Induced Disease Resistance of Satsuma Mandarins against *Penicillium digitatum* by Gamma Irradiation

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Abstract - Gamma irradiation, which is a type of ionizing radiation, can be used as a fruit inducible factor. In the present study, the effects of gamma irradiation on the resistance of mandarin fruits against *Penicillium digitatum*, the causal agent of postharvest green mold disease, were investigated. Pretreatment of a low dose of gamma irradiation effectively reduced the disease incidence and lesion diameter of mandarin fruits inoculated with *P. digatatum* during storage for 14 d. Interestingly, exposed to 400 Gy of gamma irradiation significantly maintained firmness and stimulated the synthesis of defense-related enzymes, (e.g., β-1,3-glucanase, phenylalanine, peroxidase, and polyphenol oxidase) and pathogenesis-related (*PR*) genes (e.g., *PR-1* and *PR-2*). Therefore, the gamma irradiation-induced resistance against *P. digatatum* involves both changes of phenolic compounds and the induction of expression of defense-related genes. In addition, scanning electron microscopy analysis revealed that induced disease resistance by gamma irradiation significantly inhibits the growth of *P. digatatum* in mandarin fruits. These results suggest that the exposure of gamma irradiation is a potential methods for inducing the disease resistance of fruit to postharvest fungal pathogens and for extending the postharvest life of mandarin fruit.

Key words : Gamma irradiation, Induced resistance, Mandarin, *Penicillium digitatum*

INTRODUCTION

Preharvest and postharvest diseases mainly caused by fungal pathogens during storage are major factors that affect fruit quality, shelf-life, and market value. The fungal pathogen complete its life cycle on fruit trees but enters fruit, where it remains quiescent during growth. After harvest, the pathogen existed on fruit is activated and causes disease when kept to favorable environmental conditions (Droby 2006). In addition, diseased fruits by postharvest fungal pathogens, such as *Penicillium*, *Alternaria*, and *Fusarium*, also present potential health risks, produce carcinogenic and teratogenic mycotoxins. Currently, control of postharvest diseases in fruits is mostly dependent on synthetic fungicides (Romanazzi *et al*. 2012). Given the toxicity of fungicides on the environment and growing public concerns over the human health, as well as the development of fungal resistance to fungicides, alternative strategies that possess lower costs, lower risks of fungal resistance, and lower human health and environmental risks have also been extensively examined (Feliziani *et al*. 2013). Therefore, relatively broad-spectrum, economical, and environmentally safe approaches are required. Among them, induced disease resistance has attracted attention as a straightforward and sustainable strategy for controlling fresh fruit pathogens during postharvest handling (Ouhibi *et al*. 2014; Zhu *et al*. 2016). Induced resistance involves the accumulation of antimicrobial compounds (phytoalexins), cell wall fortification, elevated levels of defense-related enzymes, and increased antioxidant activity, all of which contribute to resistance to damaging pathogens. However, compared with intact plants, relatively little is known about the defense responses

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of fresh produce.

The germicidal effect of ionizing radiation has been extensively used as an effective method for disinfecting, even though the method still possesses safety issues for consumers (Hallman 2011; Piri *et al*. 2011; Jeong *et al*. 2016). Irradiation dose is expressed in grays (Gy), which indicates the energy (J) absorbed per unit mass (kg) of the material. In case of high doses, adverse effects accumulate in the tissues in a deterministic manner, but in low doses, the effects are stochastic and depend on the efficiency of the stress response's protective mechanisms. Therefore, low doses of ionizing radiation can trigger moderate stress, which is known to induce hormesis. Recent studies have reported that irradiation causes DNA damage *via* the dimerization of thymine and other pyrimidines and that acts as a secondary intermediary process that induces both the biological stress and defense mechanisms of plant tissues (Ouhibi *et al*. 2015).

To date, studies have demonstrated the ionizing radiation can enhance host resistance to postharvest diseases. The induced disease resistance against pathogens by ionizing radiation may be involved in activating the expression of a series of resistance-related genes and triggering the activities of defense-related enzymes. For example, low doses of gamma irradiation induce the production of phenolic compounds, glucanases, chitinases, phytoalexins, and peroxidase, which contribute to resistance against *P. italicum*, and *Botrytis cinerea* in tomatoes and grapes, respectively (Nigro *et al*. 1998; Charles *et al*. 2003). However, only few studies have investigated the effect of gamma irradiation on the induced disease resistance of fruits.

