Exposing of Trichoderma spp. to Gamma Radiation for Stimulating Pesticide Biodegradation Activity

This work has been conducted to study the possibility of using fungi for degrading carbofuran. Trichoderma spp. were shown activity to convert carbofuran to 3-ketocarbofuran. Carbofuran and its main metabolite were analysed by high performance liquid chromatography. The main finding of biodegradation in the soil showed that 81.5 % and 86 % of carbofuran degraded within 14 days of incubation by T. harzianum and T. viride strains, respectively. The lowest dose of gamma irradiation 0.25 KGy enhanced the mycelia dry weight by 22.8 % and 16.2 % for T. harzianum and T. viride strains, respectively.

"The purpose of the present work is to isolate fungal strains, characterise their degradation potential and activate them with gamma radiation for bioremediation of carbofuran-contaminated soil."

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Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting contamination . Studies on microbial degradation are useful in the development of bioremediation strategies for the detoxification of insecticides (Vidali, 2001) and activate enzymes (Afify and El-Beltagi, (2011). Fungi has capability for degradation of carbofuran including Aspergillus niger (Qing et al., 2006), Fusarium graminearum (Salama, 1998), Mucor ramannianus (Seo et al., 2007) Gliocladium sp. (Slaoui et al., 2007) and Trichoderma harzianum were shown to degrade carbofuran (Wootton et al., 1993).

Gamma irradiation (1 MCi for 10 min) enhanced three isolates of Aspergillus niger which have polygalactronase, pectinmethylglacturonase, cellulase and protease (Gherbawy, 1998). Trichoderma harzianum, T. viride and T. knoingii irradiated with 0.5 KGy producing highly active exo-enzymes (Ben Akacha et al., 2008).

The purpose of the present work is to isolate fungal strains, characterise their degradation potential and activate them with gamma radiation for bioremediation of carbofuran-contaminated soil.

2. Materials And Methods

2.1. Soil sampling and characterisation

Soil samples were collected from 10 different sub-samples and taken from the areas of 25 m², (0–20 cm) depth, from heavy clay soil that had a previous history of treatment with carbofuran in the last 10 years at El-Fayoum governorate, Egypt.

2.2. Chemicals and reagents

Carbofuran (99.1% purity) was purchased from Sigma Aldrich Co.

2.3. Enrichment procedure and isolation of microorganisms

2.3.1. Soil contamination

Soil (200 g) was supplemented with carbofuran 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.

2.3.2. Cultivation in liquid medium

Mineral salt medium (MSM) contained: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 3 g; $MgSO_4.7H_2O$, 0.5 g; glucose, 3 g, micro-elements mineral solution, 2 ml (Cooney and Levine, 1972) and distilled water, 1 litre. The solid synthetic medium was obtained by addition of agar, 15 g L^{-1}. The pH was adjusted to 7 and the media were sterilised at 121°C for 15 min.

Carbofuran was introduced in a form of methanol solution to give the final concentration of 50 mg L^{-1}.

2.3.3. Cultivation and selection of isolates on agar medium

The fungal strains were isolated by plating 10-fold dilutions of the liquid medium onto MSM agar supplemented with carbofuran at concentrations of 100 mg L^{-1}, incubated at 30 ± 1 °C for 7 days. The fungal isolates were purified by using single spore or hypha tip technique.

2.4. Identification of isolates

The pure isolated fungi were identified according to Wei, 1979; Carmichael et al., 1980; Barnett and Hunter, 1998.

2.5. Exposure of isolates to ionising radiation.

Fungi (Trichoderma spp., including T. harzianum, T. viride) were selected and exposed to different doses of gamma radiation. Fungi 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 .Radiation treatments were carried out with J 6600-Cobalt-60 Irradiator.

2.6. Biodegradation studies

2.6.1. Biodegradation of carbofuran in liquid medium

Carbofuran was introduced to mediums to give the final concentration of 200 mg L^{-1}. After 24 h of shaking and solvent evaporation, 1ml of spore suspension (107 spore/ml) of each isolate was inoculated into MSM.

2.6.2. Biodegradation of carbofuran in soil

Soil 200g were placed in 500 mL and treated with carbofuran (200 mg/kg soil) in methanol solution, under sterile conditions. After mixing and solvent evaporation the fungal suspension of each carbofuran-degrading isolate was inoculated into soil. The inoculum was thoroughly mixed under sterile conditions. In addition, the samples with the mixed culture of carbofuran - degrading fungal strains (T. harzianum and T. viride) and non-sterilised soil (non-supplemented with carbofuran, earlier) were also used to compare the degradation dynamics of carbofuran in soil.

