Radiation Sensitivity of Surface Mycoflora from Fresh Strawberries (*Fragaria* x *ananassa* Duch. var. Sweet Charlie) from La Trinidad, Benguet, Philippines

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Abstract

Fungi cause postharvest diseases in a wide variety of crops around the world. Without proper identification of the prevalent fungal pathogens in crops, the efficiency of postharvest treatment is not always assured. Irradiation, a non-chemical approach, has been explored as an alternative treatment to control plant fungal pathogens. However, relevant studies on the radiation sensitivity of foodborne fungal pathogens in fresh produce and other relevant biological responses to radiation are quite limited, especially in the Philippine setting. In this study, surface fungal pathogens were isolated from fresh strawberries (Fragaria x ananassa Duch. var. Sweet Charlie) from La Trinidad, Benguet, Philippines and identified through molecular techniques. Radiation responses of fungal pathogens such as in vitro radiosensitivity based on D10 value and physio-morphological changes were determined. Molecular identification and evolutionary analysis revealed the presence of two fungal pathogens, namely Mucor circinelloides and Mucor irregularis. Gamma radiation effectively reduced the fungal population with D10 values of 0.6 and 1.4 kGy for M. irregularis and M. circinelloides, respectively, with complete growth inhibition at 3-5 kGy. Disruptions of sporangium and sporangiophores were also observed for both isolates at 1-2 kGy. The result of this study is expected to provide new data on the possible use of gamma radiation as hurdle technology for fresh fruits in the country.

Keywords*:* foodborne pathogens, fungal contamination, food irradiation, physio-morphological changes, radiation sensitivity

1. Introduction

The worldwide production of strawberries (*Fragaria* x *ananassa* Duch.) has increased by more than three-folds for the past three decades (Food and Agriculture Organization – United Nation [FAO-UN], 2021). Seventy-nine countries have cultivated this temperate fruit crop, and a combined 8.9 million tons of fresh fruits have been produced in 2019 alone (FAO-UN, 2021). Though traditionally associated with temperate countries, strawberries can also thrive in colder mountainous regions of the tropics (Darrow, 1966). In Asia, the Philippines is the only tropical country that produced it on a commercial scale, from 654 tons in 2009 to 1,102 tons in 2019 (FAO-UN, 2021). About 95% of strawberry production in the Philippines is located in the high-altitude municipality (about 1,300 masl) of La Trinidad, Benguet owing to its relatively mild temperature throughout the year, which is conducive for the fruit's growth and development (Department of Agriculture – Bureau of Agricultural Research [DA-BAR], 2006; Dumaslan and Kilakil, 2019). Several cultivars have been grown in the area including United States varieties ('Sweet Charlie,' 'Chandler' and 'Whitney') and Japan varieties ('Toyonoka' and 'Nyoho') (Ledesma *et al*., 2020). At present, the Philippine local strawberry farms commonly grow the 'Sweet Charlie' variety and consider it as most popular and treasured crop (Dumaslan and Kilakil, 2019). As the primary producer in the Philippines, strawberry industry in La Trinidad, Benguet faces issues on transportation storage and postharvest diseases (DA-BAR, 2006).

Like other fresh fruits, strawberries are highly susceptible to bruising or mechanical damage during harvest and at all stages of postharvest handling (Hussein *et al*., 2020). They are also prone to necrotrophic fungal infections such as with the ascomycetes *Botrytis cinerea* (Petrasch *et al*., 2019). Fungal diseases are prevalent in fruits leading to an estimated loss of 30-50% of the postharvest (Qadri *et al*., 2020). In 2017, approximately 5-10% of strawberry production in La Trinidad, Benguet was lost due to postharvest damage from pests and diseases (Ticbaen, 2017). The susceptibility of strawberries to bruising, leading or not leading to subsequent fungal infection, can be influenced by post-climacteric factors like temperature and humidity (Hussein *et al*., 2020). It was reported by Ferreira *et al*. (2009) that the incidence and severity of bruising for three strawberry cultivars are temperature-dependent. The bruise volume was significantly higher at 30 °C than 0-4 °C. Although there is limited information on the effects of storage humidity on bruising incidence on fresh strawberries, Banks and Joseph (1991) observed a drop in

compression bruising of bananas when stored at low relative humidity (< 75%). Thus, in general, the Philippine climate may explain the postharvest losses experienced by the fruit industry.

