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Effect of Gamma Irradiation on *Pseudomonas syringae* pv. *tomato* DC3000 – Short Communication

Rae-Dong JEONG[1], Eun-Hee CHU[1], Gun Woong LEE[1], Jeong Mee PARK[2] and Hae-Jun PARK[1]

[1]Research Division for Biotechnology, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup, Republic of Korea;
[2]Plant Systems Engineering Research Center, KRIBB, Daejeon, Republic of Korea

Abstract

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Gamma irradiation (GI) was evaluated for its *in vitro* and *in vivo* antibacterial activity against bacterial specks of tomato, *Pseudomonas syringae* pv. *tomato* (*Pst*). GI showed complete inactivation of *Pst* DC3000, especially at a dose of 200 Gy *in vitro*. Gamma-irradiated bacterial cells were found to (1) have spilled cytoplasmic contents, (2) display a damage on the surface of the cells, (3) have reduced membrane integrity, and (4) have fragmented genomic DNA, all in a dose-dependent manner. Consistent with the *in vitro* assay, a low dose of 150 Gy showed sufficient antibacterial activity on tomato seedlings. The present study suggested that the GI of bacterial cells results in substantial damage of the cell membrane, and that, along with DNA fragmentation, results in dose-dependent cell inactivation. These findings suggest that GI has potential as an antibacterial approach to reduce the severity of the bacterial speck disease of tomato.

Keywords: antibacterial activity; bacterial speck of tomato; ionizing radiation

Bacterial speck disease of tomato, caused by *Pseudomonas syringae* pv. *tomato* (*Pst*), is one of the most important diseases and is found growing epiphytically on a wide range of plants. Although bacterial speck of tomato is not considered as a high risk pathogen regarding a major economic impact, outbreaks can cause severe damage to tomato plants and reduce crop yield and fruit quality (Bashan *et al.* 1978; Colin & Chafic 1986; Hirano & Upper 2000). Currently, a variety of methods, such as cultural management, host resistance, biological control with microbial antagonists, and chemical control have been tested for control of *Pst* in young seedlings (Weaver & Wehunt 1975; Hawkins 1976; Lindow 1983). However, the primary methods for controlling bacterial speck are bactericides, such as copper compounds, streptomycin, a mixture of copper, and the fungicides maneb or mancozeb (Conover & Gerhold 1981). However, the continued use of chemical bactericides is limited because of the development of bacterial resistance and growing public concerns over the human health and environmental risks over chemical residues. Although biological control has been proposed as one of the most promising methods in the field, there are some limitations such as a slow effectiveness (Lindow 1983). Therefore, it is urgent to develop eco-friendly alternative methods to control the bacterial speck of tomato.

Recently, irradiation has become a viable alternative and an effective nonchemical treatment for the control of several pathogens (Hallman 2011). Ironically, there is a paucity of studies reporting the direct effects of irradiation on plant pathogenic bacteria, even though they are widely used for the control of pests on fruits, vegetables, and seeds. In general,

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irradiation is well known to cause cellular damage, both by direct effects and indirectly by generating reactive oxygen species, thereby likely blocking their metabolic functions. The unit of irradiation dose is the gray (Gy), which is the energy absorbed in J/kg of material. It has been reported that GI successfully inhibits the growth of pathogens on fruits or vegetables, such as *Botrytis cinerea* in sweet pepper, *Penicillium purpurogenum* in pineapple, *Rhizopus stolonifer* in sweet potato, and *Monilinia fructicola* in peach (Damayanti *et al.* 1992; Kim & Yook 2009; Jeong *et al.* 2014; Yoon *et al.* 2014). Despite many studies on the control of postharvest fungal pathogens by irradiation, the technique has not been carried out much for the control of bacterial pathogens and its potential modes of action.

In this study, we evaluated the impact of GI for the control of *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis* and tomato plants and its modes of action. We studied various biological effects, including the viability of the bacteria, morphological changes, changes in cell membrane integrity, and structural changes of DNA, to determine the possible mechanism of inactivation by GI. We also suggest a possible new approach of GI for controlling the bacterial speck on tomato seedlings through an *in vivo* assay.

MATERIAL AND METHODS

Pathogen. The bacterial strains *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (containing pVSP61, empty vector) were donated by Dr. Jeong-Mee Park (Korea Research Institute of Bioscience and Biotechnology) and grown overnight in King's B medium containing rifampicin (25 µg/ml) and kanamycin (50 µg/ml) (Sigma, St. Louis, USA). The bacterial cells were harvested, washed, and suspended in 10 mM $MgCl_2$. The cells were diluted to a final density of 1×10^5 colony forming units (CFU)/ml (A_{600}).

