Biodegradation of $^{14}$C- Diflubenzuron Pesticide by Penicillium Notatum

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ABSTRACT

Three fungi, Aspergillus flavous, Aspergillus nigar and Penicillium notatum were selected in the current study for their tendency to tolerate Diflubenzuron (DFB) pesticide. Regarding fresh and dry weight (g/100ml) P. notatum showed to be the least affected by the presence of DFB (200ppm). When Glutathione-S-Transferase (GST) was measured in the three fungi, the highest level was detected in P. notatum. These finding prompted further studies in which P. notatum tolerated up to 1000 ppm of DFB.

Another attempt was conducted to increase the production of GST using gamma irradiation. The gamma radiation showed a significant effect on the concentration of GST especially on the intercellular GST (mycelia GST). This was recognized upon applying the lowest dose of gamma radiation (10Gy). As the radiation dose increased the effect was more pronounced where both inter and extracellular enzyme (GST secreted in media) activity started showing statistically significant increase from control untreated group.

After inoculation of pesticide with $^{14}$C- DFB for 10 days the data showed that 91.2 % of the $^{14}$C- activity could be recovered. The $^{14}$C- activity was distributed, in mycelia (65.8%) and in the media (25.4%). The CHCl$_3$ extract of the mycelia contributed 41.0% of $^{14}$C radioactivity while 23.0% was in the water phase. The amount that remained without extraction (bound residues) did not exceed 1.8%. In the media, of the total recovered radioactivity, 15.4 % was in the organic phase while 10% was in the water phase. The TLC analysis of chloroform extract of external extract of fungi revealed the following: In the media, the intact parent compound (DFB) identified by co-chromatography showed a 74%, while the metabolites of concern, 4 chloro phenyl urea(CPU) showed 10.0% , 2,6difluorobenzamide (DFBAM) 6.8% and 4-chloro aniline (CA) contributed to 8.6% , however, 2,6-difluorobenzoic acid (DFBA) was not detected in the current experiment. On the other hand, the internal extract showed percent of 72.0, 7.2 and 20.6 for BFB, CPU and DFBAM, respectively. Again, DFBA was not detected in addition to CA.

Key words: Biodegradation/ Diflubenzuron/ Penicillium notatum.

INTRODUCTION

During the past decade, fungi have been used in the treatment of a wide variety of wastes, wastewaters, and the role of fungi in the bioremediation of various hazardous and toxic compounds in soils and sediments has been established. Fungi have also demonstrated the ability to remove heavy
metals and to degrade, in some cases mineralize, phenols, halogenated phenolic compounds, petroleum hydrocarbons, polycyclic aromatic compounds, and polychlorinated biphenyls and radioactive substance (1,2).

Glutathione S-transferases (GSTs) are a family of multifunctional enzymes that catalyze the conjugation of a wide variety of electrophilic compounds with glutathione (GSH) and are phase II enzymes of xenobiotic detoxification (3,4,5). GSTs have been reported from bacteria to mammals. GST’s have been reported in fungi (6,7,8). It was reported in *Fusarium oxysporum* (9), *Issatchenka orientalis* (10), *Phanerochaete chrysosporium* (11), *Yarrowia lipolytica* (12), *Cunninghamella elegans* (13), Aspergillus nidulans (14) and *Mucor circinelloides* (15).

The early published data reported on the degradation of chemicals by white rot fungi showed that these fungi can degrade DDT, Lindane, PCB, Dioxin and benzo(a)pyrene (16). Fungi as well as bacteria are used to degrade environmental pollutants like pentachlorophenol PCP, lindane DDT and 2,4 dichlorophenol (17). The zygomycete has the ability to metabolize a wide variety of xenobiotics, including polycyclic aromatic hydrocarbons and pharmaceutical drugs (18).

In more detailed study, the ability of *Aspergillus niger* and *Penicillium* sp. in the biodegradation of automotive gas oil (AGO) and premium motor spirit (PMS) and recorded that, these fungi grew at all concentrations of the petroleum products (pollutant) used (19).

Fungi are present in aquatic sediments, terrestrial habitats and water surfaces and play a significant part in natural remediation of metal and aromatic compounds. Fungi also have advantages over bacteria since fungal hyphae can penetrate contaminated soil, reaching not only heavy metals but also xenobiotic compounds. Despite the abundance of such fungi in wastes, *Penicillia* in particular have received little attention in bioremediation and biodegradation studies. Additionally, several studies conducted with different strains of imperfect fungi, *Penicillium* spp. have demonstrated their ability to degrade different xenobiotic compounds with low co-substrate requirements, and could be potentially interesting for the development of economically feasible processes for pollutant transformation (20).