In the present study, we investigated the effects of gamma irradiation on the disease resistance of fresh mandarin against *P. digitatum* by measuring several key defense-related enzymes and pathogen-related genes. We also examined whether any structural changes occurred in *P. digitatum* after the induction of resistance in mandarin fruits. The aim of the study was to investigate the possible mechanisms that underlie the gamma irradiation-induced disease resistance in fresh mandarin fruit.

MATERIALS AND METHODS

1. Fungal isolation and inoculum preparation

The fungal pathogen *P. digitatum*, was isolated from infected mandarin fruit that exhibited the typical symptoms of green mold. To gain a pure culture, monosporic conidia were transferred to potato dextrose agar (PDA; Difo Laboratories, Detroit, MI, USA). Prior to each experiment, all cultures were grown on PDA at 25°C for 14 d. To harvest the conidia, 10 ml sterile distilled water was added to a culture dish, and the conidia were gently harvested by filtration through four layers of gauze. The concentration of the conidia was measured using a hemacytometer (Warner-Lambert Technologies Inc., Buffalo, NY, USA), and the final concentration was adjusted to 10^5 conidia ml^{-1} using distilled water.

2. Source of gamma irradiation

A cobalt-60 gamma irradiator, which was housed at the Korea Atomic Energy Research Institute, Jeongeup, Korea (150 TBq capacity; ACEL, MDS Nordion, Canada) was used for the irradiation. All of the absorbed doses were calibrated using alanine dosimeters with a diameter of 5 mm (Bruker Instruments, Rheinstetten, Germany), and a Bruker EMS 104 EPR analyzer was used to determine free-radical signals. The absorbed radiation dose was 600 Gy h^{-1}.

3. Fruit treatment

Satsuma mandarin fruits were obtained from a commercial grower. All experiments were arranged in a completely randomized split-plot design, with three replicates of 10 fruits per treatment, and each experiment was repeated at least twice. The fruits were disinfected using 1% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air-dried before gamma irradiation. The fruits were then irradiated with dose levels of 100, 200, 400, 600, and 800 Gy (600 Gy h^{-1}) by manipulating exposure time, and after treatment, the fruits were stored in containers with transparent films at room temperature (25°C). After 24 h, the fruits were wounded with a sterile nail (one wound per fruit, in the equatorial zone; 2 × 2 mm) and inoculated using a suspension of *P. digitatum* conidia (10^5 conidia ml^{-1}). Finally, the mandarin fruits were placed in plastic boxes (200 mm × 130 mm × 50 mm) under high humidity (about 95%) and at room temperature for 14 d.

4. Disease evaluation

For the inoculation treatment, mean lesion diameters (per

Table 1. Primers used in real-time PCR analysis for defense gene expression

Gene name	Amplicon length (bp)	Primer sequence
CsPR-1	88 bp	F: AACTCGCCTCAAGACTACCT R: TGCAACTGTGTCGTTCCATA
CsPR-2	92 bp	F: ACTTCGCTCAGTACCTTGTTC R: GGCAGTGGAAACCTTGATTTG
β-actin	112 bp	F: TCGGGTGTTTTCACGTCTCA R: CGCCGTAGGTGAGGTAGC

fruits) were determined daily, and the incidence of green mold was calculated as the ratio of the number of disease spots to the total number of wounding points.

5. Assays for the enzyme activities

After exposure to 0 or 400 Gy gamma irradiation for 0, 3, 5, 7, and 14 d, tissue samples were collected from five randomly selected fruits from each treatment. Three replicates were performed for each measurement. All the extraction procedures were performed at 4°C, and the enzyme activities were determined using a Shimadzu UV-160 spectrophotometer (Shimadzu Co., Kyoto, Japan). The activities of β-1,3-glucanase, phenylalanine ammonia-lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO) were determined using previously described methods (Yao and Tian 2005; Shi *et al*. 2013). One unit was defined as the amount of enzyme required to catalyze the formation of 1 nmol product min^{-1}.

