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Molecular investigation of Gamma irradiated *Cladosporium herbarum* and *Trichoderma viride*

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ABSTRACT

Trichoderma viride and *Cladosporium herbarum* were isolated from clay soil and irradiated with different doses of gamma radiation (0.5, 1, 1.5, 2 kGy). The inhibitory effect on growth rate was dose dependent with maximum inhibition at 1.5 and 2 kGy for *T. viride* which showed no growth. On the other hand, *T. viride* which grown on a medium contained aqueous extract from *C. herbarum* (gcae) showed resistance than that of the wild one and exhibited growth rate of 0.22 mm / day at 2 kGy. *Cladosporium herbarum* showed resistance to gamma radiation with a growth rate of a 0.62 mm / day after irradiation with 2 kGy. This indicated the presence of resistant factor/s in the extract of *Cladosporium herbarum* which utilized by *T. viride*. Therefore, molecular studies were carried out to detect any variation in the investigated fungi. Molecular studies revealed the presence of 57 bands and 41 polymorphic bands with a total polymorphism percentage of 71.92%. The number of polymorphic bands per primer ranged from 14 bands with polymorphism percentage of 73.68% for A01 primer, 16 polymorphic bands with polymorphism percentage of 84.21% for C02 to 11 bands with polymorphism percentage of 57.89% for B07 primer. Therefore, such average polymorphism might be due to the effect of different doses of gamma radiation. The study clearly showed the possibility of improving the capability of radiation sensitive fungi to be more resistant to radiation by growing them on extract from radiation resistant ones.

Keywords: Gamma radiation, radial growth, *Cladosporium herbarum*, *Trichoderma viride*.

INTRODUCTION

Radiation is the emission of energy or particles from a source and that travels through most materials and through space (Thalita *et al*., 2014). Gamma radiation affected microbial community and leads to negative effect on microbial metabolic capacity (Jones *et al*., 2004). Khawas *et al*., (1999) reported that 2, 4 and 6 KGy of gamma rays reduced microbial count. Fungi have been successfully inactivated with gamma radiation doses ranging from 6 to 15 kGy (McNamara *et al*., 2003). The subject of fungal cell interactions with radionuclides is considerable interest for environmental remediation (Dighton *et al*., 2008). *Trichoderma harzianum*, *T. viride* and *T. knoingii* irradiated with 0.5 KGy dosage producing highly active exoenzymes (Abd El-Moneim *et al*., 2012). Gamma rays, electromagnetic waves with high penetrating power, pass through materials without leaving any residue, this considers an advantage comparing to other disinfection treatments (Adamo *et al*., 2001). Gamma irradiation produced energy causes the hydrolysis of water molecules in the substrate or irradiated material that produce free radicals and ions that attack the microorganisms DNA, dose of gamma irradiation 2.5 kGy effectively kills mild and yeast in melinjo seeds. Reze *et al.*, (2019).

Gamma radiation has shown inactivation effect on fungi that isolated from various materials. (Jyoti *et al*., 2011) reported that the effect of radiation varies among different organisms. Samy *et al*., (2016) reported that gamma radiation at doses of 3 kGy reduced the growth of *Alternaria*, *Aspergillums* and *Trichoderma* while they recorded that gamma radiation inhibited the growth of *A. alternata*, *A. solani*, *Botrytis* sp., *Cladosporium* sp., at 2.5 to 10 K Gray).

The fungal cell interacts with radio nuclides which of interest for environmental remediation, but that phenomenon is chemical in nature and is different from interactions with ionizing radiation. The radio tropism of the Chernobyl associated fungi and fungi in space represent, the first attempts to decipher the mechanism of radiation energy utilization by fungi as well as the first insights into the genetic effects of radiation on fungi (Dighton *et al*., 2008).The main objective of the present study is to compare of molecular variation between *T. viride* and *C. herbarum* as well as the isolate of *T. viride* which grown on aqueous extract from *C. herbarum (gcae).*