Understanding the fate of fluorinated aromatic compounds which use in agriculture as pesticides, herbicides and insecticides (e.g. difluobenzuron and tefluthrin) in the environment is important since the transformation products might be highly toxic (21).

Benzoylphenyl ureas (BFUS) is a class of compounds that is known to inhibit chitin biosynthesis and is often grouped together within the insect growth regulators (IGRs). Generally, the function of IGRs is to mimic the action of hormones, resulting in an incomplete moulting process (22). Diflubenzuron (DFB) belongs to this group benzoylphenyl urea (BFUS), in contrast to neuronal acting compounds the BPUS are much slower in achieving full efficacy against pest insects and the potential of its increased usage and eventually the presence of its residues (or the residues of BFUS) will increase in the environment and hence the pesticide and/or its metabolite(s) could resemble hazard to the ecosystem. The toxicity and metabolism of DFB was extensively studied by several Authors (23, 24, 25, 26, 27). However, its degradation by fungi seems to be lacking or overlooked.

The current study aims at studying the biodegradation of difluobenzuron pesticide using fungi. On the basis of growth and GST activity, an isolate identified as *Penicillium notatum* was selected for further studies. Another attempt was conducted to increase the production of GST using gamma irradiation.
MATERIALS AND METHODS

Chemicals:

\(^{14}\)C- difluobenzuron (1-(4-chlorophenyl)3-p(2,6-difluorobenzoyl)urea (DFB) labeled in both phenyl rings with specific activity 760Mbc/m mole and radiometric purity 90% (as detected by TLC analysis), was purchased from Sigma Chemical Co. St Louis Mo. USA. Technical grade difluobenzuron of purity > 92% (non-Labeled) was diluted with the labeled isotope in dimethyl sulfoxide to give the desired concentration of 200mg/l. Reference substances, (2,6-difluorobenzoic acid(DFBA), 2,6 difluorbenzamide(DFBAM)and 4-chloroaniline(4CA) were purchased from Aldrich Chemical Co Inc, Milwaukee, WI., USA. Chlorophenyl urea(CPU) was obtained from Trans World Chemicals Inc., Rockville, MD., USA. All other chemicals and reagents used were of highest available analytical grade.

Fungi and growth conditions:

Three tested fungi, Aspergillus flavous, Aspergillus nigar and Pinicillium notatum were isolated from soil. The isolated strain was identified by its color and morphology. The cells morphological properties, the presence of spore, the shape of cells and colony morphology were examined by microscope (MEIJI Model: ML2100) according to the procedure given in reference \(^{(28, 29, 30)}\).

Cultivation process (Biomass estimation):

Erlenmeyer flasks (250 ml cap.) each containing 100 ml Czapek’s sucrose liquid medium and another containing 2% malt extract were inoculated with discs (0.5 cm in diameter) in each of the tested fungi. Difluobenzuron (200 mg/l) was added to each of the selected organism in the flasks of the first set. The second set was used as control. Three replicate flasks were used for each fungus. Incubation occurred at 25ºC, after ten days of incubation, the flasks were filtered then the produced mycelia were washed thoroughly with distilled water. The collected mycelia was filtered through Whatman GF/C circles 47mm (Cat#1822 047), England for both treated and control group then weighed and GST and total protein were analyzed. The other produced mycelia were dried at oven at 80ºC till constant weight to determine the dry weight of mycelia \(^{(31)}\). Comparison of the mycelia growth and specific activity of GST were carried out to evaluate the most active organism. According to the data obtained, Penicillium notatum was found to be most efficient and thus was selected for the current work.

Cultivation of \(P.\ notatum\) on different concentrations of pesticide:

Erlenmeyer flasks (250 ml) containing 100 ml Czapek’s sucrose liquid media were incubated with different concentrations of difluobenzuron (0.0, 200, 400,800 and1000 mg/l). Fungal discs from culture of \(P.\ notatum\) was placed on each flask. Three replicate flasks were used for each dose. Incubation at 25ºC for 10 days was carried out.

