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Stimulating of Biodegradation of Oxamyl Pesticide by Low Dose Gamma Irradiated Fungi

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Abstract

This investigation has been conducted to study the possibility of stimulating *Trichoderma spp* with low dose gamma radiation for biodegradation of Oxamyl pesticides. Fungi strains capable for biodegradation of oxamyl are identified as *Trichoderma spp.,* including *T. harzianum, T. viride, Aspergillus niger, Fusarium oxysporum* and *Penicillium cyclopium*. The results indicated that *Trichoderma* spp. used Oxamyl as source of carbon and nitrogen and possesses enzyme(s), which acts on amide and ester bond in Oxamyl structure**.** Degradation of oxamyl was 72.5% within 10 days of incubation by *T. harzianum* strain. It is very important to note that degradation of oxamyl 82.05% within 10 days of incubation by *T. viride* strain. This indicated that the isolates of *Trichoderma* spp. were potentially useful for oxamyl bioremediation. The biomass of *Trichoderma spp* strain were increased and reached its maximum at 250 Gy by 21.97 and 40.0 when using *Trichoderma spp.,* as well as *T. viride*, respectively. As a general trends the gamma radiation over than 0.25 KGr reduce the growth of *Trichoderma spp* by 50.27 and 38.13, using *Trichoderma spp.* as well as *T. viride,* respectively.

Keywords: Oxamyl; *Trichoderma harzianum; Trichoderma viride*; Biodegradation; Gamma-irradiation

Introduction

Contamination of surface water by organophosphate and carbamate compounds is of concern because of the potential toxicity to aquatic organisms, especially those at lower trophic levels. Many organophosphate and carbamate compounds have acute and chronic toxicity to fish and aquatic invertebrates. *Trichoderma spp* strain improvement usually by exposing the microorganism to radiation that produces the enzyme by techniques, such as classical mutagenesis, which involves γ-rays, UV-rays [1].

Pesticides must persist long enough to control biological targets, but should not become a pollution problem [2,3]. Environmental of Oral and dermal exposure of rats to Pesticide cyanophos was characterized by studying acetylcholine esterase, aspartate transaminase, alanine transaminase and alkaline phosphatase (ALP) enzyme activities were studied by Afify and El-Beltagi [4], as biomarker for pesticides pollution. The toxicity of some pesticides, such as organophosphates and carbamate insecticides, is mainly caused by the inhibition of ChE activity of vertebrates and invertebrates. This inhibition leads to the accumulation of acetylcholine in the synaptic terminals, and therefore, to a change in the normal transmission of the nervous impulse. This interference may result in neurological manifestations, such as irritability, restlessness, muscular twitching and convulsions that may end in the respiratory failure and death of the animal [5]. Oxamyl and carbofuran, a broad-spectrum carbamate pesticide, has been used extensively in agriculture as a soil-incorporated to control a variety of insect pests of crops, including canola, corn, alfalfa, potatoes and strawberries [6]. Studies on microbial degradation are useful in the development of bioremediation strategies for the detoxification of these insecticides by microorganisms. Bioremediation is defined as the process, whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities [7].

A number of isolates capable of carrying out some form of degradation of pesticide have been isolated from soils and several fungi are have been isolated and studied, including *Aspergillus niger* [8], *Fusarium graminearum* [9], *Mucor ramannianus* [10] and *Gliocladium* sp. [11]. Recently, a strain of *Trichoderma harzianum* has been shown to degrade carbofuran [12] and organochlorines through an oxidative system [13].