6. RNA isolation and gene expression analysis

Total RNA was extracted from gamma-irradiated pear fruits using liquid nitrogen and the Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase treatment (DNA-free; Ambion, Austin, TX, USA), the RNAs were purified using the RNeasy Mini Kit (Qiagen, Korea), and quantitative real-time PCR (qPCR) analysis was performed for each sample using 40 ng total RNA, SuperScript III Platinum SYBR Green One-Step RT-qPCR Kit with ROX (Invitrogen, USA), and 20 pmol of each primer per reaction in a 7500 Real-Time PCR System (Applied Biosystems, USA). The primers of the target pathogenesis-related (*PR*) gene (*PR-1* and *PR-4*; Table 1) were designed according to previous studies (Faize *et al*. 2004). The standard curve method was used to calculate transcript abundance relative to *β-actin* as the reference gene. In addition, three replicates were performed for each treatment, and the experiment was repeated three times.

7. Pathogen structure analysis

To analyze the structure of pathogen cells located at the inoculation points of the fruit and in the fungal growth state, scanning electron microscopy (SEM) analysis was conducted according to Mares *et al*. 2004. Small pieces of peel tissue (2 mm × 5 mm × 1 mm) were torn from around the inoculation points using forceps, fixed in 6% (v/v) glutaraldehyde in a sodium cacodylate buffer, briefly post-fixed for 1 h at 4°C in OsO_4 in the same buffer and then dehydrated in acetone. The samples were then coated with gold in a sputter coater (JFC 1100 E; JEOL, Tokyo, Japan) and examined using a SEM (JEOL, Tokyo, Japan) (Mares *et al*. 2004).

8. Statistical analyses

The data were subjected to analyses of variance (ANOVA) at $P < 0.05$ using SPSS 13.0 for Windows (SPSS Inc., USA) and Duncan's multiple range tests were used to determine the significance of differences between mean values.

RESULTS

1. Effect of gamma irradiation on disease resistance of mandarin fruit inoculated with *P. digitatum*

The disease diameter and incidence of disease symptoms in the mandarin fruit cause by *P. digitatum* were significantly reduced by pre-treated gamma irradiation ($P < 0.05$; Fig. 1A, B). The mean lesion diameters of all the gamma-irradiated mandarin fruits were lower than those of the control fruits for both the 7-and 14-d treatments, and the 400 Gy irradiation treatment was most effective. Interestingly, over doses of 600 Gy of gamma irradiation decreased disease resistance gradually compared to that of 400 Gy irradiation. In addition, gamma irradiation also significantly inhibited disease development as indicated by lower incidence ratios, and the 400 Gy irradiation treatment was the most effective (Fig. 1C).