Gamma irradiation:

Cobalt-60 gamma cell located at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Giza, Egypt) was used for the irradiation treatment. The gamma source gave a dose rate of 28.8 Gy/hour at the time of experiment. A 0.5cm diameter of mycelia disc from 10 days old culture of \(P.\ notatum\) was placed on the center of Czapek’s sucrose plate according to the method described by \(^{(32,33)}\). After 10 days of incubation at 25ºC, the samples were exposed to gamma irradiation at doses of 0.0, 10, 25, 50, 100,150, 200, 300 Gy. Discs (0.5cm in diameter) of irradiated and non-irradiated culture were inoculated as mentioned before and percent of change in growth between different doses of gamma irradiation and control was calculated as the follows:
% Change of control = \frac{\text{Mean Treated} - \text{Mean Control} \times 100}{\text{Mean Control}}

Ammonia sulfate fractionation, protein and enzyme assay:

Triplicate samples, each 1 g of fungal mats was mixed with 10 ml of 0.01 M phosphate buffer (pH 7.0) and then grinded in a mortar and a pestle, the mixture was squeezed through several layers of cheese cloth and the filtrate was further clarified by centrifugation at 10,000 rpm for 20 min. at -4°C using Sigma 2k15-USA centrifuge. The resulting clear supernatant volume was measured then stored at -10°C before being used for all subsequent assays (34). A known weight of ammonium sulphate was added slowly to the measured supernatant (internal extract) and remaining media (external extract) to bring it to 85% saturation, after the precipitation process was completed, the solution was centrifuged, the precipitate was dissolved in minimum volume of phosphate buffer (pH 7.4). Finally, precipitate was placed in a dialysis bag and placed in large volume of buffer for many hours to remove the excess of salt. Protein was determined by Commassi brilliant blue (G250) using bovine albumin as a standard protein according to (35). GST activity was assayed according to (36). Fifty micro litre of enzyme extract filtrate was mixed with 1ml of phosphate buffer (pH 7.4) and 0.1ml Glutathione reduced (GSH). The mixture was incubated at 37°C for 5 minutes. Then 0.1ml of 2,4-chorodinitrobenzen (CDNB) was added. After incubation at 37°C for 5 min, the reaction was terminated by adding 0.1ml trichloroacetic acid (TCA), mixed well and centrifuge at 3000 r.p.m. for 5 min. Absorbance was measured at 340 nm using spectrophotometer (Erba-Model chem. 6, No 6135, Germany) against blank which was treated in the same way except that 2,4-chorodinitrobenzen was added after the addition of trichloroacetic. GST activity was calculated according to the following equation:

\[ \text{GST activity} = \frac{\Delta A \times 2.812}{\text{Gram tissue used}} \]

Treatment with radiolabelled difluobenzuron:

After \(^{14}\text{C}-\text{DFB}\) was added into culture medium of \textit{Penicillium} to give a final concentration of 200mg/l, for 10 days, culture was filtered, the filtrate was extracted 3x300 ml by chloroform. The biomass was homogenized and extracted. After extraction, the organic phase was evaporated (Rotary evaporator-Bibby RE-200, Ribby Sterlin,UK) till known volume. Aliquot samples from different extracts were taken for the determination of possible metabolites and \(^{14}\text{C}\) activity.

Determination of metabolites:

Extracts were analyzed by thin layer chromatography involving Silica gel Polygram Sil G/UV 254 with fluorescent indicator , Macherey Nagel,Germany, using Benzene: Dioxane : Acetic acid (90:30:1v/v/v) as the solvent system. Authentic substances were co-chromatographed as reference materials. Metabolite spots were depicted by UV-Light Lamp (Spectroline-longlife filter at 245nm) and their radioactive spots were measured by dividing the plates into 0.5 cm zones, which were scratched into vials containing 10 ml scintillator and then counted.

Radiomeasurements:

\(^{14}\text{C}-\text{activity of different fractions were determined in Liquid scintillation Counter, Packard Tri Carb2300TR, using a scintillation cocktail composed of 5.5g permablend in one liter toluene .Water phase samples were determined in a scintillation cocktail composed of 5.5 g permablend , 120 g naphthalene in 1L dioxane. Fifty gm of tissue samples (dried fungi) were solubilized in a solubilizer (solusol )for several days. Quenching correction was carried out using an external standard spec-check of known activity. \]
Statistical analysis:

The results were statistically analyzed using the program SPSS version 17. One way analysis of variance F-test was given to estimate the significant (at p <0.05) of different treatments.