Low dose of ionizing radiation on microorganisms is responsible of accelerated enzyme activity [14]. The lowest dose of gamma irradiation (1 MCi for 10 min) enhanced three isolates of *Aspergillus niger,* investigated to produce more biomass and polygalactronase, pectinmethylglacturonase, cellulase and protease [15]. *Trichoderma harzianum*, *T. viride* and *T. knoingii* irradiated with 0.5 KGy dosage resulted in the highest percentage of pathogen growth reduction by producing highly active exo-enzymes [16]. The two thermophilic isolates, *Streptomyces albaduncus* and *S. erythogresius,* were exposed to increasing doses of gamma radiation up to 5 KGy. All radiation did not affect the physiological properties, but relativity higher doses enhanced the utilization of carbon sources and increased their sodium chloride tolerance from 8 to 10%. Dose level of 2 KGy enhanced the antimicrobial activity of both isolates, either at first or second generation against bacteria, moulds and yeasts among them [17]. The low doses of gamma ray (10 and 20 Gy) significantly increased the alcohol-dehydrogenase enzyme activity of *Saccharomyces cerevisiae* [18].

The present work aimed to apply gamma radiation on *Trichoderma spp,* and to enhance effective hydrolytic enzymes in their bio-control abilities for biodegradation of oxaml pesticides. Low dose of gamma

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radiation used to enhancement *T. viride* and *T. harzianum*. Several successful attempts had been made to increase the bio-control potential of *Trichoderma* spp by exposing them to gamma radiation.

Materials and Methods

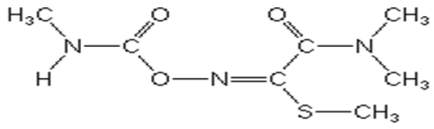
Soil sampling and characterization

Soil sample were collected from 10 different sub-samples and taken from the areas of 25 m^2, (0-20 cm) depth, from heavy clay soil had a previous history of treatment with Oxamyl in the last 10 years at field located in El-Fayoum governorate, Egypt.

Detailed physical and chemical properties of the soil are presented in Table 1. In the laboratory, the soil was gently air-dried to the point of soil moisture suitable for sieving. After sieving to a maximum particle size of <2 mm, the soil kept in a plastic bag at 4°C for 7 days before use.

Chemicals and reagents

Technical grade Oxamyl (99.1% purity) was purchased from Sigma Aldrich Co., Nasr city, Egypt. All other chemicals and solvents were ultra pure grade and obtained from El-Gomhouria CO. for Trading Chemicals and Medical Appliances, Egypt.



Chemical structure of Oxamyl (Methyl 2-(dimethylamino)-*N*-[(methylamino) carbonyl] oxy]-2-oxoethanimidothioate).

Enrichment procedure and isolation of microorganisms

Soil contamination: Prepared soil (200 g) was supplemented with Oxamyl at concentration of 50 mg/kg soil, introduced in a form of methanol solution. After mixing and solvent evaporation, the soil was incubated in the dark at 30 ± 1°C, in a thermostatic chamber for 90 days. The water content of the soil was adjusted to 50%. Throughout the incubation period, water losses exceeding 5% of the initial values were compensated by the addition of deionized water. After 30 and 60 days of incubation, the soil was contaminated again with the same dosage of Oxamyl

Identification of isolates: The pure isolated fungi were identified according to the most documented keys in fungi identification [19,20]. The morphological identification of isolated fungal strains is based on the morphology of the fungal culture colony or hyphae and the characteristics of spores.

Exposure of *T. harzianum, T. viride* to gamma radiation

The most effective Oxamyl degrading fungi (*Trichoderma spp.,* including *T. harzianum, T. viride*) selected and exposed to different doses of gamma radiation. Slants of 7 days old culture were irradiated with doses of 0.0, 0.02; 0.05; 0.1; 0.25; 0.5; 1.0; 2.0 and 5.0 KGy, and three replicates were used for each dose. Radiation treatments were carried out at Atomic Energy Authority, Abu-Zabal at dose rate of Egypt's Mega-gamma-1 type, J 6600-Cobalt-60 Irradiator.

