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Macro and micro-structural study on *Aspergillus parasiticus* inoculated in peanut kernels treated with gamma radiation (Cs137)

Estudo macro e micro estrutural de Aspergillus parasiticus inoculado em grãos de amendoim tratados com radiação gama (Cs137)

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ABSTRACT

This study investigated the micro and macro-morphology of *Aspergillus parasiticus*, CMT 00064, inoculated into peanut kernels, after gamma irradiation with Cs137. Plates containing kernels inoculated with the fungal strain were incubated in a BOD germination chamber at 25ºC for 5 days. These plates were irradiated at the dose rate of 1.6 kGy/min on the sixth day of incubation. The absorbed doses were: 0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 kGy. The observed inactivation doses ranged from 4.5 to 8.0 kGy between days 1, 7 and 15 after irradiation. Fungus growth was observed 15 days after irradiation in the plates irradiated between 0 and 7.5 kGy demonstrating that the dose of 8.0 kGy could eliminate the fungus. Subcultures in nutrient media of isolates irradiated at 7.5 kGy recovered its growth in 15 days. Kernels irradiated with 6.5 kGy grew without metula indicating elimination. Colonies that grown after irradiation up 6.0 kGy were biseriate. However, after irradiation with 6.5 kGy they were uniseriate. The survival rates of *A. parasiticus*, in irradiated substrate peanuts, decreases with increasing absorbing doses of ionizing radiation.

Keywords: Food Irradiation. Peanuts. *Aspergillus parasiticus*. Cesium 137. Micro and macro-morphology.

RESUMO

Este estudo investigou a micro e macro morfologia de *Aspergillus parasiticus*, CMT 00064, inoculado em grãos de amendoim, após irradiação com gama do Cs137. As placas contendo grãos de amendoim, inoculados com a cepa fúngica foram incubadas numa câmara de germinação de BOD, a 25 ºC, durante 5 dias. Estas placas foram irradiadas com uma taxa de dose de 1,6 kGy / min no sexto dia de incubação. As doses absorvidas foram: 0; 3,5; 4,0; 4,5; 5,0; 5,5; 6,0; 6,5; 7,0; 7,5 e 8,0 kGy. As doses de inativação observadas variaram de 4,5 a 8,0 kGy entre os dias 1, 7 e 15, após irradiação. O crescimento do fungo foi observado 15 dias após a irradiação, nas placas irradiadas entre 0 e 7,5 kGy, demonstrando que a dose de 8,0 kGy foi suficiente para eliminar o fungo. Subculturas em meio nutritivo de isolados irradiados a 7,5 kGy recuperaram seu crescimento em 15 dias. Os grãos irradiados com 6,5 kGy cresceram sem metula indicando eliminação. As taxas de sobrevivência de *A. parasiticus*, em amendoim de substrato irradiado, diminuem com o aumento das doses absorventes de radiação ionizante.

Palavras-chave: Irradiação de alimentos. Amendoim. *Aspergillus parasiticus*. Césio 137. Micro e macro-morfologia.

INTRODUCTION

Peanuts are widely cultivated worldwide and represent food of high importance as a source of protein. It is one of the agricultural products most resilient to contamination by aflatoxin as the result of traditional harvest, drying, and storage practices (ZORZETE et al., 2011).

Therefore, the health of consumers depends on its sanitary quality, which is dependent on the peanuts' naturally-peculiar ecosystem (STOEV, 2013).

Changes in ecological factors that impact the peanut's microbiota occur after the harvesting, drying, and storing processes when the *Aspergillus* spp. and *Penicillium* spp. fungi become predominant (SANTOS et al., 2013).

Kernels that are subjected to prolonged periods under high temperatures and increased humidity lose quality, by either an increase in fungal contamination or production of mycotoxins, which creates economic losses for agribusinesses (FACCA, 2010).

Fungi are present in almost all regions in the world, in various climates; however, they are predominant in warmer areas and more common and diverse in the tropics. Contamination and growth of toxigenic species in peanut kernels are enhanced in these regions and require stringent monitoring in commercial production (WANG et al., 2011).

Aspergillus is a genus of anamorphic fungi that reproduces by producing phialoconidia. They have high metabolic versatility and ability to disperse their conidia in the environment and survive under adverse conditions such as low humidity and water availability. This genus has a worldwide distribution (KLICH; PITT, 2002; RUDREW et al., 2013).