2.6.3. Chemical analyses

2.6.3.1. Extraction and Purification of carbofuran

The soil was slurry centrifuged at 6000 rpm at 25 ºC for 15 min to separate the liquid from the soil. The liquid phase was filtered through cellulose acetate paper (Whatman- number 1,) 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, carbofuran and its metabolites were extracted in a separation funnel with dichloromethane. high-performance liquid chromatography (HPLC), with column Zorbax SB-C18 column (250×4.6 mm, 5 µm), and 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. The detection wavelength of carbofuran was 275 nm.

2.7. Data analysis

Data was analysed 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.

3. Results and discussion

3.1. Isolation and Identification of isolates

Five fungi are selected and tested for their ability to degrade carbofuran pesticide. The fungal strains were identified to the species level as Trichoderma spp. including T. harzianum and T. viride, Aspergillus niger, Fusarium oxysporum and Penicillium cyclopium. The results indicate that Trichoderma spp. use carbofuran as source of carbon and nitrogen. This result suggested that the fungus possess enzyme(s) which acts on amide and ester bond in carbofuran (table 1). These results agree with Rajagopal et al., (1984) who isolated Bacillus sp., Micrococcus sp., Arthrobacter sp. and Azospirillum sp. capable of using carbofuran (Wootton et al., 1993) .

3.2. Effect of gamma radiation on the mycelia dry weight of the fungal strains

Data in Table (2) indicate that the mycelia dry weight of the fungal strains T. harzianum and T. viride decreased with the applied gamma-radiation dose up to 5000 Gy. Mycelia of T. harzianum strain was increased significantly at 100 and 250 Gy by 13.7 and 22.8 %, respectively. As for the T. viride strain, this increased in mycelia at 100 and 250 Gy, respectively. Exposure of Trichoderma spp. at dosage of 250 Gy shows increase in biodegradation of carbofuran by 14 % (79 % total biodegradation) and 9 % (83.5 % total biodegradation) by T. harzianum and T. viride, respectively (Ortega et al., 2011).

These results are in agreement with Younis (1999) who found that growth of T. viride was increased at 0.5 KGy of gamma-radiation.

3.3. Biodegradation of carbofuran in mineral salt medium (MSM)

The results (table 3) shows biodegradation of carbofuran at concentration of 200 mg/L by T. harzianum and T. viride cultivated in MSM. This result suggested that the fungus possesses enzyme(s) which acts on function groups in carbofuran. The results showed that 65% and 74.5% of carbofuran were degraded within 10 days of incubation by T. harzianum and T. viride, respectively. The results agrees with (Haggag and Mohamed 2002) cited that Trichoderma harzianum, T. viride and T. knoingii irradiated with 0.5 KGy dosage involved in carofuran degradation.

The metabolites by Trichoderma spp. were identified by HPLC. The main metabolite of carbofuran was 3-hydroxycarbofuran converted to 3-ketocarbofuran suggested by. Salama (1998).

3.4. Effect of gamma radiation on degradation ability of the fungal strains in soil

Table (4) shows the biodegradation of carbofuran by gamma irradiated Trichoderma spp. in sterile and non-sterile soil samples. The data showed that an increase on the biodegradation ability of carbofuran by Trichoderma spp. compared with non-irradiated Trichoderma spp. Degradation of carbofuran by gamma irradiated T. harzianum was 76.5 % and 85 % within 14 days of incubation in non-sterile and sterile soil, respectively. While the degradation of

Table 1: Effect of carbofuran on dry weight biomass for all tested fungi in MSM containing different concentrations of carbofuran within 7 days of incubation.

Carbofuran Concentration (mg L^{-1})	Dry Weight Biomass mg/100 ml *				
	T. harzianum	*T. viride*	*Aspergillus niger*	*Fusarium oxysporum*	*Penicillium cyclopium*
0(control)	133.2±(0.23094)	169.0±(0.57735)	154.2±(0.40414)	95.60±(0.23094)	160.6±(0.34641)
20	137.4±(0.11547)	170.8±(0.11547)	203.2±(0.51961)	100.8±(0.28867)	132.0±(0.11547)
50	143.2±(0.46188)	188.8±(0.40414)	181.4±(0.34641)	106.8±(0.69282)	128.6±(0.23094)
100	150.8±(0.34641)	195.8±(0.11547)	134.8±(0.40414)	128.8±(0.63508)	126.0±(0.57735)
200	167.6±(0.05773)	222.0±(0.28867)	95.80±(0.11547)	82.80±(0.46188)	122.6±(0.34641)
250	150.0±(0.57735)	206.0±(0.57735)	92.20±(0.46188)	71.10±(1.09696)	115.0±(0.63508)
300	120.0±(0.28867)	190.0±(0.51961)	78.90±(0.80829)	63.50±(0.28867)	102.0±(0.05773)

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

Table 2: Mycelial dry weight (mg /100 ml) of gamma irradiated Trichoderma sp. grown on MSM with 200 mg L-1 of carbofuran at 30 ºC for 7 days of incubation.