Chemical analyses

Extraction and purification of oxamyl: Oxamyl was analyzed by high performance liquid chromatography (HPLC) at Atomic Energy Authority, Abu-Zabal, Egypt. In order to extract Oxamyl from the soil and liquid phases, the soil was slurry centrifuged at 6000 rpm at 25°C for 15 min to separate the liquid from the soil. The liquid phase was filtered through cellulose acetate paper (Whatman- number 1, England), prior to the liquid–liquid partitioning extraction procedure. Briefly, 2 mL of methanol were added to 2 mL of liquid sample, and then the mixture was sonicated twice for 10 min on a 50/60 voltage cycle. After sonication, Oxamyl was extracted in a separation funnel with dichloromethane. For the method of high-performance liquid chromatography (HPLC), the supernatant was dissolved in the same volume of pure grade methanol and filtrated by membrane filters (0.45 µm). An aliquot of the residue in a 20 µL sample size was injected into a HPLC. The analytical column was Zorbax SB-C18 column (250×4.6 mm, 5 µm), and the solutes were detected using PDA detector with gradient UV-VIS detection ranging from 200 to 600 nm. The mobile phase consisted of 70% methanol and 30% water at a flow rate of 1.0 mL min^{-1}.

Data analysis: Data was analyzed by SPSS program Version 11.5.0. The significance of treatments was set at p-value less than or equal to 0.05 by the one-way ANOVA test.

Results and Discussion

The biochemical and genetic basis of microbial degradation has received considerable attention. Several genes/enzymes which provide microorganisms with the ability to degrade organo-pesticides, have been identified and characterized. The ability of these organisms to reduce the concentration of xenobiotics [21] is directly linked to their long-term adaptation to environments, where these compounds exist. Gamma irradiation may be used to enhance the performance of such microorganisms that have the preferred properties, essential for biodegradation. Therefore, gamma irradiation was used to activate several fungi and determine the activities of their growth under this condition, as well be discussed in our investigation.

Oxamyl concentration (mg/L)	Dry weight biomass mg/100 ml*				
	T. harzianum	*T. viride*	*Aspergillus niger*	*Fusarium oxysporum*	*Penicillium cyclopium*
0 (control)	133.2 ± (0.40414)	169.0 ± (0.57735)	154.2 ± (0.46188)	95.6 ± (0.51961)	160.6 ± (0.05773)
20	168.6 ± (0.28867)	178.8 ± (0.51961)	154.6 ± (0.11547)	121.8 ± (0.46188)	157.0 ± (0.28867)
50	171.2 ± (0.11547)	185.6 ± (0.057735)	156.2 ± (0.17320)	145.8 ± (0.63508)	151.8 ± (0.11547)
100	176.0 ± (0.92376)	199.2 ± (0.17320)	159.4 ± (0.69282)	137.8 ± (0.11547)	146.0 ± (0.23094)
200	182.0 ± (0.28867)	175.0 ± (0.40414)	166.0 ± (0.28867)	120.2 ± (0.86602)	141.6 ± (0.80829)
250	169.3 ± (0.17320)	160.0 ± (0.63508)	145.0 ± (0.51961)	108.7 ± (0.17320)	122.0 ± (0.40414)
300	152.8 ± (0.11547)	149.1 ± (0.51961)	132.0 ± (0.92376)	96.2 ± (0.40414)	105.8 ± (0.17320)

* The values are the means of three replicates with the standard error (in parentheses) which was within 5% of the mean.

Table 1: Effect of oxamyl on the dry weight biomass of isolated fungi in MSM containing different concentrations of oxamyl within 7 days of incubation.

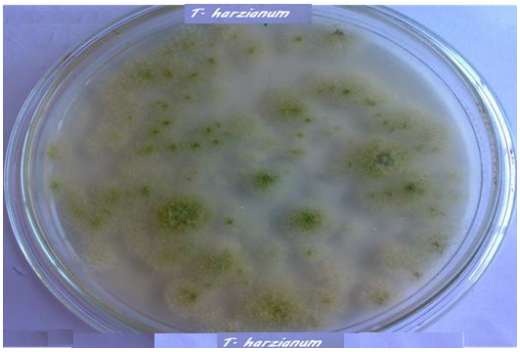


Figure 1: *Trichoderma harzianum* strain.