Gamma irradiation. A cobalt-60 gamma irradiator 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, where a Bruker EMS 104 EPR analyser (both Bruker Instruments, Rheinstetten, Germany) was used to determine the free-radical signals. The radiation absorbed dose was 600 Gy/hour.

Measurement of viable cell counts and nucleic acid, and protein amounts. After irradiation (0, 20, 60, 100, and 200 Gy), the bacteria cell suspensions were immediately serially diluted with 10 mM $MgCl_2$ using 10-fold dilutions and 100 µl were plated on King's medium plates. The plates were incubated at 28°C for 48 h, and the colony forming units of *Pst* DC3000 were counted. Survival curves were created by exponential function to the survivor CFU/ml vs the actual radiation dose. The irradiated bacterial cell suspensions were centrifuged at 12 000 *g* for 10 min and the supernatants were analysed for nucleic acid and protein. The amount of nucleic acid and protein released from the irradiated cells was measured at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and at 595 nm by the method of Bradford using a spectrophotometer (Biochrom Libra S70; Biochrom Ltd., Cambridge, UK), respectively (Bradford 1976). The Bradford assay was performed on the supernatants to quantify release of proteins by the various treatments. All experiments were carried out in triplicate.

Scanning electron microscopy. Irradiated cells were fixed with 3% glutaraldehyde overnight. The samples were dehydrated by successive treatments of ethanol in water; the ethanol concentration for each treatment was increased in concentrations of 50–100%. The samples were coated with gold in a sputter coater (JFC 1100 E) and were examined under a scanning electron microscope at 15 kV (both JEOL Ltd., Tokyo, Japan).

Membrane integrity assay. *Pst* DC3000 cells were irradiated at 0, 50, 100, 150, 200 Gy in a King's B broth. After 2 h of incubation at 28°C, cells were stained with 10 µg/ml propidium iodide (PI) (an indicator of cell membrane integrity loss) (Sigma-Aldrich, St. Louis, USA) for 5 min at 30°C (Fish *et al.* 2000). The cells were observed with a Zeiss Axoskop 40× microscope (Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set (Zeiss No. 15: excitation BP 546/12 nm, emission 590 nm).

Structural changes in DNA. Genomic DNA from bacteria exposed to GI were analysed by agarose (1%) gel electrophoresis using a 1 × TAE buffer. Standardised DNA samples from each irradiation dose were loaded with ethidium bromide and visualised under UV light. The experiments were carried out in triplicate.

Inoculations. The bacterial suspension was injected into the abaxial surface of the *Arabidopsis* (Col-0) and tomato seedling (*Lycopersicon esculentum*) leaf using a needleless syringe. Following the inoculation

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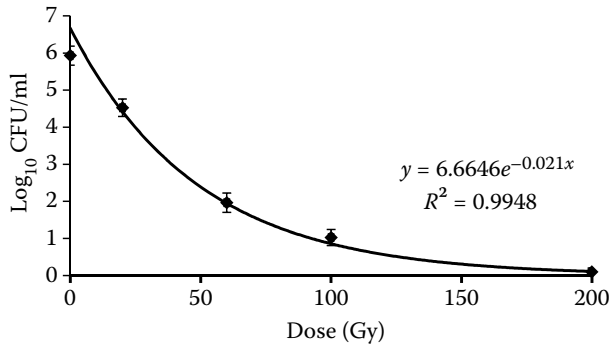

Figure 1. Inhibitory effect of gamma irradiation of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Survival curves of *Pst* DC3000. Experiments were conducted independently thrice. Error bar represents standard errors of three replications

of *Pst* DC3000, inoculated leaves were kept to dry at room temperature for 1 hour. These plants were irradiated at 0, 50, 100, 150, and 200 Gy of GI. Three leaf discs from the inoculated leaves were collected and homogenised in 10 mM $MgCl_2$ diluted 10^3- or 10^4-fold and plated on King's B medium. *Arabidopsis* plant and tomato seedlings were grown to monitor the disease symptoms for 10 days in MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and 14-h photoperiod.