MATERIAL METHODS

Isolation and identification of fungi

The investigated fungal spp. were isolated among a group of fungi isolated from soil samples located at Helwan University gardens, and preserved in mycology Lab (Botany and Microbiology Department, Faculty of Science, Helwan University). The isolated fungi were identified depending on their morphological charasteristics on different culture media (Czapeks - Dox agar, potato dextrose agar, malt extract agar), and the microscopic examination for their conidia and hyphae (Barnett and Barry, 1987; Pitt, 1979).Then confirmaon was carried out in the Regional Center for Mycology and Biotechnology, Alazhar University, Egypt for *Trichoderma viride* and *Cladosporium her-barum.*

Preparation of irradiated fungal cultures *Cladosparium herbarum*, wild culture of *T. viride* and *T. viride* (gcae) were tested for their ability to develop colonies after exposing to different doses of gamma

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radiation (0.0 (control), 0.5, 1, 1.5 and 2 kGy). For each fungal species, 10 slants containing equal amounts of dox agar medium (6 ml) were inoculated with one disc from 7 day- old culture plates. The slants (2 for each treatment) were incubated at 25-27 ºC for 5 days and then irradiated by gamma radiation as mentioned previously**.** All slants were directly transferred to mycology lab after irradiation and used for further studies. One slant from each treatment was used for studying radial growth rate and the other one was used for biomass production and molecular studies.

Source of radiation

Irradiation process was carried out at National Centre for Radiation Research and Technology, Nasr City, Cairo, Egypt. The irradiation facilities used were an experimental Co^{60} Russian Gamma Chamber. INDIA. The average dose rate of this gamma radiation source was 2.5 kGy/1hr at the time of the experiment.

Dry biomass measurement

Fungal biomass determination was carried out by filtration of liquid cultures through reweighted filter paper then washed with distilled water three times and dried in oven at 60°C until obtaining 2 successive equal weights for the same sample. Fifteen flasks were used for each fungal species, three for each treatment as well as the control one.

Molecular studies

DNA extraction

DNA was extracted from fresh mycelia according to the modified mini prep CTAB method according to (Karthikeyane *et al*., 2010). About 0.5 g fresh biomass was placed into mortar in liquid nitrogen before ground to a fine powder using disposable plastic grinders. The powder was then mixed to homogenous slurry with 100 µl extraction buffer (100 mM Tris- HCl pH 8.0, 20 mM Na2 EDTA, 1.4 M NaCl, 2% w/v CTAB, 0.2% v/v 2-mercaptoethanol)while continuing grinding. Additional, 900 µl of the extraction buffer was subsequently added before the tube was incubated at 65°C for 30 min. The sample was then cooled to ambient temperature and washed by adding 200 µl of wet chloroform (chloroform: octan-1-ol= 24:1). The mixture was gently mixed and centrifuged at 13000 rpm for 2 min. The aqueous layer was removed and subjected to second washing process, by adding 500 µl of wet chloroform. The DNA was precipitated from the aqueous layer by adding 600 µl ice-cold propan-2-ol at room temperature for 10-15 min. After centrifugation at 13000 rpm for 2 min, DNA pellet were washed with 1 of wash buffer twice with 650 µL (76% ethanol, 10 mM ammonium acetate) and stands at room temperature for 20 min, before subjected to another centrifugation. The supernatant was discarded, and the DNA was air dried by inverting the tube for 15 min. The DNA was then dissolved in 100 µl TE buffer (10 mM Tris-HCl pH 7.6; 1 mM EDTA) and stored at 4°C until used.

Random Amplified Polymorphic DNA (RAPD) was carried out using three random primers (Table S1). Primers used in the analysis were synthesized in MWG Biotech (A01, C02 and B07). The polymerase chain reaction mixture consisted of 0.8 U of Taq DNA polymerase (Bio labs, New England), 0.1 mM dNTPs, and 25 pool of random primer, 2.5 µl, 10X Taq DNA polymerase buffer and 50 ng of genomic DNA. The final reaction volume of 25 µl was placed in a DNA thermal cycler (Perkin Elmer 9700). The PCR program included an initial denaturation step at 94°C for 5 mines followed by 40 cycles with 94°C for 1 min, 36°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The samples were cooled at 4°C. The amplified DNA fragments were separated on 1. 2% agarose gel and stained with ethidium bromide. The amplified pattern was visualized on a UV transilluminator and photo-graphed.