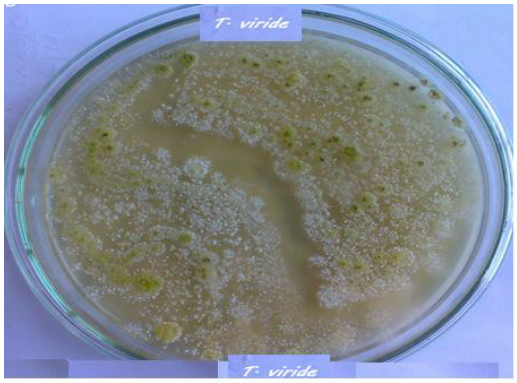


Figure 2: *Trichoderma viride* strain.

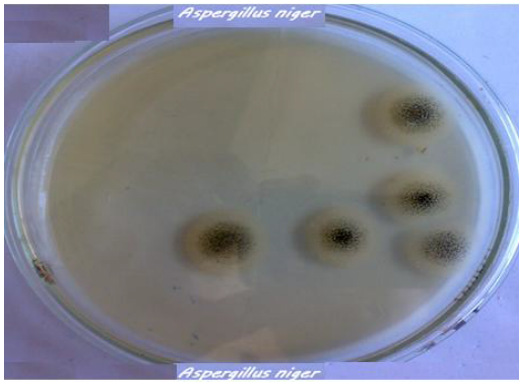


Figure 3: *Aspergillus niger* strain.

Isolation and identification of fungi

Fungi were isolated from soil collected from El-Fayoum governorate treated with different concentration of oxamyl (20-300 mg/L). After several tests of culture on synthetic medium containing the Oxamyl, five fungi were selected and tested for their ability to degrade Oxamyl, pesticide. The fungi strains were identified as *Trichoderma spp.,* including *T. harzianum, T. viride, Aspergillus niger, Fusarium oxysporum* and *Penicillium cyclopium* (Table 1) (Figures 1-5). The results showed that *Trichoderma spp.,* including *T. harzianum* and *T. viride,* reach its maximum growth using oxamyl concentration of 200 and 100 mg/L and yielded 182.0 and 199.2, respectively. On the other hand, *Aspergillus niger* reach its maximum yield 166.0 with 200 mg/L, while the growth of *Fusarium oxysporum* and *Fusarium oxysporum* were weak (Figures 2 and 6). From the above results, *Trichoderma* spp. used Oxamyl as source of carbon and nitrogen and possesses enzyme(s) which acts on amide and ester bond in Oxamyl. Degradation of oxamyl was 72.5% within 10 days of incubation by *T. harzianum* strain. While the degradation of oxamyl was 82.05% within 10 days of incubation by *T. viride* strain, this indicated that the isolates of *Trichoderma* spp. were potentially useful for oxamyl bioremediation (Table 2) (Figure 7).

These results agree with those of Rajagopal et al. [22], who isolated *Bacillus* sp., *Micrococcus* sp., *Arthrobacter* sp. and *Azospirillum* sp. capable of using pesticides as source of carbon and nitrogen. This increase in the biomass of the culture with Oxamyl could be explained by the fact that this product constitutes, an additional carbon and nitrogen contribution, which allows the synthesis of new secondary metabolites favoring the production of microbial biomass, and in consequence, support a faster use of Oxamyl. The increase in biomass (mycelial dry weight) was reported when *T. viride* strain incubated with pesticides [12]. Therefore, *Trichoderma spp* have been selected for enhancement by gamma radiation for better biodegradation of oxamyl pesticide.