Several species of *Aspergillus* are of importance to humans and animals due to their ability to produce metabolites when present in foods, especially *A. flavus*, *A. parasiticus*, and *A. ochraceus*. They are most commonly found as contaminants of food and other materials in locations with tropical and subtropical climate. Many species have great capacity for growth and metabolism at low levels of water availability (ROSSETTO et al., 2005).

This genus is considered a major cause for losses of agricultural products through degradation, particularly post-harvest. Besides food spoilage, they produce a variety of mycotoxins that affect food-processing stages. Peanuts, walnuts, sorghum, soybeans, and many other legumes can be contaminated by *Aspergillus* species (ZORZETE et al., 2013).

Aspergillus fungi are of particular importance because of its impact on agriculture and human health. Fungi growth reduces the nutritional value and digestibility of food; the emergence of mycotoxins can cause mycotoxicosis in humans and animals. Thus, the prevention of fungus growth is necessary to control food contamination with mycotoxins (DURAN, et al., 2007; COSTA, 2013; IMAMURA et al., 2014).

A. parasiticus and *A. flavus* are found in peanuts producing aflatoxin B1. This toxin produces hepatotoxic, nephrotoxic, and carcinogenic effects (BORGES, 2011; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2010).

Gamma irradiation has been used to reduce or eliminate the microbiota in different substrates such as peanuts, rice, oats, wheat and maize (BORGES, 2012; FARKAS, 2011; BENTO et al., 2012; COSTA, 2013).

Gamma irradiation is composed of electromagnetic waves with high penetrating power that pass-through food without leaving residues. Its use as a disinfecting technique presents advantages compared to other disinfection treatments such as those that use chemicals. Food irradiation is used to reduce or eliminate microorganisms, especially fungi, promoting hygienic and quality improvement. It contributes in

marketing safe products, domestically and internationally, with direct benefits throughout the supply chain (INTERNATIONAL CONSULTIVE GROUP ON FOOD IRRADIATION, 1995).

The present study evaluated the macro and microscopic effects of gamma radiation on the morphology of *Aspergillus parasiticus* strains, in naturally infected peanut kernels, to assess parameters that could improve inactivation and elimination of this fungus in peanuts.

MATERIAL AND METHODS

a) Contamination of the Samples

Samples of fresh and shelled peanut kernels were purchased at supermarkets and checked for water activity.

Aspergillus parasiticus CMT 00064 was reactivated through inoculation in Petri dishes containing PDA medium (Potato Dextrose Agar), in triplicates, and incubated for 10 days in a BOD germination chamber (Biological Oxygen Demand), Fanem, model 347 at 25°C. Mycological analysis were carried out in the Taxonomy, Biochemical, and Bioprospection of Fungi Laboratory (LTBBF) at IOC/FIOCRUZ.

b) Irradiation of the Samples

Peanut kernels were inoculated with grown cultures of *Aspergillus parasiticus* CMT 00064 and incubated in a BOD germination chamber at 25°C for 5 days. On the sixth day, peanuts were irradiated with gamma radiation at the doses of 0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 kGy in the Arm Technological Center (CTEx). The dose rate provide by irradiator was 26,66 Gy/min, at 33°C.

c) Cultivation of Aspergillus parasiticus

After irradiation were taken from each plate irradiated grains directly inoculated in Petri dishes, containing PDA culture medium, to verify the presence or absence of growth of fungal colonies and other characteristics. Plates were incubated in a BOD germination chamber at 25°C and growth was monitored on days 1, 7, and 15 after irradiation. Morphological characteristics of colonies were assigned based on the descriptions recommended by Klich (2002). *Aspergillus parasiticus* species were classified considering color, texture, colony diameter, and presence of resistance structures such as sclerotic. Grown irradiated colonies and control colonies (non-irradiated) were transferred to Petri dishes containing MEA (malt extract agar) culture medium and inoculated into three equidistant points; these plates were incubated for 7 days for the observation of growth rate and macroscopic characteristics. Growth rates were observed on the 7th day and colonies were measured using an electronic caliper. Color, overall appearance of the colony and its reverse were observed to evaluate fungal radiosensitivity through the analysis of macro-morphological characteristics of *A. parasiticus* CMT 00064.

d) Description of Micro morphological Structures

In addition, the fungus was cultived on slides mounted with coverslips, in sterile Petri dishes with filter paper and 15 ml of MEA. The characteristics of micro-morphological structures such as stipe, bladder, metula, phialide and conidia were observed, and size, shape, texture, and color of colonies were recorded. With the

growing of medium already gelled and sterilized, using a scalpel, cut small pieces of approximately 0.5 cm across. With the aid of a platinum loop, a block of culture medium (MEA) was placed into the center of a blade. After that, the fungus was seeded on one side of the piece and this face covered with a blade. This preparation was placed in a Petri dish with moistened filter paper on the bottom, in order to keep the moisture place to facilitate the growth of fungus. These plates were incubated in a BOD germination chamber at 25°C for 7 days. Subsequently, slides were mounted, stained, observed under an optical microscope, and photographed. Measurements were taken using these pictures.

