Original Research Article

Postharvest suppression of gray mold (*Botrytis cinerea*) on peach through application of *n*-propyl dihydrojasmonate

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**ABSTRACT**

Control of gray mold disease (*Botrytis cinerea*) and change of endogenous plant hormones, ethylene, jasmonic acid (JA), and abscisic acid (ABA) in peach (*Prunus persica* L. Batsch 'Akatsuki') treated with *n*-Propyl dihydrojasmonate (PDJ) were investigated. Peaches were compared between non-PDJ dipping (control) and 400 µM-PDJ dipping before inoculation with *B. cinerea* conidial suspension stored at 25°C for 6 days. The results showed that PDJ application significantly decreased (*P* < 0.05) disease symptoms and lesion diameter, meanwhile, this application induced the accumulation of ethylene production, JA, and ABA concentration. The upstream expression level of *PpACS1* (1-aminocyclo-propane-1-carboxylic acid synthase) and *PpAOS* (allene oxide synthase) genes in PDJ application were significantly higher in the peaches inoculated with *B. cinerea* than the control. In contrast, PDJ application decreased ester and lactone compounds which are the major volatile compound of peach. However, alcohol and aldehyde compounds were increased by the PDJ application. These results suggested that PDJ application delayed the infection of *B. cinerea* through accumulation of ethylene, endogenous JA, and ABA in peach.
**INTRODUCTION**

*Botrytis cinerea* is one of the most important postharvest diseases resulting in economic loss on peach. Infection by *B. cinerea*, so-called gray mold disease, is initiated at flower stage, latent during fruit stage, and eventually occurs during retail stage (Elad et al., 2004). Fungicides are widely used to control pathogens at pre and post-harvest stage, especially benzimidazoles and dicarboximides. However, pathogens have developed resistance to these. In recent years, consumers are concerned about environmental pollution and human health risks. Alternative methods for control of postharvest diseases such as biological and cultural method, Jasmonic acid (JA) derivative and methyl jasmonate (MeJA) has been reported to be effective to enhance disease resistance in peach infected with *Penicillium expansum, Botrytis cinerea*, and *Rhizopus stolonifer* (Jin et al., 2009). In Japan, n-Propyl dihydrojasmonate (PDJ) is JA synthetic analog which is convenient to use. PDJ application effectively delays postharvest diseases infection, for example; *Colletotrichum gloeosporioides* in Japanese apricot (*Prunus mume* Sieb.) and *Glomerella cingulata* in grape berry (*Vitis labrusca* × *Vitis vinifera*) respectively (Nimitkeatkai et al., 2011 and Wang et al., 2015).

JA plays an important role during plant development, ripening, pathogenesis-related (PR) proteins, and response to abiotic and biotic stress (Concha et al., 2013; Wasternack, 2007; Wei et al., 2017). Exogenous JA has been reported to enhance disease resistance, that is, activation of defense enzymes, increase phenol compound and antioxidant capacity, and accumulation of hydrogen peroxide, resulting in delaying postharvest diseases in peach, strawberry, and grape (Jiang et al., 2015; Jin et al., 2009; Saavedra et al., 2016). PDJ application also effectively delayed ripening through decreased ethylene production and expression level of *PpACO1* in peach (Ziosi et al., 2008). JA and ethylene signal transduction pathways act synergistically in a plant’s immunity through accumulation of *ethylene response factor (ERF)* gene (Pieterse et al., 2009). Moreover, peach aroma volatile compounds are lactones and esters which provide fruity notes, and C6 aldehydes and alcohols contribute green sensory notes in the ripening fruit (Zhang et al., 2010).

Antagonistic interaction between the abiotic stress hormone, abscisic acid (ABA), and JA have been observed in *Arabidopsis thaliana* inoculations with *Fusarium oxysporum* (Jonathan et al., 2004). Another study found that high concentration of ABA inhibits ethylene production in tomato and strawberry (LeNoble et al., 2004). For volatile compounds, on the other hand, 2-Hexanal, C6 aldehydes, had been reported to inhibit postharvest pathogens such as *Monilinia laxa* and *B. cinerea* (Fiorella et al., 2006; Tsao and Zhou 2000). In contrast, ester and alcohol volatile compounds developed as a result of *B. cinerea* infection from the latent phase to growth phase after wounding in strawberry (Neri et al., 2015).