Effect of gamma radiation on *Trichoderma spp*

Results in Table 3 and Figures 8 and 9 indicated the effect of different gamma-radiation doses 0.0, 0.02; 0.05; 0.1; 0.25; 0.5; 1.0; 2.0 and 5.0 KGy, on biomass of *Trichoderma spp.,* as well as *T. viride* grown on MSM with oxamyl at concentration of 200 mg L^{-1} within 7 days of incubation. The biomass of *Trichoderma spp* strain were increased

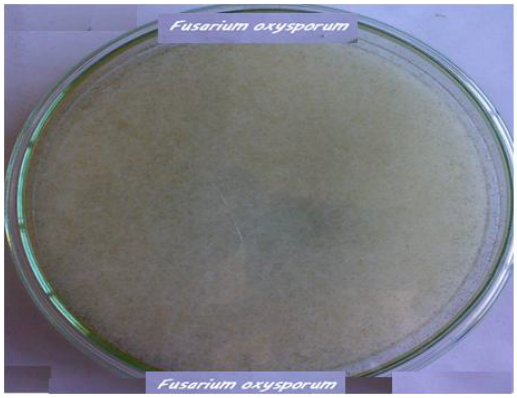


Figure 4: *Fusarium oxysporum* strain.

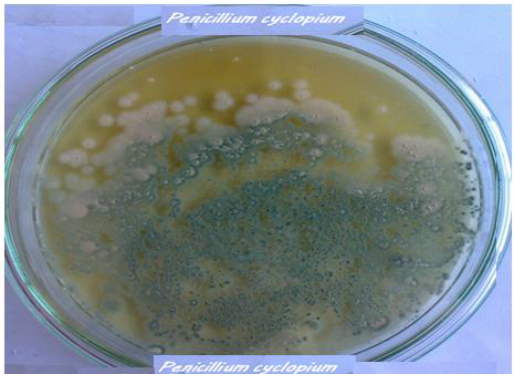


Figure 5: *Penicillium cyclopium* strain.

Fungal strain	Time of incubation (d)	Oxamyl control	Oxamyl inoculation	Oxamyl loss (%)
T. harzianum	1	198.0	175.9	12.05
	2	196.2	148.6	25.7
	3	193.2	129.0	35.5
	4	190.3	111.0	44.5
	5	189.3	101.2	49.4
	6	184.9	90.6	54.7
	7	178.6	81.9	59.05
	8	172.1	72.0	64
	9	167.2	64.0	68
	10	160.0	55.0	72.5
T. viride	1	198.0	181.8	9.1
	2	196.2	168.2	15.9
	3	193.2	142.0	29
	4	190.3	122.2	38.9
	5	189.3	95.7	52.15
	6	184.9	76.5	61.75
	7	178.6	61.0	69.5
	8	172.1	52.1	73.95
	9	167.2	44.2	77.9
	10	160.0	35.9	82.05

Table 2: Biodegradation of oxamyl (200 mg L^{-1}) in mineral salt medium by *Trichoderma spp*.

Gamma irradiation dose (Gy)	Mycelial dry weight (mg/100 ml)*			
	T. harzianum		*T. viride*	
	Growth mg/100 ml	*% of Change*	*Growth mg/100 ml*	*% of Change*
0	182.0 ± (0.057735)	0.0	165.0 ± (0.57735)	0.0
20	198.5 ± (0.02886)	9.07	174.0 ± (0.14433)	5.45
50	204.0 ± (0.37527)	12.08	189.1 ± (0.23094)	14.60
100	217.8 ± (0.23094)	19.67	209.5 ± (0.40414)	26.96
250	222.0 ± (0.40414)	21.97	231.0 ± (0.51961)	40.0
500	175.2 ± (0.23094)	-3.74	151.0 ± (0.40414)	-8.84
1000	129.6 ± (0.17320)	-28.79	129.0 ± (0.46188)	-21.81
2000	108.0 ± (0.63508)	-59.34	119.0 ± (0.11547)	-27.87
5000	91.50 ± (0.11547)	-50.27	102.0 ± (0.28867)	-38.13

*The values are the means of three replicates with the standard error (in parentheses) which was within 5% of the mean.

Table 3: Mycelial dry weight (mg/100 ml) of gamma irradiated *Trichoderma* spp. grown on MSM with 200 mg L^{-1} of oxamyl at 30ºC for 7 days of incubation.