Scoring and analysis of RAPDs

DNA bands were scored for their presence (1) or absence (0) in the RAPD profile of the samples and data were analysed using the MVSP software (with Jaccard coefficient). The index of similarity between samples was calculated using the formula:

$$Bab=2\ Nab/\ [Na + Nb],$$

where Nab is the number of common fragments observed in individuals a and b.

Na and Nb are the total number of fragments scored in a and b, respectively.

The BS values were calculated for each primer separately and the average for all primers was carried out with each comparison. Dendogram was constructed using the Average Linkage between Groups.

Statistical analysis

Stander error for the mean values was estimated by used Microsoft Office Excel.

RESULTS

Effect of gamma radiation on fungal biomass

From table 1 in case of *C. herbarum*, gamma irradiation stimulated growth with a maximum biomass at 1.5 kGy (4.4 g). However, in case of *T. viride* (wild), gamma irradiation was inhibitory with complete inhibition at dose 2 kGy, on the other hand , *T. viride* which grown on a medium contained aqueous extract from *C. herbarum* (gcae) showed elevated radio resistance and tolerate growth under all radiation doses Table 1).

Effect of gamma radiation on radial growth rate

In case of *C. herbarum*, as shown from table 2, gamma irradiation stimulated the growth with a maximum radial growth rate at 0.5 kGy (1 mm/ days). However, in case of *T. viride* (wild), gamma irradiation had an inhibitory effect and reached its maximum complete inhibition at dose 1.5 and 2kGy, respectively. On the other hand, *T. viride* which grown on a medium contained aqueous extract of *C. herbarum* (gcae) showed an elevated radio resistance and tolerate growth under all radiation doses.

Molecular characterization

Three different RAPD primers (A01, C02 and B07) were used in this study to detect any variation among the treated samples and their controls. These prim

generated clear and reproducible bands. RAPD analysis produced amplified fragments that can produce informative and polymorphic products resolvable by gel electrophoresis (1.2% Agarose gel) and succeeded to give polymorphism among the different treatments. These results were represented in figures (1A, B and C). A total number of 57 bands including 41 polymorphic bands were estimated with a total polymorphism percentage of 71.92%. The number of polymorphic bands per primer ranged from 14 bands with polymorphism percentage of 73.68% for A01 primer, 16 polymorphic bands with polymorphism percentage of 84.21% for C02 and 11 bands with polymorphism percentage of 57.89% for B07 primer. From these observed data, C02 primer generated the highest percentage of polymorphism (84.21%) compared to other primers. Such average polymorphism might be due to the effect of the different fungal treatments or the effect of the different doses of gamma radiation (Figure 1, A-C). The purity of DNA preparations (as determined by spectrophotometer reading at 260 nm and 280nm) ranged between1.8-2.0, the average size of the genomic DNA as deduced from electrophoresis on 1.2% agarose gel was about 50kb as judged from comparison with an undigested λ DNA sample run parallel to the tested samples.

Table (1): Effect of gamma radiation on biomass dry weight (g/50ml) of studied fungal species.

Fungal Species	Gamma radiation dose (kGy)†				
	control	0.5	1.0	1.5	2.0
Cladosporium herbaru m	2.77± 0.06	2.97± 0.07	3.50± 0.23	4.4± 0.090	4 ± 0. 24
Trichoderma viride (wild)	1.85± 0.03	1.71± 0.01	1.39± 0.06	No growth	No growth
Trichoderma viride (gcae)	2.1± 0.110	2.5± 0.19	2.8± 0.07	2.4± 0.100	2.1± 0.15

†All data are in mean (Mean ± Standard error).

Table (2): Effect of gamma radiation on radial growth rate of tested fungal species (mm/days).

