Material and Methods

MATERIALS AND METHODS

Source of fungal isolates:

Fungal populations were isolated from different sources of foods namely potato, tomatoes, orange, mandarin, bread, fenugreek, wheat, lupine, as well as chicken feed, air and soil.

Isolation, purification and identification of fungi:

Under aseptic conditions, a small portion of rotted lesions of the above mentioned foods or feeds and known amount of soil were placed on Czapek's-yeast extract agar medium (yeast extract, 5.0g;; sucrose, 30.0; sodium nitrate, 3.0g; magnesium sulphate, 0.5g; potassium chloride, 0.5g; ferrous sulfate, 0.01; dipotassium hydrogen phosphate, 1.0g; agar-agar, 20.0g; distilled water, 1 litre; chloramphenicol, 100 mg; pH 6.4 ± 0.2 **(Pitt & Hocking; 1997)** in petri-dishes and incubated at 25-28 °C for 3-7 days according to the type of fungi. The emerged fungi were purified, using single spore technique at the same medium **(Smith, 1961)**, then grown on slants to make stock cultures. The fungal isolates were identified according to their morphological characters and microscopical examination in Mycological Centre, Faculty of Science, Assiut University, Egypt.

Preparation of spore suspension:

The studied fungi were individually grown on Czapek's-yeast extract agar medium in 250 ml Erlenmeyer flasks at 25-28 °C for 10 days.

The method of harvesting of fungal conidia from the cultures was done according to **Appiah *et al.* (1980)**. Twenty ml. sterile solution of tween-80 (2.9 %) were added to each flask. For facilitation the spores to

suspend in the solution, the flasks were placed in automatic electric shaker for 1 min. The spore suspension was decanted into a sterile centrifuge tubes and centrifuged twice at 4000 r.p.m. for 15 minutes. Each time the supernatant liquid was decanted and the pellet resuspended in tween solution. The prepared spore suspension was diluted, using sterile water to contain about 10^7 spores/ml, with the aid of haemicytometer.

Measuring the fungal growth diameter:

The isolated fungi were individually grown on Czapek's-yeast extract agar medium in Petri-dishes at 25-28 °C for 10 days. The dishes were exposed to various doses of gamma radiation (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 kGy). Discs (5 mm in diameter) were taken from the different treatments and transferred to pre-poured Petri-dishes with Czapek's-yeast extract agar medium.

Three replicate dishes were used for each treatment, dishes of the different treatments were kept in the incubator adjusted to 25-28 °C till the maximum growth (the mycelium of any treatment reach the edge of Petri-dish). Through the period of incubation, the radial growth of the three replicates was measured daily in mm, and the average was calculated.

Determination of decimal reduction dose (D_{10}-value):

D_{10}-value is defined as the radiation dose in kGy which kills 90 % of the initial count of the cells. It is calculated as the regression line **(WHO, 1981)**. The D_{10}-value was calculated for each fungus in both physiological saline solution and substrate (lupine seeds or chicken feed). In this method, 1 ml of the stock fungal spores suspension (10^7 spores/ml)

was added to 9 ml of sterile saline solution (8.5 g sodium chloride /L) in test tubes or to 9 gm of sterilized substrate (lupine seeds or chicken feeds exposed to 10.0 kGy gamma radiation for inhibition of natural fungi) in 100 ml conical flask. The tubes and flasks were then exposed to incremental doses of gamma radiation (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0 kGy). Three replicate tubes and flasks were used for each treatment.

After irradiation process, serial dilutions were made from each replicate to obtain 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 spores/ml saline solution, one ml of each dilution (as well as the original suspension, 10^7) was poured onto Czapek's-yeast extract agar medium in Petri-dishes. Two dishes were used for each dilution.

The dishes of different treatments were incubated at 25-28 °C for 5-7 days. The colonies were counted in each dish and the survival curve for each fungus was plotted. The D_{10}-value was calculated for each fungus from the regression line

$Y = a + b\,x$

$$D_{10} = -\frac{1}{b}$$

$$b = \frac{\Sigma xy - n\bar{x}\bar{y}}{\Sigma x^2 - n\bar{x}^2}$$

$$r = \frac{\Sigma xy - x\Sigma\frac{y}{n}}{\sqrt{\left(\Xi x^2 - \frac{(\Sigma x)^2}{n}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right)}}$$

a = log of microbial count when x equal zero.

b = Regression factor.

x = dose levels (kGy).

y = microbial count (log count).

n = Number of treatment.

$$\bar{x} = \frac{\Sigma x}{n}$$

$$\bar{y} = \frac{\Sigma y}{n}$$

Irradiation process:

Irradiation process was carried out at National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Co-60 gamma Chamber 4000 A (Indian) was used for irradiation. The dose rate of that source was 0.9 kGy per hour at the time of experiments.

Mycelium dry weight (biomass):

Fungal growth in liquid medium was measured by mycelial dry weight according to **Madhyastha & Bhat (1984)**. The Erlenmeyer flasks containing 50 ml of Czapek's-yeast extract liquid medium were inoculated with unirradiated and irradiated (0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 kGy) fungal spore suspension and incubated at 25-28 °C for 10 days. The cultures were autoclaved at 121 °C for 1/2 min. to kill the spores and vegetative mycelia. The mycelium mats were separated from the cultures through preweighted filter paper, and dried at 60°C overnight, cooled in desiccator and weighted to determine the mycelial dry mass as a measure of fungal growth.