RESULTS AND DISCUSSION

To determine the effect of GI on *Pst* DC3000, the *Pst* DC3000 suspensions (10^5 CFU/ml) were irradiated at a dose of 0, 20, 60, 100, and 200 Gy (Figure 1). The number of colonies of *Pst* DC3000 was significantly decreased with increasing doses of GI. The treatment of 100 Gy resulted in an approximate 5-log reduction of the viable count compared to the initial counts. Moreover, *Pst* DC3000 was completely inactivated at 200 Gy, which was the lethal dose (Figure 1). It is well known that ionising radiation deals with the inactivation of microorganisms including insects, fungi, bacteria, and viruses (Aquino 2012). Intriguingly, bacteria such as *Escherichia coli*, *Salmonellae*, and *Campylobacter jejuni* were also reduced with increasing doses of GI, but they were completely inactivated at 2.5 kGy as a lethal dose, higher dose than that of *Pst* DC3000 due to microorganism sensitivity to radiation (Mayer-Miebach *et al.* 2005; Lee *et al.* 2006). Therefore, this high sensitivity of *Pst* DC3000 to GI might be useful for its applications to the control of bacterial pathogens in crops.

Morphological changes of bacterial cells after irradiation were examined using a scanning electron microscope (SEM). SEM was employed with cells with irradiation doses of 0, 50, 100, 150, and 200 Gy, and the shapes of their surface were compared. It was found that non-irradiated cells had a smooth surface, while irradiated cells with increasing doses appeared more rugged and their surfaces became irregular (Figure 2). It is obvious from the images that the shapes of higher-dose irradiated cells showed significant structural changes compared to non-irradiated spores (Figure 2). The present study demonstrates the remarkable dose-dependent alterations in the surface of the *Pst* DC3000. GI also strongly affects the surface changes of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) in a dose-dependent manner (unpublished data). These structural changes can be caused by oxidative reactions that can destabilizse the bacterial chromosomal DNA-membrane interactions (Rosen *et al.* 1990).

The damages to the membranes of *Pst* DC3000 by GI prompted us to examine the membrane integrity. A fluorescent dye, propidium iodide (PI), was used to determine whether GI led to the loss of membrane integrity in *Pst* DC3000. Cells that have lost their membrane integrity showed red staining under a fluorescence microscope. It was shown that, compared with the control, more cells were stained with PI after irradiation (Figure 3A). This indicates that an increase in GI increased the number of strongly PI-stained cells in a dose-dependent manner. Irradia-

Figure 2. Scanning electron microscope image illustrating the effects of gamma irradiation (0, 50, 100, 150, and 200 Gy) on the morphology of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000

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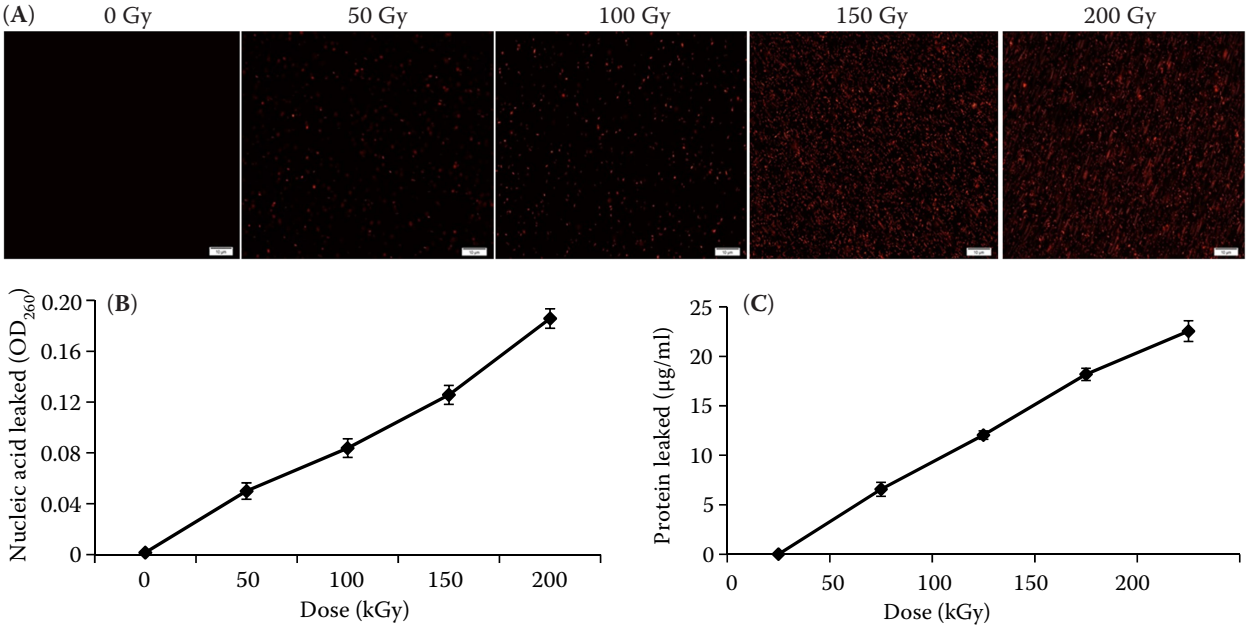

