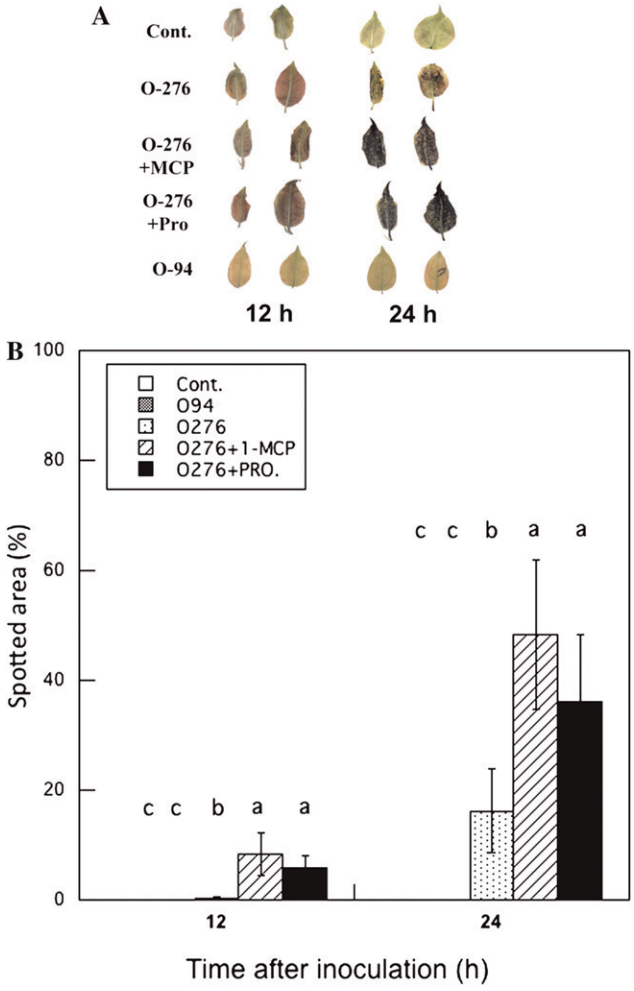

Fig. 1. Changes in disease symptoms after inoculation with spore suspensions of nonpathogenic *A. alternata* strain O-94 (O-94) and virulent *A. alternata* strain O-276. Water-sprayed leaves were as a control (Cont.). O-276-inoculated leaves were exposed to air (O276), 1-methylcyclopropene (1-MCP) (O276 + MCP), or propylene (O276 + Pro). (**A**) Typical symptoms at 12 h and 24 h after inoculation and treatment. (**B**) Changes in the percentage of black-spotted area to total leaf area after inoculation and treatment. Error bars indicate the SD of each mean value (n = 5). For the same hour after inoculation, values with the same letters are not significantly different ($P < 0.05$).

Results

Early symptoms of foliar disease include the formation of necrotic lesions that later expand. Necrotic lesions were observed on leaves exposed to both 1-MCP and propylene at 12 h (Fig. 1A–B) and increased greatly at 24 h. At 24 h, 1-MCP and propylene treatment showed an average of 48% and 36% lesions, respectively. No significant differences were observed in the percentage of black-spotted areas between leaves exposed to 1-MCP and propylene at 12 h and 24 h. On the other hand, necrosis was not observed at 12 h on O-276-inoculated leaves without 1-MCP and propylene treatment, although it was observed at 24 h. Foliar disease symptoms greatly increased on 1-MCP- and propylene-treated leaves in comparison with untreated O-276-inoculated leaves. Nonpathogenic O-94-inoculated and control leaves showed no necrotic symptoms even at 24 h.

Control leaves showed no detectable ethylene production during the experimental period (Fig. 2), whereas nonpathogenic O-94 inoculation resulted in a slight increase. In both 1-MCP- and propylene-treated leaves, on the other hand, ethylene production was markedly increased from 6 h to 12 h compared with O-276-inoculated leaves without treatment.

After reverse transcription PCR amplification and 5′ and 3′ RACE cloning, *PpACS4* cDNA 1866 bp in length was obtained encoding a putative polypeptide of 488 amino acids (data not shown). The coding region of *PpACS4* was 96% identical to that of apple ACS (MdACS5A: AB034992). To analyze the expression of ACC synthase and ACC oxidase genes, we prepared total RNA from leaves 0 h, 1 h, 6 h, and 12 h after inoculation and treatment. For ACC synthase genes, expression of *PpACS1* was not observed after any treatment at any time (Fig. 3). *PpACS2* was expressed only at 12 h after O-276 inoculation with 1-MCP and propylene treatment, but its mRNA accumulation level was low. Expression of *PpACS3* and *PpACS4* increased from 1 h to 6 h after inoculation with nonpathogenic *A. alternata* strain O-94. Expression of *PpACS3* and *PpACS4* was also induced at 6 h by pathogenic O-276, but expression of *PpACS4* decreased at 12 h. 1-MCP exposure led to a marked increase in mRNA levels of *PpACS3* and *PpACS4* at 12 h. Propylene exposure induced *PpACS4* expression at 6 h with a significant increase at 12 h, whereas the expression of *PpACS3* was greatly increased after 6 h. At 12 h after inoculation, expression levels of *PpACS3* and *PpACS4* in both 1-MCP- and propylene-treated leaves were much higher than those in O-276-inoculated