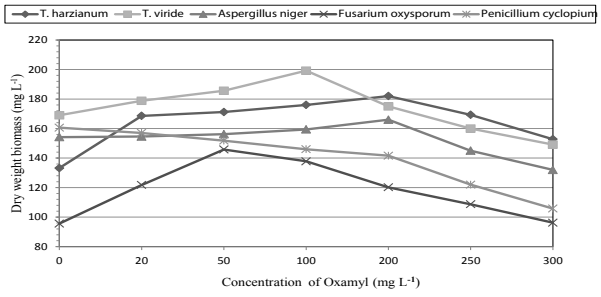


Figure 6: Effect of oxamyl on the dry weight biomass of isolated fungi in MSM containing different concentrations of oxamyl within 7 days of incubation.

and reached its maximum at 250 Gy by 21.97% and 40.0% when using *Trichoderma spp.,* as well as *T. viride*, respectively. As a general trend, the gamma radiation over than 0.25 Kg reduce the growth of *T.* spp by 50.27% and 38.13% using *Trichoderma spp.,* as well as *T. viride*, respectively. Previous studies have shown that relatively low dose of ionizing radiation on microorganisms is responsible of accelerated enzyme activity [14]. The low doses of gamma ray (10 and 20 Gy) significantly increased the alcohol-dehydrogenase enzyme activity of *Saccharomyces cerevisiae* [18]. *Trichoderma harzianum, T. viride* and *T. knoingii* irradiated with 0.5 KGy dosage resulted in the highest percentage of pathogen growth reduction by producing highly active exo-enzymes (cellulosae and chitinase isoenzymes), as confirmed by Haggag and Mohamed [16]. These results are in agreed with stated that growth of *T. viride* was increased at 0.5 KGy of gamma-radiation. Mycelial dry weight increased in isolates of *Aspergillus tamaru, A. flavus* and *A. niveus,* when exposed to gamma-irradiation doses of 0.2 and 0.5 KGy [23]. Previous studies have shown that relatively low dose of ionizing radiation on microorganisms is responsible of accelerated enzyme activity [14]. The low doses of gamma ray (10 and 20 Gy) significantly increased the alcohol-dehydrogenase enzyme activity of *Saccharomyces cerevisiae* [18]. *Trichoderma harzianum, T. viride* and *T. knoingii* irradiated with 0.5 KGy dosage resulted in the highest percentage of pathogen growth reduction by producing highly active

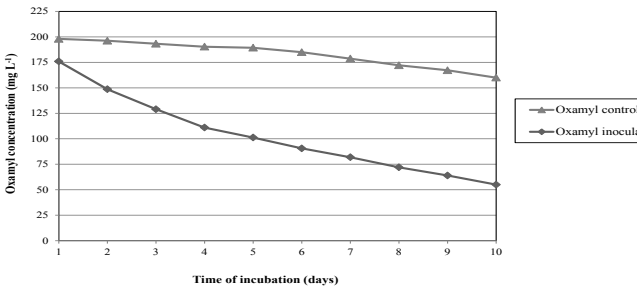


Figure 7: Biodegradation of Oxamyl (200 mg L^{-1}) in MSM by *T. harzianum strain* within 7 days of incubation at 30°C.

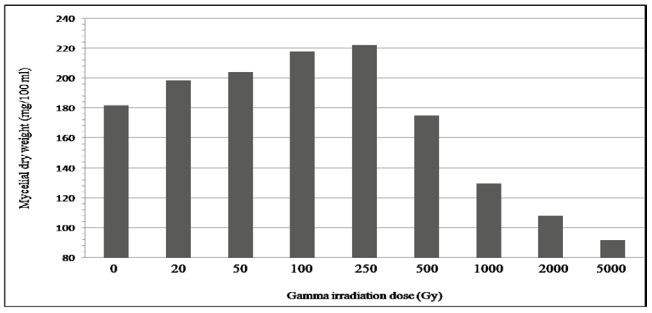


Figure 8: Mycelial dry weight (mg/100 ml) of gamma irradiated *T. harzianum* grown on MSM with 200 mg $L^{-1}$of Oxamyl at 30ºC for 7 days of incubation.

