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EFFECTS OF POSTHARVEST PHYSICAL TREATMENTS SUCH AS UV-C OR LOW OXYGEN ON FRUIT QUALITY IN APPLE AND JAPANESE APRICOT FRUITS

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千葉大学学位申請論文

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Orathai Phonyiam

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ABSTRACT

Nowadays, fruit rot decay caused by pathogen fungi has more affected quality and shelf life storage in agricultural commodities. This research focused on examination of the effect of physical treatment on controlling postharvest fruit decay and on enhancing plant hormones and antioxidant activity in harvested apple and Japanese apricot fruit.

In experiment 1, effects of UV-C irradiation before or after inoculation with *Colletotrichum gloeosporioides* affected phytohormone and antioxidant in apple fruit were determined. The result showed that both UV-C irradiations inhibited lesion diameter. Total phenolic and flavonoid concentrations, 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity were induced by the UV-C irradiation, but malondialdehyde (MDA) concentrations were declined. Moreover, UV-C irradiation before inoculation (UV-BI) promoted the accumulation of jasmonic acid (JA) concentrations and *allene oxide synthase 1* (*MdAOS1)* compared to other treatments but UV-BI decreased ethylene production, 1-aminocyclo-propane-1-carboxylic acid (ACC) accumulation and down-regulated their related genes. Furthermore, UV-BI up-regulated pathogenesis-related (PR) genes; *MdPR1a, MdPR2,* and *MdPR4*. It is possible that UV-BI inhibited *C. gloeosporioides* compared to UV-C after inoculation (UV-AI) through the increase of endogenous JA concentrations and up-regulation of PR genes, thus the crosstalk between JA and ethylene may also play the roles in defense mechanism against pathogen infection.

In experiment 2, the effect of short anoxic treatment on change of abscisic acid (ABA), antioxidant and enzymatic antioxidant activities in Japanese apricot was investigated. This study aims to investigate the influence of short anoxic treatment on phytohormone and antioxidant activity in Japanese apricot fruit. Mature green Japanese apricot were exposed to nitrogen gas with flow rate of 200–250 mL min^{-1} for 6 h at 20°C and untreated fruit was used as a control treatment. Fruit were kept at 20°C (90–95 % RH). The result showed that anoxic treatment delayed the changes of fruit color. Moreover, DPPH scavenging activity, ferric reducing antioxidant power (FRAP), and total phenolic concentrations were induced by anoxic treatment but MDA accumulation was decreased. Additionally, Anoxic treatment promoted ABA

accumulation and peroxidase (POD) and delayed the decrease of superoxide dismutase (SOD) and catalase (CAT). In conclusion, anoxic treatment may delay the ripening in Japanese apricot through inducing ABA concentrations and antioxidant and enzymatic antioxidant activities.

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CHAPTER 1

General introduction

1.1 Background

Presently, the total global fruit production has been the continue rising to 867 million tons in 2018 (FAO, 2018). Among this increasing, postharvest decay is considered to a main impact in terms of the nutritional quality and particularly to reduce production systems of several produces. The major causes influencing postharvest quality of fruit include species or cultivar type, postharvest environment, maturity and ripening stage, harvesting method and handling, as well as postharvest treatment (Bekele, 2018). These factors may lead to the large scale of postharvest losses if not manage properly. In general, the losses of individual fruit due to postharvest decay can start in any process during postharvest handling. Among these losses, the necrotrophic fungi are largely discovered in wide hosts, resulted in reducing agricultural crop yields in plants (Pandey et al., 2016). In recent years, many studies observed that more than 50% of fresh crops in developing countries are lost before consumption due to pathological colonization (Feliziani and Romanazzi, 2013). It has been showed that the most important pathogens caused postharvest diseases including *Aspergillus*, *Botrytis*, *Fusarium*, *Penicillium*, *Rhizopus*, and other species (Nunes, 2011). With the controlling of pathogen infection, using of chemical treatment as a fungicide was carried out for commercially inhibit decay in crops (Yamaga et al., 2016; Mamarabadi et al., 2018). However, the frequent use of some fungicides has resulted in environmental contamination and risk contributed to human health, as well as the occurrence of a fungicide-resistant pathogen.

On the other hand, after harvest, climacteric fruits are then becoming into the ripen and senescence stage (Atkinson et al., 2011). In addition, there are different technique used to control postharvest decay and extend the shelf-life of crops include chemical treatment (Promkaew et al., 2019), biological treatment (Bazioli et al., 2019), and physical treatment (Duan et al., 2020). Ultraviolet C (UV-C)

radiation has been reported to suppress fruit fungal decay (Sripong et al., 2019), promoted the synthesis of phenolic substances (Sheng et al., 2018) as well as delayed postharvest senescence and maintained fruit quality during their postharvest storage. (González-Aguilar et al., 2010). On the other hand, postharvest physical treatment such as anoxic treatment was used in maintaining the quality and prolong storage life in peach (*Prunus persica*), tomato (*Lycopersicon esculentum Mill.*) and asparagus (*Asparagus officinalis*) (Fallik et al., 2003; Lara et al., 2011; Techavuthiporn and Boonyaritthongchai, 2016).

Nevertheless, even if there has been reported the effect of postharvest physical treatments on controlling postharvest decays and maintaining acceptability of fruit, but a few studies focused on the crosstalk between plant hormones, pathogenesis-related (PR) genes involving antioxidant activity.

1.2 Objective

1.2.1 To study the effect of UV-C radiation on controlling postharvest decay in satsuma mandarin and apple fruit.

1.2.2 To investigate the effect of UV-C irradiation before or after inoculation with *C. gloeosporioides* on inducing plant hormones, PR genes, and antioxidant activity in apple fruit

1.2.3 The study the effect of anoxic condition on the change in ABA concentrations and antioxidant system during ripening in Japanese apricot.

1.3 Expected outcome

1.3.1 Clarify the effect of UV-C irradiation on controlling postharvest decay in satsuma mandarin and apple fruit.

1.3.2 Clarify the effect of UV-C radiation before and after inoculation with *C. gloeosporioides* on inducing plant hormones, PR genes, and antioxidant activity in apple fruit

1.3.3 Clarify the effect of short anoxic treatment on ABA concentrations and antioxidant system during ripening in Japanese apricot.

CHAPTER 2

Literature review

2.1 Botany Description of apple

Around 85 million tons of apples are global produced and continue growing (KNOEMA, 2019). Apples are grown produce in the temperate climates and the major grown is *Malus* species. In 2019, 41 million tons of apple production were produced by China which is the largest producer followed by United States of America, Turkey, India, Iran and other countries (Table 2.1). According to the apple cultivars, Golden Delicious, Red Delicious, Gala, Fuji, Granny Smith, and Jonagold are the main economically in global commercial market (Spadaro et al., 2020). Apples are one of the most popular fruit for a good effective for health benefits because they are nutritious, loaded of vitamins, antioxidants, and immunity (Teixeira et al., 2020).

Table 2.1 Top of apple production country in 2019

Rank	**Country**	**Production quantity** (million Tons)
1	China	41,000,000
2	United States of America	4,820,690
3	Turkey	3,000,000
4	India	2,370,00
5	Iran	2,096,700
11	Japan	735,200

Source: KNOEMA, 2019

Fruit morphology and Characteristics

‘Tsugaru’ apple is classified as a *Mulas domestica* of the Rosaceae family. This cultivar is another popular apple cultivar in commercial market of Japan, available in between early summer to early winter. Tsugaru apple fruit has a moderate size with a round shape. Due to Tsugaru apples have a great sweet flavor, crisp, and juicy this Japanese apple is considered to one of a sweetest cultivar. The colors of Tsugaru apple are green at immature and become green-red base at a commercial mature stage (Azumino, 2020).

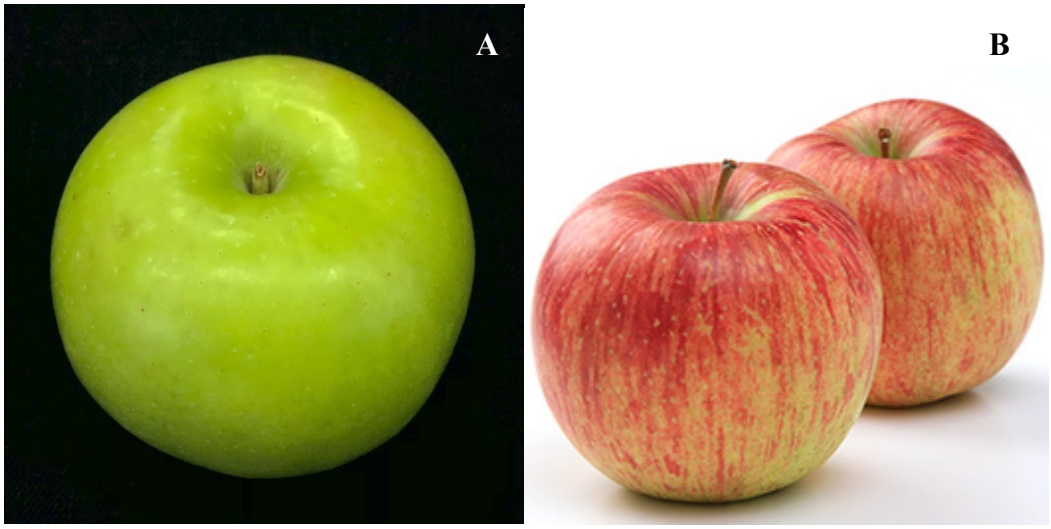

Figure 2.1 The morphology of ‘Tsugaru’ apple fruit (*Mulas domestica*) at immature (A) and at a commercial maturity stage (B).

2.3 Botany Description of apricot

Apricots are cultivated in the temperate regions, and were grouped as a stone fruit beside peach, cherry, plum, nectarines, mangoes, and lychees (Álvarez-Hernández et al., 2020). Today, the world production of apricot has increased from 2.8 million tons in 2000 to 3.8 million tons in 2018 (FAO, 2018). According to the production of apricots, Turkey can produce 750,000 tons which accounted as the biggest producer followed by Uzbekistan, Iran, Algeria, Italy, and other regions (Table 2.2). Apricots are contributed to the importance dietary sources of vitamins, phenolics, minerals, and carotenoids (Zhou et al., 2020b).

Table 2.2 Top 10 producers of apricot in 2018

Rank	**Country**	**Production quantity** (Tons)
1	Turkey	750,000
2	Uzbekistan	493,842
3	Iran	342,479
4	Algeria	242,243
5	Italy	229,020
9	Japan	112,400

Source: FAO, 2018

Fruit morphology and Characteristics

Japanese apricot (*Prunus mume*) is originated in China and has been introduced to other East Asian regions such as Japan, Taiwan, Korea, and Vietnam (Bailly, 2020). Maturity and ripening of Japanese apricot are associated with the changing of skin color from green at unripe and turn yellow at ripen stage, firmness, and flavor (Wang et al., 2019). However, the raw fresh fruits are too sour to consume due to its rich of citric acid. In addition, Japanese apricots are generally harvested at unripe stage, then were processed as a pickle, liquor, syrup, and juice (Tsuchida, et al., 2019).

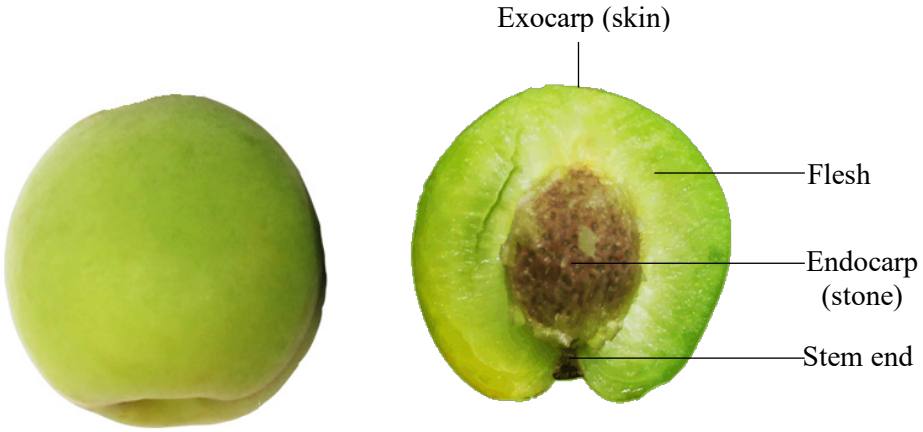

Figure 2.2 'Inazumi' Japanese apricot at maturity stage and anatomy.

2.3 Postharvest decays and pathogens in fruits

Nowadays, postharvest disease that cause decay to durable and perishable fruits and vegetables (Figure 2.3) were greatly spread out. Nabi et al. (2017) reported that 20–25% of postharvest crops were decayed due to pathogen infection during postharvest handling process. These decays and pathogens infected by microorganism involving bacteria, fungi, and viruses, contributing to substantial losses of crop productions. Pathogen infects fruit by wound through injuries occurring between pre and postharvest handling, transportation, and all stage to market and point of consumption (Narayanasamy, 2006). For example, postharvest diseases of citrus were not only occurred in the field during growing season but also when the pathogen infected before, during or after harvest (Smilanick et al., 2020). In addition, Gomes et al. (2015) shown that postharvest decay through the supply chain process is a highest losses segment in many crops, which accounted in a significantly economic loss. On the other hand, ripe fruits are more susceptible to the attack of several microorganisms as they are fully rich in nutrients, moisture, and easily injured that pathogen required for facilitating their infection (Rivka, 2001).