RESULTS AND DISCUSSION

Table (1): Growth of various fungi on media containing diflubenzuron (200mg/l) presented in fresh and dry weight(g/100ml)

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Biomass growth (g/100ml)</th>
<th>% change of control</th>
<th>Control</th>
<th>Treated</th>
<th>Control</th>
<th>Treated</th>
<th>% change of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight</td>
<td>Fresh weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. notatum</td>
<td>5.90±0.58</td>
<td>5.70±0.70</td>
<td>-3.38</td>
<td>0.87±0.07</td>
<td>0.83±0.09</td>
<td>-4.59</td>
<td></td>
</tr>
<tr>
<td>A.Flavus</td>
<td>8.80±0.80</td>
<td>6.58±0.55*</td>
<td>-25.22</td>
<td>0.69±0.09</td>
<td>0.53±0.03*</td>
<td>-23.18</td>
<td></td>
</tr>
<tr>
<td>A.nigar</td>
<td>9.10±0.70</td>
<td>8.70±0.85</td>
<td>-4.39</td>
<td>0.75±0.06</td>
<td>0.70±0.05</td>
<td>-6.66</td>
<td></td>
</tr>
</tbody>
</table>

*Significant p <0.05

Table (1) represents the growth of three different fungi on media containing 200mg/l diflubenzuron. P. notatum showed to be least affected by the presence of 200mg/l as represented by fresh and dry weight. Data obtained in Table (1) illustrated that P. notatum was the best fungi for its capability to grow in media containing 200 ppm of diflubenzuron compared to the other two fungi (A.Flavus and A.nigar). The potential of Penicillium strains as a naturally, abundant and cheap source for heavy-metal environmental reduction, oxidize, mineralize hydrocarbons, such as phenol, halogenated compounds, PAHs and different xenobiotic compounds(20). These results supported the choice of the P. notatum as the best fungi for its capability to grow in media containing diflubenzuron for processing the mentioned categories.

Table (2) shows the effect of diflubenzuron (200 mg/l) on GST activity secreted by different fungi (U/mg protein). P. notatum showed the highest GST activity, both intracellular and extracellular, 1.29±0.15 U/mg protein and 7.34±0.80 U/mg protein for both internal and external GST in the control group, respectively. In case of the group treated with the pesticide the fungi secreted 1.19±0.19 and 6.98 ± 0.70 U/mg protein for internal and external media, respectively.

Table (2): Effect of diflubenzuron (200mg/ml) on glutathione-S –transferase activity (U/mg protein) secreted by different fungi.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Internal GSTg</th>
<th>% change</th>
<th>External GSTh</th>
<th>% change</th>
<th>Sumi</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
<td>Treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. notatum</td>
<td>1.29±0.15</td>
<td>1.19±0.19</td>
<td>7.34±0.80</td>
<td>6.98±0.70</td>
<td>-4.90</td>
<td>8.63±2.40</td>
</tr>
<tr>
<td>A.Flavus</td>
<td>1.12±0.10</td>
<td>0.91±0.10</td>
<td>5.50±1.20</td>
<td>3.50±0.45</td>
<td>-36.36</td>
<td>6.62±3.22</td>
</tr>
<tr>
<td>A.nigar</td>
<td>0.97±0.10</td>
<td>0.62±0.02</td>
<td>2.80±0.30</td>
<td>2.50±0.50</td>
<td>-10.71</td>
<td>3.77±2.07</td>
</tr>
</tbody>
</table>

* Significant p<0.05,  a GST extracted from mycelia,  bGST in media,  c Total GST from mycelia and media
Table (3): Effect of different concentration of diflubenzuron on growth and glutathione activity (U/mg protein) secreted by *P. notatum*.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Biomass growth (g/100ml)</th>
<th>GST(U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight</td>
<td>Dry weight</td>
</tr>
<tr>
<td>Control</td>
<td>5.90±0.90</td>
<td>0.87±0.07</td>
</tr>
<tr>
<td>200</td>
<td>6.10±0.70</td>
<td>0.89±0.08</td>
</tr>
<tr>
<td>400</td>
<td>4.90±0.90</td>
<td>0.69±0.09</td>
</tr>
<tr>
<td>800</td>
<td>5.60±0.60</td>
<td>0.79±0.09</td>
</tr>
<tr>
<td>1000</td>
<td>6.30±0.40</td>
<td>0.90±0.05</td>
</tr>
</tbody>
</table>