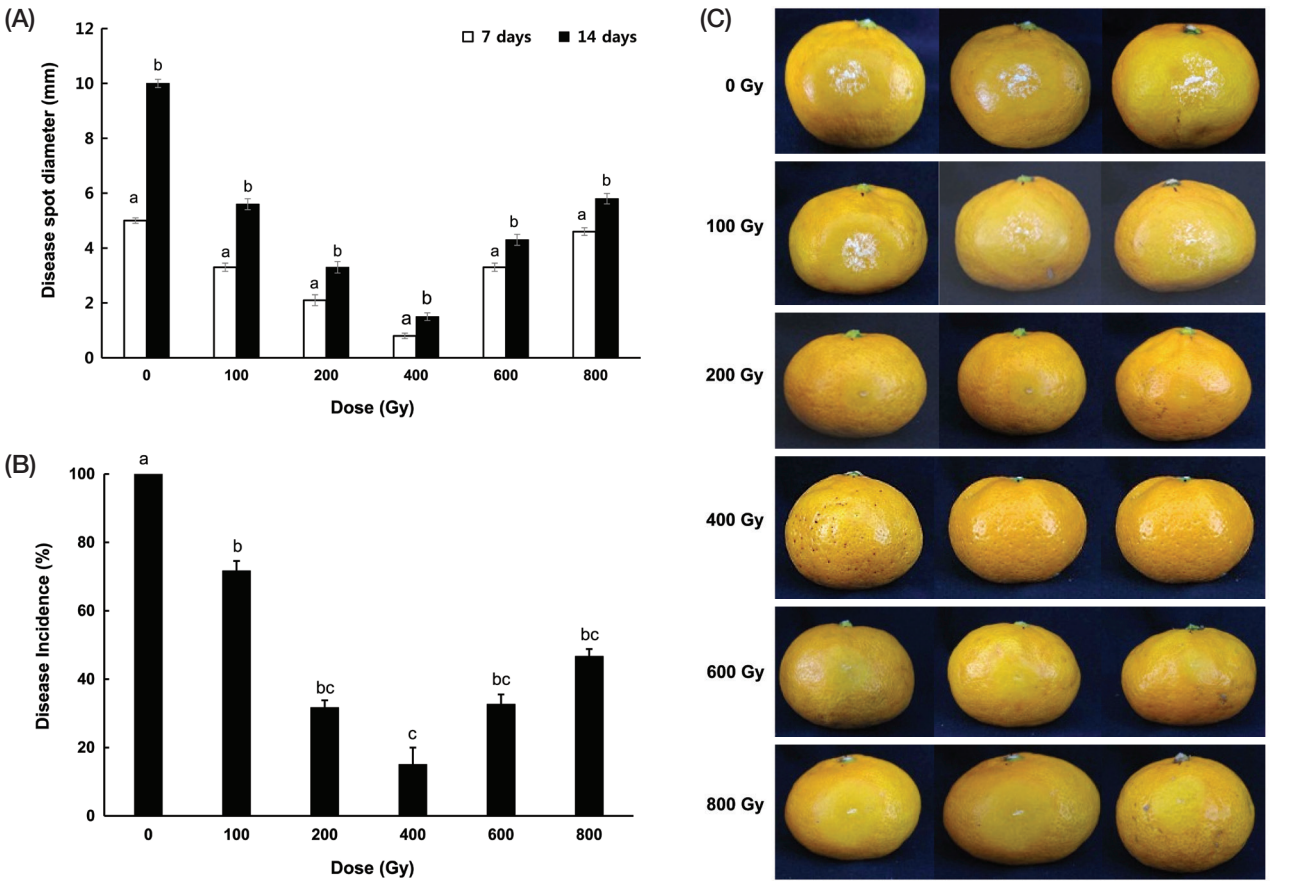

Fig. 1. Effect of gamma irradiation on green mold disease in harvested mandarin fruit. (A) Effect of gamma irradiation dose on lesion diameters after wounding inoculation. (B) Effect of gamma irradiation dose on green mold incidence after wounding inoculation. (C) Effect of gamma irradiation dose on disease severity. Values followed by the different letters are significantly different ($P<0.05$) based on Duncan's multiple range test (n = 3). The experiment was performed in three independent sets.

2. Effect of gamma irradiation on enzyme activity

Because the 400-Gy irradiation treatment was the most effective in improving the resistance of mandarin fruits against *P. digitatum*, we subsequently examined the effect of the 400-Gy irradiation treatment on the activities of several resistance-related enzymes. β-1,3-glucanase, POD, PAL, and PPO were induced rapidly by gamma irradiation during the early stages of storage; it peaked on 7 d and then declined gradually. The activity of β-1,3-glucanase was significantly higher in the irradiated fruit than in the control fruit 5 d after inoculation (Fig. 2A) and gradually decreased at 5 d after inoculation. Meanwhile, the activity of POD in the irradiated fruit dramatically increased for 5 d after inoculation and was gradually decreased at 7 d and 14 d after inoculation (Fig. 2B). In addition, the activities of PAL and PPO in the irradiated fruits gradually increased during the first 5 d after inoculation but then gradually decreased afterward (Fig. 2C, D). The patterns of enzyme activities in both the irradiated and control fruits were similar. However, under prolonged storage periods, the irradiated fruit consistently exhibited higher enzyme activities than the control fruit. When over dose of 600 Gy irradiation was treated the mandarin fruits, the β-1,3-glucanase, POD, PAL, and PPO were dramatically decreased compared to control fruits (data not shown). This results indicated that decreased disease resistance by over dose of 600 Gy is critically correlated with the reduction of enzyme activity.

3. Effect of gamma irradiation on defense-related gene expression

Quantitative real-time PCR indicated that gamma irradiation increased the expression of three genes; *PR-1* and *PR-2* were carried out with defense-related genes. The transcript level of three genes, *PR-1* and *PR-2*. The expression of *PR-1* and *PR-2* was gradually induced by gamma irradiation during 14 d compared compared to control (Fig. 3A, B).