Figure 3. *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 viability and leakage of nucleic acid and protein from irradiated bacterial cells. (**A**) Loss of membrane integrity of *Pst* DC3000 after gamma irradiation (bar represents 10 µM). The released amount of nucleic acid (**B**) and protein (**C**) in the supernatant of the cell suspension were measured (Error bar represents standard errors of three replications)

tion inhibits the growth of *Pst* DC3000 by directly damaging the membrane and causing the cell death of the bacterial pathogen. Damage to the membrane leads to the loss of osmotic balance and an influx of fluids and ions, as well as a loss of proteins and ribonucleic acid, eventually causing cell death (Woo *et al.* 2000). To verify this hypothesis of whether irradiation caused cell damage of *Pst* DC3000, the leakage of the nucleic acid and protein was determined. Nucleic acid and its related compounds, such as pyrimidines and purines, are well known to absorb UV light at a wavelength of 260 nm (Khalil & Villota 1988). Furthermore, similarly damaged cells are also known to release intracellular proteins into a suspension. The loss of cell cytoplasmic content was found to be correlated with the GI dose. The amount of nucleic acid released into the cell suspension was analysed by measuring the absorbance at 260 nm (Figure 3B). The amount of leaked nucleic acid from the cells grew relative to an increase in the increasing doses of the cell suspension. In addition, the amount of protein released into the cell suspension was also analyzed. Moreover, the amount of leaked protein was shown in a dose-dependent manner (Figure 3C). These data suggest that irradiation might directly act on the cell membrane of the bacteria, leading to the breakdown of the cell membrane and release of cell materials. These data verify the destruction of cells and provide clear evidence of the antibacterial activity of irradiation. A previous study suggested that e-beam irradiation increases the inactivation of bacteria through DNA fragmentation (Fiester *et al.* 2012). To test this hypothesis, genomic DNA extracted from irradiated cells was measured to determine the effect of GI on the DNA integrity. DNA concentrations were standardised at 1.2 µg per well prior to electrophoresis. The increasing dose

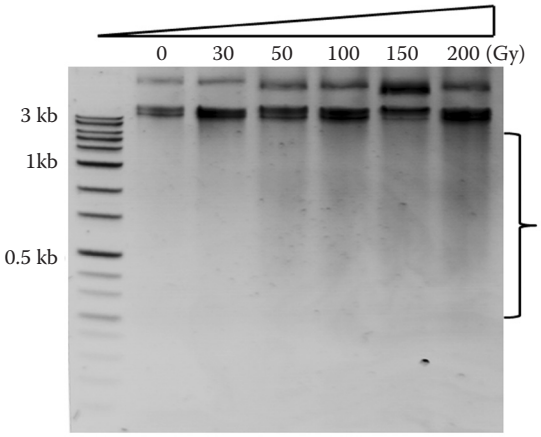

Figure 4. Effect of gamma irradiation (GI) on the genomic DNA of bacteria. Visualisation of the effect of GI on the electrophoretic pattern of DNA extracted from 0, 30, 50, 100, and 150 Gy