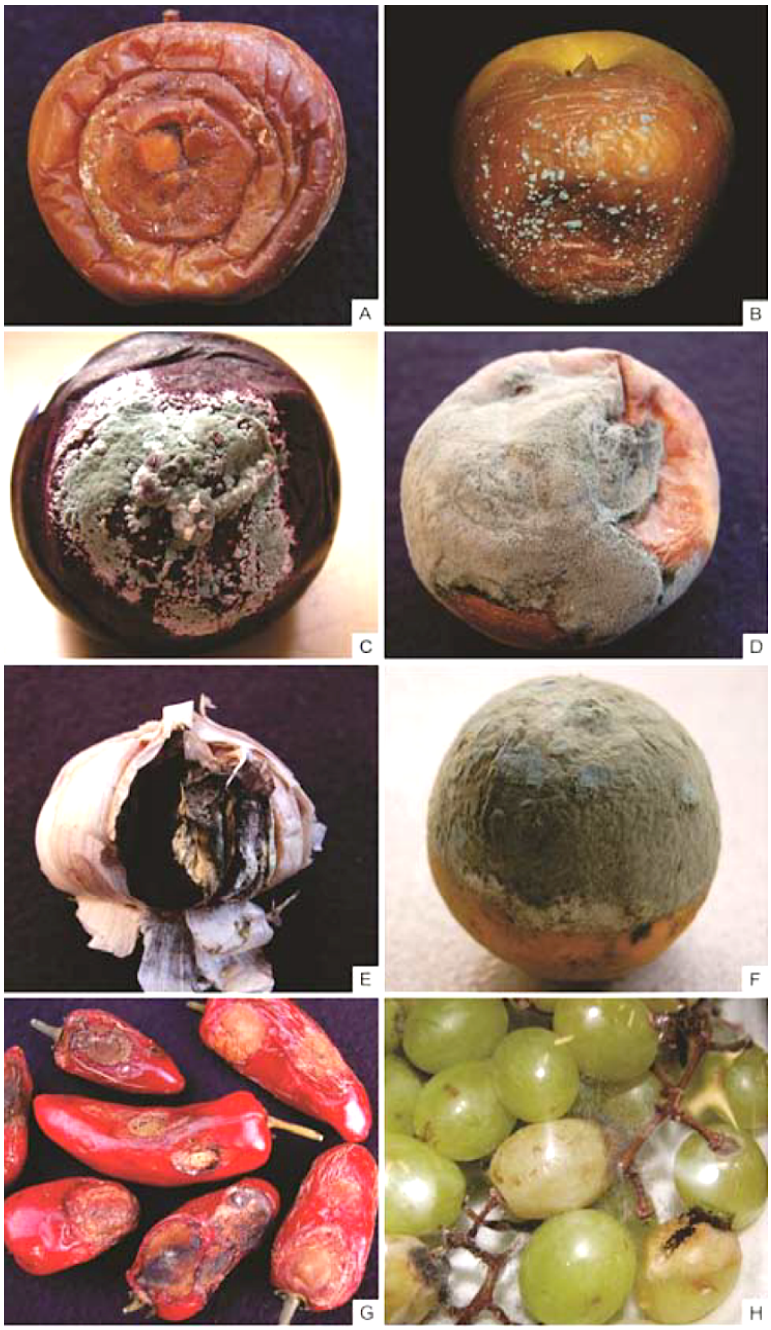

Figure 2.3 Examples of postharvest ddecays in crops. Bitter rot (A) and blue mold (B) of apple fruit, decay of nectarine fruit (C), brown rot of peach fruit (D), black mold of garlic (E), green mold of citrus fruit (F), anthracnose of pepper fruit (G), and decay of table grapes (H).

2.3.1 Postharvest decays and pathogens in apple fruit

Apples are also susceptible to infection caused by pathogenic fungi such as *B. cenerea, Colletotrichum acutatum*, and *C. gloesporioides* species, which affected storage shelf-life and quality of fruit (Suktawee et al., 2019). It has been reported that around 50% of apple decay was found during pre and postharvest stages (Ogawa et al., 1995). Bitter rot disease caused by *C. gloesporioides* has been reported as one of a serious postharvest decay in apple fruit, where largely borne since by the orchard management until market storability (Spadaro et al., 2020). In general, apples were originally infected by *C. gloesporioides* in the field and the decays were existed during postharvest period (Sutton, 1990). The symptoms of decay are showed the soft circular rotted spots on fruit surface. Soon after, the lesions are finally developed and produced cream to salmon spores (Figure 2.4) around the center of infected area (Sharma et al., 2017).

C. gloeosporioides is grouped to hemibiotrophic of infection phase, where necrotrophic stage was later occurred (Shama and Kulshrestha, 2015). The pathogen normally penetrates injured or senescence tissue and develop itself as a saprophyte which obtain nutrients from dead matter (Sharma et al., 2017). As the spread of pathogen, the spores of *C. gloeosporioides* were colonized on a young fruit by air, wind, rain, irrigation, as well as required high humidity and suitable temperature (Shama and Kulshrestha, 2015). After colonization, germination of *C. gloeosporioides* spores can perform a dormant structure until fruit gets injury or damage. Finally, the hyphae are formed and developed to necrotrophic phase to kill the host tissue (Much et al., 2008).

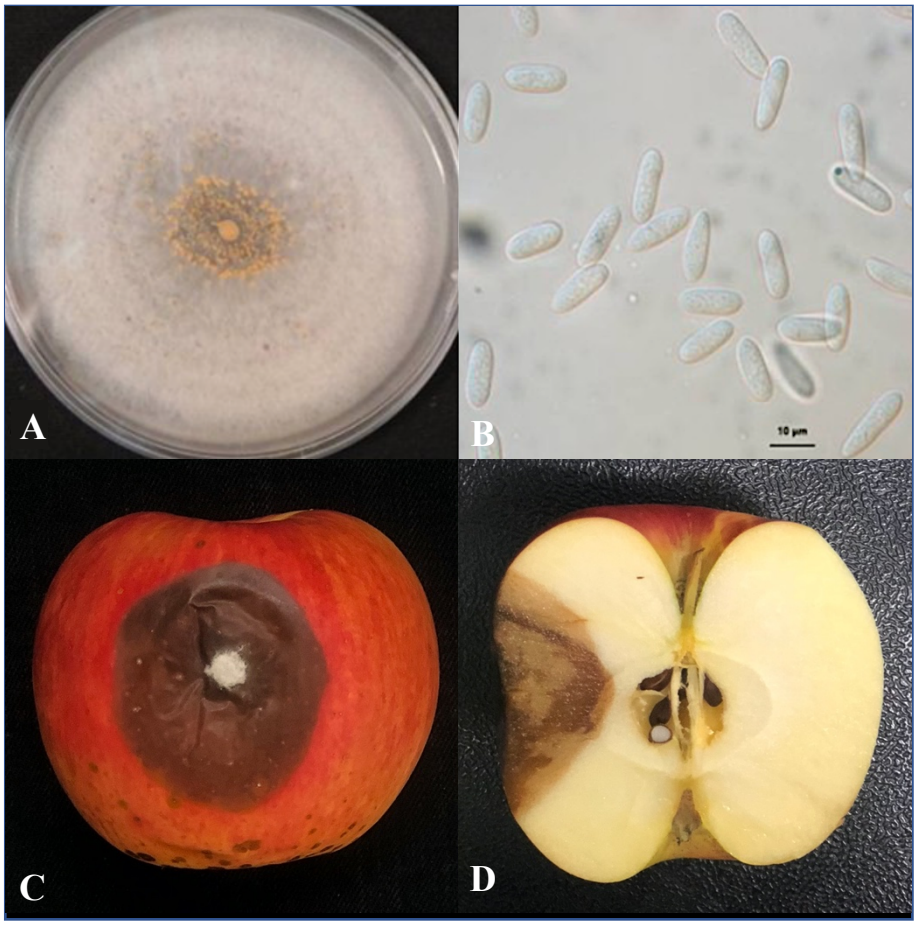

Figure 2.4 Morphological characteristics of *Colletotrichum gloesporioide*. One week of colonies cultured on potato dextrose agar (A), Conidia (B), decay development on 'Tsugaru' apple fruit at 15 days (C) and cross section of infected wound (D) of incubation at temperatures around 25°C.

2.3.3 Postharvest decays and quality management

Several ways of postharvest management strategies have been used and aimed at controlling and eliminating decay symptoms during postharvest handling process of fruits and vegetables (Palou, 2014). Among the management of postharvest decay, the treatments were mainly classified to chemical, biological, and physical treatment (Nabi et al., 2017).

Chemical treatment

Postharvest treatment with the synthetic chemicals has been broadly applied to control microorganism infection by use as a fungicide. Naqvi (2004) reported that postharvest losses of fungicide treated fruit can detect only 2–4%, while 15–30% of losses were occurred in fruit without treatment during 3 weeks after harvest. In citrus fruit, postharvest fungicide is one of the most common method to control green and blue molds (Smilanick et al., 2020). In Table 2.3, the ranking of fungicides and active ingredients for the inhibition of plant pathogens were showed.

It has been reported that benzimidazole and strobilurin fungicides are widely used to reduce *C. gloeosporioides* and they affect broad spectrum against a large type of pathogens in a variety of crops (Ishii, 2006). The fungicides effect pathogen by forming the binding site to attack microtubules and spindle microtubules, thus the nuclear division and hyphae development were stopped. Lui et al. (2005) demonstrated that 0.5 mM of benzimidazole treatment reduced the severity of blue mold cause by *P. expansum* in peach fruit. Moreover, application of bayleton fungicide has been reported for *C. gloeosporioides* management (Kimaru et al., 2018). However, besides the undesirable effect of chemicals treatment on environment and human health, useless chemical strategies are subjected to consider.

Table 2.3 Ranking of fungicide for the control of plant pathogens

Fungicide	Curative control	Protective control	Sporulation control	Other target diseases
Imazalil	Excellent	Good	Excellent	Alternaria rot Diplodia rot
Thiabendazole	Excellent	Fair	Good	Anthracnose Diplodia rot
Sodium orthophenylphenate	Excellent	Poor	Fair	-
Pyrimethanil	Excellent	Poor	Poor	-
Fludioxonil	Fair to good	Fair to good	Fair to poor	Diplodia rot
Propiconazole	Fair	Poor	Poor	Sour rot
Azoxystrobin	Poor	Fair	Fair to poor	-

Biological treatment

Nowadays, the global trend toward to minimize the use of fungicide for controlling postharvest decay and pathogens on crops, thus biological agents or antagonistic microorganisms were alternatively interested in term of safer and eco-friendly to human and environment (Nabi et al., 2017). In the mode of action of postharvest biological management, there are 4 major mechanisms of action; (1) antibiosis, (2) competition for limiting nutrients and space, (3) parasitism or direct interaction with the pathogen, and (4) inducing of host resistance (Droby et al., 2020). Previous studies demonstrated that many postharvest pathogens such as bacteria, yeasts, and fungi were greatly inhibited by microbial antagonists. As shown in Table 2.4, the example of different microbial antagonists for the control of postharvest diseases of fruits were showed (Pimenta et al., 2008; Nabi et al., 2017). In citrus fruit, the microbial antagonists such as *Bacillus pumilus and Pseudomonas fluorescens* were reported to control *P. digitatum* (Thonglem et al., 2007; Wang et al., 2018)

Table 2.4 Microbial antagonists used for the control of postharvest diseases of several fruits

Microbial antagonist	Disease and its causal agent	Fruit
Candida oleophila	Penicillium rot (*Penicillium expansum*)	Apple
Candida sake	Penicillium rot (*Penicillium expansum*), grey mold, Rhizopus rot	Apple, Pear
Cryptococcus laurentii	Bitter rot (*Glomerella cingulata*) Brown rot (*Monilinia fructicola*) Rhizopus rot (*Rhizopus stolonifer*) Gray mold (*Botrytis cinerea*)	Apple Cherry Peach Pear
Pantoea agglomerans	Penicillium rot (*Penicillium expansum*)	Apple
Meyerozyma caribbica	Anthracnose *(Colletotrichum gloeosporioides)*	Passion fruit
Saccharomycopsis schoenii	Green mold (*Penicillium digitatum)* *Blue mold (Penicillium italicum)*	Orange

Physical treatment

Reduction of fruit decay using physical postharvest treatments are interested worldwide. Several methods of physical postharvest management, such as heat treatments, low temperature storage, magnetic fields, radiation, as well as modified or controlled atmosphere (CA) storage have been considered as an effective means for the control of pathogens. For instance, heat treatment in form of hot water dip, rinsing, and brushing, vapor, hot air, and curing were effectively inactivated microorganism by inducing resistance chemical molecules in the host to destroy and kill the pathogen (Spadoni et al., 2015). Tahir et al. (2009) reported that 'Aroma' and 'Ingrid Marie' apple fruits exposed to 40°C in combination with CA storage inhibited bitter rot disease caused by *C. gloeosporioides*. Moreover, hot water dipping treatment at 56°C for 20 seconds significantly reduced spore

germination of *P. digitatum* (Strano et al., 2004). However, low temperature treatments can also be considered eliminating or delaying postharvest decays and pathogens in various fresh fruits and vegetables (Usall et al., 2016). On the other hand, the ionizing radiations involve ultrasound, radio, microwave, infrared, visible light, ultraviolet, X-rays, and gamma rays are a promising alternative means in directly destroying the nuclear DNA of microorganism, where pathogens were stopped their functions (Nabi et al., 2017). Therefore, physical methods may not only directly inhibit pathogen infection but also enhance biochemical substances such as antioxidants, plant hormones, and corresponded to up regulate the expression of pathogen-related (PR) genes, thus the pathogen defense mechanisms in plant were promoted.