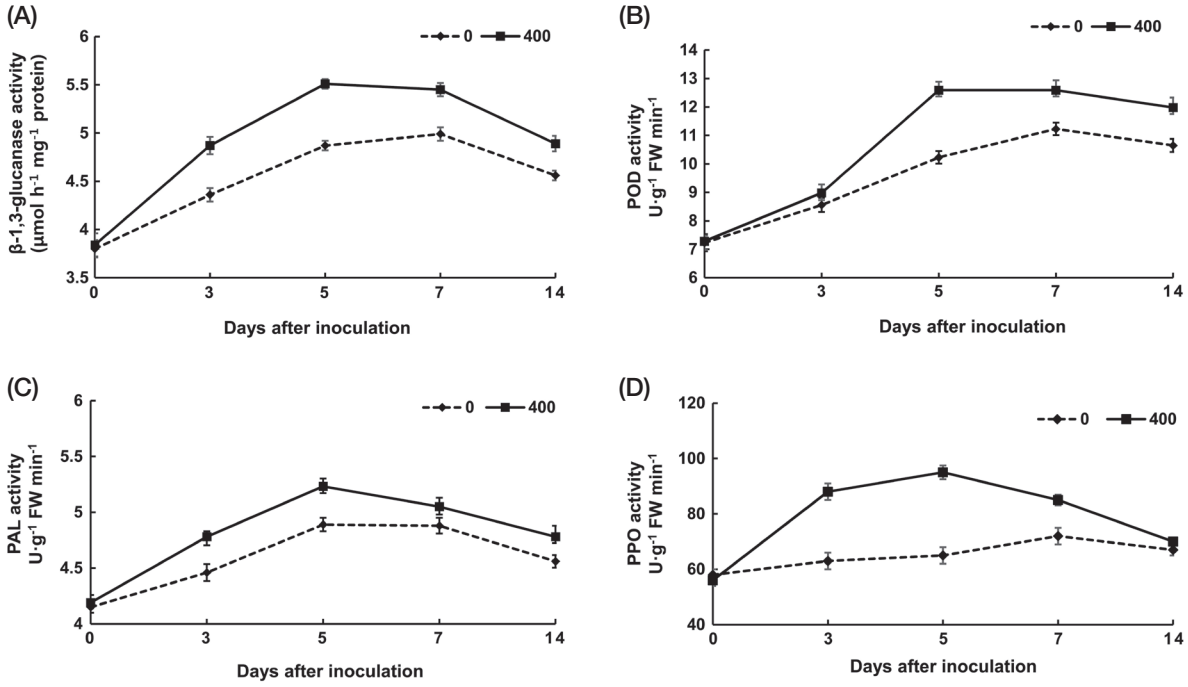

Fig. 2. Effect of gamma irradiation on post-inoculation enzyme activities. (A) Effect of 400-Gy irradiation on β-1,3-glucanase activity. (B) Effects of 400-Gy irradiation on peroxidase activity. (C) Effect of 400-Gy irradiation on phenylalanine ammonia-lyase activity. (D) Effects of 400-Gy irradiation on polyphenol oxidase activity. Values represent means ± the standard error (n = 3).

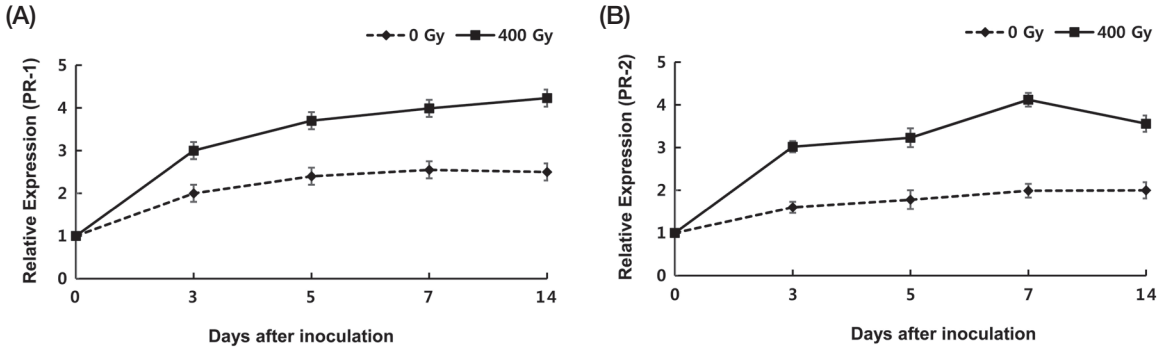

Fig. 3. Effect of gamma irradiation (400 Gy) on the relative expression of defense-related genes. (A) *PR-1* and (B) *PR-2*. Values were normalized to those at time 0, which were arbitrarily set to unity. Values represent the means ± the standard error of nine replicates (20 fruits each) pooled from three experiments.