Fungal Species	Gamma radiation dose (kGy)†				
	Control	0.5	1.0	1.5	2.0
Cladosporium herbarum	0.85	1.00	0.75	0.64	0.62
Trichoderma viride (wild)	2.00	1.57	1.38	0.00	0.00
Trichoderma viride (gcae)	1.87	2.25	1.61	0.80	0.22

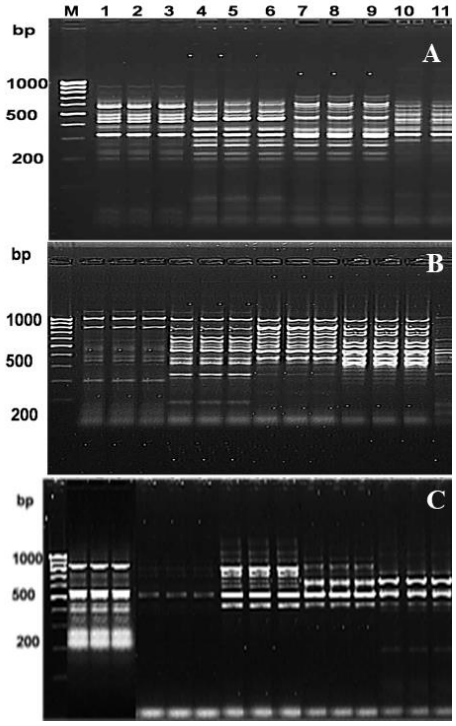

Figure (1): Electrophoresis gels (1.2% agarose) of the pure genomic DNA of Fungal isolates, irridiated with different doses compared to untreated control, using different primers; A, primer A0; B, primer C02 and C, primer B07.

The primer gave amplified bands (representative of these PCR products are shown in Fig 1, A-C). Primer A01 gave 5 monomorphic bands and 14 polymorphic bands. Primer C02 gave 3 monomorphic bands and 16 polymorphic bands. Primer B07 gave 5 monomorphic bands and 11 polymorphic bands.

In general, the three primers gave amplified fragments with total bands 57. The corresponding efficiency reported, among the three primers, was ranging from 73.6%, 84.2% and 57.8% for A01, C02 and B07, respectively (Table S2)

Dendrogram analysis revealed that the 15 samples were grouped in two main groups; group I which contains the three control samples, and group II which contains all treated samples. Group II was divided into 2 divisions; division 1 which includes samples 10, 11, 12 and division 2 that includes the other treated samples. In turn division II was subdivided into 2 main clusters; cluster I which included treatment (7, 8, 9) and cluster II which diverged into 2 separate sisters clades; one includes treatment (4, 5, 6) and the other includes treatment (13, 14, 15). The side scale indicates the relative similarity and dissimilarity between differ -rent treatments (Figure 2). It is clear that the treatment (10, 1 and 12) is the most different from control and from other treatments, as well.

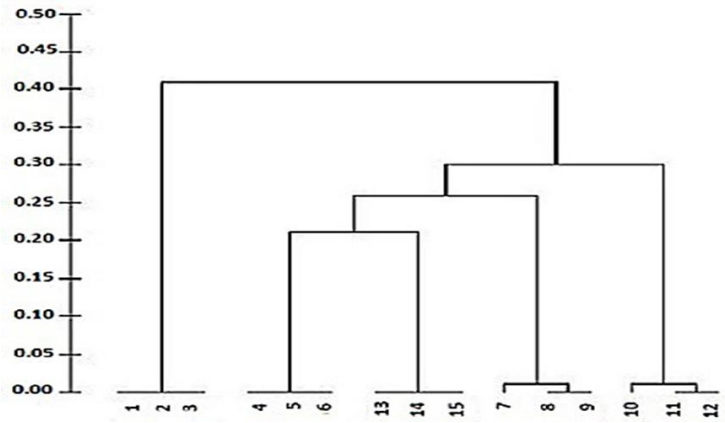

Figure (2): The genetic dendrogram of the three fungal species revealed by their RAPD markers technique

