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Effect of Gamma Irradiation on the Growth and Patulin Production of *Penicillium griseofulvum* in an Apple Model System

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Abstract The effect of gamma irradiation on the prevention of breeding a patulin-producing mold and reducing patulin content was evaluated in an apple model system. *Penicillium griseofulvum*, a patulin-producing standard mold strain was artificially inoculated into apples and a gamma irradiation was performed. The D_{10}-values of the conidia of *P. griseofulvum* in an aqueous suspension and the apple model system were calculated at 0.28 and 0.48 kGy, respectively. The viable cell counts of the inoculated conidia in the apples showed 2 decimal point reductions at a dose of 1 kGy. Breeding and growth of the survived conidia was prevented during 10 weeks of post-irradiation storage period, especially at 4°C. The concentration of patulin in the non-irradiated apples was gradually increased and reached about 950 ppm at 25°C and 410 ppm at 4°C, but the production of patulin was not observed during storage after 1 kGy of gamma irradiation.

Keywords: patulin, *Penicillium griseofulvum*, apple, gamma irradiation

Introduction

Patulin [4-hydroxy-4H-furan(3,2C)-pyran-2(6H)-one] is a food mycotoxin which is generally produced from mold strains of *Penicillium* and *Aspergillus* spp. (1-4). Patulin has the features of an anti-microbial and a mutagenic activity, and it also influences the nervous and immune systems (5-7). Especially, patulin causes an acute poisoning to the digestive system of infants (5-7). Although patulin has been detected in various grains and fruits, the most frequent contaminations have been reported in apple and apple juice. Therefore, patulin has been controlled by an important quarantine and food safety standard for apple and processed apples (8,9). The quarantine limitation of a patulin concentration in apple juice has been regulated at less than 50 μg/L (50 ppb) by World Health Organization (WHO) (10) and Food and Agriculture Organization (FAO) (5). For several decades, patulin has been studied as a matter of concern in the fields of detection (11), epidemiological investigation (12), and isolation as well as the characterization of a patulin-producing mold (13).

There are two typical technical approaches for reducing a patulin level. The first method is the decomposition of patulin by chemical, biochemical, and physical treatments. The other one is the sterilization and/or control of a patulin-producing mold. Various methods such as charcoal treatment, filtration, ascorbic acid treatment, and fermentation have been partially introduced to reduce the levels of patulin in apple and apple juice (14). However, these processes are generally expensive, time-consuming, and inefficient. Therefore, there is the need to develop an economical and easily adaptable industrial process to control patulin levels. Cold storage and pasteurization methods have been widely used in the preservation of apple and apple juice, respectively. However, a few microorganisms can grow at a low temperature after these treatments (15), and the patulin present in the apple juice has been known to be stable against a heat treatment (16).

Food irradiation has been described to be a powerful process for a food preservation and sterilization (17,18). Generally, fungal strains are more sensitive to a radiation than bacteria, and it is generally accepted that about 1 kGy of a radiation dose is sufficient for a fungal removal in fruits (19). In addition to that, it has been reported that a gamma irradiation could reduce a patulin level in apple juice (20). This effect is caused by the strong activity of the free radicals induced from water molecules by an ionizing radiation. Therefore, a gamma irradiation may be highly effective for both reducing the contaminated patulin level and controlling the patulin-producing mold in apple.

In the present study, the effects of a gamma irradiation on the control and/or sterilization of patulin-producing fungus, *P. griseofulvum*, inoculated into an apple model system were determined. In addition, the microbial growth of the aerobic bacteria, lactobacilli, and yeasts after a gamma irradiation was investigated.

Materials and Methods

Reagents and samples Standard patulin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and the media for a microbiological determination were purchased from Difco Laboratories (Detroit, MI, USA).

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Apples (*Malus pumila* var. *dulcissima*) were purchased at Jeongup, Korea at November 2005. All other chemicals were commercially available products of analytical grade.