Ultraviolet radiation

Application of ultraviolet (UV) radiations are frequently used as a specific radiation for the inhibiting of postharvest pathogens on broadly fresh agricultural crops. Regarding the wavelength of UV light, Table 2.5 showed the radiations have characterized into 3 spectrums: UV-A (wavelength between 330–400 nm), UV-B (wavelength between 330–270 nm), and UV-C (wavelength between 270–200 nm) (Koutchma, 2019). Yang et al. (2019) revealed the beneficial effects of UV-A/-B/-C by increasing antioxidant capacities in blueberry fruit.

Among the UV radiations, UV-C established the most efficient wavelength on direct capability affected pathogens and induced plant defense response. Presently, UV-C irradiation has been successfully experimented of decreasing decay and physiological disorder, maintaining quality, and delaying ripening in a variety of fruits and vegetable (Pombo et al., 2009; Park and Kim, 2015; Sripong et al., 2019). Mohamed et al. (2017) reported a potentially of UV-C radiation in suppressing severity of crown rot disease on banana (*Musa* AAA). In another study, Charles et al. (2008) showed that UV-C treatment at a dose of 3.7 kJ m^{-2} promoted fruit against pathogens to *B. cinerea* in tomato. Furthermore, application of UV-C was greatly effective in eliminating the severity of blue mold by inducing a defensive mechanism in the flavedo tissue of satsuma mandarin fruit (Yamaga et al., 2019).

Table 2.5 Type of electromagnetic spectrum and sources of spectrum

Spectrum type	Wavelength (nm)	Source
Gamma rays	< 0.1	Radioactive substances
X-rays	0.1-50	X-ray tubes
Ultraviolet (UV)	50-400	Sunlight, mercury vapour lamps
UV-A	330-400	Sunlight, mercury vapour lamps
UV-B	270-330	Sunlight, mercury vapour lamps
UV-C	200-270	Ozone atmosphere, welding torches, mercury lamps, and UV sanitizing bulbs
Visible	400-700	The sun, hot objects, fires, light bulbs, fluorescent tubes
Infrared	400-10	Hot bodies, the sun and fires
Microwave	$\sim 10^7$–10^8	Oscillating electrical charge in a microwave transmitter
Radio waves	$\sim 10^8$–10^{13}	Electrical oscillating circuit

Reducing of postharvest decay in fruits and vegetables stimulated by the UV-C irradiation is associated with enhancing defense mechanisms (Usall et al., 2016). In tomato, UV-C treatment promoted a formation of biochemical structural barriers and induced PR proteins, that was contributed to the induced resistance to *B. cinerea* (Charles et al., 2008; Charles et al., 2009). Also, the use of UV-C illumination significantly regulated biosynthesis of ethylene, associated to the delay ripening in tomato and mango (*Mangifera indica*) (Bu et al., 2013; Pristijono et al., 2018). Therefore, depending of UV-C dosage, the UV-C exposure condition required optimal intensity for each individual produce without causing undesirable and harmful effects.

Table 2.6 Effect of UV-C treatments on disease development of fresh commodity

Commodity	Investigated dose	Germicidal effects	Reference
Orange	7.92 kJ m^{-2}	Inactivate spores of *P. digitatum* under *in vivo* and *in vitro* study	Gündüz and Pazir (2013)
Pear	5 kJ m^{-2}	Inhibited the *Monilina Fructicola*	Li et al. (2010)
Strawberry	4.1 kJ m^{-2}	Reduced the growth of *Botrytis cinerea*	Pombo et al. (2011)
Tomato	8 kJ m^{-2}	Eliminated growth of *penicillium digitatum*	Obande et al. (2011)

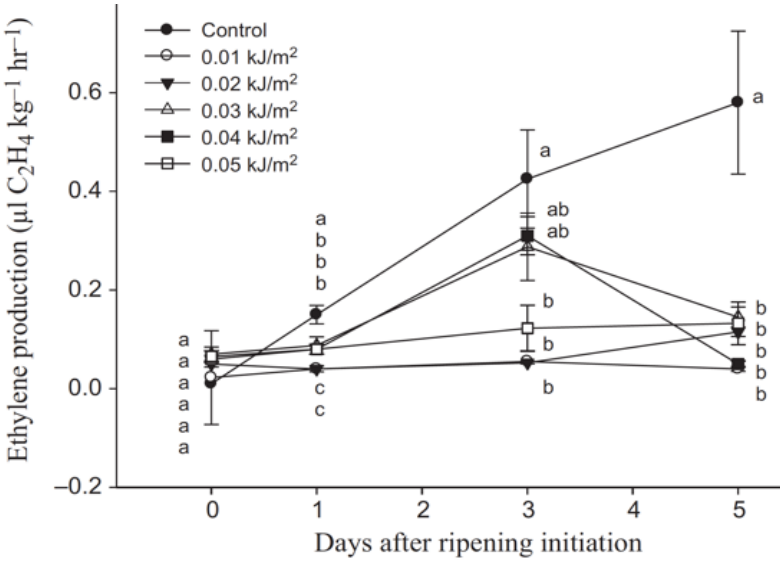

Figure 2.5 Effect of UV-C radiation on ethylene production rates of banana fruit during ripening days at 25°C (b) influenced by UV-C irradiations (Mohamed et al., 2017).

Anoxic treatment

Anoxic treatment is recently conducted as an alternative physical treatment by modification or reduction of oxygen composition around produce from normal air (20.95%) to be approximately 0.05% at period time. Due to it is a stressor, anoxic treatment can accelerate biochemical compounds such as plant hormones and antioxidant enzymes in commodities. For example, application of anoxic treatment can be greatly reduced undesirable physiological disorder in Chinese water chestnut *(Eleocharis tuberosa*) (You et al., 2012), maintained postharvest acceptability in kiwifruit (*Actinidia deliciosa*) (Song et al., 2009), and induced defense resistance to *B. cinerea* in tomato (Fallik et al., 2003). In case of pineapple, Phonyiam et al. (2016a) demonstrated that anoxic treatment for 24 hours promoted enzymatic and non-enzymatic antioxidants, thus leaded to inhibited physiological disorder (Figure 2.6B). Moreover, Yi et al. (2006) noted that the optimal postharvest anoxic treatment for regulating ethylene production and reducing respiration rate in 'Brazil' banana was at 9 hours prior to shelf life storage (Figure 2.6C-D). Thus, this practice has potential to be employed during postharvest management of fresh fruits and vegetables, where the optimal time of treatment should be appropriately integrated with cultivar dependent of commodity.

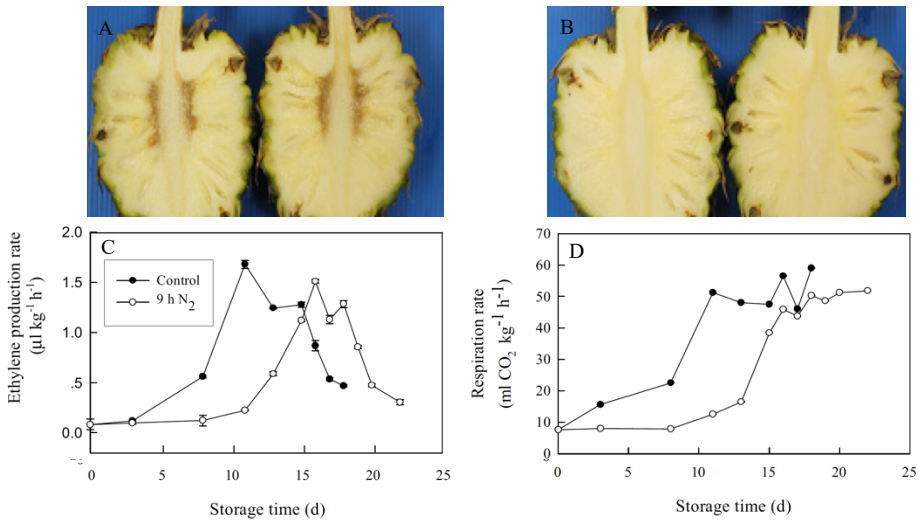

Figure 2.6 Effect of 24 hours anoxic treatment on internal browning appearance in pineapple stored at 10°C; untreated (A) and after anoxic treatment (B) and effect of anoxic treatment on changes in banana; ethylene production (C) and respiration (D).

CHAPTER 3

UV-C irradiation before or after inoculation with *Colletotrichum gloeosporioides* affected phytohormone and antioxidant in apple fruit (*Malus domestica* borkh.)

3.1 Preliminary study of UV-C radiation against green mold decay caused by *Penicilium digitatum* in satsuma mandarin fruit *(Citrus unshiu)*

In general, green mold decay caused by *P. digitatum* is the major contributor to postharvest losses in mandarin fruit. The green mold induces soft water-soak peel and white colony (Chen and Peng, 2016). The UV-C radiation has been reported to control microbial developments in fruits and vegetables (Mohamed et al., 2017; Sripong et al., 2019). For instance, irradiation of UV-C decreased gray mold development caused by *B. cinerea* in strawberry fruit (*Fragaria ananassa*) (Jin et al., 2017), regulated the expression level of gene which is related to lipid oxidation and cell wall degradation in tomato fruit (*Lycopersicon esculentum*) (Lui et al., 2011). Jasmonic acid (JA) plays a role of enhancing secondary metabolite production and defense against pathogens in plant (Shikano et al., 2018). Thus, the objective of this research aimed to study the effect of UV-C irradiation on cellular structure, JA, and green mold development in satsuma mandarin infected with *P. digitatum*.

3.1.2 Materials and method

3.1.2.1 Plant material and pathogen

'Aoshima unshu' satsuma mandarin (*Citrus unshiu*) fruit at commercial maturity stage were harvested from an orchard in Shizuoka prefecture, Japan. Prior to treatment, fruit were disinfected with 150 ppm of sodium hypochlorite (NaOCl) for 1 min, thoroughly rinsed in tap water and drained at 25°C overnight.

P. digitatum (Registration No. MAFF 242809) was used as a pathogenesis agent for green mold decay in this study. The spore suspension was pure cultured in potato dextrose agar (PDA) at room temperature for 7 d.

3.1.2.2 Inoculation of green mold and UV-C irradiation

Satsuma mandarin fruits were divided into 3 groups of ninety fruits per treatment. The inoculation was performed according to the method of Suktawee et al., 2019. Fruits were uniformity wounded (5 mm diameter × 3 mm deep) at the apteral and pedicel part of single fruit with a sterilized knife. The spore suspension (20 µL) of *P. digitatum* at the concentration of 1.2×10^6 spores mL^{-1} was then applied onto the wound.

To assess the effect of UV-C treatment, the irradiation dosages were varied at 0 (untreated control), 3 and 10 kJ m^{-2}. Non-irradiated fruit were set as untreated control group. The UV-C light was performed by six UV lamps with a peak at 253 nm (GL-20, Toshiba, Japan) from a distance of 25 cm. All fruits were kept at 25°C consisted with 95% relative humidity. The lesion diameter, cellular structure, and JA concentration were measured and sampled every day after treatment (DAT).

3.1.3 Results and suggestion

As shown in Figure 3.1, UV-C treatments strongly inhibited the lesion diameter of green mold in comparison with the untreated control, particularly at 6 DAT (10 kJ m^{-2} UV-C was 4.13 cm, 3 kJ m^{-2} UV-C was 4.93 cm, and the untreated control was 5.13 cm, respectively). Moreover, the UV-C treatment especially for 10 kJ m^{-2} significantly reduced mycelia development with maintained albedo structure at 5 DAT (Figure 3. 2). On the other hand, both 3 and 10 kJ m^{-2} UV-C significantly enhanced the increase of JA concentrations at 3 and 4 DAT, then were slightly decreased in all treatments at 5 DAT (Figure 3.3).

Therefore, our study suggests that UV-C radiation in which the optimal dosage between 3 and 10 kJ m^{-2} were greater maintain the integrity of cellular structure and enhanced jasmonic acid accumulation in satsuma mandarin fruit. These results might have inhibited postharvest decay in fresh fruits.

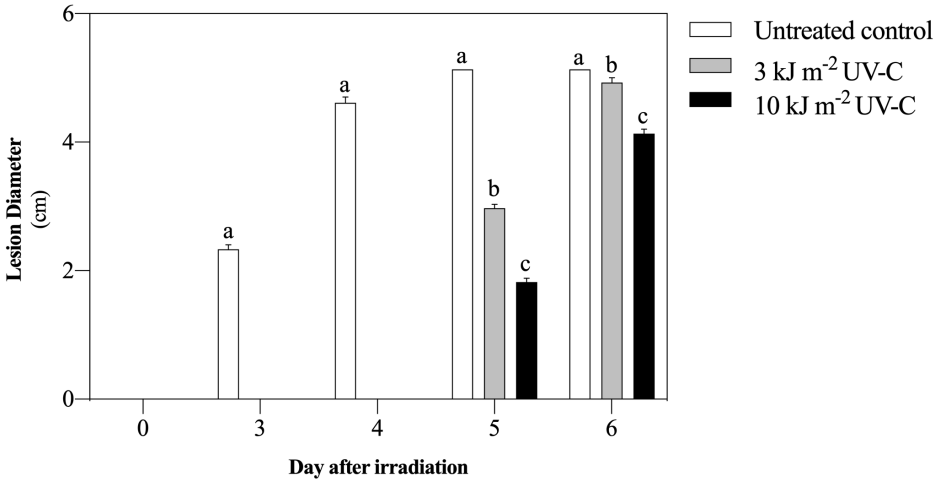

Figure 3.1 Effect of UV-C irradiation on inhibition of green mold development in satsuma mandarin fruit in untreated control, 3 and 10 kJ m^{-2} UV-C groups. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications.