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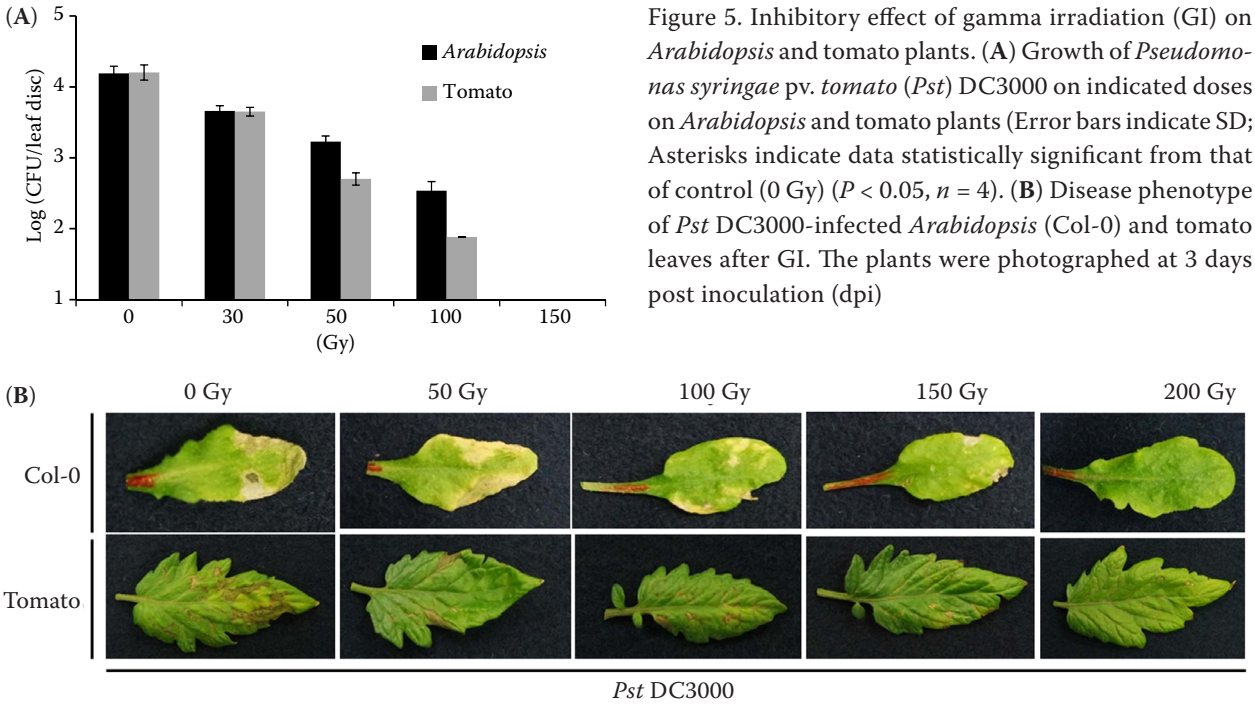

Figure 5. Inhibitory effect of gamma irradiation (GI) on *Arabidopsis* and tomato plants. (**A**) Growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 on indicated doses on *Arabidopsis* and tomato plants (Error bars indicate SD; Asterisks indicate data statistically significant from that of control (0 Gy) ($P < 0.05$, $n = 4$). (**B**) Disease phenotype of *Pst* DC3000-infected *Arabidopsis* (Col-0) and tomato leaves after GI. The plants were photographed at 3 days post inoculation (dpi)

of GI caused a degradation of the genomic DNA of bacteria (Figure 4).

To examine whether GI has antibacterial activity *in vivo*, we introduced GI to *Arabidopsis* (Col-0) and tomato seedlings infected with *Pst* DC3000 (Figure 5A). Doses of 30, 50, 100, and 150 Gy were used to inhibit the bacterial development. The complete inhibitory effect was shown at 150 Gy (Figure 5A). There were no disease symptoms when irradiated at 150 Gy in *Arabidopsis* and tomato seedlings (Figure 5B). Consistent with a previous study, up to 200 Gy of GI did not significantly affect the physiological and morphological changes on *Arabidopsis* plants and tomato seedlings (Norfadzrin *et al.* 2007; Kim *et al.* 2011). These results suggest that 150 Gy of GI efficiently control bacterial speck disease of tomato seedlings without any phytotoxicity.

In the present study, GI could directly inhibit the growth of *Pst* DC3000 (*in vitro*) and reduced the disease severity of bacterial specks on tomato seedlings. It is important to note that *Pst* DC3000 sterilisation can be due to a loss of membrane integrity, a loss of DNA integrity, or both. Thus, we conclude that cell membrane damage, altered membrane permeability, and subsequent spore leakage play a critical role along with DNA fragmentation resulting in bacteria inactivation. Our results suggest that GI is a promising approach to the control of bacterial diseases on fruits and vegetables.

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Corresponding author.

Dr Rae-Dong Jeong, Research Division for Biotechnology, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 56212, Republic of Korea, E-mail: jraed2@kaeri.re.kr