Physical, chemical and biological treatments have been used to manage postharvest diseases with the end goal of keeping fruits disease- or symptom-free until it is marketed or consumed (Gachango *et al*., 2012; Qadri *et al*., 2020). The increasing global demand for chemical-free fresh produce, the development of fungal resistance and the rising treatment cost have likewise opted for the transition from the use of synthetic fungicides to more eco-friendly and sustainable alternatives (Korsten, 2006; De Costa and Gunawardhana, 2012).

Recent studies consistently showed that irradiation kills bacterial pathogens on fresh and fresh-cut produce (Smith and Pillai, 2004; Niemira and Fan, 2005; Fan *et al*., 2008). It is also efficacious with other microbiologically important food hazards such as the antibiotic-resistant strains of *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* (Dharmarha *et al*., 2019), *Salmonella*, *Listeria monocytogenes* (Smith and Pillai, 2004; Niemira and Fan, 2005); yeast, molds, fungal toxins (Fan *et al*., 2008) and foodborne viruses (Bidawid *et al*., 2000; Pexara and Govaris, 2020). Special attention has been given to fungal pathogens, not just for their economic impact on crop productivity but also in the context of food safety. Fungal pathogens that infect crops in the Philippines include anthracnose and rotting diseases like *Athelia rofsii* in banana (Acabal *et al*., 2019), *Colletotrichum* sp. and *Fusarium chlamydosporum* in soursop ('*guyabano*') (Alberto and Otanes, 2016) and *Neoscytalidium dimidiatum* and *Colletotrichum* sp. in dragon fruits (Pascual *et al*., 2016). Mycotoxigenic fungi like *Aspergillus flavus*, *Fusarium* and *Penicillium* spp. are also found in other commodities such as coffee, rice, corn, banana, sorghum, peanut and soybean (Balendres *et al*., 2019). As these biological agents can be found in soil, contamination with other fresh produce is prevalent. Hence, proper identification of fungal pathogens is important to ensure the efficiency of postharvest treatment like gamma-radiation.

Likewise, the sensitivity and survival of various fungal pathogens after irradiation varies depending on the fungal species, the source of ionizing radiation and the nature of the material in which the fungal pathogen thrives. Both in vitro and in vivo studies on the sensitivity and survival of the most common foodborne fungal pathogens in the local setting are limited, especially on the dose range of gamma radiation required to achieve D10 values of pathogenic fungi in vitro as well as the radiation response to some

of the physio-morphological properties of the exposed fungi. Thus, this study investigated the in vitro radiation sensitivity of fungal pathogens present on the surface of locally produced, healthy-looking Strawberry (*Fragaria* x *ananassa* Duch. var. Sweet Charlie) fruits from La Trinidad, Benguet and assessed some physio-morphological properties of the identified fungal pathogens. The results suggest how to optimize food irradiation as a hurdle technology for fresh fruits in the Philippines.

2. Methodology

2.1 Sample Collection and Preparation

A total of 5-kg ripe strawberries (*Fragaria* x *ananassa* Duch. var. Sweet Charlie) were harvested from a strawberry farm in La Trinidad, Benguet, Philippines (located in high altitude area; February 2019). These were packed in rectangular slotted plastic baskets and transported in an air-conditioned vehicle back to the Philippine Nuclear Research Institute (PNRI), Quezon City, Metro Manila for fungal isolation and storage. Upon arrival at PNRI, fruits with visible damage were sorted out; only healthy-looking ones were used.