* Significant p<0.05, a GST extracted from mycelia, b GST in media, c Total GST from mycelia and media

The data presented in Table (3) shows that the growth and GST activity secreted by *P. notatum* at different concentration of diflubenzuron. The increase of pesticide concentration (0-1000ppm) was not reflected by a significant increase in growth on GST activity except when 1000 ppm was used. The data illustrated that even upon using the highest concentration 1000 ppm it had a slight insignificant increase in growth 6.30±0.40 and 0.90±0.05 g for fresh and dry wt, respectively. GST activity showed 1.42±0.07 and 8.1±0.85 u/mg protein for both internal and external, respectively. On the other hand, the high levels of the GST activity found in *P. notatum* may be one of the reasons that this organism could tolerate up to 1000 ppm of DFB. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low (37,38,39).

GST’s could display various functions in different organisms such as protection against antibiotics in bacteria or lignin degradation by cleaving b-aryl ether linkages (40,41) and in *Saccharomyces cerevisiae*, the role of ScGTO1 in the peroxisomes could be related to the redox regulation of the Str3 cystathionine b-lyase protein (42).

Low doses of gamma rays stimulated spore germination and growth of some fungi and produce stimulatory effects but doses causing inhibitory effects were that which increased with the increasing of gamma radiation dose, this was attributed to the fact that the high doses greatly suppressed the essential metabolic processes (43). The response of the fungus differed according to the fungal species, stages of growth and doses of irradiation (44).

The effect of gamma irradiation doses on mycelium growth was investigated. The results in table (4) revealed that the highest yields were observed at dose 200Gy (5.48 and 0.92 g/100ml for fresh and dry weight, respectively) then the growth declined to minimum values (3.11 and 0.63 g/100 ml) at dose 300 Gy. Numerous studies supported the current results that showed that low doses of ionizing radiation induced fungal growth (45,46). Low experimental doses of gamma radiation up to 100 Gy were significantly stimulatory to spore germination of *Penicillium expansum* and was completely inhibited at 1000 Gy, which was considered as the lethal doses (44).

From the results in Fig (1) it is clear that the highest total activity of GST activity (activity for intracellular and extracellular GST) was detected after exposing *P. notatum* to 200 Gy gamma radiation (11.81±0.71 mg/protein). On the other hand, it should be mentioned that, intracellular GST activities reached maximum activity after exposing fungus to 150 Gy while extracellular activity reached maximum after exposing to 200 Gy. Furthermore, the gamma radiation doses affected the secretion of GST especially on the intercellular GST. This was recognized upon applying the lowest
dose of gamma radiation (10Gy). As the radiation dose increased the effect was more and more pronounced where both inter and extra cellular enzyme activity started to show a statistically significant increase from control untreated groups. It is worthy to mention that, fungal GSTs protect cells against damage resulting from oxidative stress, heavy metals, and antifungal compounds, glutathione is involved in cellular defense mechanisms for xenobiotic and reactive oxygen species and this may explain the increase that occurred in the current experiment after the application of gamma radiation\(^{46,47,48,14}\).

Table (4): Effect of gamma radiation dose on biomass of \(P.\ notatum\).

<table>
<thead>
<tr>
<th>Doses (Gy)</th>
<th>Biomass growth (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight</td>
</tr>
<tr>
<td>0.0</td>
<td>4.10±1.09</td>
</tr>
<tr>
<td>10</td>
<td>4.62±1.83</td>
</tr>
<tr>
<td>25</td>
<td>4.91±1.51</td>
</tr>
<tr>
<td>50</td>
<td>4.66±1.00</td>
</tr>
<tr>
<td>100</td>
<td>4.12±1.90</td>
</tr>
<tr>
<td>150</td>
<td>4.65±1.60</td>
</tr>
<tr>
<td>200</td>
<td>5.48±1.31*</td>
</tr>
<tr>
<td>300</td>
<td>3.11±0.92*</td>
</tr>
</tbody>
</table>

*Significant p<0.05

![Figure 1](image.png)

Fig. (1): The variation in GST activity of \(P.\ notatum\) due to different doses of gamma irradiation