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DISCUSSION

The results of effect of gamma radiation on the mycelial dry weight of [*T. viride*, *T. viride* (gcae) and *C. herbarum*] are agreement with (Ortega *et al*., 2011) who found that growth of *T. viride* was decreased at 0.5 kGy of gamma radiation. Mycelial growth of the tested fungal species was affected by gamma irradiation of spores. The lower doses of gamma rays significantly increased radial growth rate and dry weight of *T. viride* (gcae) and *C. herbarum* and the highest increase (2.25 and 1.57 mm/day) were observed at 0.5KGy respectively. As radiation dose increased over 0.5KGy, the growth rate parameter gradually decreased to reach a minimum at (1.5 and 2KGy) were the lethal dose in case of *T. viride*. The obtained lethal dose value was in accordance with Geweely *et al*., (2006), who reported that lower doses of gamma radiation increased the growth of *Aspergillus flavus* and complete inhibition was observed at 2KGy. Non-irradiated and irradiated cultures especially at high dose DNA fingerprinting (RAPD) managed to distinguish between mutants (with different doses of gamma radiation) and wild type (control). By comparing these results with physiological results, it was found that by increasing the dose of gamma radiation, the resistance of these different fungi to gamma radiation increase. But still the resistance of *Cladosporium herbarum* is higher than *Trichoderma viridi*. Also in case of *Trichoderma viridi* (gcae) the resistance to gamma radiation increase; and this could be explained due to the transfer of resistance factor from *Cladosporium herbarum* into *Trichoderma viridi*. The different three primers used in this study illustrated that their differences in response of these different fungi to the different doses of gamma radiation; and this is due to the mutation caused by the action of radiation on the nucleotides sequences. This appeared clearly due to appearance and absence of some bands patterns with the different radiation doses with the different primers used. However, there was no obvious difference within the different fungi with each other in each treatment independently; and this could be due to the chosen of RAPD primers which were complementary with the same sequences in different fungi (i.e. attached in the conserved sequence). The dendrogram of combined results of RAPD primers showed that the wild species (control) are in separate clade from the mutant (treated with different gamma radiation doses). However, the lowest and highest gamma radiation doses are closer to each other than the other doses; and this could refer that higher doses did not cause much mutation like in lower one. While in doses of 1 and 1.5 KGy the sub clades separated the results obtained from both *C. herbarum* and *T. viridi* grown on extract of *C. herbarum* closed to each other and this genetically approved that the resistance factors transfer from *Cladosporium herbarum* to *Trichoderma viridi*; and approved with results obtained from physiological bioassays.

High efficiency of a primer is indicative of a large area of the genome that complements and allows base pairing between the primer and the genomic DNA. Moreover, it has been suggested that high efficiency positively correlates with the high GC content of the primer. This suggestion, however, is not supported by results of the present work that the three primers (with 70% GC) have equivalent efficiencies as shown in table 4.

As the primer efficiency depends on the total number of bands amplified by the primer and this could include a number of common (monomorphic) ones (representing conserved sequences among various species, efficiency would not be very informative in identifying species. A more informative parameter in this respect is the primer discriminatory power (Grrundman *et al*., 1995).

Discriminatory value of a primer depends only on the number of polymorphic bands produced by the primer relative to the total number of polymorphic bands produced by all primers.

These obtained results was confirmed by the results of (Abbasi *et al*., 2016) who mentioned that gamma rays have very high energy, causing gene mutations by replacement of nucleotides (by oxidative deamination) or chromosome breakage. This was clear in this study as different doses of gamma radiation affect both physiological and molecular treats of the selected

fungi. Also, this study is supported by (Baharvand *et al*., 2014) who showed that it is possible to improve the antagonistic capability of *Trichoderma* for biological control of plant diseases through mutation with gamma radiation. Microarray analysis revealed that both X-rays and gamma rays up regulated genes related to cell cycle and DNA processing, cell rescue defense and virulence, protein and cell fate, and metabolism. Likewise, for both type of rays, the down regulated genes belonged to mostly transcription and protein synthesis, cell cycle and DNA processing, control of cellular organization, cell fate, and C-compound and carbohydrate metabolism categories (Dadachova and Casadevall, 2008). All studies since (Salama *et al*., 1977) till to (Calado *et al*., 2014) approved that *Cladosporium herbarum* is resistant to gamma radiation. As lower doses of gamma (5 Krad) stimulated the spore germination and growth of *Cladosporium herbarum*, where, higher doses (500 Krad) revealed that *Cladosporium herbarum* was of moderate resistance. Abdollah *et al*., (2014) and Tauxe (2001) reported that the high energy rays of irradiation directly damage the DNA of living organisms, inducing cross linkages and other changes that make an organism unable to grow or reproduce. When these rays interact with water molecules in an organism, they generate transient free radicals that can cause additional indirect damage to DNA. Moreover, other factors may involve to the sensitivity or tolerance of fungi on γ- ray. Sommer (1964) mentioned that multi cellular spore or bicellular spores are more tolerant to γ-radiation than the unicellular spore. Thus, this may be /one reason that unicellular spore of *T. viride* showed more the sensitive to γ- radiation.