2.2 Fungal Isolation and Morphological Characterization of Colonies

The surface microflora was determined by immersing 50 g of fruits in 450 mL of phosphate-buffered solution (pH 7.4). About 100 µL of the solution was inoculated in potato dextrose agar (PDA) (HiMedia Lab Pvt. Ltd., India) using spread plate technique and incubated at 30 °C for 72 h. Chloramphenicol (50 µg/L) was utilized to inhibit bacterial growth in the medium. All fungal colonies were isolated using the single spore touch method with some modification (Goh, 1999; Senanayake *et al*., 2020). Briefly, a portion of the sporulating colony was transferred aseptically onto the surface of a clean dish with 1 mL of 0.05% Triton X-100 (Boehringer Mannheim GmbH, United States). Using an inoculating needle, the colony was spread out and a single spore was transferred to PDA plates to obtain axenic mycofloral culture. Morphological observation based on the mycological typing methods of Guarro *et al*. (1999) was performed on fungi grown on PDA mounted on Shear's solution. Fungal structures such as hyphal elements, sporulating structures and other nonspecific structures were observed through a laboratory microscope (BB Evo Model, Euromex Microscopen bv, Netherlands).

2.3 Deoxyribonucleic Acid (DNA) Sequencing and Phylogenetic Analysis

The five-day-old axenic cultures in PDA plates were sent to Macrogen Co., Ltd. (Seoul, South Korea) for DNA extraction and sequence analysis of the partial internal transcribed spacer (ITS) regions. DNA extraction was performed by boiling lysis method following Queipo-Ortuno *et al*. (2008) with some modifications. Briefly, distilled water was poured on culture plates. The fungal mycelium from 1-mL cell suspension was pelleted in centrifuge tubes at 15,000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 40-µL distilled water, subjected to boiling at 100 °C in a water bath for 10 min, cooled on ice and centrifuged at 15,000 g for 10 s. An aliquot of 2 µL of template DNA was used for polymerase chain reaction (PCR). The primers ITS1 5'(TCC GTA GGT GAA CCT GCG G)3' and ITS4 5'(TCC TCC GCT TAT TGA TAT GC)3' were mixed with a cocktail mixture: 10X Taq PCR buffer, 2.5-mM dNTP mixture, KOMA - Taq (2.5 U/µL) and high-performance liquid chromatography (HPLC)-grade distilled water under cycle condition as stated: initial denaturation at 95 °C for 5 min, 30 cycles (denaturation at 95 °C for 0.5 min, annealing at 55 °C for 2 min and extension at 68 °C for 1.5 min) and final extension at 68 °C for 10 min. PCR products were purified using the Montage PCR Clean-up Kit (Millipore) and sequenced in both directions using the respective PCR primers. The BigDye™ Terminator kit v 3.1 Cycle Sequencing Kit (Applied Biosystems, United States) was used and sequencing products were resolved through an automated DNA sequencing system (Model 3730XL, Applied Biosystem, United States).

The sequence obtained from each strain was aligned with the Basic Local Alignment Search Tool (BLAST) algorithm to obtain related sequences. The evolutionary history of the partial ITS sequences of the isolates were analyzed using the maximum likelihood (ML) method and Tamura 3-parameter model (Tamura, 1992) conducted in MEGA X (Kumar *et al*., 2018; Stecher *et al*., 2020). The analysis revealed a tree (Figure 2) that has the highest log likelihood (-3412.73). Initial trees for the heuristic search were obtained by Neighbor-Join and BioNJ algorithms to a matrix of pairwise distance estimated using the Tamura 3-parameter model. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories [+G parameter = 0.4584]). The tree is drawn to scale with branch lengths measured in the number of substitutions per site.

2.4 Determination of Radiation Sensitivity of Fungal Isolates

Pure culture of the fungal isolates was incubated at 30 °C for three to five days in Saboraud's dextrose agar medium (HiMedia Lab Pvt. Ltd., India) supplemented with 50-µg/L chloramphenicol. The fungal inoculum was prepared by flooding the subculture plates with 0.05% Triton X-100 and shaking with sterile glass beads to harvest sporangiospores. The concentration was estimated initially by adjusting the turbidity using sterile water and comparing the final inoculum with 0.5 McFarland standard. The actual concentration was later confirmed using a hemocytometer (Improved Neubauer Counting Chamber, Ingram and Bell Ltd., Germany). Adjustment of the spore concentration was performed as needed to obtain approximately log10 8.2 CFU/mL. This suspension was employed as the final inoculum solution.