RESULTS AND DISCUSSION

a) Macroscopic observation of irradiated A. parasiticus (CMT 00064)

Growth was observed on day 1 and 7 after irradiation in samples that received doses between 0.0 and 4.5 kGy suggesting possible inactivation through doses between 5.0 and 8.0 kGy. On day 15 samples irradiated with all selected doses (from 0.0 to 7.5 kGy) showed growth suggesting possible inactivation at doses of 8.0 kGy and up (Table 1). Some flinched colonies were observed, these resumed colonies growth after approximately 15 days.

Table 1. Growth of irradiated *A. parasiticus* (CMT 00064).

Dose kGy	Day 1	Day 7	Day 15
0.0	+	+	+
4.0	+	+	+
4.5	+	+	+
5.0	-	-	+
5.5	-	-	+
6.0	-	-	+
6.5	-	-	+
7.0	-	-	+
7.5	-	-	+
8.0	-	-	-

+ GROWTH
- NO GROWTH

The diameter of *A. parasiticus* colonies cultured on MEA culture medium after 7 days measured between 60 and 70 mm. According to Klich (2002), diameters larger than 45 mm are considered acceptable.

Table 2 demonstrate the diameters of *A. parasiticus* CMT 00064 colonies grown after irradiation with doses between 0.0 and 4.5 kGy. There colonies behaved as expected for this irradiation range showing decreased diameters when exposed to higher doses. The diameter reduction of colonies was significant at 5.0 kGy and extreme at 7.5 kGy. Colonies irradiated with doses between 5.0 and 7.5 kGy showed diameters below the normal range, (between 60-70 µm), however, smaller than the values described by Ribeiro et al. (2008). No growth was observed in cultures irradiated with 8.0 kGy.

Table 2. Diameter of *A. parasiticus* (CMT 00064) colonies on MEA.

Dose (kGy)	Diameter (mm)
0.0	65.30
2.0	55.93
3.0	51.28
4.0	48.13
5.0	37.50
6.0	19.43
7.0	10.56
8.0	NG

NG = NO-GROWTH

According to Klich (2002), the macroscopic characteristics observed in *A. parasiticus* colonies cultured on MEA for 7 days are thick, with non-dense flaky texture, and dark green. Usually discreet mycelium and reverse opaque yellow or opaque green color is observed. The sclerotic, when present, assumes a dark brown or black color (Table 3).

Morphological variations among strains, irradiated and control, are presented in Table 3, such as color, texture and reverse of colonies. Plates irradiated with doses between 0.0 and 5.0 kGy showed presence of brown sclerotia. Receiving doses of 5.5 kGy to 6.5 kGy, the plates were left with a discrete presence of brown sclerotium, which disappeared completely with dose application above 7.0 kGy. Macroscopic changes were observed in colonies irradiated with doses between 5.5 and 8.0 kGy, which are not within alterations already observed for the species according to Ribeiro et al. (2008).

Table 3. Macro-morphologic characteristics of *A. parasiticus* (CMT 00064) colonies inoculated on peanuts in MEA medium at 25ºC.

Dose kGy	Morphological and characteristics of colonies of *A. parasiticus* in MEA
0.0	Flaky texture, olive green color, mycelium (discreet) white with reverse olive yellowish green and dull yellow. Presence of brown color sclerotia.
3.0	Flaky texture, olive green color, mycelium (discreet) white with reverse olive yellowish green and dull yellow. Presence of brown color sclerotia.
4.0	Flaky texture, olive green color, mycelium white, with reverse olive yellowish green and dull yellow. Presence of brown color sclerotia.
5.0	Flaky texture, olive green color, mycelium white, with reverse brownish green and dull yellow. Presence of brown color sclerotia.
5.5	Flaky texture, olive green chesnut brown color, mycelium chesnut brown green with reverse brownish green to whitish yellow. Discreet presence of brown color sclerotia.
6.0	Flaky texture, olive green chestnut brown color, mycelium chestnut brown green with reverse brownish green to pastier green. Discreet presence of brown color sclerotia.
6.5	Flaky texture, olive yellowish green color, mycelium yellowish white with reverse olive greyish green to grayish yellow. Discreet presence of brown color sclerotia.
7.0	Velvetish texture, dull green color, mycelium white with reverse greyish yellowish green to dull yellow.
7.5	Velvetish texture, dull green color, mycelium white with reverse yellowish green to dull yellow.
8.0	NG