Gamma radiation induce certain enzymes, which leads to recovery of radiation damage after irradiation\(^{49}\). Gamma radiation increase the activities of polygalacturonase and tissue macerating enzymes of \(Penicillium\ expansum\) than those in the controls \(^{50}\). Gamma radiations reported as an effective mutagenic agent for improvement of extracellular lipase production by \(Penicillium\ spp\) \(^{51,52}\). \(Aspergillus\ niger\) showed the highest extracellular lipase activity at dose140 Gy of gamma irradiation, \(Rhizopus\ microsporus\) (80 Gy) and \(Penicillium\ atrovenetum\) (60 Gy) \(^{53}\).
Diflubenzuron breakdown by hydrolysis, soil degradation, or plant and animal metabolism initially yields 2,6-difluorobenzoic acid and 4-chlorophenylurea. Ultimately, the end products are either conjugated into mostly water soluble products or are biologically acylated and methylated, further conversion of these compounds to 2,6-difluorobenzamide and 4-chloroaniline (54,55). Diflubenzuron, applied to sandy loam soil, was calculated to have a half-life of 2-14 days (depending on soil texture) when incubated at 24 ± 1°C and maintained at 77% of 0.33 bar moisture capacity. The major degradate, 4-chlorophenyl urea, reached a maximum concentration of 30.8% to 37% of the applied radioactivity at 7 to 14 days post-treatment. The other major degradate, CO₂, reached a maximum concentration of 26.3 % of applied radioactivity by day 21 post-treatment. Three minor degradates, 2,6difluorobenzoic acid, 2,6, difluorobenzamide, and 4-chloroaniline, which each had a maximum concentration of <10 % of applied radioactivity were identified, as well. (MRIDs 00039473, 00039474, and 41722801 EPA).

The data presented in table (5) shows that, after inoculation of media with 14C- DFB for 10 days the data showed that 91.2 % of the 14 C- activity could be recovered. The 14 C- activity was distributed, in mycelia (65.8%) and in the media (25.4%). The CHCl₃ extract of the mycelia contributed 41.0% of 14C radioactivity while 23.0% was in the water phase. The amount that remained without extraction (bound residues) did not exceed 1.8%. In the media, of the total recovered radioactivity, 15.4 % was in the organic phase while 10% was in the water phase. In the current study, the incomplete degradation of DFB maybe a result of its interference with chitin presence in the cell wall of fungi and hence the secretion of enzymes responsible for the biodegradation was altered.

Table (5): Distribution of diflubenzuron and or its metabolite(s) in different extracts of the P. notataum

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mycellia</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform(%)</td>
<td>41.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Water(%)</td>
<td>23.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Bound Residue(%)</td>
<td>1.8</td>
<td>0.00</td>
</tr>
<tr>
<td>Total(%)</td>
<td>65.8</td>
<td>25.4</td>
</tr>
<tr>
<td>Total % Recovered</td>
<td>91.2</td>
<td></td>
</tr>
</tbody>
</table>

ND: Non detected

Table (6) demonstrates the Rf values as well as the percentages of 14C-Difluobenzuron in the media and mycellial extract of P. notatum after 10 days inoculation with 14C- DFB. Ten days of incubation was chosen since it was proved by the earlier studies that 10 days was the optima for the fungus (56,57). The TLC analysis of chloroform extract of external extract of fungi revealed the following: In the media, the intact parent compound (DFB) identified by co-chromatography showed a 74%, while metabolites of concern, 4 chloro phenyl urea (CPU) showed 10.0%, 2,6difluorobenzamide (DFBAM) 6.8% and 4-chloro aniline (CA) contributed to 8.6%, while 2,6-difluorobenzoic acid (DFBA) was not detected in the current experiment. On the other hand, the internal extract showed % change of 72.0, 7.2 and 20.6 for BFB, CPU and DFBAM, respectively. DFB was not completely degraded during current experiment and it might take a longer time for its complete degradation. Intriguing enough, to find that, United States Environmental Protection Agency, cited 2 mushrooms metabolism studies, In the first metabolism study, residues of parent, CPU, and 2,6-difluorobenzoic acid (DFBA) were qualitatively identified in mushrooms grown in [14C]diflubenzuron treated compost. In a related residue study conducted at 0.5X and 1X the label rate, parent and CPU residues were found at comparable levels (0.07 ppm) while DFBA residues (0.6 ppm) were about 10 times higher than the other metabolites. In the other mushroom metabolism study, residues of parent, CPU, DFBA and PCA were detected at up to 0.18, 0.6, 3.96, and 0.02 ppm, respectively, in mushrooms.
treated at less than 1X. A separate mushroom metabolism study was submitted for reregistration purposes. Mushrooms were grown in either compost or casing soil which had been treated with diflubenzuron at 1X and 5X the maximum label rate respectively. For mushrooms grown in compost treated with diflubenzuron, the major identified radioactive residue was DFBA, accounting for 33% to 138% of Total Radioactive Residue (TRR) (0.04 to 0.33 ppm). CPU and PCA were identified, ranging from 0.2% to 0.4% TRR (0.005 - 0.001 ppm) and 0.6% to 2.7% TRR (0.001 to 0.004 ppm) respectively. Although bound residues accounted for up to 19% TRR (0.03 ppm), no efforts were made to release the bound residues. For mushrooms grown in casing treated with diflubenzuron, the major identified radioactive residue was DFBA, accounting for 82% to 224% TRR with ppm levels ranging from 5.3 to 8.8 ppm. CPU and PCA were identified, ranging from 0.3 to 2.3% TRR (0.02 - 0.08 ppm) and 0.04% to 4.7% TRR (0.002 to 0.16 ppm) respectively. No efforts were made to release the bound residues that were present at up to 0.42 ppm.