A total of 15 mL of the inoculum were aseptically transferred to a sterile cell culture flask (Corning Culture Flask, Sigma Aldrich Inc., United States). The samples (n = 3 per treatment) were then subjected to gamma radiation emitted from 60Co-source (Ob-Servo Sanguis-04, Institute of Isotope Co., Ltd., Hungary) at doses 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 kGy with a dose rate of 2.25 kGy/h. The absorbed dose was calibrated using an alanine dosimeter. After irradiation, the samples were stored at 4 °C before the analysis. The number of surviving sporangiospores (in colony-forming units/CFU) was counted after 24-72 h of incubation at 30 °C. The analysis was carried out in triplicate and three repeats (n = 3). The survival ratio, presented as log N/No, where N is the number of surviving spores in irradiated samples and No is the initial number of spores in control samples, was plotted against radiation dose per fungal isolate to obtain the survival curves. Simple linear regression analysis was done to obtain the best-fit model for both isolates. Radiation sensitivity was expressed in terms of D10 value – a measure of the treatment required for a 1-log reduction on the survival ratio. Data analysis at $\alpha = 0.05$ was performed to verify whether the constructed survival curve is a good model for the linear relationship between the survival ratio and radiation doses.

2.5 Fungal Morphology and Colony Characterization

After subjecting to various radiation doses, the fungal isolates were inoculated in SDA plates using the spread plate technique and incubated at 30 °C for 24-72 h. The characteristics of the asexual states such as the sporangiophores and sporangia were assessed under a compound light microscope using Lactophenol cotton blue stain (Loba Chemie Inc., India).

3. Results and Discussion

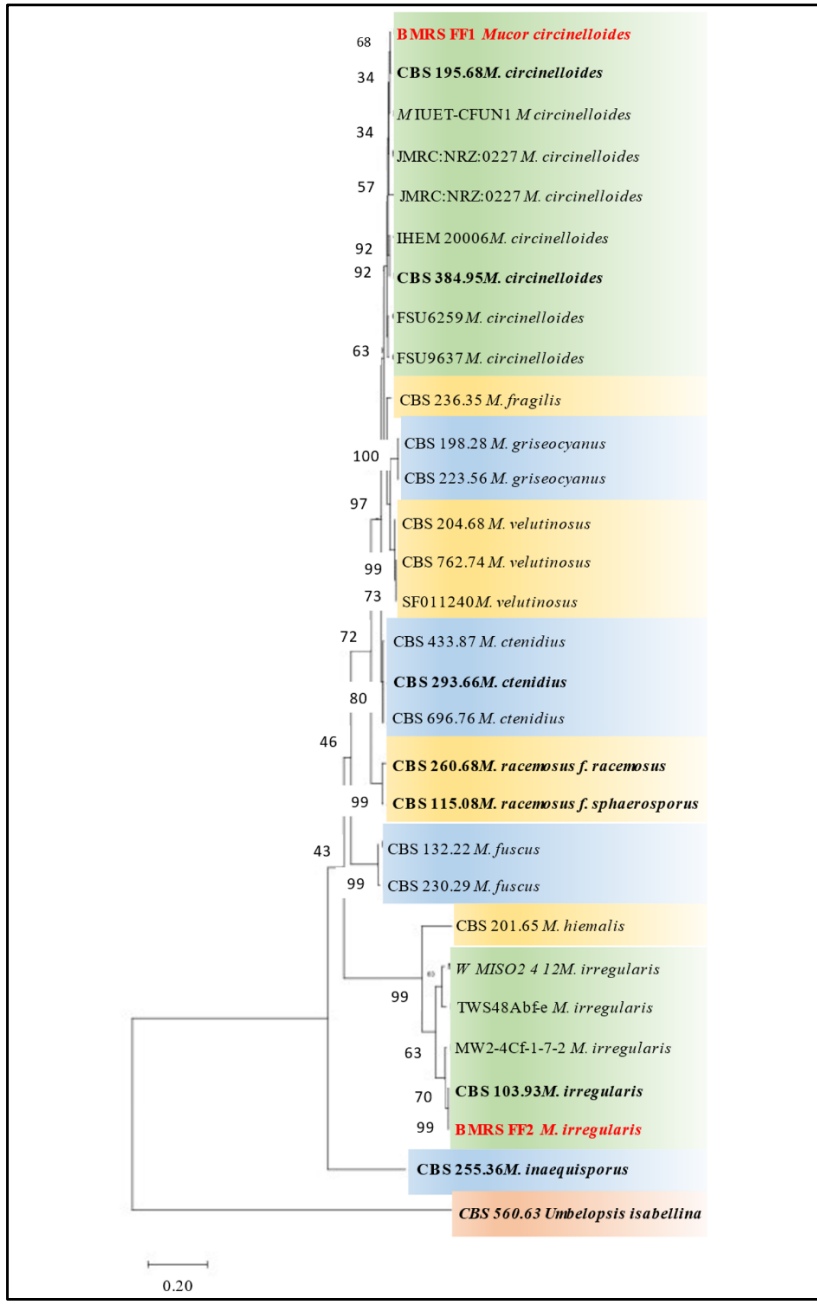
3.1 Isolation and Species Identification of Surface Mycoflora