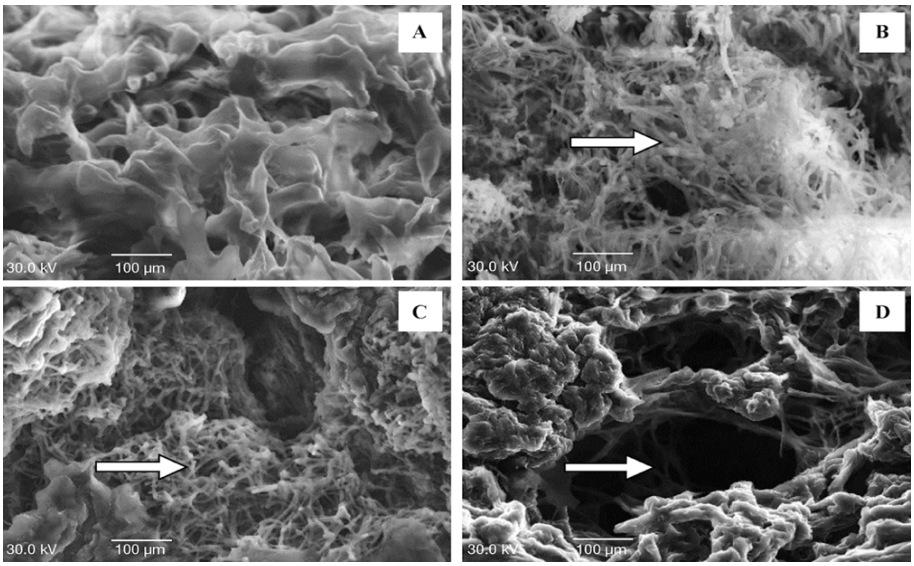

Figure 3.2 Effect of UV-C irradiation on cellular structure of albedo section in satsuma mandarin fruit; before treatment (A), untreated control (B), 3 kJ m^{-2} (C) and 10 kJ m^{-2} UV-C (D) groups. The arrows indicate conidiophores of *P. digitatum*.

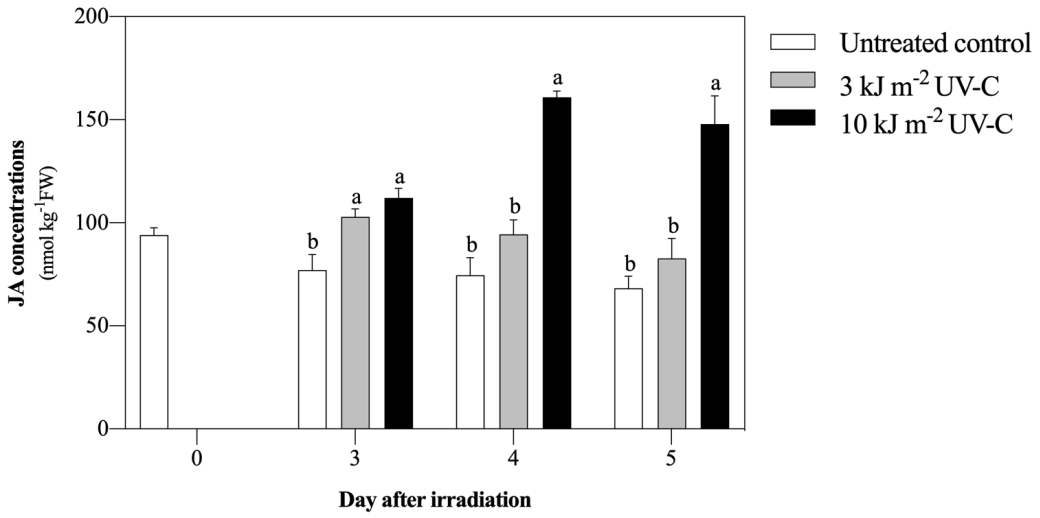

Figure 3.3 Effect of UV-C irradiation on jasmonic acid concentration in satsuma mandarin fruit in untreated control, 3 and 10 kJ m^{-2} UV-C groups. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications.

3.2 UV-C irradiation before or after inoculation with *Colletotrichum gloeosporioides* affected phytohormone and antioxidant in apple fruit (*Malus domestica* borkh.)

3.2.1 Introduction

The development of decay after harvest caused by *Colletotrichum gloeosporioides* contributes to major postharvest losses in apples. Wounding or infection with a pathogen affected the signaling molecules of plant hormones such as jasmonic acid (JA) and ethylene (Khan et al., 2014). In addition to induce defense response after pathogen attack, a rapid expression of local defense genes occurs in plant and subsequently resulting to induce in systemic resistance (ISR) through the hormone JA (Poveda, 2020). For example, endogenous JA and the ethylene production and ethylene-related genes; 1-aminocyclo-propane-1-carboxylic acid (ACC) *ACC synthase 1* (*MdACS1), ACC oxidase 1* (*MdACO1*), and the ethylene signal transduction genes; *ethylene receptor 1* (*MdETR1*), *ethylene response sensor 1* (*MdERS1*), and *constitutive triple response 1* (*MdCTR1*) were induced in apple fruit (*Malus domestica*) infected by *Botrytis cinerea* (Suktawee et al., 2019). JA is regarded as an important plant signal molecule in the defense mechanism against necrotrophic phytopathogens (Ali et al., 2018). JA application can enhance disease resistance against *Fusarium oxysporum* in *Solanum quitoense* plants (Avila et al., 2019) and can up-regulate the expression level of pathogenesis-related (PR) genes in apple leaves (Bai et al., 2013).

PR proteins are classified in various families such as antifungal proteins (PR1), *β*-1,3-glucanases (PR2), and chitinases (PR4), and they have properties related to plant defense against biotrophic and necrotrophic pathogens (Sels et al., 2008). For example, enhanced activity of *β*-1,3-glucanase and chitinase enzymes was found as part of the defense against *Alternaria alternata* in tomato fruit (*Solanum lycopersicum*) (Cota et al., 2007). Besides, plants exhibiting high disease resistance constitutively express PR genes and this has been reported in various pathosystems such as apple-*Venturia inaequalis*, potato-*Phytophthora infestans*, and tobacco-*Phytophthora nicotianae* (Jain and Khurana, 2018). Furthermore, the expression of

PR genes has been shown to be correlated with the activation of JA and ethylene, with up-regulation of their biosynthetic-related genes (Ali et al., 2018).

Ultraviolet (UV)-C irradiation is considered to be a low-cost non-chemical treatment against postharvest diseases (Sripong et al., 2019). It has been demonstrated that UV-C radiation at a dose of 5 kJ m^{-2} inhibited *Monilinia fruticola* growth in pear (*Pyrus bretschneideri* Rehd.) through induction of the defense-related enzymes activities (Li et al., 2010). Sheng et al. (2018) reported that UV-C up-regulated phenolic and flavonoid biosynthetic-related genes such as *phenylalanine ammonia-lyase* (*PAL*), *chalcone synthase*, and *flavanone 3-hydroxylase* in grapes (*Vitis vinifera*). Moreover, the application of UV-C enhanced the signaling and biosynthesis of JA in *Arabidopsis thaliana* (Xu et al., 2016). In addition, Gangopadhyay et al. (2016) reported that UV-C priming radiation reduced the root rot disease caused by *Macrophomina phaseolina* in Indian Coleus (*Coleus forskohlii*). Therefore, UV-C irradiation may induce antioxidants, higher JA concentrations, and PR genes, and can result in the inhibition of postharvest disease. However, there are few reports on the effect of applying UV-C irradiation before pathogen infection on postharvest disease. This study investigated the effects of UV-C irradiation on *C. gloeosporioides* infection, PR genes, ethylene, and JA.

3.2.2 Materials and methods

3.2.2.1 Plant material

Apple fruit 'Tsugaru' were harvested at a pre-climacteric stage (5 months after full bloom) from the experimental field at Chiba University located at 36°N latitude, 139°E longitude and 747 m altitude. After harvest, apples were thoroughly cleaned in tap water and disinfected with 0.002 M of sodium hypochlorite (NaOCl) for 1 min. Fruit were then rinsed with distilled water and dried at 25°C overnight.

3.2.2.2 Pathogen inoculation and UV-C treatment

Colletotrichum gloeosporioides (Registration No. MAFF 239927) spore suspension at a concentration of 6×10^5 spores mL^{-1} was cultured in potato dextrose agar (PDA) at room temperature for one week. The inoculation was performed

according to Suktawee et al. (2019). A wound (5 mm diameter × 3 mm deep) was made on each apple fruit. The *C. gloeosporioides* mycelia agar block (6 mm diameter) was put on each wound.

The UV-C radiation lasting 1.27 min for the dose of 3 kJ m^{-2} was designated according to our preliminary study on satsuma mandarin fruit. Apples were divided into three groups with 60 fruit each. The UV-C treatments were performed as followed; UV-C before inoculation (UV-BI): UV-C irradiation was performed before inoculation of fruit with *C. gloeosporioides*. UV-C after inoculation (UV-AI) group: UV-C irradiation was performed after inoculation of fruit with *C. gloeosporioides*. An Inoculated fruit without exposing to UV-C light was the untreated control. Following the UV-C treatment, the treated apples were kept at 25°C with 95% relative humidity for 16 days. The peels from 15 apples in each treatment were sampled at 0, 4, 12, and 16 days after treatment (DAT), the samples were immediately collected by liquid nitrogen and transferred to a freezer at –80°C.

3.2.2.3 Determination of lesion diameter and measurement of fruit firmness

The lesion diameter (15 fruits per treatment) was measured using caliper after inoculation every day. Fifteen apples per each treatment were analyzed by texture analyzer with a cylindrical probe (2 mm diameter) and the speed of 100 mm/min (Rheo Meter CR-100, Sun Sciencetific, Japan). The measurement was performed after peeling.

3.2.2.4 Evaluation of total phenolic and total flavonoid concentrations and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant capacity

Three grams of fresh sample (3 replications of 15 fruits per treatment) were extracted with 20 mL of 80% methanol and centrifuged at 15,000 g for 10 min at 4°C. Total phenolic concentrations of peel were evaluated using Folin-Ciocalteu's method according to Sheng et al. (2018). The phenolic concentrations were monitored at 765 nm by spectrophotometer (U-2910, Hitachi, Japan). Total flavonoid concentration was evaluated according to Sheng et al. (2018) with modifications. The peel extract (250 µL) was added into 1 mL of deionized water, 75 µL of 5% sodium nitrite ($NaNO_2$). Then, 75 µL of 10% aluminium chloride

($AlCl_3$) was added to the mixture. After 5 min, 1 mL of 4% sodium hydroxide (NaOH) was added. This reaction mixture was measured at 510 nm. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant capacity was determined according to Phonyiam et al. (2016a) with slight modifications. A 500 µL of sample extract was mixed with 1.5 mL of 0.1 mM DPPH-methanolic solution. The decrease of absorbance was monitored at 517 nm after incubation in dark for 30 min.

3.2.2.5 Measurement of malondialdehyde (MDA) concentration

Lipid peroxidation was examined by the production of malondialdehyde (MDA) according to Phonyiam et al., (2016a) with modifications. One gram of fresh sample (3 replications of 15 fruits per treatment) was homogenized with 7 mL of 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 10 min at 4°C. Thereafter, the supernatant of 500 µL was mixed with 1.5 mL of 0.5% thiobarbituric acid (TBA) in 10% TCA and thereafter incubated in 85°C for 30 min. The cooled mixture was measured at 532 nm by spectrophotometer. The non-specific turbidity of MDA was determined at the absorbance of 600 nm.

3.2.2.6 Measurement of JA concentration

Endogenous JA concentration was determined according to Segarra et al. (2006). The 0.5 gram of fresh sample (3 replications of 15 fruits per treatment) was homogenized with 5 mL of MeOH–H_2O–CH_3COOH (90:9:1, v/v/v) and 100 µL of 10^{-4} M of ibuprofen which was used as the internal standard. The homogenate was separated after centrifugation at 15,000 g, 10 min at 4°C. The supernatant was dried under 37°C and filtered with 1 mL of methanol absolute HPLC by a Omnipore 0.45 µm filter (Millipore, Bedford, USA). Analysis and quantification of extract containing JA was carried out under liquid chromatograph mass spectrometer (LC-MS) (LCMS-2010EV; Shimadzu, Kyoto, Japan). The 5 µL of injected volume was monitored through RP-18 octadecylsilyl column (5 µm, 2 mm I.D. × 150 mm; Kanto Chemical, Tokyo, Japan). JA concentration was calculated from the ratio of peak area for *m/z* 206/161 (ibuprofen).

3.2.2.7 Determination of ethylene and ACC concentration

The ethylene production was examined according to Suktawee et al. (2019). Apple fruits (3 replications of 15 fruits per treatment) was sealed in a plastic jar of 1.7 L and incubated at 25°C for an hour. A 1 mL gas sample was analyzed by gas chromatography (GC-2014; Shimadzu, Kyoto, Japan). ACC concentrations were evaluated based on Suktawee et al. (2019) with slight modifications. One gram of peel (3 replications of 15 fruits per treatment) was extracted with 10 mL of 0.1 M HCl. The reaction mixture which contained 2 mL crude extract, 0.2 mL of 0.1M $HgCl_2$ and 0.2 mL of 5% NaOCl was rapidly mixed and monitored by gas chromatography.