The relationship of plant hormones, JA, ethylene, and ABA, and volatile compounds during infection with postharvest pathogens in peach is unclear. The aim of this study was, first, to evaluate the effect of PDJ application on controlling *B. cinerea* in peach, then to investigate relationship between PDJ application, on endogenous JA, ethylene production, ABA, and change of volatile compounds associated with defense pathogen responses.

**MATERIALS AND METHODS**

**Peach fruit and treatment**

Peach trees (*Prunus persica L. Batsch ‘Akatsuki’) grown from *Prunus persica* (MAFF, 712208) was used and cultured on potato dextrose agar (PDA) at 25°C for 10 days. Spore suspension was prepared by removing the conidia from fungal culture on PDA and suspending in sterile distilled water. The spore suspension was adjusted to 1 × 10⁶ conidia•mL⁻¹. For pathogen inoculation, all groups were wounded with a sterilized nail (3 mm depth and 5 mm in diameter). Both groups were inoculated with 50 µL of the spore suspension after 24 h. PDJ group and inoculation group without PDJ treatment were PDJ⁻*Ino*⁻ and PDJ⁺*Ino*⁺, respectively. The untreated control group was inoculated with 50 µL of sterile distilled water with 0.5% agar.

After inoculation, fruit were placed into a container covered with polyethylene bag and then stored at 25°C with 95% relative humidity (RH) for 6 days. Peel was collected from 15 fruit (3 fruit per replication) at 0, 1, 2, 3, and 6 days after treatment. Peach peel was frozen immediately in liquid nitrogen and stored at -80°C until analysis. The frozen tissues were analyzed for 1-aminocyclopropane-1-carboxylic acid (ACC) concentration, ethylene-related gene (*PpACS1, PpACO1, PpCTR1, and PpCTR1*), and expression levels of *PpAO5* gene. Ethylene production rate was calculated as shown in the equation as show below.

\[
\text{Disease incidence} = \frac{\text{number of fruit diseased}}{\text{total number of fruit}} \times 100
\]

**Ethylene production and ACC concentration**

Ethylene production was analyzed as previously described by Kondo et al. (1991). One peach fruit was incubated in 1.67 L closed plastic box for 1 h at 25°C and then 1 mL of headspace gas was
collected. Ethylene production was analyzed from 1 mL of headspace gas using gas chromatography (model GC-2014; Shimadzu, Kyoto, Japan) with a flame-ionisation detector (FID). The ethylene production rate was expressed as microliter of ethylene per kilogram per hour.

For ACC concentration, 2 grams of frozen peel were homogenized with 0.1 M of hydrochloric acid then centrifuged and the supernatant was used for analysis. The supernatant was mixed with 0.1 M mercuric chloride and 5% sodium hypochlorite with saturated sodium hydroxide in glass tube with lid and 2 mL headspace gas was injected into a gas chromatography (model GC-2014; Shimadzu, Kyoto, Japan) with FID and then the peak area was converted to ACC concentration.

**Jasmonic acid (JA) concentration**

Endogenous JA concentration determination was performed as previously described by Kondo et al. (2005) with GC-MS-SIM (QP 5000; Shimadzu, Kyoto, Japan; 25 m×0.25 mm I.D. column). The frozen peels (1 g) were homogenized with 1μL as an internal standard in 10 mL of saturated NaCl and 20 mL of diethyl ether containing 0.005% butylated hydroxytoluene (BHT) as an antioxidant. The ether phase was removed after centrifugation at 10,000 rpm and then extracted 3 times with diethyl ether containing 0.005% BHT. The pooled ether extract was dried under warm air, after that the residue was dissolved in 200 μL of chloroform/isopropylethylamine, 1:1 (v/v), and derivatized at 50°C for 60 min with pentafluorobenzyl bromide. The ions were measured as m/z 390, 264, and 209. The concentration of JA was determined from the ratio of peak areas for m/z 209 (jasmonic acid)/264 (ibuprofen).