Two distinct fungal isolates, labeled as BMRS_FF1 and BMRS_FF2, were obtained from the surface of the harvested strawberry fruits. The isolates were deposited at the Mycology Laboratory of the Polytechnic University of the Philippines with accession numbers of PUPML-2021296 and PUPML-2021297, respectively. Using the DNA sequence of the ITS regions of the fungal isolates, a phylogenetic tree was reconstructed using the ML method (Figure 1). Molecular identification of fungal isolates revealed that BMRS_FF1 is *Mucor circinelloides* and BMRS_FF2 is *Mucor irregularis* (formerly *Rhizomucor variabilis*). The ITS sequence data were deposited to the National Center for Biotechnology Information (NCBI, 2017) with an accession number MZ227502 (*Mucor circinelloides* isolate PH_BMRS_FF1) and MZ227503 (*Mucor irregularis* isolate PH_BMRS_FF2). The analysis involved 30 nucleotide sequencing, including *Umbelopsis isabellina* as the outgroup. For clarity, the species name of the fungal isolates and not the sample labels were used in the succeeding text.

It was observed that a total of four fungal colonies were still isolated from a single batch of the harvested strawberries. After three to four days of incubation, predominant fungal colonies were observed to be light yellow to grayish-white. After microscopic evaluation of fungal isolates (= 5 passages of pure cultures), only two were submitted for molecular identification because of distinct similarity among colonies in terms of fungal growth pattern and sporulating structures. The colony and photomicrographs of the fungal species recovered from the strawberry samples are shown and described in Figures 2-3.

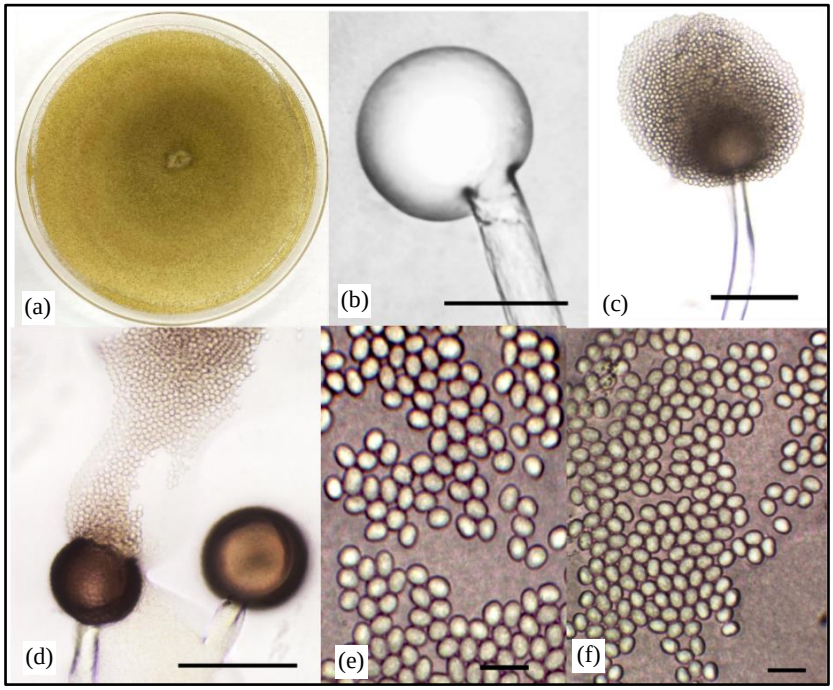
3.2 Radiation Sensitivity of Fungal Isolates

