# Effect of Gamma Radiation on Growth and Mycotoxin Production of *Alternaria alternata*

**Braghini R[1]\*, Rocha LO[1], Pozzi CR[2], Frizzarin A[2], Reis TA[1] and Corrêa B[1]**

[1]*Laboratório de Micotoxinas, Instituto de Ciências Biomédicas - ICB II, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, CEP 05508000, São Paulo, SP, Brazil*

[2]*Instituto de Zootecnia, Rua Heitor Penteado, 56, CEP13460000, Nova Odessa, São Paulo, SP, Brazil*

\***Corresponding author:** Braghini R, Laboratório de Micotoxinas, Instituto de Ciências Biomédicas - ICB II, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, CEP 05508000, São Paulo, SP, Brazil, Tel: +55 11 30917295; Fax: +55 11 30917354; E-mail: raquelbraghini@yahoo.com.br

## Abstract

The ubiquitous genus *Alternaria* contains many species that are able to invade cereals, oleaginous plants and other crops. *Alternaria alternata* is considered one of the most important species and can produce several mycotoxins under favourable conditions of temperature and humidity, including the economically important toxins: alternariol (AOH) and alternariol monomethyl ether (AME). The aim of this study was to evaluate the radio-sensitivity of *Alternaria alternata* spores through different gamma radiation doses. *A. alternata* growth and the production of AOH and AME were then analyzed. After fungal irradiation with 2 kGy, 5 kGy and 7 kGy, the spores were suspended with sterile distilled water followed by inoculation on wheat grains. The count of the colony-forming units per gram (CFU/g) was performed using Dichloran Rose Bengal Chloramphenicol (DRBC) and Dichloran Chloramphenicol Agar Malt Extract (DCMA); AOH and AME were analyzed using Liquid Chromatography coupled with Mass Spectrometer (LC-MS). Results showed that fungal growth and toxin production increased with the increase of radiation dosage. The implications of these findings in relation to the resistance of *A. alternata* spores to gamma irradiation are discussed.

**Keywords:** *Alternaria alternata*; Spore; AOH; AME; Gamma radiation; Wheat

## Introduction

The genus *Alternaria* is a ubiquitous fungus in nature and its species are considered both plant-pathogenic and saprophytic that may affect crops in the field or cause harvest and post-harvest decay of plant products [1]. Some species are able to produce mycotoxins in plants and food. This has several implications due to toxic effects of *Alternaria* toxins in humans and animals, therefore compromising food safety and posing important risks to public health [2].

*Alternaria alternata* is considered the most mycotoxigenic species within this group, and it is able to produce several toxins, including alternariol (AOH) and alternariol monomethyl ether (AME). These toxins frequently occur in wheat, other grains and seeds as well as fruits and processed fruit products [1,3-9]. Furthermore, AME and AOH have been reported to be genotoxic, mutagenic and carcinogenic [1,5,10].

Previous studies suggested that the increased incidence of human oesophageal cancer in China was correlated with the contamination of cereals with *A. alternata* toxins [11]. Lehmann et al. [12] have reported the estrogenic potential, inhibition of cell proliferation, and clastogenicity of AOH in Ishikawa and V79 cells in vitro. Although various studies have demonstrated the toxicity of AOH and AME [1,5,10,12], currently, there is a lack of regulation for the presence of *Alternaria* toxins in food worldwide. The European Food Safety Authority (EFSA) [10] has recently released an opinion on *Alternaria* toxins for European countries, with the human dietary exposure to AOH and AME exceeding the threshold of toxicological concern (TTC) value, indicating a need for more toxicological data and regulation for the presence of these toxins in food.

Up to date, many measures have been taken in order to decrease fungal contamination and mycotoxin production in food, including radiation methods. Radiation is a physical treatment, which consists in the exposition of packed or bulk food to an ionizing radiation, during enough time to effectively reduce microbial contamination [13-15]. The treatment efficacy depends on several factors, including food composition, irradiation doses and the number or type of microorganism [16]. Radiation methods can inactivate microorganisms that decompose food, including bacteria, filamentous fungi and yeasts. As well as destroy other organisms that can cause diseases, including parasites and insects [17]. However, a previous study demonstrated the importance of radio-resistance of fungal species, such as *Fusarium* and *Alternaria* [18]; this might lead to serious implications in the control of filamentous fungi through radiation process, for this reason, studies of radio-sensitivity of species of fungi are warranted for developing better strategies to control fungal and mycotoxin contamination in food.