**Abscisic acid (ABA) concentration**

One gram of frozen peach peel (three replications) was homogenized in 20 mL cold 80% (v/v) methanol including 0.5 g polyvinylpyrrolidone with 0.2 μg ABA-d6 as an internal standard. Endogenous ABA was extracted and analyzed as previously described by Setha and Kondo (2009). The methyl ester of ABA was analyzed by gas chromatography-mass spectrometry-scan ion monitoring (QP 5000; Shimadzu, Kyoto, Japan; 25 m×0.25 mm I.D. column). The ions were measured as m/z 190, 194, 260, and 264. The concentrations of ABA was determined from the ratio of peak areas for m/z 190 (d0)/194 (d6).

**Total RNA extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR) analysis**

The frozen tissue (0.3 g fresh weight; three replications) was extracted using cetyltrimethylammonium bromide buffer and column-based extraction method (Henderson and Hammond, 2013). cDNA was synthesized from RNA using ReverTra Ace®qPCR RT Master Mix following the manufactures instructions (Code No. FSQ-201; Toyobo co.,LTD, Osaka,Japan). The specific primer of each gene was used for qRT-PCR analysis (Table 1). Transcription level was estimated by qRT-PCR (model: Steo One Plus, Life Technology, Tokyo,Japan) with a KAPA SYBR FAST Master Mix (Kapa Biosystem, Boston, MA, USA) according to instruction manual. The expression level of each gene was calculated as the coefficient of variation, and normalized to the transcript level of the average of UBIQUITIN (UBQ), and Actin gene.

**Volatile compounds analysis**

Volatile compounds were extracted and identified by gas chromatography-mass-spectrometry (GC–MS) and the quantification of selected volatile compounds according to Wang et al. (2015). The frozen peel (0.5 g) was put into 4 mL vial with lid and incubated with 10 μL cyclohexanol as an internal standard at 40°C for 30 min. The headspace gas were aborbed and extracted using 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane solid-phase micro-extraction fiber (Supelco, Bellefonte, PA) and then reabsorbed at the injection port of GC-FID (GC-4000 plus, GL Sciences, Kyoto, Japan, DB-Wax; 60 m × 0.25 mm I.D. capillary column; Agilent, Santa Clara, CA, USA), which was set at 250°C. The concentrations of individual compound were determined using the peak of the internal standard as a reference value and calculated based on standard curves of pure compounds.

**Statistical analysis**

The statistical analysis was performed with SAS analysis (version 8.2, SAS institute, Cary, NC, USA). The data were analyzed by one-way analysis of variance (ANOVA). The treatment effect and the mean were separated by Tukey-Kramer test at P≤0.05. The data were presented the mean values of the three replications ± standard error (SE).

**RESULTS AND DISCUSSION**

**Disease incidence and lesion diameter of B. cinerea on peach**

PDJ\textsuperscript{+} Ino\textsuperscript{+} treatment prevented the infection of gray mold on peach for 5 days and lesion diameter was observed at 6 days after treatment (DAT). However, the disease incidence and lesion diameter of PDJ\textsuperscript{−} Ino\textsuperscript{−} treatment was observed at 4 DAT and significantly higher than PDJ\textsuperscript{+} Ino\textsuperscript{+} treatment. Disease incidence and lesion diameter was not observed in the untreated group. On the other hand, the untreated group showed no infection of gray mold (Figure 1A, B). The results showed PDJ application at 400 μM concentration retarded disease incidence and suppressed B. cinerea infection as well as lesion diameter on peaches (Figure 1). In agreement with our results, JA derivative, methyl jasmonate (MeJA), and PDJ have been reported to suppress postharvest diseases of fruit such as strawberry (Gabriela et al., 2017), grape (Jiang et al., 2015; Wang et al., 2015), and Japanese apricot (Nimitkeatkai et al., 2011), which practicable enhanced activity of defense resistance enzyme and related genes.
Ethylene production, ACC content, Jasmonic acid concentration, and expression level of ethylene related genes and PpAOS gene on peaches inoculated with B. cinerea

Ethylene production rapidly increased in PDJ+ Ino- treatment at 3 DAT. ACC concentration and expression of PpACO1 gene were induced in PDJ+ Ino- treatment and significantly higher in later stages compared with other treatment, especially PpACO1 gene (Figure 2B, D). PDJ- Ino+ treatment showed a marked increase in ACC concentration at 6 DAT while PDJ- Ino- treatment increased ACC concentration and expression of PpACO1 gene at 2 and 1 DAT, respectively. PDJ- Ino+ treatment up-regulated expression of PpACS1 and PpCTR1 gene, thus, ethylene production was increased and significantly higher than PDJ- Ino+ treatment and the untreated group (Figure 2A, C, F). The highest expression of PpETR1 gene was observed in untreated control group between 3 to 6 DAT (Figure 2E).