Using regression analysis, the proposed linear model for *M. irregularis* and *M. circinelloides* were found to be $Y = –0.73X + 0.47$, $r^2 = 0.9001$ and $Y = –1.71X – 0.25$, $r^2 = 0.9668$, respectively. Graphical analysis showed that the D10 values of *M. irregularis* and *M. circinelloides* were 0.6 and 1.4 kGy, respectively (Figure 4a). These D10 values can be translated to 90% microbial population reduction after the treatment. Likewise, the total inhibition (< 10 CFU/mL) of growth was observed to be 3 kGy for *M. irregularis* and up to 5 kGy for *M. circinelloides*.



The percentage of trees in which the associated taxa clustered together was shown next to the branches. The scale bar denoted the expected substitution per site. Strain accession numbers were followed by the species name. The tree was rooted to *Umbelopsis isabellina*.

Figure 1. Phylogenetic analysis of fungi isolated from strawberry using the loci ITS by maximum likelihood method and Tamura three-parameter model



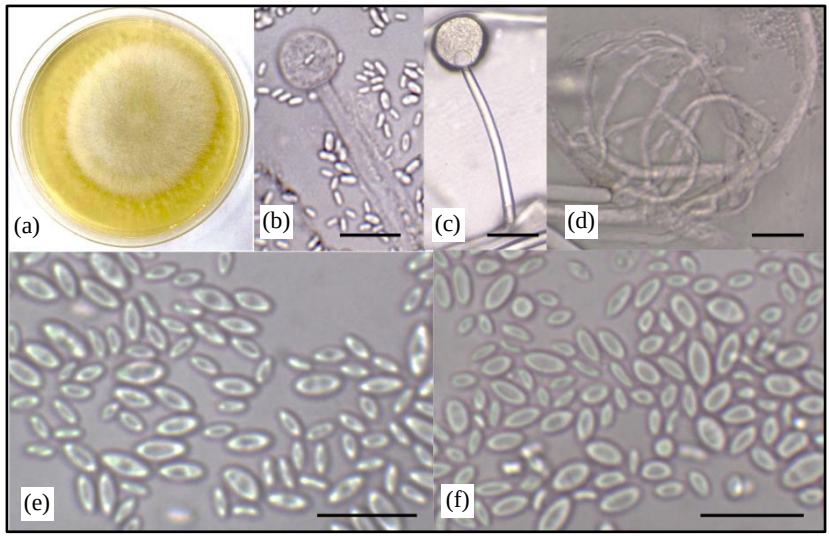
Colonies with well-defined woolly tuft mycelium (nonseptate to lightly septate) (a); white from the front turning dark grayish brown after 72-h incubation (b); transparent sporangiophores, hyaline with straight long branches (c-d); upright shorter branches that developed into circinate (e-f); sporangiospores were hyaline, smooth-edged, rounded to ellipsoidal.

Figure 2. *Mucor circinelloides* Tiegh., Annales des Sciences Naturelles Botanique sér. 6, 1: 94 (1875); seven-day culture (a); sporangiophores and immature sporangium (HPO) scale bar = 29 µm (b); sporangium (HPO) scale bar = 32 µm (c-d); sporangiospores (HPO) scale bar = 12 µm (e-f)

Irradiation not only affects the viability of fungal pathogens but also the structural integrity of the viable fungi (Figures 4b and 4c). Microscopic evaluation revealed that gamma radiation affected both the sporangium and sporangiophores, which can indirectly influence the germination of fungal pathogens. It was observed that the sporangiospores *of M. circinelloides* were visibly released from the sporangium leaving empty sporangia sacs after 2-kGy treatment, while the disruption of the structure of sporangiophores was observed after 3-kGy treatment. On the other hand, the structure of both sporangium and sporangiophores of *M. irregularis* were seen to be disrupted just after 1-kGy treatment.