Gamma irradiation dose (Gy)	Mycelial dry weight (mg/100 ml)*	
	T. harzianum	*T. viride*
0	168 ± (0.11547)	222 ± (0.57735)
20	176 ± (0.57735)	236 ± (0.11547)
50	178 ± (0.51961)	240 ± (0.57735)
100	191 ± (0.28867)	248 ± (1.15470)
250	206 ± (0.17320)	258 ± (1.73205)
500	188 ± (0.98149)	215 ± (1.15470)
1000	144 ± (0.63508)	205 ± (2.30940)
2000	122 ± (0.40414)	187 ± (0.57735)
5000	083 ± (0.28867)	144 ± (1.21243)

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

Table 3: Biodegradation of carbofuran (200 mg L-1) in mineral salt medium by Trichoderma spp.

Fungal strain	Time of incubation (days)	Carbofuran control	Carbofuran inoculation	Carbofuran loss (%)	Main metabolite (3-ketocarbofuran)
T. harzianum	0	200.0	200.0	0.0	0
	1	197.0	178.0	11.0	9
	2	195.0	148.2	25.9	13
	3	191.3	121.0	39.5	18
	4	188.8	109.0	45.5	21
	5	185.0	99.0	50.5	28
	6	181.0	91.0	54.5	35
	7	175.9	85.0	57.5	39
	8	169.2	80.0	60.0	45
	9	165.0	75.0	62.5	56
	10	163.0	70.0	65.0	60
T. viride	0	200.0	200.0	0.0	0
	1	197.0	181.0	9.50	4
	2	195.0	151.0	24.5	11
	3	191.3	125.0	37.5	15
	4	188.8	112.0	44.0	22
	5	185.0	104.0	48.0	31
	6	181.0	87.0	56.5	40
	7	175.9	77.0	61.5	46
	8	169.2	67.0	66.5	54
	9	165.0	58.0	71.0	62
	10	163.0	51.0	74.5	70

Table 4. Biodegradation of carbofuran in sterile and non-sterile soil by gamma irradiated Trichoderma spp.

Fungal strain	Time of incubation (d)	Carbofuran control in sterile soil (mg)	Carbofuran control in non-sterile soil (mg)	Carbofuran inoculation in sterile soil (mg)	Carbofuran inoculation in non-sterile soil (mg)	3-Keto carbofuran in sterile soil (mg)	3-Keto carbofuran in non sterile (mg)
T. harzianum	0	200	200	200	200	0	0
	2	195	193	165	185	14	12
	4	189	185	142	152	22	23
	6	183	180	119	129	35	28
	8	176	172	98.0	107	41	33
	10	168	162	78.0	89	47	41
	12	162	152	59.0	63	59	50
	14	156	143	30.0	47	66	55
T. viride	0	200	200	200	200	0	0
	2	195	193	181	185	17	12
	4	189	185	162	171	29	22
	6	183	180	138	134	37	31
	8	176	172	91.0	105	48	39
	10	168	162	64.0	82	52	46
	12	162	152	34.0	52	62	54
	14	156	143	18.0	41.0	75	59

carbofuran by non-gamma irradiated T. harzianum was 75 % and 81.5 % within 14 days of incubation in non-sterile and sterile soil, respectively (Afify and Shosha 1988).

4. Conclusion

The radiation enhanced Trichodermaspp. for biodegradation of carbofuran by hydroxylation at the three position and oxidation to main metabolite 3-ketocarbofuran by Aspergillus niger and Fusarium graminearum (Salama et al., 1998). Therefore this point needs further investigation to study the activity of enzymes needed to degrade carbofuran to the main metabolite. In the future one could even apply Trichoderma spp. directly under special condition or synthetise these enzymes and apply in the field for biodegradation of carbofuran to clean environments from pollutants.

Acknowledgments

This work was supported by Scientists Next Generation Program, Academy of Scientific Research and Technology, Egypt and Cairo University, Faculty of Agriculture Biochemistry Department.

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