Based on this information, this work aimed to evaluate the radio-sensitivity of *A. alternata* spores through different gamma radiation doses. The growth of *A. alternata* and the AOH and AME production in wheat samples artificially contaminated with *A. alternata* were then analysed.

## Materials and Method

### Fungal strain

The toxigenic *A. alternata* strain used in the study was isolated from sunflower seeds cultured at the Experimental Station of the

Department of Zoo Technology, Nova Odessa, São Paulo, Brazil. This strain (5 CP) is part of the culture collection of the Department of Zoo Technology and part of the culture collection of the Institute of Biomedical Sciences, ICBII, University of São Paulo, Brazil.

## Irradiation process

The isolate was irradiated at three different doses (2, 5 and 7kGy) in the Institute of Energy and Nuclear Research (Instituto de Pesquisas Energéticas e Nucleares - IPEN-CNEN/SP) using a Gammacell 220 cobalt-60 source (MDS Nordion, Ottawa, Canada). A dose rate of 2.58 kGy/h was applied, with the temperature ranging from 25ºC to 28ºC.

## Spore suspension

A toxigenic *A. alternata* strain isolated from CATI sunflower seeds cultured at the Experimental Station of the Department of Zoo Technology, Nova Odessa, São Paulo, Brazil, was inoculated into a Roux flask containing V8 agar [19] and incubated under continuous cold light illumination for 12 days. After this period, the surface of the colony was gently scraped off with a cell scraper and the inoculum transferred into a tube containing 50 mL distilled water. To prevent radiolysis, this suspension was filtered through sterile filter paper and the spore mass retained on the paper was transferred into another tube. The tubes were then centrifuged at 10,000 rpm for 20 min to remove remaining water. The supernatant was discarded and the spore mass was transferred into sterile test tubes and then irradiated with doses of 2, 5 and 7 kGy. After irradiation, the spore mass was re suspended in sterile distilled water and 2 drops of Tween 80 per 100 mL were added. Spores were counted in a Neubauer chamber and the final concentration of the suspension was adjusted to $1 \times 10^6$ spores/mL. The control group (non-irradiated) was prepared after scraping off the surface of the colony with sterile distilled water and Tween 80 and the concentration was also adjusted to $1 \times 10^6$ spores/mL.

## Wheat samples

A total of 5 kg of wheat seeds were used for the determination of the number of colony-forming units per gram (CFU/g) and investigation of mycotoxins. The seeds were divided into four groups of 8 samples each.10 g of each sample were used for the investigation of fungal growth and 2.5 g for the detection of mycotoxins (AOH and AME). The samples were ground and stored in sealed polypropylene bags and then irradiated with 20 kGy for the elimination of contaminating microbiota. After this procedure, the samples were inoculated with *A. alternata* spore suspensions previously irradiated with 2, 5 and 7 kGy. The samples were then divided into four groups: group 1 (control group inoculated with non-irradiated suspensions) and groups 2, 3 and 4 (inoculated with irradiated suspensions). After 8 and 15 days of incubation at 25°C, mycotoxins and the number of CFU/g were analysed.

## *A. alternata* spore inoculation on the grain samples

The grain samples were ground and stored in autoclaved beakers. Next, 1 mL of the spore suspension was inoculated into 10 g of wheat for analysis of the effect of radiation on the fungal growth. The samples were stored in a plastic container and incubated in a BOD oven at an adjusted temperature of 25ºC and relative humidity of 97.5% obtained using 200 mL of 30% potassium sulphate solution, resulting in a water activity (Aw) of 0.98 [20]. The container was sealed with adhesive tape and the samples were incubated for 15 days until analysis.