The biological response to radiation was notably different between the fungal isolates examined in this study. *M. irregularis* was more radiosensitive than

M. circinelloides. Previous studies on other common fungal food-borne pathogens revealed D10 values ranging from 0.04 to 2.74 kGy (Maity *et al*., 2011; Jeong and Jeong, 2018).



Colonies with cottony mycelium (a), originally white then turned light yellow (b-d); simple or branched sporangiophores that often arose from hyphae with scarce archaic rhizoids (e-f); sporangiospores were mostly subspherical to ellipsoidal, hyaline, smooth-edged and very variable (c); sporangia was subglobular to globular.

Figure 3. *Mucor irregularis Stchigel*, Cano, Guarro & Ed. Álvarez, Medical Mycology 49 (1): 71 (2011); seven-day culture (a); sporangiophores (LPO) scale bar = 40 µm (b-c); archaic rhizoids (HPO) scale bar = 40 µm (d); sporangiospore (HPO) scale bar = 8 µm (e-f)

Understanding the physiological response of pathogens, such as the D10 value, to ionizing radiation is critical for postharvest control in fresh produce. The complexity and repair mechanism by which a cell, in general, can reverse radiation damage is sometimes associated with its radiosensitivity (Jeong and Jeong, 2018). Another interesting adaptive strategy that further explains the differences between the sensitivity of various fungal pathogens to extreme environments like radiation exposure is the presence of fungal melanin (Nosanchuk *et al*., 2015). Previous works demonstrated the broad-spectrum effects of melanin on fungal pathogenesis including the reduction of the susceptibility of fungal species on microbicidal treatment and reactive oxygen species (ROS) (Nosanchuk *et al*., 2015), which are produced after exposure to ionizing radiation (Choe and Min, 2005).