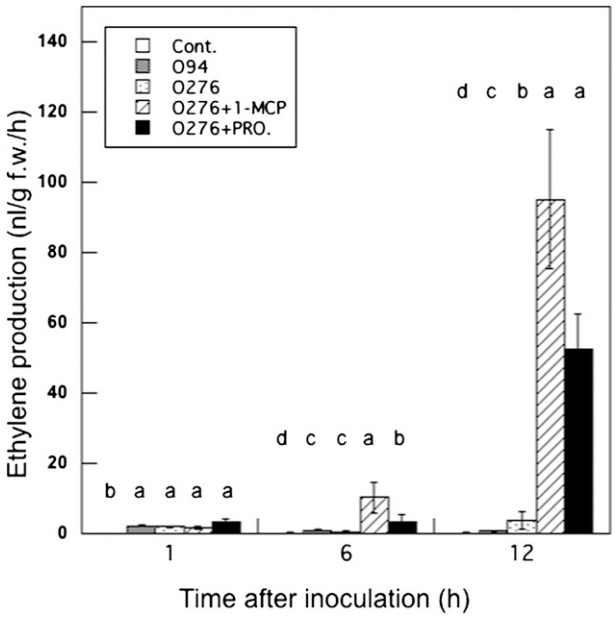

Fig. 2. Changes in ethylene production of leaves with water only treatment (Cont.), strain O-94 inoculation (O-94), strain O-276 inoculation (O-276), strain O-276 inoculation and 1-methylcyclopropene (1-MCP) exposure (O276 + MCP), and strain O-276 inoculation and propylene exposure (O276 + Pro). Error bars indicate the SD of each mean value (n = 5). For the same hour after inoculation, values with the same letters are not significantly different ($P < 0.05$).

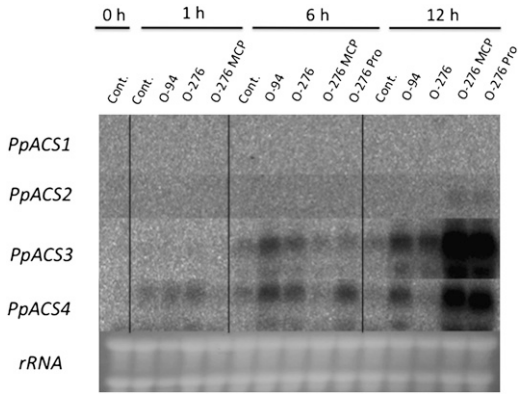

Fig. 3. Expression analysis of 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*PpACS1, 2, 3,* and *4*) in leaves with water-only treatment (Cont.), strain O-94 inoculation (O-94), strain O-276 inoculation (O-276), strain O-276 inoculation and 1-methylcyclopropene (1-MCP) exposure (O276 + MCP), and strain O-276 inoculation and propylene exposure (O276 + Pro). Northern blot analysis of total RNA (10 µg per lane) extracted from leaves. Ethidium bromide-stained *18SrRNA* is shown as a control for equal loading.

leaves without treatment. For ACC oxidase genes, the mRNA of *PpACO2* and *PpACO4* was already present in control leaves at 0 h (Fig. 4). In particular, *PpACO2* showed higher levels after all treatments from 0 to 6 h with a decrease at 12 h in control leaves. *PpACO4* expression was slightly elevated at 6 h after O-276 inoculation. Expression of *PpACO1* increased from 1 to 6 h after inoculation with both O-94 and O-276 and remained at higher levels in 1-MCP- and propylene-treated leaves at 12 h.