The expression of *PR-1* and *PR-2* was relatively stable in the control fruit over the 14 d period.

4. Effect of gamma irradiation on *P. digitatum* structure

P. digitatum infected the surface of both the control and irradiated mandarin fruits in the spore and hyphae states (Fig. 4A, B). SEM analysis revealed that fungal spores and hyphae abundantly colonized the infected the surface of the control fruits. However, the growth of *P. digitatum* began was significantly inhibited in the inoculated areas of the irradiated fruits at 14 d after inoculation. In addition, the restriction of fungal growth was also correlated with discrete structural changes.

DISCUSSION

So far, gamma irradiation, a type of ionizing radiation, has been mainly used to be an effective method for controlling fungal diseases in fruits by directly killing patho-

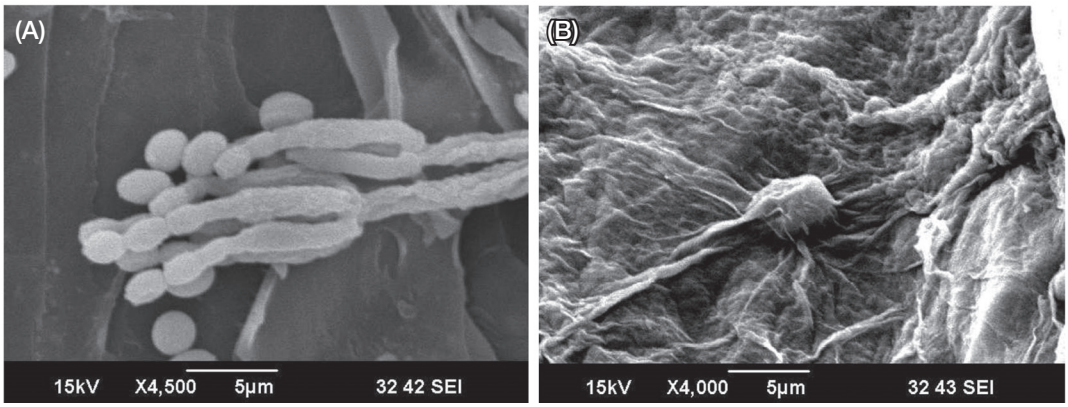

Fig. 4. Effect of gamma irradiation (400 Gy) on *P. digitatum* growth state on the surface of mandarin fruits. (A) SEM of peel from control fruit. (B) SEM of peel from irradiated fruit. Scale bars = 5 μM.

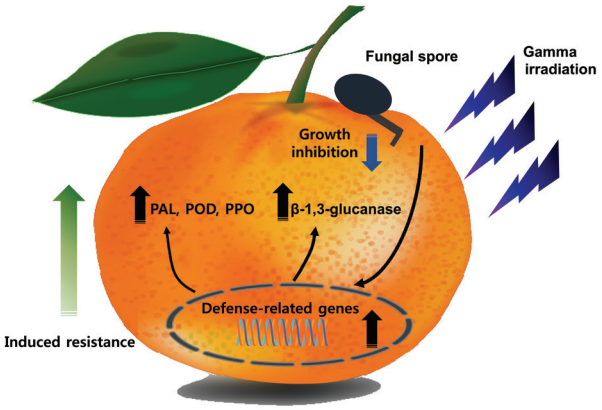

Fig. 5. Induced disease resistance in mandarin fruits by gamma irradiation. Gamma irradiation can enhance the activities of disease resistance-related enzymes (1,3-glucanase, PAL, POD, and PPO) and pathogen-related genes (*PR-1* and *PR-2*), thereby inducing disease resistance.