Moreover, the number or density of spore in the inoculum exposed to radiation may affect the radiation dose required for the inactivation of microorganism. Increased spore density in the inoculum generally needs to elevate radiation dose (Barkai- Golan 1992). Changes or non-changes of biocontrol activity of *T. viride* after irradiated treatment may depend on level of tolerance and the doses of ray. Rezi *et al*., (2019) reported that the minimum inhi-bitory dose (MID) dose of gamma irradiation for 108 *Aspergillus* ochraceus spore is approximately 2.5 kGy. 29.

CONCLUSION

In the present work, low doses of gamma radiation produced stimulatory effects on growth, represented by biomass dry weight and radial colony diameter, but high doses had inhibitory effects. Therefore, its effect on tested fungi is dose dependent. Among the fungi tested, *C. herbarum* and *T. viride* (gcae) were recorded as radio-resistant, meanwhile the wild isolate (*T. viride*) was radio-sensitive. Generally, fungi appear to be very resistant to radionuclides in the environment, this fat of resistance may be due to the presence of smaller amount of DNA per nucleus compared to mammalian cells. Nevertheless, the evidence from studies done as a comparison between *T. viride* - sensitive and *T. viride* (gcae) – resistant, suggest that there may be other factors that confer radio resistance. These factors may provide some protection against ionizing radiation and be integral to the absorption and retention of radionuclides.

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دراسه جزيئيه لفطرتى التريكوديرم فيردى وكلادوسبيريوم هيربارم مشععه بأشعة جاما

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الملخص العربـــــي

تم عزل *Trichoderma viride* و *Cladosporium herbarum* من التربة وإشعاعهما بجرعات مختلفة من إشعاع جاما (0.5 ، 1 ، 1.5 ، 2 كيلوجراي). كان التأثير التثبيطي على معدل النمو يعتمد على الجرعة المعطاه وتم الحصول على الحد الأقصى من التثبيط بالإشعاع عند 1.5 و 2 كيلوجرام في *T. viride* والذي لم يظهر أي نمو. ومن ناحية أخرى ، أظهر *T. viride* الذي نما على وسائط تحتوي على مستخلص مائي من *C. herbarum* وأظهر مقاومة أكثر من تلك الموجودة في العينةالاصلية. كما أظهر *C. herbarum* مقاومة لأشعة جاما اثبت ذلك بوجود نمو. هذا يدل على وجود عامل مقاومة في مستخلص *Cladosporium*. الذي استخدم T. viride لذلك ، تم إجراء بصمة الحمض النووي للكشف عن أي اختلاف في الفطريات التي تم فحصها. أوضحت الدراسات الجزيئية أن البادئات الثلاثة لـ RAPD أعطت عددًا إجماليًا من 57 نطاقًا و 41 نطاقًا متعدد الأشكال مع إجمالي نسبة تعدد الأشكال 71.92٪. وتتراوح عدد النطاقات المتعددة الأشكال لكل برايمر من 14 نطاقًا بنسبة تعدد الأشكال 73.68٪ للطبقة الأولى من A01 ، و 16 فرقة متعددة الأشكال مع نسبة تعدد الأشكال من 84.21٪ لـ C02 إلى 11 نطاقًا بنسبة تعدد الأشكال 57.89٪ للطبقة الأولى من B07. و لذلك أنتج C02 أعلى نسبة من تعدد الأشكال (84.21 ٪). هذا بسبب تأثير العلاجات الفطرية المختلفة أو تأثير الجرعات المختلفة من إشعاع جاما. أوضحت الدراسة إمكانية تحسين ت قدرة الفطريات الحساسة للإشعاع عن طريق زراعتها على مستخلصات من تلك المقاومة للإشعاع.