3.2.2.8 Measurement of total RNA extraction and quantitively RT-PCR analysis

The total RNA was extracted from the apple peel. A 500 mg of peel (3 replications of 15 fruits per treatment) was extracted by the modified cetyl trimethylammonium bromide (CTAB) method (Henderson and Hammond, 2013). The purified RNA quality and integrity was determined by UV spectrophotometer (SmartSpec Plus, BIO RAD, USA) in the ratio of A260/A280 and agarose gel electrophoresis, respectively. The first strand cDNA was synthesized by the reversion of RNA using Revertra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) with the final concentration of 500 ng 50 μL^{-1} was used as the reaction mix for quantitative RT-PCR analysis. The gene specific primers were used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) examination (Table 3.1). The analysis was performed by qRT-PCR (StepOnePlus™; Applied Biosystems, Foster City, CA, USA) using KAPA SYBR FAST qPCR Master Mix ABI Prism (Kapa Biosystems, Wilmington, MA, USA). The expression of each gene was examined by the quantitation comparative cycle threshold (ΔΔCT) to normalized to the CT values of *MdSAND*, *MdUBQ and MdHISH3*.

3.2.2.9 Statistical analysis

All results were shown as the mean ± standard error (SE). The statistical analysis of variance was performed by least significant difference (LSD) test at $P \leq 0.05$ (SPSS 16; IBM Corp., Armonk, NY).

Table 3.1 Primer sequences used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analyses

Gene name	Primer sequences (5`-3`)	References/ Accession No.
MdACS1	(F) TCACCTCAATATATCTCCTGGA (R) CCTCAGGGACGTTGTAATAC	U89156
MdACO1	(F) GTTCTACAACCCAGGCAACG (R) TCTCAGAGCTCAGGCAGTTG	NM001294118
MdETR1	(F) GGGGCGACTCATCTTATCAA (R) CACCACCGCAGTTAAAACCT	AF032448
MdERS1	(F) CAGATGAGCTGCTGGTGAAA (R) TATGAGCTCCAAGGGAATGG	AY083169
MdCTR1	(F) ACTTCTTGGATCCAGTGCCG (R) GACAGACAGCCATTCACCCA	DQ847149
MdAOS1	(F) GGGAGAAGCTGTTGAAGCAC (R) TCCAGCACACTGTTTGTTCC	XM008366758
MdPR1a	(F) GCTCAGCCGTAATACAATCCTCTC (R) TACCCCCACTACTGCACCTCACT	DQ318212
MdPR2	(F) CTTCACAGTCACCATCTTCAACA (R) GGTGCACCAGCTTTTTCAA	AY548364
MdPR4	(F) ATACCACCTCTACAATCCACA (R) GTCCAAGTCCAATCCTCC	XM008372483
MdSAND	(F) CCCAGGACTTTGAGCTTTATGC (R) TATCACCATGAAAAGGGGCTTG	Mimida et al. (2015)
MdUBQ	(F) CTCCGTGGTGGTTTTTAAGT (R) GGAGGCAGAAACAGTACCAT	U74358
MdHISH3	(F) GTCAAGAAGCCCCACAGATAC (R) CTGGAAACGCAGATCAGTCTTG	AY347801

3.2.3 Results

3.2.3.1 Lesion diameter and fruit firmness

UV-C irradiation significantly reduced the lesion diameter compared to the untreated control throughout the storage period with a profound effect at 16 DAT (UV-BI was 4.51± 0.15 cm, UV-AI was 4.49±0.11cm and untreated control was 5.04±0.08 cm, respectively) (Figure 3.4A). UV-C irradiation significantly delayed the decrease of firmness in apple fruit at 12 and 16 DAT compared with the untreated control (Figure 3.4B). The soluble solid content (SSC) and titratable acidity (TA) did not show any significant difference in each treatment (data not presented).

3.2.3.2 Total phenolic and total flavonoid concentrations, DPPH antioxidant capacity and MDA concentrations

The total phenolic concentrations were generally increased by UV-BI throughout storage period compared with other treatments (Figure 3.4C). UV-BI significantly enhanced the total flavonoid concentration at 4 and 16 DAT compared with other groups (Figure 3.4D). In the UV-AI, total flavonoid concentration was highest increased at 12 DAT and decreased at 16 DAT. UV-BI irradiation significantly increased DPPH antioxidant capacity compared to the untreated control (Figure 3.4E). The DPPH antioxidant capacity in UV-AI at 12 DAT was higher than that in the untreated control but decreased at 16 DAT. The MDA concentration was significantly decreased by both UV-BI irradiation and UV-AI at 4 DAT. There were no significant differences at 16 DAT between each treatment (Figure 3.4F).

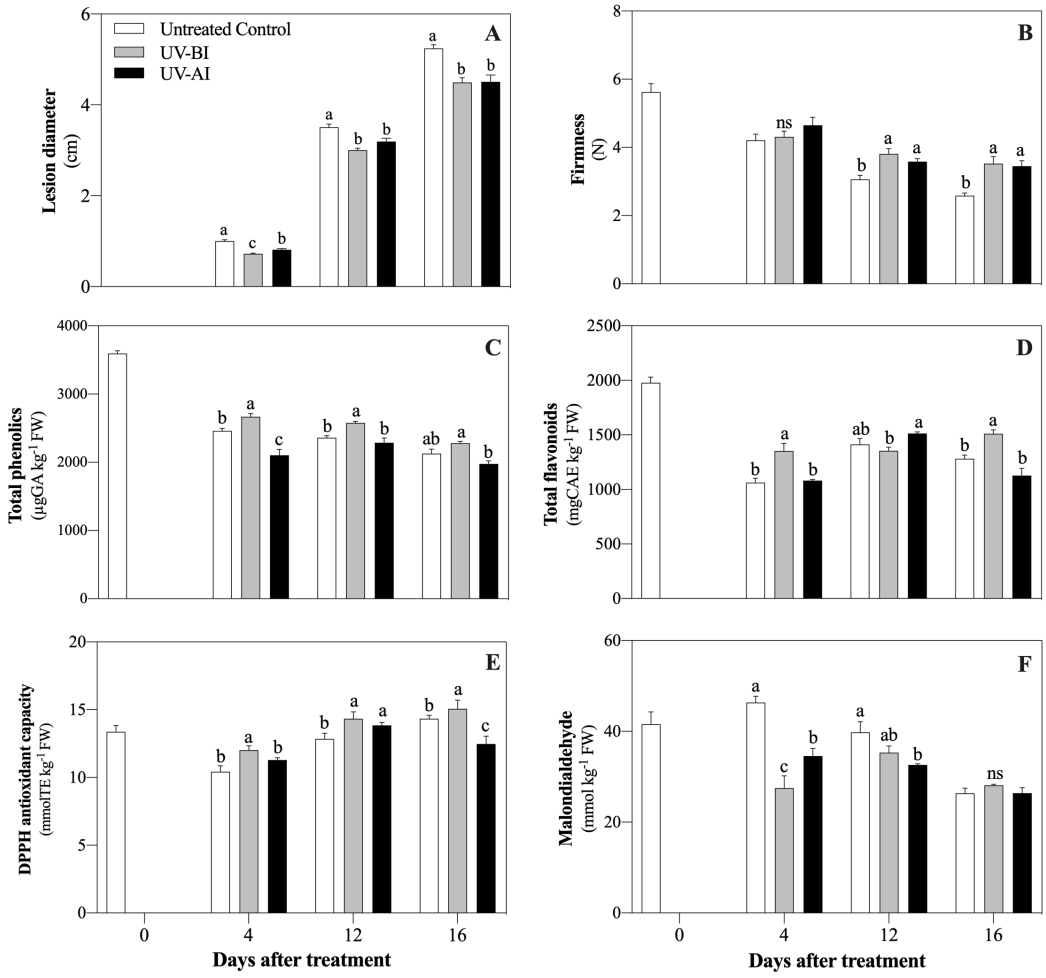

Figure 3.4 Effect of UV-C irradiation on lesion diameters (A), fruit firmness (B), total phenolic (C), total flavonoid (D), DPPH antioxidant capacity (E) and malondialdehyde (F) in untreated control, UV-BI and UV-AI treatments. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications of 15 fruits per each treatment, "ns" indicates no significant difference.

3.2.3.3 JA concentrations and expression of *MdAOS1*

The UV-BI significantly increased JA concentrations at 4, 12 and 16 DAT compared with UV-C (AI) and untreated control groups (Figure 3.5A). UV-AI also showed higher concentrations than the untreated control. The expression of *MdAOS1* in both UV-BI and UV-AI was significantly up-regulated compared to the untreated control at 4, 12 and 16 DAT. (Figure 3.5B).

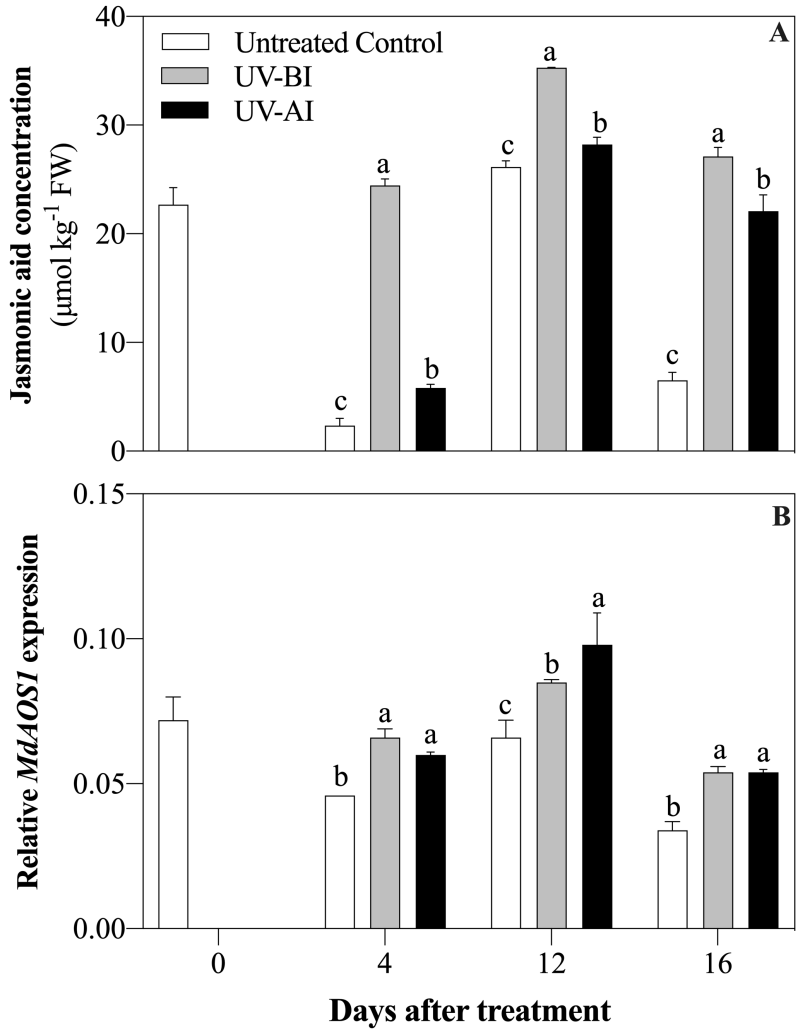

Figure 3.5 Effect of UV-C irradiation on jasmonic acid (A) and expression of *MdAOS1* (B) in untreated control, UV-BI and UV-AI treatments. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications of 15 fruits per each treatment, "ns" indicates no significant difference.

3.2.3.4 Ethylene production, ACC concentration and ethylene related genes

In general, ethylene production in the untreated control was higher than that of UV-C irradiation during storage (Figure 3.6A). Ethylene production in the untreated control was highest at 16 DAT. The ACC concentration in the untreated control was highest at 12 DAT (Figure 3.6B). The UV-BI and UV-AI generally down-regulated the expression levels of *MdACS1* compared with those of the untreated control (Figure 3.6C). The *MdACO1* expression levels in UV-BI and UV-AI decreased significantly at 4 and 16 DAT (Figure 3.6D). The expression levels of *MdETR1*, *MdERS1* and *MdCTR1* by both UV-BI and UV-AI were significantly lower than those in the untreated control (Figure 3.6E–G).

3.2.3.5 PR genes

The expression levels of *MdPR1a* were significantly increased by UV-BI and UV-AI irradiation compared to the untreated control at 4 and 12 DAT (Figure 3.7A). In general, *MdPR2* in each treatment gradually decreased during storage (Figure 3.7B). The expression of *MdPR2* was up-regulated by the UV-C (BI) at 4 and 12 DAT compared to that of untreated control group. The expressions of *MdPR4* were significantly up-regulated by UV-AI treatment (Figure 3.7C).