gens (Geweely and Nawar 2006). In the present study, we have attempted to demonstrate potential mechanisms by which gamma irradiation can induce disease resistance in mandarin fruit to green mold caused by *P. digitatum*. Interestingly, even though treatment over 600 Gy of gamma irradiation did not increase the inhibitory effect compare to the treatment with 400 Gy of gamma irradiation. This raised the possibility that gamma irradiation seems to be both through direct interactions with the fungus itself, and via physiological responses of the mandarin tissue. It was great to test compare the effectiveness of control of fungal disease by gamma irradiation or fungicides. However, the fungicides were not used as a comparable method because of toxicity. Some previous studies have provided evidence that preharvest or postharvest application of irradiation results in decreased decay incidence and lesion areas in several fruits (Sumira *et al*. 2012). The results from the present study show that pre-treatment of gamma irradiation significantly reduced both the disease diameter and incidence of mandarin fruits inoculated with *P. digitatum*. The results of the present study indicate that gamma irradiation can effectively decrease the incidence of green mold. Gamma irradiation can also enhance the activities of disease resistance-related enzymes (PAL, POD, and PPO) and pathogen-related genes (*PR-1* and *PR-2*). These results indicated that gamma irradiation could remarkably enhance postharvest disease resistance in fruits.

The temporary moderate stress induced, by gamma irradiation can inflict repairable damage to DNA, which can subsequently activate repair mechanisms. This suggests that sub-lethal radiation can trigger a positive outcome in the homeostasis of plants, i.e., hormesis (Shama and Alderson 2005).

The disease resistance of fruit is largely due to the activation of a highly coordinated biochemical and structural defense response that inhibits the growth of pathogens. For example, the temporary accumulation of phenolic compounds has been implicated in disease resistance in plant-pathogen interactions (Daayf *et al*. 2012). The PAL enzyme plays a critical role in the phenylpropanoid pathway, which affects the synthesis of polyphenols, flavonoids, phytoalexins, and lignin, which accumulate during plant-pathogen defense responses (Daayf *et al*. 2012). PODs are oxide-reductive enzymes involved in cell wall polysaccharide processes in plant-pathogen defense responses, such as the oxidation of phenols and lignification (Nicholoson

and Hammerschmidt 1992). PPOs trigger the oxidation of polyphenols into antimicrobial compounds and lignification of plant cells (Mohammadi and Kazemi 2002). Induction of defense-related enzymes and genes by irradiation is correlated with fruit firmness, changes in pH, cell wall composition, and an increase in soluble sugars and secondary metabolite. Consistent with other studies, our experiments showed that the pre-treatment of gamma irradiation induced higher activities of these defense-related enzymes in mandarin fruits during the whole storage period, which suggests that the enzymes might be involved in the activation of the defense response in mandarin fruits, as well as the subsequent inhibition of pathogen growth (Fig. 5).

Although many studies have previously demonstrated that UV irradiation, a type of non-ionizing radiation, can induce disease resistance, only a few studies have shown that exposure to ionizing radiation can enhance plant resistance against pathogens, especially fungal pathogens. Patil *et al*. (2004) demonstrated that low doses ($\leq$200 Gy) of gamma irradiation coupled with 35 d of storage were useful in enhancing antioxidant enzymes, flavanones, lycopene, and terpenoids in grapefruit. In contrast, Salem and Moussa (2014) reported that doses of over 1000 Gy of gamma irradiation significantly decrease the quality of pear fruits, including their firmness, weight, acidity, and soluble protein content, whereas doses of 1000 Gy did not. Together, results of these studies indicate that treatment with low doses of gamma irradiation reduces the produced a reduction in postharvest decay of fruits *via* induced disease resistance.

CONCLUSION

Our results demonstrate that pre-treatment of gamma irradiation has a positive effect on enhancing disease resistance against postharvest blue mold in mandarin fruit. Pre-treatment of gamma irradiation induced defense-related enzymes such as β-1,3-glucanase, POD, PAL, and PPO and defense-related genes, *PR-1* and *PR-2*. These parameters were all correlated with the onset of induced resistance in fruit. Induced disease resistance by gamma irradiation dramatically inhibited the growth of postharvest *P. digitatum* on mandarin fruits. However, further studies on the molecular mechanism of gamma irradiation-induced resistance by defense response in mandarin fruit are in necessary. Our results suggest that the exposure of gamma irradiation is a potential methods for inducing the disease resistance of fruit to postharvest fungal pathogens and for extending the postharvest life of mandarin fruit.

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