## Water activity

Water activity was determined with an AQUALAB CX-2 apparatus (Decagon, Pullman, WA, USA).

## Analysis of the fungal growth on grain samples

After the incubation period, 10 g of each sample was transferred to Erlenmeyer flasks containing 90 mL sterile distilled water. Samples were shaken for 30 min and 1 mL was divided into serial dilutions of $10^{-2}$ to $10^{-4}$ in test tubes. Petri dishes containing Dichloran Rose Bengal Chloramphenicol (DRBC) Agar, recommended for the enumeration of common fungi in foods [21], and Dichloran Chloramphenicol Malt Extract Agar (DCMA), recommended for the isolation of *Alternaria* species [22], were prepared for each dilution. An aliquot (0.1 mL) of each dilution was transferred to a Petri dish and spread over the surface with a Drigalski spatula. The plates were incubated at 25°C for 7 days and the number of CFU/g was determined [23].

## Determination of AOH and AME toxins by LC-MS

### Inoculation of the spore suspension

A total of 0.5 mL of each spore suspension was inoculated into 2.5 g of wheat grains. Samples were stored and incubated as described in item *A. alternata* spore inoculation on the grain samples for a period of 8 days.

### Extraction of AOH and AME

The determination of AOH and AME was performed based on the methodology recommended by Visconti et al. [8], with modifications. Samples were transferred into a flask with 15 mL of methanol. After shaking for 40 minutes, the material was transferred to Falcon tubes and centrifuged for 20 minutes at 3000 rpm, at 4°C. The supernatant was transferred into another Falcon tube with 6 mL of ammonium sulfate 20%. After 1 minute, they were centrifuged for 20 minutes at 3000 rpm, at 4°C. After centrifugation, the supernatant was transferred into another Falcon tube with 6 mL of dichloromethane for the extraction of toxins. The dichloromethane was removed from samples using Pasteur pipette and evaporated. The residues were resuspended in 1 mL of methanol for analysis.

### Chromatographic conditions

A total of 20 µL of the methanol solution was injected into Liquid Chromatography (CTO-10AVP, Shimadzu, Kyoto, Japan) coupled with a Mass Spectrometer (Quattro LC, Waters / Micromass, Manchester, UK). A column Luna C18, 5 µm, 150 × 4.60 mm was used at a temperature of 40ºC, isocratic mobile phase of methanol: water (70:30, v/v) and flow of 1.4 mL/minute.

The quantification was determined with calibration curves using standard solutions of the respective mycotoxins (Sigma, St. Louis, MO). For the calibration curves, concentrations from 62.5 to 5000 ng/mL of standard solutions of AOH and AME were used. The correlation coefficient was 0.995036 for AOH, and 0.999347 for AME. The limit of detection of the method was 1.25 ng/g, and the recovery tests presented results of 70% for AOH, and 84% for AME.

## Statistical analysis

The results were analysed using the nonparametric Mann–Whitney test. In order to obtain a confidence level of 95%, the level of

significance was corrected based on Bonferroni's inequality [24]. For the evaluation of the number of CFU/g and toxin levels, the individual level of significance was $p=0.0125$ when the radiation dose was compared, and $p=0.004$ when the type of culture medium and type of toxin were compared.

In the current study, *A. alternata* spores were irradiated with 2, 5 and 7 kGy and the potential of *A. alternata* to grow in DRBC and DCMA was analyzed. In general, at higher radiation doses, there was an increase in *A. alternata* growth for DRBC medium ($p<0.0125$).