NG = NO-GROWTH

b) Microscopic observation of irradiated A. parasiticus (CMT 00064)

The changes observed in the structures of *A. parasiticus* (CMT 00064) are described in Table 4 according to:

a. Stipe of plates irradiated with doses between 5.5 and 7.5 kGy are not in agreement with the data presented in the literature; therefore, become smaller than the minimum value described;

b. Vesicles of plates irradiated with doses between 5.0 and 7.5 present values smaller than the minimum value described in the literature according Ribeiro et al. (2008);

c. Metula of plates irradiated with doses 2.5 and 6.5 kGy presented values slightly below the minimum described in the literature, however, irradiation with doses between 7.0 and 7.5 kGy showed no metula;

d. Phialide of plate of non-irradiated samples (0.0 kGy) showed values similar to the minimum value described by Klich (2002), while measurements in samples irradiated with doses between 2.5 and 7.0 kGy remained under the minimum value described in the literature; samples irradiated with 7.5 kGy presented no phialide;

e. Conidia of plates irradiated with doses between 0.0 and 4.0 kGy remained within the range of values described in the literature, however, the ones irradiated with doses between 4.5 and 7.5 kGy remained under the minimum values described by Klich (2002).

Table 4. Average Length of Structures (in µm) of Colonies of *A. parasiticus* (CMT 00064) incubated for 7 days in MEA medium at 25ºC.

Dose kGy	Estipes (conidiophores)	Vesicules (Horizontal/Vertical)	Metula	Phialides	Conidia
0.0	605.64	31.61 / 33.49	11.83	9.99	6.73
1.0	486.65	23.51 / 24.27	9.72	8.85	5.72
2.0	283.50	15.97 / 16.17	7.36	7.22	4.88
2.5	239.04	13.94 / 14.56	6.13	6.02	4.42
3.0	219.81	11.93 / 12.46	5.64	5.53	4.01
4.0	177.59	11.13 / 11.20	3.96	3.77	3.28
4.5	123.15	10.23 / 10.47	3.18	2.87	2.50
5.0	116.43	8.94 / 9.09	2.48	2.29	2.00
5.5	91.44	7.47 / 7.61	2.26	2.02	1.59
6.0	79,43	6.26 / 6.47	2.01	1.87	1.13
7.0	54.35	4.40 / 4.69	NP	1.38	0.93
7.5	38.39	2.52 / 2.88	NP	NP	0.74

NP = NO PRESENT

Aspergillus parasiticus (CMT 00064) infecting peanut kernels irradiated with doses of 7.0 and 7.5 kGy grew without metula. Therefore, the same was no longer viewed, indicating that this structure had been eliminated. Colonies from fungus irradiated with 7.0 kGy became uniseriate. The phialide was no longer seen in fungus irradiated with 7.5 kGy, which became non-graded. Colonies from fungus colonies irradiated with doses between 0 and 6.5 kGy were biseriate (Table 5).

Table 5. Micro morphological characteristics *A. parasiticus* (CMT 00064) colonies.

Dose kGy	Microscopic Characteristics of Colonies of *Aspergillus parasiticus* after incubation for 7 days at 25°C in MEA medium
0.0	Thick, smooth and unsepted conidiophores; Stipes with walls finely roughened to very rough. Vesicles wide, spherical or slightly and elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark yellowish green color.
3.0	Thick, smooth, mostly unsepted conidiophores; Stipes with walls finely roughened to very rough. Vesicles wide, spherical or slightly e elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark yellowish green color.
3.5	Thick, slightly wrinkled and unsepted conidiophores; Stipes with walls finely rough-ened to very rough. Vesicles wide, spherical or slightly e elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark yellowish green color.
4.0	Thin, smooth, slightly wrinkled and unsepted conidiophores; Stipes with walls finely roughened to very rough. Vesicles wide, spherical or slightly elongate, biseriate and hyaline. Conidia globose and distinctly rough walled dark yellowish green color.
4.5	Thick slightly wrinkled and unsepted conidiophores; Stipes with walls finely rough-ened to very rough. Vesicles wide spherical or slightly elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark yellowish green color.
5.0	Thick, slightly wrinkled and unsepted conidiophores; Stipes with walls finely rough-ened to very rough. Vesicles wide, spherical or slightly elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark green color.
5.5	Thick, slightly wrinkled and unsepted conidiophores; Stipes with walls finely and smooth. Vesicles wide, spherical or slightly elongate, biseriate and hyaline. Conidia globose and distinctly rough walled, dark green color.
7.0	Thick, slightly wrinkled and unsepted conidiophores; Stipes with walls finely and smooth. Vesicles wide, spherical or slightly elongate, no seriate and hyaline. Conidia globose and smooth, green color.