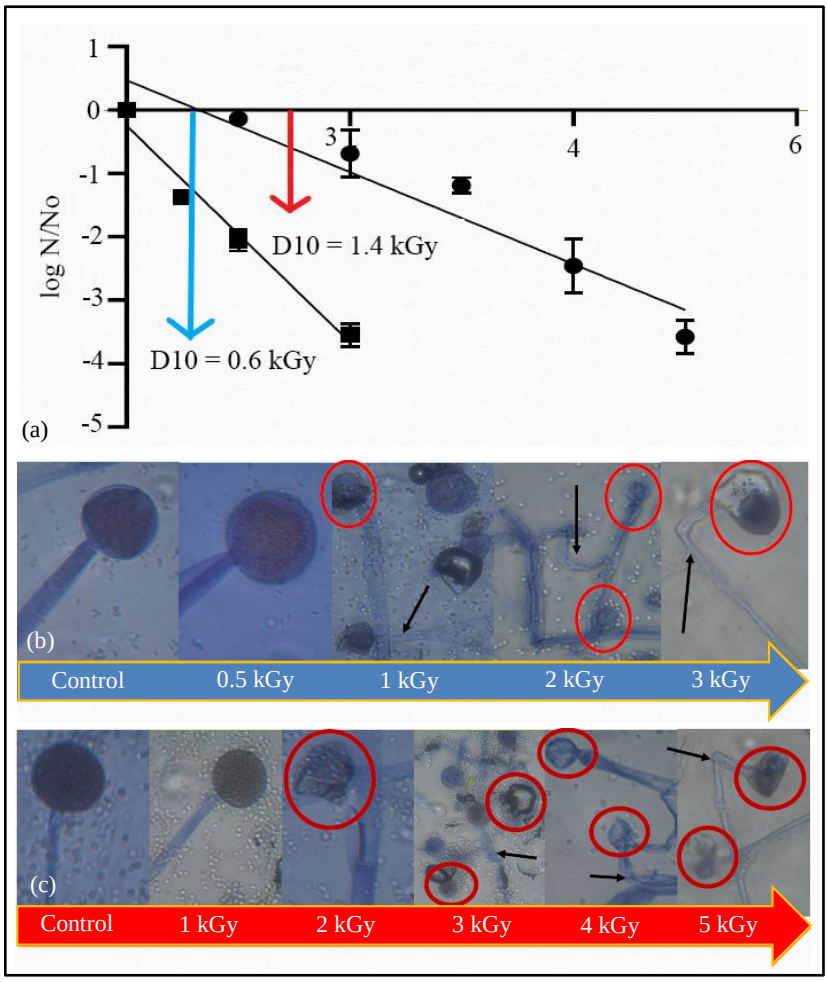


Figure 4. Responses of fungal pathogens to gamma radiation: radiation sensitivity of *M. irregularis* (circular data points) and *M. circinelloides* (square data points) showing the best-fit line to describe the linear relationship between radiation dose and log survival ratio and the interpolated D10 values for *M. irregularis* (blue arrow) and *M. circinelloides* (red arrow) (a); disruption of sporangia (in circle) and sporangiophore (arrow) was seen at 1 to 3 kGy for *M. irregularis* (b); disruption of sporangia (in circle) was observed at 2 to 5 kGy and sporangiophore (arrow) was noticed at 3 to 5 kGy for *M. circinelloides* (c).

Furthermore, Jeong and Jeong (2018) highlighted other important factors affecting fungal responses to ionizing radiation such as the presence of mycelial water content as a natural radioprotector, the ability to repair DNA breaks, the induction of specific enzymes associated with the recovery from

radiation damage and the presence of chemical substances suppressing the process of growth.

Inhibition of spore germination with or without structural morphological damage is directly related to the strength of radiation dose. It was observed that exposing fungal species to increasing radiation levels not only inhibited their germination but also altered the structural integrity of their sexual or asexual apparatuses. Although there were significant changes in the hyphal length and conidial germination after radiation exposure in the 17 isolates from four genera of microfungi, according to Tugay *et al*. (2006), up to 60% showed radiostimulation of conidial germination and hyphal length.

Since the deformation or damage on sporangia and sporangiophores were already detected at lower radiation doses, it was surmised that further increasing the absorbed dose can lead to the total inhibition of sporangiospores germination. Thus, the minimum dose leading to complete inhibition may be considered as the sublethal dose. The results of this study are consistent with that of works that explored spore germination with gamma irradiation (Barkai-Golan *et al*., 1968; Geweely and Nawar, 2006; Tugay *et al*., 2006; Jeong *et al*., 2016).

As fungal spoilage can be a severe problem in fruits and vegetables, international organizations such as FAO and International Atomic Energy Agency (IAEA) have endorsed the application of ionizing radiation, despite scientific and consumer concerns on its use, from various sources to control the microbiological and chemical integrity of fresh produce and other food commodities. The required dose of 1.0-7.0 kGy to control spoilage and growth of pathogenic microorganisms has been established since 1988 by the World Health Organization (WHO). Due to differences in radiation sensitivities among microorganisms linked to their inherent chemical and physical structures and ability to recover from radiation injury (Farkas, 2006), spores from fungi are resistant to the effects of ionizing radiation (Calado *et al*., 2014). They must be requiring higher radiation doses to control their growth in food. However, irradiation of vegetables and fruits is recommended to be less than 1 kGy to avoid nutritional losses and chemical changes (Aziz *et al*., 2006; Braghini *et al*., 2009).

4. Conclusion and Recommendation

This study focused on the two fungal pathogens, *M. circinelloides* and *M. irregularis*, on the surface of fresh strawberry fruits. Gamma radiation effectively reduced the fungal population with D10 values of 0.6 and 1.4 kGy for *M. irregularis* and *M. circinelloides*, respectively. Complete growth inhibition was observed at 3-5 kGy. Disruptions of sporangium and sporangiophores were observed for both isolates at 1-2 kGy. For better application of the food irradiation approach for hurdle technology, there is a need to study in vivo radiosensitivity study and other radiation sources such as EB and X-ray on the fungal pathogens. Moreover, other parameters to assess the growth rate, viability and morphology, namely germ tube length and rate of germination, may be considered. It should be noted that this study explored the surface mycoflora of fresh, healthy-looking strawberry fruits from only one site and sampling time since the soil and climatic conditions can also significantly impact the growth of fungal contaminations in fresh produce.

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