Determination of some cellular components:

Total protein:

Total nitrogen content of fungal isolates was determined by using the micro-Kjeldahl method **(AOAC, 1990)**. The results were calculated as gm nitrogen /100 gm dry weight. The protein content was calculated by multiplying the nitrogen content by a factor of 6.25.

Amino acids:

Amino acid determination was performed according to the method of **Winder & Eggum (1966)**. Oxidation with performic acid, to protect methionine and cysteine from destruction, followed by acid hydrolysis was carried out in a closed conical flask for determining all amino acids other than tryptophan. Sample of 20-30 mg of dried and defatted fungal mat of the studied isolates were weighted in scrow-tubes, and 5 ml of performic acid were added. The tubes were closed and placed in ice water bath for 16 hour, sodium metabisulfate and HCL (6N) were added to the oxidized mixture. The tubes were placed in an oven at 110 °C for 24 hours. The tubes were then opened and the content was then evaporated for dryness in a rotary evaporator. A suitable volume of sodium citrate buffer (pH 2.2) was added to the dried film of the hydrolyzed sample. After all soluble material completely dissolved, the sample was ready for analysis. The system used for the analysis was High Performance Amino acid Analyzer, Biochrom 20 Pharmacia Biotech at National Centre of Radiation Research and Technology (NCRRT), Nasr City, Cairo.

Lipids:

Lipids were extracted from fungal isolates using a mixture of chloroform and methanol according to the method described by **Bligh & Dyer (1959)**. To obtain the crude lipids, chloroform layer was filtered over anhydrous sodium sulphate and then evaporated under vacuum by using a rotary evaporator.

Fatty acids:

For preparing the fatty acids, the extracted lipid were saponified by boiling under reflux with an excess of dilute aqueous ethanolic alkali. The ether containing the water-soluble hydrolysis products (mainly soap

solution and glycerol) was acidified by sulphuric acid to liberate the free fatty acids. The free fatty acids were then extracted with diethylether recovered, dried over anhydrous sodium sulphate and transformed to their methyl esters for GC-MS analysis **(Varso, 1972)**. Fatty acids were determined quantitatively using a Gas chromatograph-mass selective detector instrument "GC-MS" type HP 6890 series at National Centre of Radiation Research and Technology (NCRRT), Nasr City, Cairo.

Condition of analysis:

Column: Capillary Hp-Innowax column; 30 m length; 250 μ; 0.25 μ film thickness.

Oven: Programmable with initial temperature of 150 °C for one minute, then raised in three ramps as follows:

Rate °C / min.	Final temp. °C	Final time / min.
17	235	2
1	242	1
8	255	6

Nucleic acids:

Nucleic acid contents were extracted from fungal isolates according to the modified method of **Shibko *et al.* (1967)**. Two ml of RNA solution was added to 3 ml of HCl-$FeCl_3$ and 0.3 ml of alcoholic orcinol solution, then heated for 4 minutes in boiling water bath. After cooling, the absorbance was read at 670 nm against orcinol blank **(Ashwell, 1957).** DNA was determined by diphenylamine calorimetric method. Two ml of DNA solution, incubated for 17 hrs at 30 °C and read at 600 nm against water blank **(Burton, 1968)**.

Solution and reagents for nucleic acid analysis:

a- Hydrochloric acid-ferric chloride mixture, 0.1 gm of $FeCl_3$ was added to 100 ml of concentrated HCl.

b- Alcoholic orcinol, 0.6 gm of orcinol was added to 9.4 ml of absolute ethyl alcohol.

c- Diphenyl amine reagent, 1.5 gm of diphenyl amine was added to 100 ml of glacial acetic acid and 1.5 ml of concentrated H_2SO_4.

d- Aqueous acetaldehyde solution, 1.5 ml of acetaldehyde was added to 98.4 ml of distilled water.

e- Working solution, 0.1 ml of aqueous acetaldehyde solution was added to 20 ml of diphenylamine reagent to give the working solution.

Artificial fungal contamination:

The samples of lupine or chicken feed (100 gm / polyethylene bags, three replicates for each treatment) were firstly sterilized by irradiation at 10.0 kGy, thereafter a known amount of sterilized water was added to lupine or chicken feed to adjust its moisture content at 15%. The amount of water plus spore suspension was theoretically calculated referring to the moisture content of seeds **(Christensen & Kaufmann, 1965).** The sample of lupine was inoculated with spores of *Curvularia lunata*, while chicken feed was inoculated by spores of *Alternaria alternata* and *Fuarium oxysporum*. The inoculated samples were irradiated at different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 kGy). The samples were stored at ambient temperature for three months. Total fungal count was determined immediately after irradiation (zero time) and periodically every one month of storage.

Total fungal count:

Enumeration of fungal counts for each treatment was done according to **Koburger & Marth (1984)** by suspending 10g of each sample in 90 ml, sterilized saline solution (0.85 % NaCl) to give dilution 1: 10, other decimal dilutions (1:100, 1: 1000, .. etc) were prepared by sterilized saline solution. One ml of the serial dilutions (in dublicates) was put in the center of sterilized Petri-dishes (10 cm diameter). The plates were poured by Czapek's-yeast extract agar medium and incubated at 25-28 °C for 3-5 days. All the fungal colonies were counted and recorded.