The increment of JA concentration and expression level of PpAOS gene were shown in PDJ- Ino- treatment and significantly higher than PDJ- Ino+ treatment and untreated control group, respectively (Figure 3A, B). PDJ- Ino+ treatment increased JA concentration and expression level of PpAOS gene especially at 3 and 6 DAT and related with the increment of ethylene production. Also, JA concentration affected to decrease lesion diameter of B. cinerea on PDJ- Ino- treatment. On the other hand, PDJ- Ino- treatment rapidly increased JA concentration at 2 DAT, but it was not significantly different with PDJ- Ino- treatment.

Ethylene and JA play an important role in plant defense response to pathogen as induced systemic resistance (ISR). The ISR can promote mitogen-activated protein kinase (MAPK), the octadecanoid pathway (oxylipins biosynthesis), the phenylpropanoid pathway, and cell wall metabolism (Gianfranco et al., 2016; Lloyd et al., 2011; Shoresh et al., 2010). Our results showed that PDJ- Ino+ treatment significantly increased ethylene production and endogenous JA at 3 and 2 DAT, respectively. Previous reports have revealed that PDJ application involved in enhancing ethylene production on pears (Kondo et al., 2007), grapes (Wang et al., 2015), and Japanese apricots (Nimitkeatkai et al., 2011). In addition, the current study indicated that PDJ- Ino+ treatment induced expression of PpACO1 and PpCTR1 gene in peach. Tong et al. (2017) suggested JA application up-regulated both ACS1 and ACO1 gene pass through induction of transcription factor of the JA signaling pathway, MY2 gene, in apple. However, PDJ- Ino+ treatment showed significantly lower ACC content and down-regulated expression of PpACO1 gene than PDJ- Ino- treatment. This result is supported by previous study which showed that down-regulated expression of PpACO1 by JA in peaches at harvest (Ziosi et al., 2008). On the other hand, PDJ application up-regulated expression of PpAOS gene resulting in increment of endogenous JA. AOS gene has been reported to regulate JA biosynthesis and increased at ripening stage in apple, peach, and tomato (Fan et al., 1998; Kondo et al., 2000; Terrigiani et al., 2012; Ziosi et al., 2008). Ethylene positively regulates the induction of AOS gene (O’Donnell et al., 1996), while JA induces expression of ACO, resulting in enhanced ethylene production (Hudgins and Franceschi, 2004). The defense related genes, such as plant defensin (PDF2.1) and β chitinase (β-CH) gene, were induced by both JA and ethylene hormone against necrotrophs pathogen (Po-Wen et al., 2013; Zhu et al., 2011). Moreover, Lorenzo et al. (2003) reported Ethylene response factor1 (ERF1) as a functional transcription factor which is a key gene regulating ethylene or JA or both, and responding to pathogen resistance through active expression of PR gene. Our results suggest that PDJ application may inhibit B. cinerea growth through the induction of ISR by JA/ethylene signaling defense response pathways.

Abscisic acid concentration on peaches inoculated with B. cinerea

ABA had the highest concentration at harvest time of peach (0 DAT). PDJ- Ino+ treatment slightly decreased ABA concentration when compared with PDJ- Ino+ treatment and untreated control group which was observed a profound decline at 1 DAT (Figure 4). ABA concentration increased in PDJ- Ino+ treatment and was significantly higher than PDJ- Ino+ treatment at 3 DAT. However, ABA concentration was sharply decreased at 16 DAT in PDJ- Ino- and PDJ- Ino+ group. ABA plays an important role in response to biotic and abiotic stress in fruit. Nevertheless, the increment of ABA are depended on time (Brigitte and Felix, 2005). ABA has been reported to regulate plant resistance to pathogens (AbuQamar et al., 2017). Our result found that B. cinerea infection in peach increased endogenous ABA at 3 DAT. The result was supported by previous research which reported that ABA concentrations were induced in sugar beet leaves during fungal infection, Cercospora beticola (Schmitd et al., 2008). Moreover, Kazan and Manners (2013) reported MYC gene, regulator of the JA signaling pathway, positively regulated ABA signaling pathway.