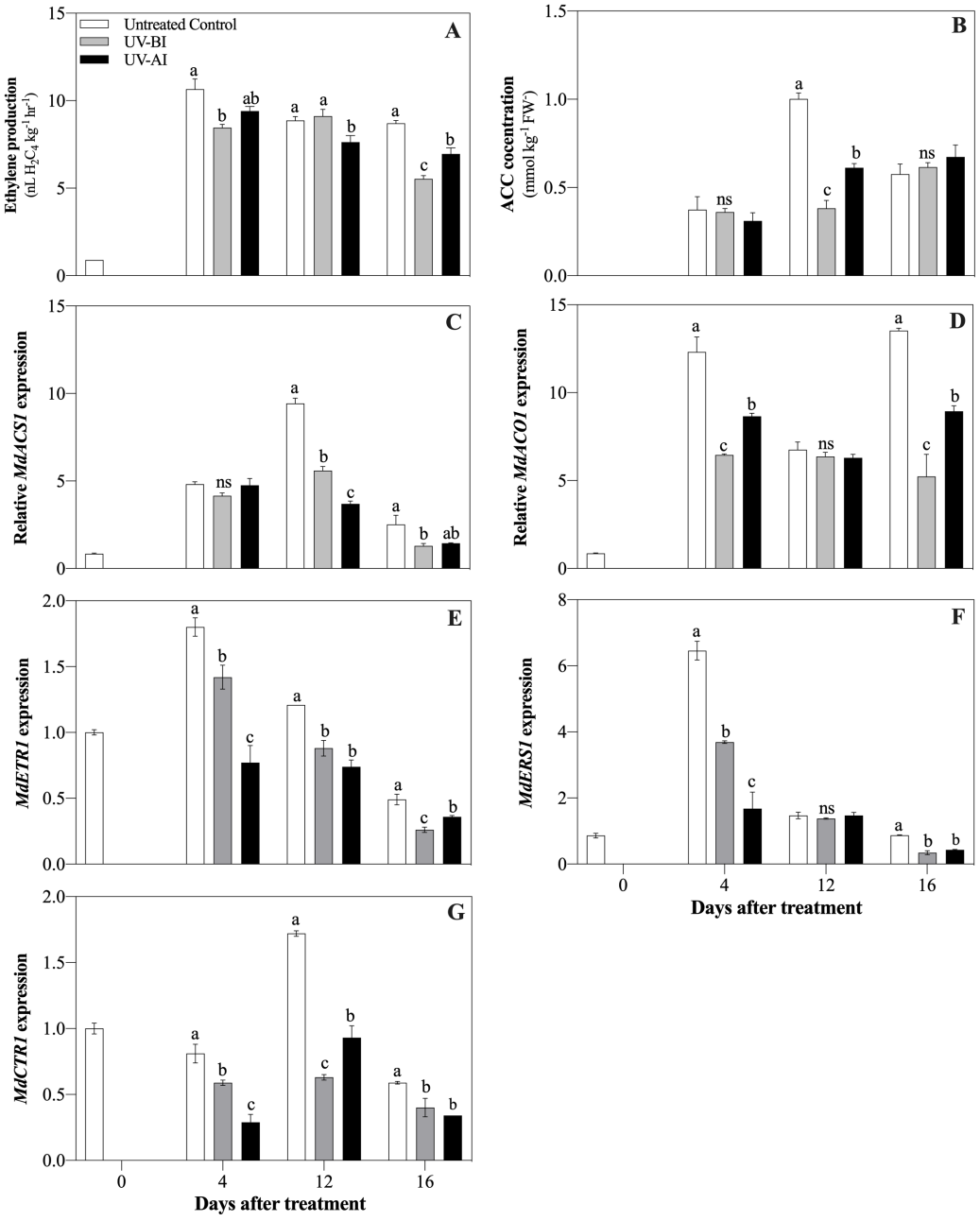

Figure 3.6 Effect of UV-C irradiation on ethylene production (A), ACC concentration (B), expressions of *MdACS1* (C), *MdACO1* (D), *MdETR1* (E), *MdERS1* (F), and *MdCTR1* (G) in untreated control, UV-BI and UV-AI treatments. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications of 15 fruits per each treatment, "ns" indicates no significant difference.

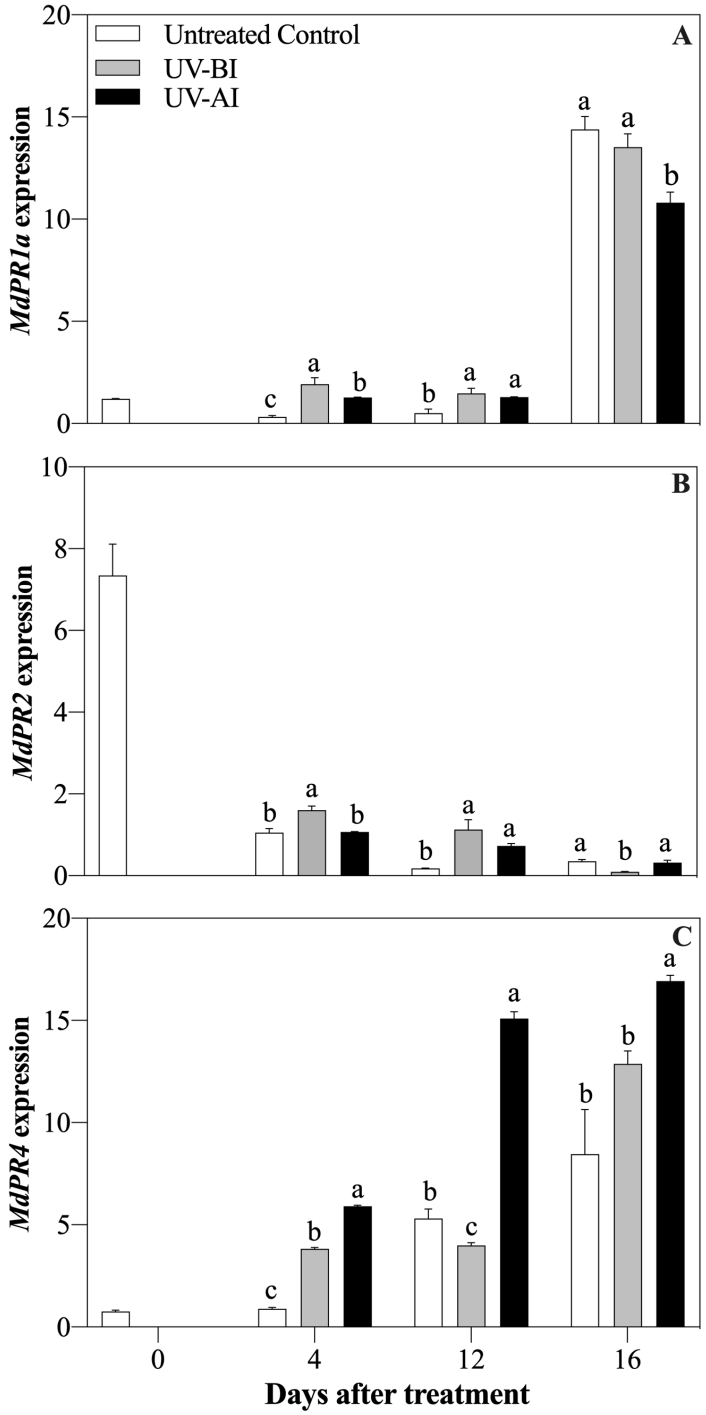

Figure 3.7 Effect of UV-C irradiation on expressions of *MdPR1a* (A), *MdPR2* (B), *and MdPR4* (C) in untreated control, UV-BI and UV-AI treatments. The different letters indicate significant differences at $P \leq 0.05$ by LSD test at each day with the mean ± SE of three replications of 15 fruits per each treatment, "ns" indicates no significant difference.

3.2.3 Discussion

UV-C irradiation limited the proliferation of foodborne pathogens including *Escherichia coli, Listeria innocua or Salmonella enterica* in apples (Graça et al., 2013) and inhibited decay caused by *Botrytis cinerea* in strawberry (*Fragaria ananassa)* (Jin et al., 2017). In our study, both UV-BI and UV-AI at a dose of 3 kJ m^{-2} decreased the lesion diameter of *C. gloeosporioides* in apple fruit. This is comparable to earlier researches of Charles et al. (2009) and Sripong et al. (2019) who demonstrated that UV-C could induce disease resistance through increase of plant defense related-enzymes like PAL, peroxidase (POD) and chitinase involved in activating of PR protein such as PR2 in mangosteen (*Garcinia mangostana* L.) and tomato. On the other hand, Severo et al. (2015) indicated that UV-C delayed fruit softening might be due to the decrease in expression of firmness-associated genes such as *pectate lyase a* (*PLa*), *PLb* and *PLc* in strawberry. These results agree to our study that both UV-BI and UV-AI significantly delayed softening in infected apple fruit. Moreover, Kesari et al. (2010) reported that a decrease of fruit firmness could promote the activity of *PR1a*.

UV-C radiation stimulated total phenolic and flavonoid concentrations in wheat (*Triticum aestivum)* and lettuce (*Lactuca sativa*) (Ouhibi et al., 2014; Badridze et al., 2015). Our study showed that UV-BI increased total phenolic and flavonoid concentrations compared to UV-AI and the untreated control in apple infected with *C. gloeosporioides*. These results suggest that the increase in bioactive substances such as phenolic and flavonoid were immediately induced after irradiation with UV-C. UV-C irradiation may enhance the synthesis of secondary metabolites associated with the inhibition of pathogen infection. Furthermore, our study also revealed that UV-BI increased DPPH antioxidant activity. Jin et al. (2017) confirmed that UV-C treatment promoted antioxidant enzyme activities and inhibited of gray mold development in strawberry fruit. Li et al. (2019) and Xu et al. (2019) showed phenolic, flavonoid and overall antioxidant activities were increased by stimulation of their key enzymes; PAL, cinnamate-4-hydroxylase (C4H) and 4-coumarate coenzyme A ligase (4CL) after exposure to UV-C radiation. Our results suggest that UV-BI treatment induced disease

resistance prior to infection with *C. gloeosporioides* through enhanced accumulation of phenolic and flavonoid concentrations as well as increased DPPH antioxidant activity.

Malondialdehyde (MDA) is part of the secondary end-product of lipid peroxidation which results in membrane structure damage (He et al., 2019). The previous research showed that UV-C irradiation suppressed the production of MDA and lipoxygenase activity which occurs lipid membrane degradation and resulted in control of crown rot disease in banana (*Musa* AAA) (Pongprasert et al., 2011). In our study, UV-C treatment decreased MDA concentrations. Therefore, lower MDA production may preserve integrity of cell membrane structure and subsequently promote resistance against the infection of *C. gloeosporioides*.

It has been shown that JA promoted plant resistance against pathogens by inducing antimicrobial compounds and activating plant signaling molecules (Shigenaga and Argueso, 2016). Previous study demonstrated that UV-C treatment induced JA accumulation in strawberry fruit during storage (Xu et al., 2019). Moreover, Xu et al. (2017) reported that JA affected the antioxidant activity in plant. These results agree with our results which showed that apple fruit exposed to UV-BI significantly promoted endogenous JA, total phenolic and flavonoid concentrations as well as antioxidant activity and PR gene expression. Our result suggests that increase of JA accumulation may have influenced high antioxidant activity involved in the up-regulation of PR genes. The *allene oxide synthase (AOS)* is a dominant key gene for the biosynthesis of JA (Garrido-Bigotes et al., 2018) and our study found that the expression of *MdAOS1* was stimulated in UV-C irradiation. JA concentrations may have increased throgh the increase of *MdAOS1* expression.

Ethylene plays a role in fruit ripening and in plant defense against pathogens. For example, it has been reported that UV-C radiation suppressed ethylene biosynthesis in tomato, banana and mango (*Mangifera indica*) (Bu et al., 2013; Pristijono et al., 2018). Our result also showed that UV-BI decreased the ethylene production as well as decreased ACC concentrations. Moreover, UV-BI also down-regulated transcript levels of ethylene biosynthesis genes (*MdACS1 and MdACO1)* and signal transduction genes (*MdETR1*, *MdERS1* and *MdCTR1*). These results

may be associated with the JA accumulation. Sun et al. (2016) reported that both ethylene and JA signaling play the role in regulating necrotrophic pathogen. For example, the interaction between ethylene and JA revealed that *JASMONATE ZIM DOMAIN PROTEIN* (JAZ) down-regulated the transcripts of *ETHYLENE INSENSITIVE 3* (EIN3) and EIN3-like 1 (EIL1), resulted in stimulation of defense protein such as *PLANT DEFENSIN* 1.2 (Yang et al., 2019). Therefore, this suggests that the reduction of ethylene biosynthesis is required during JA signaling to enhance plant defense against *C. gloeosporioides* by UV-BI.

The accumulation of PR proteins is frequently used as a defensive marker against phytopathogens such as viruses, bacteria, and fungi (Sels et al., 2008). The induction of the *PR1* and *PR2* genes was considered to activate phytohormones such as JA and ethylene (Ali et al., 2017). For example, enhanced expression of *MpNPR1* promoted pathogen defense against apple diseases caused by *Erwinia amylovora* (Malnoy et al., 2008). Furthermore, Sarowar et al. (2005) also reported that high resistance to oomycete and bacterial pathogens in tobacco (*Nicotiana tabacum)* were stimulated by the overexpression of *CABPR1.* Besides, *PR2* family genes played a role in plant defense through the deconstruction of the cell walls of pathogenic fungi (Balasubramanian et al., 2012). Our results also showed that the expressions of *MdPR1a and MdPR2* were up-regulated by UV-C irradiation. Jin et al. (2017) reported that UV-C treatment prior to *B. cinerea* inoculation induced defense-related genes such as *cinnamoyl CoA reductase-1 allele, chitinase, polyphenol oxidase,* and *PAL6* in strawberry fruit. *PR4* is categorized as a chitinase enzyme, which particularly function in the plant defense response through hydrolyzing chitin-containing pathogen cell walls (Hamid et al., 2013). Our results showed that the expression level of *MdPR4* was up-regulated by UV-C application. These results agree with those of Bai et al. (2013), who demonstrated that an up-regulated expression of *MdPR4* may be related to ribonuclease activity that is associated with antifungal mechanism.

3.2.5 Conclusion

The UV-BI was more efficiency on suppression of *C. gloeosporioides* than UV-AI through increase of JA concentrations and expression of *MdAOS1*, decrease of ethylene production and down-regulation of *MdACS1, MdACO1, MdETR1, MdERS1 and MdCTR1* expressions, as well as up-regulation of PR genes. In addition, total phenolic and flavonoid concentrations and DPPH antioxidant activity were increased by UV-BI irradiation. UV-BI may induce the apple defense mechanism against *C. gloeosporioides* through the synergistic interaction of PR gene and crosstalk between JA and ethylene.