Gamma irradiation to apples The apples were inflicted with an artificial wound with a 2 mm diameter on the surface. The apples were put into a polyethylene (PE) vinyl bag, sealed, and irradiated in a cobalt-60 irradiator (point source AECL, IR-79; MDS Nordion International Co., Ltd., Ottawa, ON, Canada) at the Korea Atomic Energy Research Institute (KAERI), Jeongup, Korea. The source strength was approximately 100 kCi with a dose rate of 10 kGy/hr at 10±0.5°C. Dosimetry was performed by using 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany), and the free radical signal was measured by a Bruker EMS 104 EPR analyzer. The dosimeters were calibrated against an international standard set by the International Atomic Energy Agency (IAEA, Vienna, Austria). The applied dose was 1 kGy, which is the recommendation dose for a fruit irradiation by the IAEA (21). Non-irradiated sample was also prepared as a control. The gamma irradiated and non-irradiated apples were stored at 4 and 25°C, and they were analyzed at 0, 1, 3, 5, and 10 weeks of storage period.

Preparation of the sample inoculated with *P. griseofulvum* For the artificial inoculation test, the apples were sterilized by a gamma irradiation (5 kGy). Radiation-sterilized apples were found to be devoid of any viable microorganism. The type strain of the patulin-producing mold, *P. griseofulvum* (ATCC 46037), was obtained from American Type Culture Collection (Manassas, VA, USA). *P. griseofulvum* was cultivated on potato dextrose agar (PDA) plates for 7 days at 30°C. The conidia on the plates was washed twice with a sterile Tween 80 (0.1%, v/v) solution and they were centrifuged (1,500×g for 10 min at 4°C) in a refrigerated centrifuge (VS-5500; Vision Scientific, Co., Seoul, Korea). The pellets of the conidia were washed twice with sterile saline and they were resuspended in a cell density of 10^7 cells/mL. The test culture suspension (10 μL) was uniformly and aseptically inoculated on the artificial wound of the apple. The apples inoculated by *P. griseofulvum* were put into a PE vinyl bag, and then they were irradiated in a cobalt-60 irradiator at a dose of 1 kGy. The gamma irradiated and non-irradiated apples were stored at 4 and 25°C, and they were analyzed at 0, 1, 3, 5, and 10 weeks of storage period.

Microbiological analysis Three apples per each treatment were sliced, thoroughly mixed, and then each sample was homogenized for 5 min at 4°C with an equal volume of sterile saline solution (NaCl, 0.85%). The samples for the microbiological count were prepared in a series of decimal dilutions by a sterile saline solution. The media used for the total aerobic bacteria and *Lactobacillus* group were a plate count agar and an MRS agar, respectively. Selective media for the enumeration of the yeast and mold were prepared by adding chloramphenicol (100 mg/L) and 10% tartaric acid (17 mL/L) on PDA. Each diluent (100 mL) was spread in triplicate on each agar plate and the plates were incubated for 72 hr, and then the colony formation units (CFU) per gram were calculated. The incubation temperatures for the total aerobic bacteria, *Lactobacillus* spp., yeast, and mold were 37, 30, 25, and 25°C, respectively.

Estimation of the radiation sensitivity of *P. griseofulvum* An aqueous suspension of *P. griseofulvum* conidia was washed twice with a sterile Tween 80 (0.1%, v/v) solution and they were centrifuged (1,000×g for 10 min at 4°C) in a refrigerated centrifuge (VS-5500; Vision Scientific, Co.). The pellets of the conidia were washed twice with sterile saline and they were resuspended in a cell density of 10^7 cells/mL. The suspension was transferred to a 5-mL conical tube, and then it was irradiated in a cobalt-60 gamma irradiator at doses of 0, 0.5, 1.0, and 2.0 kGy. Viable counts of the irradiated and non-irradiated samples on the PDA plates were examined. The radiation sensitivities were calculated as D_{10}-values, an expression of the radiation dose needed to reduce the number of microorganisms by 10-fold. The value was calculated from the equation of the survival plot (22).