*Magnification of 100x, 200x, and 400x with each interval slit = 2.5 micrometers.

According to Bracarense (2014), genetic evaluation indicated that mutants could have the conidiogenesis affected by the lack of acquisition of competence or development of conidiophores. A mutant of this type may require special nutritional condition and could be more sensitive to situations of stress.

Differences in radio-sensitivity between fungal genera are discussed in the literature, however, incubation time after irradiation for mycological analyzes has not been discussed. The assessment of radio-sensitivity in fungi is of utmost importance because unclear information could lead to conclusive errors about inactivation or elimination.

Blank and Corrigan (1995) verified that spores of *Alternaria* spp., *Curvularia* spp. and *Cladosporium* spp. were at least three times more resistant to irradiation compared to the genera *Aspergillus* and *Penicillium* spp. The different response of these fungi suggests the presence of thick walled macronidia, which can confer protection. Maity (2004), irradiated peanut kernels and observed that *Aspergillus* subjected to doses of 4.0 kGy did not survive while Alternaria did. This author demonstrated the radiosensitivity of the fungi of the genus *Aspergillus* spp., using a dose of 4.0 kGy. Borges (2007) observed radio-sensitivity on the seventh day after inoculation in *Aspergillus flavus* and *Aspergillus parasiticus* in irradiated peanut kernels, oats, rice, and wheat. This author describes *A. parasiticus* as the most radio-resistant species with D_{50} and D_0, with doses of 3.1 and 4.7 kGy, respectively, and reported that *A. flavus* was observed with D_{50} and D_0 with doses of 3.5 and 4.5 kGy, respectively. These results are consistent with the results obtained in the present study.

Endogenous growth of *Aspergillus* spp. is reported by Alves et al. (2011) who obtained several apparently healthy corn isolates. The lower production of conidia, with slower growth and large presence of sclerotia (resistance structure in irradiated isolates), was observed by Ribeiro et al. (2008) when they irradiated chopped corn contaminated with *Aspergillus* spp. with doses of 0.0, 2.0; 3.5, and 5.0 kGy. A significant fungus reduction using the dose of 2.0 kGy and complete inhibition with the dose of 4.0 kGy was observed.

According to Alves et al. (2011), grinded corn subjected to a dose of 3.5 kGy allowed the growth of *Aspergillus* spp., *Eurotium* spp., and *Penicillium* spp., however, when analyzed for species identification, only the growth of sterile mycelium, and no growth after 15 days of incubation, were observed.

Fungal growth in peanut kernels subjected to irradiation was also observed by Ribeiro et al. (2011), using the dose of 5.0 kGy, which provided a reduction in infection rates from 82% to 17.3%. In the present work, there was also similar reduction. Since the larger dose to the smaller becomes colonies reduction and smaller structures until the complete reduction.

CONCLUSIONS

This study has expanded the macro- and micro-morphology overview in *Aspergillus parasiticus* (CMT 00064) irradiated with different doses of gamma radiation. *A. parasiticus* lost some structures (metula and phialide) after irradiation. This causes the fungus to lack in production of conidia because of the loss of phialides. The macro- and micro-morphologic changes in the fungus' characteristics that occurred after gamma irradiation showed that the peanut kernels that were severely contaminated. had their microbiota reduced or eliminated according to the absorbed dose. Gamma irradiation at the dose of 8.0 kGy showed to be effective in inactivating or eliminating microbiota in peanut fresh kernels improving the quality of this food to the consumers

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AUTHOR CONTRIBUITION

Accompanied the studies of nuclear radiation, irradiation of samples, participated in the sequence alignment, and drafted the manuscript: The author MCLS. Use the laboratory CCAMP/LABZOO/IOC/FIOCRUZ, and supporting of samples analysis, as well contributed to the suggestion of scientific references: The authors SSD e WTCE; Responsible for the statistical analysis of the results of the present study. Elaboration of the graphics with statistical results: The author ABMF; Carrying out the article, the review and approval of the final manuscript proof: The author MMBBSX; and project advisor and reviewer activities: The author RMF.

COMPETING INTERESTS

The authors declare there are no competing interests.

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