CHAPTER 4

Changes in abscisic acid, antioxidant concentration and the activities of antioxidant enzymes in Japanese apricot (*Prunus mume*) under short-term anoxic storage conditions

4.1 Introduction

Apricot (*Prunus mume*) is classified as a climacteric fruit, and its shelf-life is generally limited. Ripening of the fruit can be delayed with low temperatures, controlled atmospheres or chemical treatments (Fagundes et al., 2015; Park et al., 2018; Wang et al., 2018). An anoxic treatment is an alternative technology that can be used for regulating the ripening process (Pesis, 2005), reducing decay development in tomatoes (*Lycopersicon esculentum*) (Fallik et al., 2003) and maintaining postharvest quality in peaches (*Prunus persica*) (Lara et al., 2011). Furthermore, Li et al. (2013) reported that application of a low oxygen treatment promoted antioxidant enzyme activities by reducing reactive oxygen species (ROS) levels and lipid peroxidation in mushrooms (*Pleurotus eryngii*). It is widely considered that anoxic treatment is attractive because of its effectiveness and the relatively low cost of implementation.

ROS such as hydrogen peroxide (H_2O_2), the hydroxyl radical (·OH) and the superoxide radical ($\cdot O_2^-$) generally originate in the mitochondria, chloroplast, and apoplast during respiration and photosynthesis (Tovar-Mendez et al., 2011). The formation of ROS causes cell damage by interfering with the activity of proteins, nucleic acids, and the lipid membrane (Choudhary et al., 2012). An excess of ROS also influences the concentrations of some plant hormones such as abscisic acid (ABA), which respond to various environmental stresses (Hu et al., 2005). Recently, Kowitcharoen et al. (2015) showed that endogenous ABA concentrations and antioxidant activities increased significantly under abiotic stress in sugar apple (*Annona squamosa*). Moreover, the application of ABA induced an increase in the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD),

ascorbate peroxidase (APX), and glutathione reductase (GR) (Choudhary et al., 2012).

An anoxic treatment was shown to significantly delay fruit softening in parallel with suppression of ethylene production in Japanese apricots (Phonyiam et al., 2016b). However, there are no reports on the role of an anoxic treatment on the ripening processes and antioxidant systems in Japanese apricots. The study, therefore, aimed to investigate the effect of anoxic conditions on the changes in ABA concentrations and antioxidant systems during ripening in 'Inazumi' Japanese apricot.

4.2 Materials and Methods

4.2.1 Fruit materials and treatment

Fruit of Japanese apricot *(Prunus mume*) 'Inazumi', 105 days after full bloom (DAFB) (before the ripening stage), were obtained from an experimental field located at 35°N Lat., 140°E Long. at Chiba University, Japan.

The fruit were randomly assigned to two groups of 150 fruits each. The apricots were placed into plastic chambers and then exposed to pure nitrogen (N_2). The oxygen concentrations in the chambers was measured in the headspace with a gas analyzer to be 0.05% (v/v). The treatments were left for 6 h with humidified N_2 flow (200–250 mL min^{-1}). Untreated fruit were used as a control. Fruit were kept in corrugated boxes and then subsequently stored at 20°C (90–95% RH). Samples were randomly collected every 2 days. Fresh samples were freeze dried and stored at -30°C until analysis. Sample weight was expressed on a dry weight basis.

Changes in skin color were measured with a Chroma meter (Mini Scan EZ 45/0 Hunter Lab). Following Ziosi et al. (2008), a* and b* values were calculated and expressed as hue angle ($h° = tan^{-1}$ b*/a*). In order to interpret the color, red was determined as an angle of 0°, yellow as 90°, and green as 180°.

4.2.2 Measurement of Lipid peroxidation

Lipid peroxidation was determined by the production of malondialdehyde (MDA) following Song et al. (2009) with some modifications. A freeze-dried sample (0.4 g) was mixed with 3 mL of 10% trichloroacetic acid (TCA). The sample was incubated overnight at 4°C. The homogenate was centrifuged at 6,600 *g* for 10 min at 4°C. A supernatant of 0.4 mL was mixed with 1.6 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 10% (w/v) TCA, incubated for 30 min in boiling water and then cooled quickly. Absorbance was measured at 532 nm by spectrophotometer (GENESYS 10S, USA). The non-specific turbidity was corrected by deducting the absorbance at 600 nm. An extinction coefficient of 155 mM cm^{-1} was used for determining the MDA concentrations.

4.2.3 Measurement of antioxidant activity and total phenolic concentrations

Freeze dried samples (500 mg) were extracted in 10 mL of 80% methanol and then shaken overnight at room temperature. The mixtures were filtered using Whatman filter paper No.1 and the methanolic extract was stored at -30°C until analysis.

Free radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was assayed following Phonyiam et al. (2016a) with modifications. A sample of the extract (10 µL) was mixed with 3 mL 0.1 mM DPPH-methanolic solution and was then incubated in the dark for 30 min at room temperature. The decrease of absorbance was assayed at 517 nm by spectrophotometer (GENESYS 10S, USA) and scavenging of DPPH radical activity was expressed as mmol of trolox kg^{-1}.

Ferric reducing antioxidant power (FRAP) activity was investigated according to Phonyiam et al. (2016a). A sample of the extract (400 µL) was added to 2.6 mL of FRAP reagent and the mixture was incubated for 30 min at 37°C. After incubation, the mixture was monitored at 595 nm and concentrations of FRAP were expressed as mmol of Fe^{2+} kg^{-1}.

Total phenolic concentrations were determined following Salem et al. (2013) with slight modifications. A methanolic extract of 250 µL and 1.25 mL of the Folin-Ciocalteu's phenol reagent were mixed. A 1 mL aliquot of 7.5% (w/v) sodium

carbonate (Na_2CO_3) was added to the mixture after incubation for 3 min. The mixture was then incubated for 1 h at room temperature. The phenolic concentrations were determined at 765 nm and results were expressed as g of gallic acid kg^{-1}.

4.2.4 Extraction and determination of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities

A 500 mg sample of the freeze-dried sample was dissolved in 5 mL of extraction buffer containing 1% (w/v) polyvinyl pyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA). The mixture was incubated for 3 h at 4°C, and then centrifuged at 15,000 *g* for 10 min at 4°C. The supernatant was used for determining the SOD, CAT, and POD activities by spectrophotometer (GENESYS 10S, USA) assay.

The SOD activity was evaluated by inhibiting the nitrobluetetrazolium (NBT) reduction, which was monitored at 560 nm according to the recent method of Wu et al. (2014) with slight modifications. Sodium phosphate buffer of 0.1 M (pH 7.8) was used for extraction. The reaction mixture (3 mL) contained 200 µL of crude extract, 1.6 mL of 50 mM phosphate buffer (pH 7.8) and 300 µL for each of 100 mM EDTA, 130 mM *L*-methionine, 750 µM NBT and 20 µM riboflavin. The mixed solutions were illuminated with luminescent lamps for 10 min and monitored at 560 nm. The reaction mixture was placed in the dark, and it served as a blank. The SOD activities were expressed as enzyme units per kg protein.

CAT activity was evaluated by measuring the decomposition of H_2O_2 according to the assay developed in Wu et al. (2014) with slight modifications. A 0.1 M sodium phosphate buffer (pH 7.0) was selected for extraction. The assay mixture (2 mL) was comprised of 1.5 mL of 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.5 mL of crude extract. The spectrophotometric measurement was monitored at 240 nm after incubation for 10 min. The specific activities of CAT were expressed as enzyme units per kg protein by using an extinction coefficient of 0.0436 mM^{-1} cm^{-1}.

The POD activity was assayed according to the method of Wu et al. (2014), using guaiacol as the substrate. The reaction mixture consisted of 0.5 mL of crude

enzyme, 1 mL of 100 mM Na-phosphate buffer (pH7.0) and 0.5 mL of 8 mM guaiacol. The mixture was incubated for 3 min at room temperature. A 1 mL aliquot of 24 mM H_2O_2 was then added to the mixture. The increase in absorbance at 470 nm was recorded every 30 sec for 3 min using a spectrophotometer. The POD activity was expressed as enzyme units per kg protein.

Protein content was evaluated following the method of Bradford (1979) using bovine serum albumin (BSA) that served as the standard.

4.2.5 Extraction and determination of abscisic acid (ABA)

ABA concentration was analyzed according to the method of Kowitcharoen et al. (2015). Freeze dried sample (1 g) was homogenized in 20 mL of 80% (v/v) methanol with 0.1 g butylatedhydroxy toluene (BHT) antioxidant, 0.1 g ascorbic acid, and 0.5 g polyvinylpolypyrrolidone (PVPP). A 200 µL of ABA-d_6 (an internal standard) was then added. The homogenate was centrifuged at 15,000*g* for 15 min at 4°C. The solution was filtered to obtain an aqueous solution. The extracted sample was adjusted to a pH of 2.5 by 0.1 M hydrogen chloride (HCl) and partitioned 3 times with 100% (v/v) ethyl acetate. The ethyl acetate was removed through evaporation. The dried sample was re-dissolved using 1.5 mL of 100% ethyl acetate. The samples were purified by high performance liquid chromatography (HPLC; Spectroscopic, Tokyo, Japan) and identified using gas chromatography mass spectrometry (GC-MS-SIM; model QP5000; Shimadzu, Kyoto, Japan).

The statistical analyses were carried out using SPSS version 16.00 software (IBM Institute, NC). The analysis of data was expressed as mean ± standard deviation (SD). The differences were compared with t-tests at $p < 0.05$.

4.3 Results

4.3.1 Coloration

The anoxic treatment was able maintain a light green peel color for up to 6 days of storage (DAS). The hue value was measured at 107.7 (Figure.4.1) whereas the untreated fruit, which turned yellow, had a hue value of 89.4.

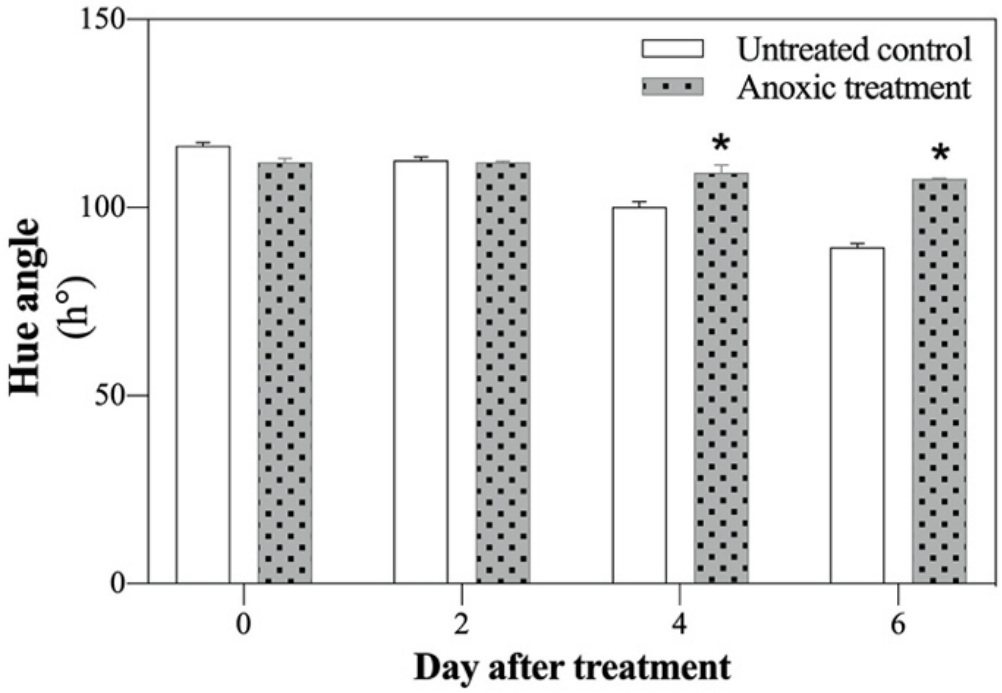

Figure 4.1 Changes of hue angle in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ±SD of three replications, *: significant at $p < 0.05$.

4.3.2 MDA concentration

The concentration of MDA in the untreated fruit gradually increased throughout the storage period (Figure. 4.2A). In contrast, the MDA levels in the anoxic-treated fruit significantly declined to 4 DAS and then increased but remained below the untreated value.

4.3.3 DPPH, FRAP activities, and TPC concentrations

The DPPH radical scavenging activities in the anoxic treated fruit were higher than those in the untreated fruit (Figure 4.2B). FRAP did not show significant differences except for 4 DAS (Figure 4.2C) when the FRAP level in the anoxic-treated fruit was higher than that in the untreated fruit. The total phenolic concentration in both treatments gradually increased during storage (Figure 4.2D). However, total phenolic concentration did not show any clear or consistent differences between anoxic-treated fruit and the untreated fruit.

4.3.4 SOD, CAT, and POD activities and ABA concentration

SOD activities in both treated and untreated fruit gradually decreased during storage (Figure 4.3A). However, SOD activities in the anoxic-treated fruit were higher than those in the untreated fruit throughout. CAT activities in anoxic-treated fruit were also higher than those in the untreated control (Figure 4.3B) except at 6 DAS. POD activities continuously increased throughout the storage period (Figure 4.3C) and were higher in the anoxic-treated fruit than in the untreated control at 4 and 6 DAS.

The anoxic treatment significantly increased ABA concentration (Figure 4.3D) except at 6 DAS where values were low. The highest concentrations of ABA in both the anoxic-treated and untreated fruit were observed at 4 DAS.