Analysis of the patulin Extraction and analysis of the patulin was performed by the method of AOAC (23). Three apples per each treatment were sliced and thoroughly mixed. Fifty mL of apple juice was extracted with 50 mL of ethyl acetate. Each extract was mixed with 20 g of anhydrous sodium sulfate and incubated at room temperature for 30 min, and then it was filtrated using filter paper (Whatman No. 4). The filtrated samples were concentrated at 40°C by a nitrogen stream, cooled at room temperature, dissolved in distilled water (pH 4.0), and then stored at −70°C. Quantitative detection of the patulin was carried out using a high performance liquid chromatography (HPLC). A 10 μL aliquot was injected into an HPLC (Waters Associates, Milford, MA, USA) with a built-in ultraviolet (UV) detector at 276 nm. HPLC analyses were performed with a Shiseido column (3.9 mm i.d., 300 mm length) and the extracts were separated with a distilled water-acetonitrile (95:5) solvent at a flow rate of 0.7 mL/min. The column temperature was maintained to be constant at 40°C. Identification of the patulin produced from the isolated fungal strain was performed by the retention time with the confirmation of a standard patulin.

Results and Discussion

Microbial analysis of the gamma irradiated apple The artificially wounded apples were treated by gamma irradiated (1 kGy) and the growth rates of the total aerobic bacteria, lactobacillus, yeast, and mold were evaluated during 10 weeks of storage period at 4 and 25°C (Fig. 1). The natural contamination state of the total aerobic bacteria, lactobacillus, yeast, and mold in the apples were on the levels with 9.52×10^3, 8.27×10^2, 6.84×10^1, and 8.74×10^1 CFU/g, respectively. After the gamma irradiation with a dose of 1 kGy, the viable cells of total aerobic bacteria and the lactobacillus were decreased by 1 decimal point reduction, and the yeast and mold groups were decreased by 2 decimal point reductions (Fig. 1). This result was comparable to the previous result described by Aziz and Moussa (24).

The population of the total aerobic bacteria in the non-irradiated apples gradually increased and reached about 10^6