Discussion

Ethylene is a major modulator of many aspects of plant life, including various mechanisms by which plants react to pathogen attack (Broekaert et al., 2006). Ethylene evolution occurs concomitantly with the progression of disease symptoms in response to a number of virulent pathogen infections (Lund et al., 1998). However, no report has yet documented the correlation between ethylene production and black spot disease in pears. We used propylene, an analog of ethylene, and 1-MCP, an inhibitor of ethylene action, to clarify the role of ethylene in defense against the pathotype *A. alternata*, which causes black spot disease in pears. Propylene is a useful tool for investigating the effects of ethylene and measuring ethylene production. Surprisingly, respective treatment of pear leaves with propylene and 1-MCP resulted in decreased resistance to *A. alternata* with rapid expansion of necrotic lesions. Both the ethylene analog and ethylene perception inhibitor accelerated disease symptoms. The present article is, to our knowledge, the first report indicating that both ethylene action and 1-MCP enhanced disease symptoms. Many reports have shown that the salicylic acid (SA) and jasmonic acid (JA) signaling pathways play an important role in plant defense systems (Glazebrook, 2005). However, we previously showed that SA and MeJA do not influence lesion formation on 'Nijisseiki' leaves after O-276 infection (Egusa et al., 2009). The present results indicate that ethylene perception is associated with disease resistance and susceptibility to black spot in pears. In tomato, ethylene enhances resistance to the fungus *Botrytis cinerea* and 1-MCP results in increased susceptibility (Diaz et al., 2002), whereas ethylene promotes susceptibility to *A. alternata* (Moussatos et al., 1994), *Colletotrichum gleosporioides* (Cooper et al., 1998), and *Xanthomonas campestris* pv. *vesicatoria* (Lund et al., 1998). Ethylene has also been shown to both inhibit and promote the symptoms of *Verticillium* wilt in tomato (Robinson et al., 2001). Robinson et al. proposed that post-infection ethylene enhanced *Verticillium* wilt development, whereas its presence at the time of infection inhibited disease development. The discrepancy of the double signaling function of ethylene in black spot disease also indicates that ethylene has different effects at different stages during disease establishment of *A. alternata*. The infection behavior of the pathogen was equal on the leaf surface of susceptible and resistant cultivars, but penetration was detected only in susceptible leaves (Otani et al., 2002). All virulent isolates of *A. alternata* release AK-toxin from spores after germination, whereas avirulent isolates produce no AK-toxin (Hayashi et al., 1990). Pre-inoculation with nonpathogenic O-94 or pretreatment with an elicitor prepared from O-94 reduced disease symptoms caused by O-276 by changing defense-related genes (Egusa et al., 2009). By combining the present data with previous observations, it is proposed that ethylene performs two separate functions during disease establishment. At the time of inoculation, ethylene may induce resistance responses by elicitation of defense proteins, whereas after successful pathogen attack, ethylene may increase AK-toxin susceptibility and promote disease symptoms by inducing necrosis.

Enhanced disease symptoms were in accordance with increased ethylene production. *A. alternata* infection caused upregulation of the two ACC synthases, *PpACS3* and *PpACS4*, as shown by a significant increase at 12 h with both 1-MCP and propylene. *PpACS3* has also been shown to be strongly induced by wounding in fruit (Itai et al., 2003b). Negative feedback control of ethylene production has been recognized in a variety of fruits and vegetative tissues (Yang and Hoffman, 1984). Wound-induced ethylene production in avocado and persimmon fruit was enhanced by 1-MCP treatment (Owino et al., 2002; Zheng et al., 2006). Two other members

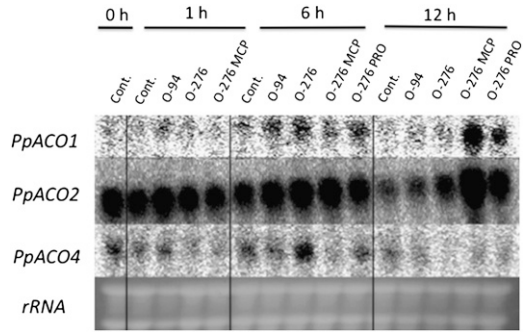

Fig. 4. Expression analysis of 1-aminocyclopropane-1-carboxylate (ACC) oxidase genes (*PpACO1, 2* and *4*) in leaves with water-only treatment (Cont.), strain O-94 inoculation (O-94), strain O-276 inoculation (O-276), strain O-276 inoculation and 1-methylcyclopropene (1-MCP) exposure (O276 + MCP), and strain O-276 inoculation and propylene exposure (O276 + Pro). Northern blot analysis of total RNA (10 µg per lane) extracted from leaves. Ethidium bromide-stained *18SrRNA* is shown as a control for equal loading.

of the ACS gene family, *PpACS1* and *PpACS2*, the expression of which increases during fruit ripening (Itai et al., 2003a), were not induced at 6 h by *A. alternata*, although *PpACS2* expression was slightly induced at 12 h with both propylene and 1-MCP. On the other hand, expression of *PpACO2* was already very high at 0 h and remained high throughout the experimental period. Expression of *PpACO1*, which is upregulated during fruit ripening (Itai et al., 1999), increased greatly at 12 h with pathogenic O-276 infection after propylene and 1-MCP treatment, respectively. A gene encoding ACC synthase was shown to increase after tobacco mosaic virus infection (Knoester et al., 1995) and ACC synthase transcription was also increased after inoculation of citrus plants with *Xanthomonus campestris* pv. *citri* (Dutta and Biggs, 1991). On the other hand, infection of tomato with *Pseudomonas syringae* pv. *tomato* was shown to increase two ACC oxidase genes in tomato plants (Cohn and Martin, 2005). These data suggest that ACC synthases rather than ACC oxidases are the key enzymes in the infection-associated ethylene biosynthetic pathway in the development of pear black spot disease.

In summary, both propylene, an analog of ethylene, and 1-MCP, an inhibitor of ethylene action, enhanced symptoms of black spot disease in Japanese pear. Both treatments also increased ethylene production in accordance with disease symptoms through altered gene expression of ethylene biosynthetic enzymes, in particular the two ACC synthase genes *PpACS3* and *4*.

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