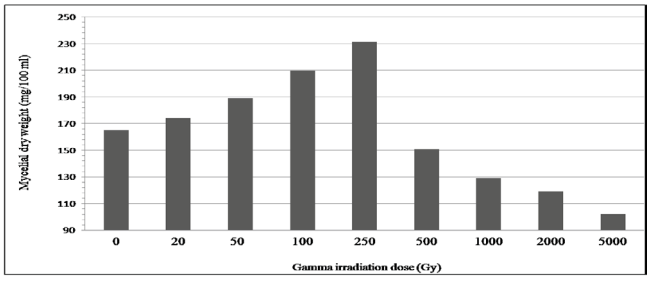


Figure 9: Mycelial dry weight (mg/100ml) of gamma irradiated *T. viride* grown on MSM with 200 mg L^{-1} of Oxamyl at 30ºC for 7 days of incubation.

exo-enzymes. The results showed that *Trichoderma* sp. presented a good growth in the presence of DDD and 21% of the pesticide was degraded. In the experiments where DDD was added after 5 days of *Trichoderma* sp. growth, and with the addition of H_2O_2, the total biodegradation occurred [24]. Haggag and Mohamed [16] found that mutagenesis of three *Trichoderma* species by gamma irradiation exhibited high capabilities to produce efficient antibiotics, enzymes and phenols. On the other hand The tested UV-induced mutants were higher in their production of enzymes (cellulases, chitinases and β-1,3-glucanases) than their parental wild type strain (*T. viride*). Cellulase was the greatest enzyme production by the tested *T. viride* strains, followed by β-1,3-glucanase then chitinase. Therefore, the enhancement of *Trichoderma spp* by gamma radiation induce the activation of the main enzymes cellulases, chitinases and β-1,3-glucanases, which depend mainly on the dose of radiation [25]. The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyzes the conversion of cellulose to glucose in an enzymatic hydrolysis. This agrees with previous reports that the amylolytic potential of *T. viride* was increased at 0.5 KGy of gamma-radiation [26]. *Trichoderma harzianum*, *T. viride* and *T. knoingii* irradiated with 0.5 KGy dosage resulted in the highest percentage of pathogen growth reduction by producing highly active exo-enzymes. Therefore, *Trichoderma* spp. mutants were effective in reducing the pathogen growth in rhizophere soil, as compared to the wild type strains.

From the results above, *Trichoderma* strains is attributable to increase biodegradation of the oxymyl pesticide according to one or more complex mechanisms, including nutrient competition, antibiosis, the activity of cell wall-lytic enzymes, induction of systemic resistance and increased plant nutrient availability, as confirmed by Ene and Alexandru [27].

Conclusion

The proposal that our enhancement *Trichoderma* spp. will involve in the biodegradation of oxamyl pesticides well be belongs to produce of several active enzymes by the two spp of *Trichoderma,* as confirmed by fungi of *Aspergillus niger* and *Fusarium graminearum* [9]. Data in this work indicate the possibility of applying gamma-radiation doses to increase oxamyl degradation by enhancement *T. harzianum* and *T. viride* with low dose gamma radiation. An enrichment procedure allowed isolating of two effective fungal strains belonging to *T. harzianum* and *T. viride,* that may participate in efficient degradation of the oxamyl. Obtained results have implicated for the development of a bioremediation strategy of oxamyl-polluted soils. However, use of pesticide-degrading microbial systems for removal of pesticide compounds from the contaminated sites requires an understanding of ecological requirements of degrading strains. There is a need for further research on the biochemical and genetic aspects of oxamyl degradation by the isolated fungi. Therefore, this point needs further investigation to study the activity of enzymes needed to degrade oxamyl to its main metabolite and further biodegradable products. In the future, it could even apply *Trichoderma* spp. directly under special condition or synthetize these enzyme and applied in the field for biodegradation of oxamyl pesticides to clean environments from pollutants.

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