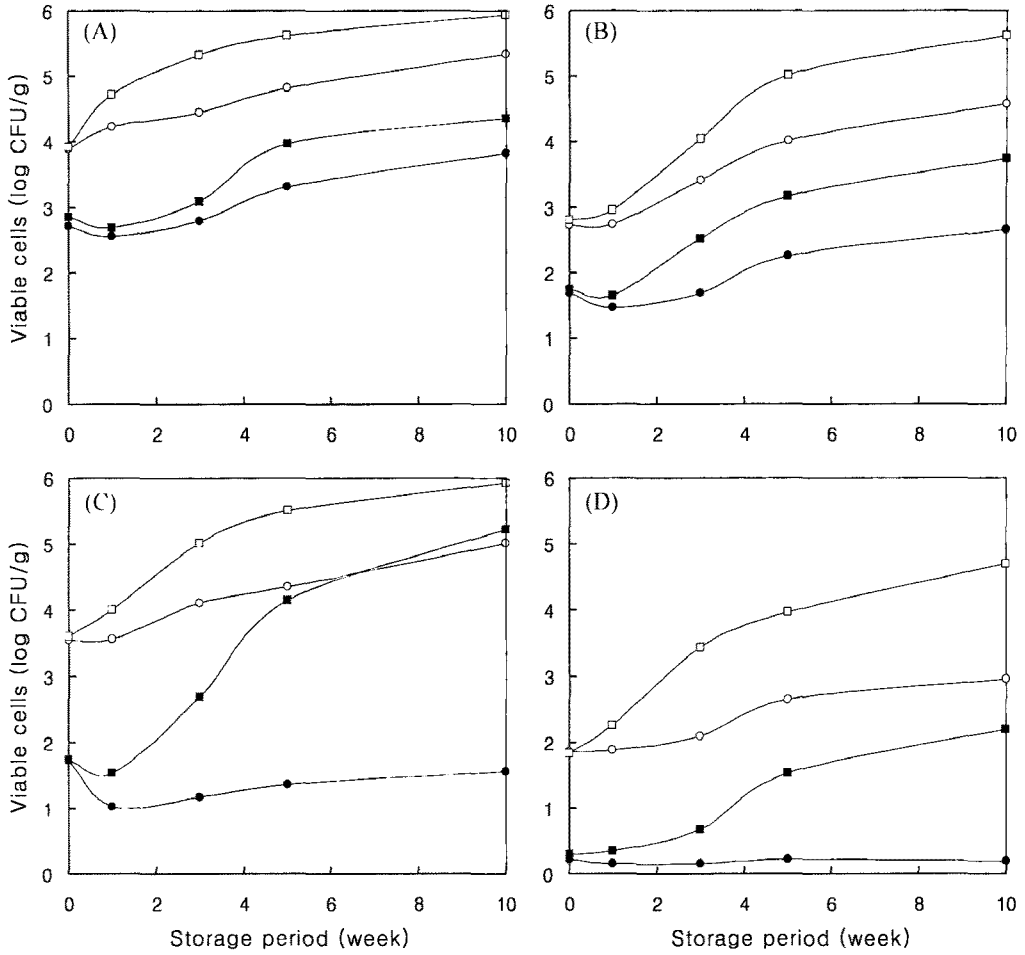

Fig. 1. The growth of aerobic bacteria (A), lactobacillus (B), yeast (C), and mold (D) in apples during the storage for 10 weeks at 4 and 25°C. (■) Irradiated (1 kGy) and stored at 25°C, (●) irradiated (1 kGy) and stored at 4°C, (□) non-irradiated and stored at 25°C, (○) non-irradiated and stored at 4°C.

CFU/g at 25°C and 10^5 CFU/g at 4°C after 10 weeks of storage time. However, those of the 1 kGy-irradiated sample were maintained at about 2 decimal points lower (Fig. 1A) when compared with the non-irradiated control at the same storage temperature. The growth patterns of the lactobacillus in the samples had some similarities with the total aerobic bacteria (Fig. 1B). During the storage periods, the yeasts were detected with a level of 10^1 CFU/g (Fig. 1C) and mold was rarely detected (Fig. 1D) in the sample stored at 4°C after a gamma irradiation. Despite a gamma irradiation treatment, the yeast grew logarithmically after the first week's storage point and reached 10^5 CFU/g after 10 weeks of a storage at the condition of 25°C. This result is caused by the nutritional and physicochemical properties of the apples. Composition of sugars and organic acids, a low pH (3-4) and high water activity provide more appropriate growth conditions for yeast group. Regardless of the storage conditions, the microbial populations of the irradiated samples were decreased slightly during the first week's storage. It was considered that the decrease of the microbial population was due to a post-irradiation effect where surviving cells that had been damaged by a gamma irradiation gradually died, by not adapting to the surrounding environment (25). However, after 3 weeks of a storage period, the viability of the microbes was recovered, and the microbes grew gradually, especially at the storage condition of 25°C. These results indicate that the apples should be stored at a low temperature to avoid a decay and/or microbial growth even after the gamma irradiation.

Effects of gamma irradiation on the apple inoculated with the *P. griseofulvum* After inoculating *P. griseofulvum* into apples, the samples were irradiated by gamma irradiation (1 kGy) and the growth rates of the *P. griseofulvum* were evaluated during the 10 weeks of a storage period at 4 and 25°C (Fig. 2). The initial contamination state of the *P. griseofulvum* in the apples was 2.36×10^3 CFU/g, and it was decreased by 2 decimal point reductions by a gamma irradiation with a dose of 1 kGy. *P. griseofulvum* in the non-irradiated apples was gradually increased and reached about 10^5 CFU/g at 25°C and 10^4 CFU/g at 4°C. However, those of the 1 kGy-irradiated sample were maintained about 2 decimal point reduction (Fig. 2) when compared with the non-irradiated sample at the same storage temperature. During the storage periods, the *P. griseofulvum* was detected with a level of 10^1 CFU/g or less in the sample stored at 4°C after a gamma irradiation. However, in spite of a gamma irradiation the *P. griseofulvum* grew logarithmically from the first week's storage and reached 10^3 CFU/g after 10 weeks storage at 25°C.

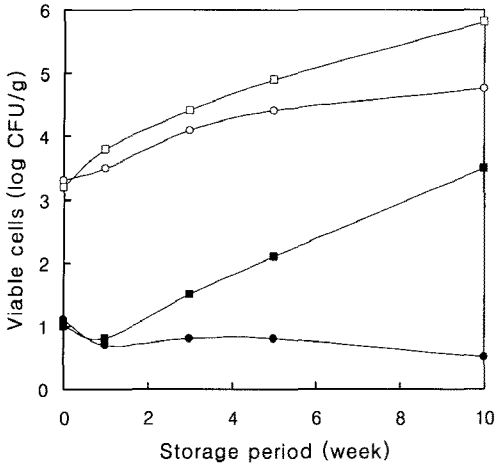

Fig. 2. The fungal growth of apples inoculated with *P. griseofulvum* during the storage for 10 weeks at 4 and 25°C. (■) Irradiated (1 kGy) and stored at 25°C, (●) irradiated (1 kGy) and stored at 4°C, (□) non-irradiated and stored at 25°C, (○) non-irradiated and stored at 4°C.