## Results and Discussion

### Effects of gamma radiation on growth of *A. alternata*

| | Number of CFU/g ($\times 10^4$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dose | 0 kGy | | 2 kGy | | 5 kGy | | 7 kGy | |
| Sample | DRBC[a] | DCMA[b] | DRBC | DCMA | DRBC | DCMA | DRBC | DCMA |
| 1 | 0.1 | 0.0 | 3.8 | 4.0 | 15.0 | 16.0 | 24.0 | 22.0 |
| 2 | 0.1 | 0.1 | 1.9 | 6.0 | 14.0 | 11.0 | 7.0 | 9.0 |
| 3 | 0.1 | 0.5 | 3.0 | 7.0 | 16.0 | 15.0 | 11.0 | 10.0 |
| 4 | 0.2 | 0.3 | 3.5 | 2.0 | 12.0 | 11.0 | 21.0 | 12.0 |
| 5 | 0.0 | 0.0 | 4.0 | 7.0 | 10.0 | 9.0 | 22.0 | 12.0 |
| 6 | 0.1 | 0.1 | 3.1 | 10.0 | 10.0 | 10.0 | 13.0 | 19.0 |
| 7 | 0.0 | 0.0 | 2.5 | 2.0 | 10.0 | 15.0 | 11.0 | 14.0 |
| 8 | 0.0 | 0.1 | 2.4 | 8.0 | 9.0 | 7.0 | 12.0 | 11.0 |
| Mean | 0.1 | 0.1 | 3.0 | 5.8 | 12.0 | 11.8 | 15.1 | 13.6 |
| Standard deviation | 0.1 | 0.2 | 0.7 | 2.9 | 2.7 | 3.2 | 6.3 | 4.6 |

**Table 1:** Number of Colony-Forming Units in samples of wheat grains, in control group (0 kGy), irradiated with 2, 5 and 7 kGy, in Dichloran Rose Bengal Chloramphenicol and Dichloran Chloramphenicol Malt Extract Agar media, a: DRBC: Dichloran Rose Bengal Chloramphenicol, b: DCMA= Dichloran Chloramphenicol Malt Extract Agar media.

There was also an increase in *A. alternata* growth for DCMA medium at higher radiation doses ($p<0.0125$). Following the analyses in the referred culture media. *A. alternata* growth was evaluated in wheat samples. Interestingly there was an increase in the number of CFU/g that was proportional to the increase in radiation dose ($p<0.0125$).

| Dose | 0 kGy | | 2 kGy | | 5 kGy | | 7 kGy | |
|---|---|---|---|---|---|---|---|---|
| Sample | AOH[a] | AME[b] | AOH | AME | AOH | AME | AOH | AME |
| 1 | 6794.6 | 452.4 | 686.6 | 762.1 | 626.3 | 838.9 | 4165 | 4634.3 |
| 2 | 15569.3 | 422.1 | 1929.3 | 635.1 | 434.6 | 648.2 | 4755.5 | 4584.8 |
| 3 | 14476.8 | 388.8 | 647.2 | 579.6 | 486.2 | 819.6 | 5954 | 7461 |
| 4 | 4868.4 | 228.4 | 756.4 | 650.9 | 443.6 | 566.9 | 5068.2 | 5049.5 |
| 5 | 23699.5 | 523.1 | 705.5 | 561.7 | 458.7 | 677.9 | 6445.5 | 5963.8 |
| 6 | 11301.8 | 468 | 524.4 | 321 | 418.9 | 697.5 | 3830.9 | 7982.9 |
| 7 | 10723.4 | 299.6 | 445.5 | 400.2 | 462.9 | 795.8 | 5134.2 | 7323.8 |
| 8 | 16986.5 | 673 | 630.1 | 519.2 | 358.8 | 605.3 | 5844.6 | 7070.5 |
| Mean | 13052.5 | 431.9 | 790.6 | 553.7 | 461.3 | 706.3 | 5149.7 | 6258.8 |

| Standard deviation | 5998 | 135.8 | 470.9 | 141 | 76.7 | 101.6 | 900.8 | 1373.3 |
|---|---|---|---|---|---|---|---|---|

**Table 2:** Levels of alternariol and alternariol monomethyl ether (ng/g) in wheat grains, in the control group (0 kGy) and irradiated groups with 2, 5 and 7 kGy, Detection limit of the method=1.25 ng/g, a: Alternariol, b: Alternariol monomethyl ether

Indeed, studies have demonstrated the effect of gamma radiation on fungal growth [25-27]. Saleh et al. [28] studying the radio resistance of fungi of the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium* and *Penicillium* observed that the species *A. alternata*, *Cladosporium cladosporioides*, *Curvularia lunata* and *Curvularia geniculata* were the most resistant to the effects of gamma radiation. Blank and Corrigan [29] and Maity et al. [30] revealed similar results, with the genus *Alternaria* presenting the highest resistance to gamma radiation. Another study revealed that *A. alternata* was resistant to the dose of 5 kGy and was able to grow in both corn and wheat samples after direct irradiation of the samples contaminated with *A. alternata* [31].