Table (6): Percentages and Rf of Difluobenzuron and its metabolites of concern in External and Internal Extract of P. notatum.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Percentages of Difluobenzuron and its metabolites</th>
<th>Rf</th>
<th>External extract</th>
<th>Internal extract</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difluobenzuron</td>
<td></td>
<td>(0.82)</td>
<td>74</td>
<td>72</td>
<td>90</td>
</tr>
<tr>
<td>2,6-difluorobenzoic acid</td>
<td></td>
<td>(0.73)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4-Chlorophenyl urea</td>
<td></td>
<td>(0.6)</td>
<td>10.0</td>
<td>7.2</td>
<td>3</td>
</tr>
<tr>
<td>2,6 difluorobenzamide</td>
<td></td>
<td>(0.4)</td>
<td>6.8</td>
<td>20.6</td>
<td>2</td>
</tr>
<tr>
<td>4-chloroaniline</td>
<td></td>
<td>(0.23)</td>
<td>8.6</td>
<td>ND</td>
<td>1</td>
</tr>
</tbody>
</table>

It is worthy to mention that, *Penicillium spp.* have demonstrated their ability to degrade different xenobiotic compounds with low co-substrate requirements, and could be potentially interesting for the development of economically feasible processes for pollutant transformation (20).

The results obtained in the current study were similar to the results reported by Zhao et al (59) that showed that *Penicillium oxalicum* could degrade methamidophos, folimath, phoxim and glyphosate with glucose as carbon source, but could not degrade chlorpyrifos, phosdrin, trichlorphrom, and dichlorvos. *Penicillium lilacinum* BP303 was able to degrade various organophosphorus pesticides by cleaving P-O in the phosphotriesters bond and P-S linkage in the phosphothiolesters effectively (60). The novel fungal enzyme hydrolyzing methyl parathion, parathion, paraoxon, coumaphos, demeton-S, phosmet, and malathion has been purified to homogeneity and characterized. *Penicillium camemberti* are used to degrade Lindane that is known to exist in the pulp and plant effluents (61). When *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. sydowii*, *A. terreus*, *Emericella nidulans*, *Fusarium oxysporum* and *Penicillium chrysogenum* were isolated from pesticide-treated wheat straw which utilize organophosphate pesticides, viz. phosphorothioic (pirimiphos-methyl and pyrazophos), phosphorodithioic (dimethoate and malathion), phosphonic (lancer) and phosphoric (profenfos) acid derivatives. Pesticide degradation was studied in vitro and in vivo (soil). *Aspergillus flavus* and *Aspergillus sydowii* hydrolyzed pesticides at 300 ppm in soil all added pesticides except profenfos were degraded within 3 weeks (62). Brown-rot fungi (*F. pinicola* and *D. Dickinii*) degraded approximately 63% and 47% of total DDT in potato dextrose broth (PDB) medium, respectively (63). On the other hand, *Aspergillus niger* showed tendency to degrade endosulfan in cultures containing up to 400 mg/ml with complete disappearance of endosulfan after 12 days (64).

It could be concluded that *P. notatum* was able to grow in a media containing up to 1000 ppm of DFB and showed to be a potential organism for the uptake DFB from the media. In the future, a
construction of a good mycoremediation technique using this fungi is possible as it took most of
radioactivity in the media and the increase of GST is possible by gamma irradiation which might be a
good tool for increase the production of this crucial enzyme.

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