Volatile compound on peaches inoculated with B. cinerea

Twenty seven volatile compounds, i.e., 8 alcohols, 5 aldehydes, 8 esters, 2 ketones, and 4 lactones, were identified from peach inoculated with B. cinerea using GC-MS (data not show). Alcohol, aldehyde, and ester concentrations accumulated significantly in PDJ- Ino+ treatment than in PDJ- Ino+ treatment at 2 and 3 DAT (Figure 5A, B, C). In contrast, lactone concentration was induced higher in PDJ- Ino+ than PDJ- Ino+ treatment at 3 and 6 DAT (Figure 5D). PDJ- Ino+ treatment showed lower concentration of ester compound and significantly lower compared to other treatments.

In our study, alcohol and aldehyde were detected to be the most abundant compounds in peach treated with PDJ application, as they were in previous research (Nimitkeatkai et al., 2011; Wang et al., 2015). Further still, they showed that alcohol and aldehyde compounds mainly increased on Japanese apricot and grape treated with PDJ. Hexanal and (E)-2-hexanal were the main aldehyde compounds in this research on peach. Cs aldehyde (hexanal, (E)-2-hexanal, and (Z)-3-hexenal) has been shown to induce plant defense resistance to inhibit postharvest pathogen such as C. gloeosporioides, B. cinerea, and Alternaria alternata (Anusha et al., 2016; Gomi et al., 2003; Kishimoto et al., 2006). The lactone compounds, i.e., γ-decalactone and δ-decalactone, were enhanced in peach infected with B. cinerea and had been found to be associated with ripening aroma compounds (Li et al. 2015; Sánchez et al. 2013). The result showed the relationship between increment of volatile compounds and enhancement of ethylene production and JA concentration while peach was applied with PDJ application. The unsaturated fatty acids, 9- or 13-hydroperoxid, were synthesized by lipoygenase (LOX) pathway which is a main substrate for oxylipin pathway (Hatanaka, 1993; Wasternack and Kombrink, 2010). The oxylipin pathway is responsible for biosynthesis of methyl jasmonate and volatile compounds, which is a different pathway including AOS pathway and hydroperoxidase lyase (HPL) pathway, respectively (Ismanian et al., 2011). These results are in agreement with previous research finding that methyl jasmonate induced ethylene production although ethylene accelerated accumulation of volatile compounds (Wei et al., 2017).
Figure 1. Disease incident (A) and lesion diameter (B) on peaches treated with PDJ or without (untreated) and inoculated with *Botrytis cinerea* during storage at 25°C for 6 days.

Figure 2. Changes of ethylene production rate (A), ACC content (B), expression of *PpACS1* (C), *PpACO1* (D), *PpETR1* (E), and *PpCTR1* (F) gene on peaches treated with PDJ or without (untreated) and inoculated with *Botrytis cinerea* during storage at 25°C for 6 days.
Figure 3. Changes of jasmonic acid concentration (A) and expression of PpAOS gene (B) on peaches treated with PDJ or without (untreated) and inoculated with *Botrytis cinerea* during storage at 25°C for 6 days.

Figure 4. Change of abscisic acid concentrations on peaches treated with PDJ or without (untreated) and inoculated with *Botrytis cinerea* during storage at 25°C for 6 days.

Figure 5. Changes of volatile compounds, alcohol (A), aldehyde (B), ester (C), and lactone (D) concentration, on peach fruits treated with PDJ or without (control) and inoculated with *Botrytis cinerea* during storage at 25°C for 6 days.
CONCLUSIONS

The increment of endogenous JA and ethylene production as well as the expression levels of \( \text{PpACS1}, \text{PpCTRL}, \) and \( \text{PpAOS} \) gene inhibit the growth of \( \text{B. cinerea} \) in peach. The defense resistance system in peach against \( \text{B. cinerea} \) depends on the synergistic relationship between JA and ethylene. In addition, alcohol and aldehyde enhance by PDJ application also inhibit fungal development.

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