The greater *A. alternata* growth at higher radiation doses might be explained as a consequence of the radio-resistance of *A. alternata* spores. It has been demonstrated that *A. alternata* presents multicellular spores with thick wall and melanin, which promotes the persistence of viable spores and the radio-resistance of the fungus [29,32,33]. Mechanisms of DNA repair may be involved in the resistance to ionizing radiation [34]. Under favourable conditions, especially in a susceptible substrate, spores will germinate and the fungus will be able to grow in abundance.

Melanin is a group of pigments with high molecular weight, formed by oxidative polymerization of phenolic and indole compounds. it usually presents dark brown or black color [35]. One of the melanin properties is to protect organisms against various environmental factors such as UV radiation, high temperatures and ionizing radiation [35-36]. Previously, Dadachova et al. [32] observed that the exposure of melanized fungi to ionizing radiation would promote their abundant growth, when compared to the non-irradiated fungal group.

Another study has correlated radio-resistance to the total concentration of lipids in microorganisms´ cells [37]. This hypothesis is based on the increase of double carbon-carbon bonds in the lipid membrane, which possibly increases the radio-resistance of microorganisms [38]. The reasons why *A. alternata* is resistant to gamma radiation is complicated to determine, however, we assume that a high melanin content in association with multicellular spores could be essential to promote radio-resistance and to allow *A. alternata* to overcome the effects of gamma radiation. Therefore, enhancing the fungus growth in wheat grains as well as in DRBC and DCMA.

### Effects of gamma radiation on the production of AOH and AME

The use of radiation greatly affected the mycotoxin levels in the experiment. The results showed that the levels of AOH were higher than those of AME, in the control group and lower at the dose of 5 kGy (p<0.0125). No difference was found in the levels of AOH at doses of 0 and 7 kGy and 2 and 5 kGy or in the levels of AME at doses of 0 and 2 kGy and 2 and 5 kGy (p>0.004). The results obtained in this study corroborate the findings of Niles [39] who observed that the radiation of wheat grains contaminated with *Aspergillus flavus* at doses of 10, 25 and 40 kGy promoted *A. flavus* growth and aflatoxin B1 production when compared to non-irradiated wheat. O'Neill et al. [40] observed that *Fusarium culmorum* was able to produce higher levels of deoxynivalenol (DON) and zearalenone in corn samples after

radiation doses of 1 and 3 kGy with the highest production at a dose of 3 kGy, Ferreira-Castro et al. [41], examining corn grain samples artificially contaminated with *Fusarium verticillioides* and irradiated with 2 kGy, determine that *F. verticillioides* was able to produce higher levels of fumonisin B1 after the radiation process.

Although previous studies have reported an increase in mycotoxin production by various genera of fungi after exposure to gamma radiation [40,41]. Several studies have shown controversial information [42-44]; such conflicts could be attributed to the use of different experimental conditions (e.g. humidity levels, radiation process, growth conditions) as well as different species of fungi [45]. The inoculum size may also affect mycotoxin production; in *A. flavus* and *A. parasiticus* the suppression of aflatoxin production occur when the level of spores in the substrate exceeds certain levels [46-48].

In conclusion, *A. alternata* spores demonstrated to be resistant to the radiation doses applied in this study. Under favourable conditions the fungus was able to germinate and produce AOH and AME. Further studies should be conducted to better understand the *A. alternata* spore resistance to gamma radiation as well as the cause for producing higher levels of toxins after radiation process. The increase in AOH and AME production after the radiation process may indicate the need for choosing an appropriate mechanism to effectively control *A. alternata* and its toxins in food.

### Acknowledgement

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