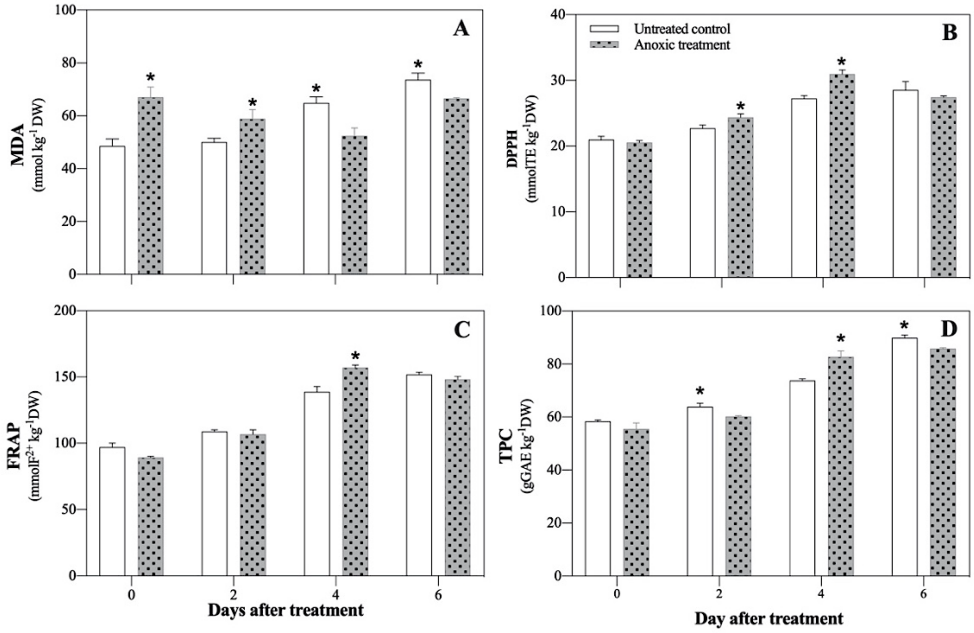

Figure 4.2 Changes of (A) MDA, (B) DPPH, (C) FRAP and (D) TPC in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ±SD of three replications, *: significant at $p < 0.05$.

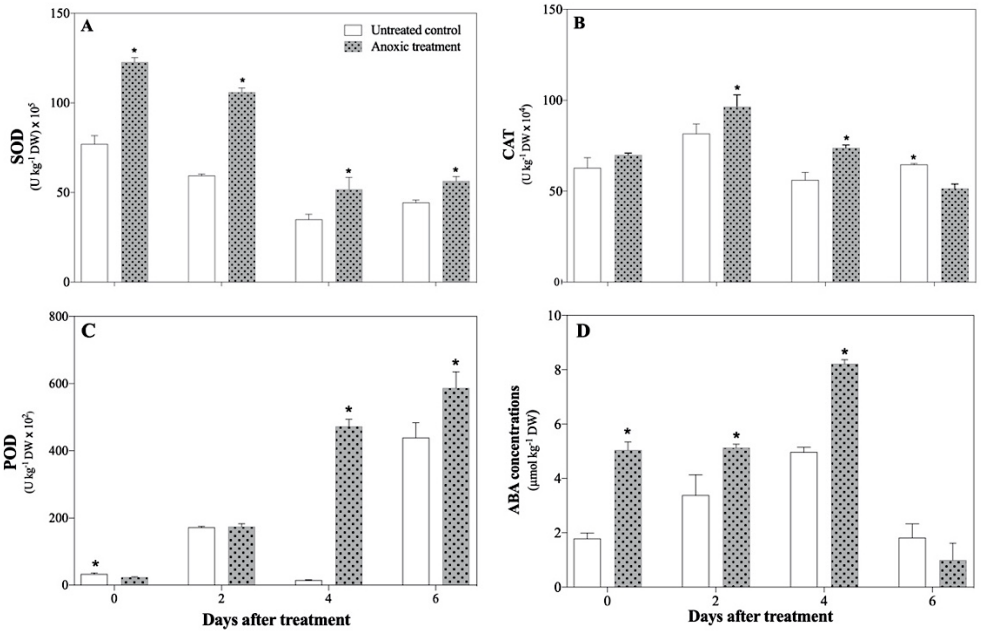

Figure 4.3 Changes of (A) SOD, (B) CAT, (C) POD and (D) ABA in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ±SD of three replications, *: significant at $p < 0.05$.

4.4 Discussion

In general, color development, due to chlorophyll degradation, is the primary physiological change that occurs during ripening of various fruits and vegetables (Kasampalis et al., 2020). Recently, it has been shown that anoxic conditions could effectively delay color development in tomatoes and pineapples *(Ananas comosus)* when compared with untreated fruit stored at room temperature (Techavuthiporn et al., 2017). The results in this study showed that anoxic treatment greatly delayed fruit color development. This finding may relate to the enhanced concentrations of antioxidants and the activities of enzymatic antioxidants, resulting in a delay in the ripening of Japanese apricots during storage at 20°C. These results correlate with previous research which showed that anoxic application for 6 h delayed ripening of Japanese apricot via inhibition of ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) production (Phonyiam et al., 2016b).

MDA is a secondary by-product of lipid peroxidation caused by abiotic stress, which is used as an indicator of cell oxidative damage (Xu and Liu, 2017). A previous study of jujube fruit (*Ziziphus Mauritiana* Lamk.) indicated that MDA concentration increased at the ripening stage with a concommitant increase of oxidative stress (Kumar, 2008). The results presented here show that, although suppressed by the anoxic treatment, an increase in MDA was found not only in anoxic-treated fruit, but also in the untreated fruit at the ripening stage. These results suggest that the increase in MDA concentration was induced by both anoxic stress and senescence.

It has been shown previously that anoxic application enhances the increase in antioxidant activity, as determined by measures such as ascorbic acid, glutathione, DPPH and FRAP values, in pineapple fruit during storage at room temperature (Phonyiam et al., 2016a; Techavuthiporn et al., 2017). The concentrations of total phenolic compounds are enhanced in plants as a resistance mechanism against many stress conditions (Setha, 2012). In this study, the increase in antioxidant activities and phenolic concentrations were higher at 4 DAS in the anoxic treated fruit. These results are consistent with those of You et al. (2012) who reported higher levels of antioxidant activity and phenolic concentrations in anoxic-treated Chinese water chestnuts (*Eleocharis tuberosa*) slices. In addition, the high

antioxidant activities and phenolic concentrations may be associated with resistance to lipid peroxidation under short anoxic conditions.

The activities of both enzymatic and non-enzymatic antioxidants are affected by the disruption of ROS activities. This study showed that SOD and CAT activities in the fruit decreased in the later stages of ripening during storage. Previous studies have also shown that SOD and CAT activities decrease during ripening (Kumar, 2008; Murshed et al., 2013). Furthermore, Resende et al. (2012) showed that the decrease of CAT activity coincided with an increase in MDA concentrations during ripening in papaya (*Carica papaya*). The result presented here show that POD activity in anoxic treated fruit was generally higher than that in untreated fruit. Song et al. (2009) similarly showed that anoxic stress stimulated an increase in POD activity in kiwifruit (*Actinidia deliciosa*) during low temperature storage. Lotfi et al. (2015) reported that an increase of POD activity under anoxic conditions may be related to the protection of cell walls against lignification and crosslinking damage. These results in this study, overall, indicate that several enzymatic antioxidants, such as SOD, CAT and POD, may have a role in regulating ROS levels in Japanese apricot under short anoxic conditions.

ABA is correlated to the ripening process in both climacteric and non-climacteric fruit (Saito et al., 2018) and Bulgakov et al. (2019) reported that ABA plays a role in the regulation of plant stress defenses. In this study, the ABA concentrations in Japanese apricot were enhanced, together with induction of SOD, CAT and POD activities, under anoxic conditions. It is possible, therefore, that the increase in ABA concentration was correlated with enzymatic antioxidant activities which may be essential for activating plant defenses against stress.

4.5 Conclusion

The anoxic treatment increased ABA concentrations, enzymatic antioxidants (SOD, CAT and POD), antioxidant activities and total phenolic concentrations, while decreasing MDA production and delaying the changes of fruit color during storage.

CHAPTER 5

General discussion

In general, postharvest decays in harvested fruits and vegetable are among the major losses that considered to limit nutritional values involving shelf life extension. Citrus fruit is the first importance production for human consumption recognized worldwide (Smilanick et al., 2020). However, the citrus decay caused by fungal pathogen is considered the most serious factor influenced quality deterioration and market losses (Strano et al., 2017). UV-C radiation has been conducted as a postharvest physical technology to prevent decay in the fruits and vegetables (Severo et al., 2015). The impact of low dose UV-C or hormetic dose (0.01 to 39 kJ m^{-2}) has been employed to promote disease defense resistance, induce phytochemical compounds as well as delay the progress of ripening and senescence of treated produce (Duarte-Sierra et al., 2020). Recently, UV-C radiation was confirmed to be a beneficial treatment of eliminating disease and decay in fresh crops caused by natural pathogenic fungi such as *Guignardia citricarpa*, *C. musae* and *Lasiodiplodia theobromae* (Canale et al., 2011; Bokhari et al., 2019; Sripong et al., 2019). Furthermore, several reports explained that a single UV-C consequently retarded fungal development in crops might contribute to the induction of bioactive antioxidant capacities such as total phenol, anthocyanin and flavonoid involving stimulation of plant defense-related enzymes such as PAL, POD and chitinase in plants exposed to UV-C radiation (Wang et al., 2009; Sripong et al., 2019).

JA and ethylene play the role in stimulation of plant defense resistance to both biotrophic and necrotrophic fungi pathogens (Ali et al., 2018). Recent studies reported that enhanced expression of JA signature gene can induce PR genes which substantially promoted plant against pathogenic fungi (He et al., 2008; Wally and Punja, 2010). Previous researches clearly demonstrated that UV-C irradiation promoted β-1,3-glucanase, chitinase activities and especially induced PR proteins in crops (Yalpani et al., 1994; Charles et al., 2009). This research showed that UV-

BI treatment significantly promoted endogenous JA concentrations with decreasing of ethylene production and its related genes such as *MdACS1, MdACO1, MdETR1, MdERS1 and MdCTR* in apple fruit. Yang et al. (2019) reported the crosstalk between JA and ethylene that the transcription of *ETHYLENE INSENSITIVE 3* (EIN3) and EIN3-like 1 (EIL1) were suppressed by *JASMONATE ZIM DOMAIN PROTEIN* (JAZ) participated in the JA pathway, thereby enhancing defense against pathogen. Bu et al. (2013) also supported that UV-C irradiation inhibited ethylene production of tomato. Our results may suggest that application of UV-BI induced the crosstalk between JA and ethylene, leading to stimulate the tolerance to fungal pathogen *C. gloeosporioides*. These defensive mechanisms may correlate to the induced expression of PR genes such as *MdPR1a, MdPR2,* and *MdPR4* in apple fruit after exposure to UV-C radiation. On the other hand, high expression of antioxidant defense related enzymes like PAL, POD, FLS, ANR and chitinase can also exhibited the potential effect on inducing pathogen defense response (Severo et al., 2015; Yang et al., 2019). UV-C irradiation has been reported to enhance biochemical substances in a variety of crops for instance lettuce, strawberry and mangosteen (Ouhibi et al., 2014; Jin et al., 2017; Sripong et al., 2019). The present study supported that UV-BI irradiation stimulated the induction of total phenolic and flavonoid concentrations as well as DPPH antioxidant activity. Thus, our results demonstrated that the UV-BI treatment clearly elicited disease resistance against *C. gloeosporioides* through stimulation the crosstalk between JA and ethylene with their related genes, promotion of PR genes and associating with induction of antioxidant activities in 'Tsugaru' apple.

Fruit ripening revealed the dramatic changes in physiological, biochemical, and molecular levels that are importance regulator factor affect acceptability and marketability of fruit (Zhou et al., 2020a). This study aimed to investigate the potential effect of anoxic treatment in order to evaluate the change of physiological, plant hormone and antioxidant systems during ripening of 'Inazumi' Japanese apricot. The current study found that anoxic treatment delayed the physiological change which indicated by significantly higher hue angle value. Recently, the anoxic treatment has been demonstrated to be induce antioxidant activities involving FRAP and DPPH values and total phenolic concentrations (You et al.,

2012; Techavuthiporn et al., 2017). Similar to previous studies, short anoxic treatment also promoted the increase of antioxidant activities and total phenolic concentrations in Japanese apricot fruit. Moreover, the enzymatic antioxidants including SOD, CAT and POD and ABA concentrations in short anoxic treated fruit were significantly maintained and promoted compared to those untreated control. This suggest that the ripening process in Japanese apricot is more extended may involve in retarding of color development, increasing of ABA concentrations and promoting of enzymatic antioxidants, antioxidant activities and total phenolic concentrations after exposure to short anoxic treatment.

In conclusion, application of different postharvest physical treatment resulted in different effect on commodities involving their physiological and biochemical changes during storage. In this study, each UV-C treatment effectively exhibited their beneficial effects on enhancing defense mechanisms and inducing biochemical activities without undesirable symptom in satsuma mandarin and apple fruit. On the other hand, short anoxic treatment revealed inducing enzymatic antioxidant and antioxidant activities that eventually resulted in delaying of ripening process in Japanese apricot. Thus, these physical treatments are a low-cost and non-chemical resource which have developed to be a successful treatment in order to control postharvest decay and maintain the quality of stored fresh commodities as a substitute for use of chemical treatment.

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