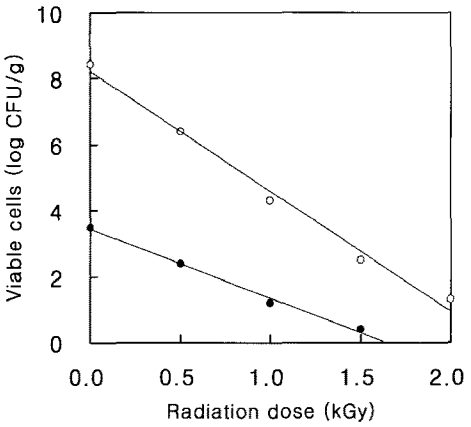

Fig. 3. Survival rate and D_{10}-value of *P. griseofulvum* by gamma irradiation in an apple model system (●) and potato dextrose broth (○).

Radiation sensitivity of *P. griseofulvum* The D_{10}-value of 0.28 kGy was calculated for a gamma irradiated aqueous conidia suspension of *P. griseofulvum* (Fig. 3). The radiation sensitivity of the conidia of *P. griseofulvum* was similar to that of *Aspergillus* and *Penicillium* spp., generally in the range of 0.2-0.4 kGy (26). Conidiospore of *P. griseofulvum* had a D_{10}-value of 0.48 kGy in the apple model system. It is generally recognized that the radiation survival rate of a microorganism is affected by the extracellular environment, e.g., the chemical composition of the foods. D_{10}-values of mold conidia are generally higher, when they are irradiated in a dry state food system than those in a regulated aqueous system (27).

Patulin analysis Apples were inoculated with conidia of the patulin-producing fungus, *P. griseofulvum* (10^3 conidia per gram apple), gamma irradiated (1 kGy), and then were evaluated for production rates of the patulin during the storage periods at 4 and 25°C. Patulin in the non-irradiated apples gradually increased and reached about 950 ppm at 25°C and 410 ppm at 4°C, but a patulin production was not

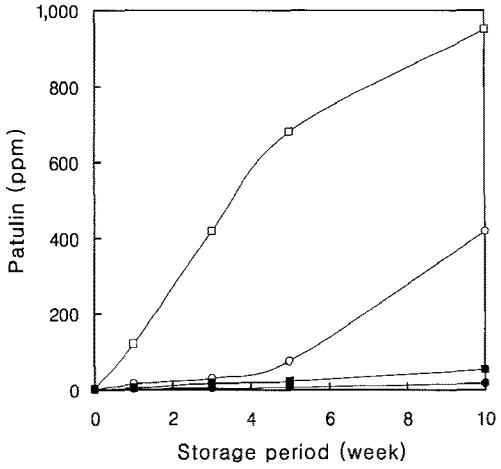

Fig. 4. The patulin production of apples inoculated with *P. griseofulvum* during the storage for 10 weeks at 4 and 25°C. (■) Irradiated (1 kGy) and stored at 25°C, (●) irradiated (1 kGy) and stored at 4°C, (□) non-irradiated and stored at 25°C, (○) non-irradiated and stored at 4°C.

detected within the post irradiation storage period (Fig. 4). Previous reports showed that a gamma irradiation is effective in preventing a mycotoxin production in food and grains by sterilization of the mycotoxin-producing moulds (24). When barley was inoculated with the conidia of *A. alutaceus* (10^6 conidia per gram), ochratoxin was not detected after 3.0 kGy of an electron beam irradiation and 4.0 kGy of a gamma irradiation (28).

In conclusion, since the natural contamination levels of the molds in apples were reported in the range of 10-10^3 CFU/g (29), it is suggested that 1.0 kGy of a gamma radiation is enough to prevent the growth of a patulin-producing mold and the production of patulin in apples, especially in the conditions of a cold storage after a irradiation treatment.

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