



## Culture Parameters Act on Phenylurea Herbicides (Chlortoluron and Isoproturon) Degradation by Fungi

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### ABSTRACT

To point out the effects of culture conditions on phenylurea herbicides (chlortoluron and isoproturon) degradation by five selected fungi and in an attempt to enhance the degradation process, three parameters (initial chemical concentration, amounts of glucose and nitrogen) were varied. The levels of xenobiotics, in the culture media, were monitored by HPLC analysis, after five days of cultivation. The best results were obtained at low initial concentrations (20 mgL<sup>-1</sup> vs 100) and with medium amounts of glucose (5 gL<sup>-1</sup> vs 10) and nitrogen (2.4 mM vs 24). When these two elements were lacking from the culture media, biodegradation was not suppressed, but took place at a lesser extent. Thus, initial chemical concentration and amounts of glucose and nitrogen, in the culture media, were found to strongly influence the level of phenylurea herbicides biodepletion by fungi.

### 1. Introduction

Since the discovery and marketing of phenylurea herbicides, shortly after the Second World War, this group has grown to be one of the most important classes of herbicides used worldwide [1]. The phenylurea herbicides are mostly used for either pre- or post-emergence control of many annual and perennial broadleaved weeds in cotton, fruit or cereal production. The phenylurea compounds generally have relatively high water solubilities and low tendencies to sorb to soil, rendering them mobile in soil. Due to their extensive use, the phenylurea herbicides are detected in surface water and groundwater [2-4] in concentrations exceeding the European Union limit value of 0.1 mgL<sup>-1</sup> for pesticides in drinking water [5]. Thus, they are on the list of 33 priority substances, which seriously threaten surface and groundwater, set up by the European Union in the Water Framework Directive [6]. On the other hand, several phenylurea herbicides have been shown to be endocrine disruptors or have genotoxic or ecotoxic effects [7-9].

Studies on fungal degradation of phenylurea herbicides are found in the literature [10, 11, 5], but those that deal with the efficient degradation of a series of phenylureas by the same strain are scarce. It had been proven, somewhere else, that *Rhizoctonia solani* could efficiently degrade chlortoluron, diuron and isoproturon [10], unfortunately, this strain is a plant pathogen. Therefore, in an attempt to speed up and increase the bioconversion of a series of phenylurea herbicides by the same fungi strains, we undertook this study to examine how varying some culture parameters (initial concentration of the chemicals, glucose and nitrogen amounts in the media) can influence their removal by five selected strains of Micromycetes (*Aspergillus parasiticus*, *Aspergillus ustus*, *Dicyna ampullifera*, *Embellisia annulata* and *Mortierella bainieri*), otherwise efficient in the biodegradation of, at least, one of the two herbicides (chlortoluron and isoproturon).

### 2. Experimental Methods

#### 2.1 Micro-Organisms

The strains used in this study belong to the collection of the Mycology laboratory (CMPG: Collection Mycologie Pharmacie Grenoble). Stock cultures were maintained at 4 °C on solid Malt Extract Agar (MEA) or Potato Dextrose Agar media (PDA).

#### 2.2 Chemicals

The phenylurea herbicides were from Ciba (Aigues-Vives, France) and recrystallized from ethanol. Their purity was checked by HPLC (High Performance Liquid Chromatography), prior to use and was higher than 99%. Other products, except agar and malt extract which were, respectively, from Cooperation Pharmaceutique Française (Melun, France) and Difal (Villefranche sur Saône, France), were from Prolabo (Paris, France).

#### 2.3 Media and Culture Conditions

##### 2.3.1 Solid Media

MEA used for stock cultures contained (per liter): malt extract, 15 g; agar, 15 g. PDA was prepared (per liter) with potato extract, 200 g; dextrose, 20 g and agar, 15 g.

##### 2.3.2 Liquid Media

Two synthetic liquid media were used in this work: Galzy and Slonimski (GS) medium [12] and ammonium tartrate (ATM) medium [13]. All the media were adjusted to pH 4.5.

##### 2.3.3 Culture Conditions

They were the same as described elsewhere [10]. Stock solutions of the chemicals, at 25 gL<sup>-1</sup> and dissolved in a DMSO/ethanol mixture (50/50, v/v), were sterilized by filtration through 0.22 µm-pore size Millipore membranes and added to the two-day-old cultures to a final concentration of 20 or 100 mgL<sup>-1</sup>. The depletion of the two substrates was evaluated after

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seven days of cultivation (five days with the herbicides). The temperature was 24 °C and light 1200 lux, with a photoperiod of 12 hours per day. Each series of experiments was run, at least, in triplicate and cell-free flasks were included for abiotic degradation assessment.

## 2.4 Method of Analysis

The disappearance of the two products was monitored by HPLC. Aliquots from the culture media were spiked with a syringe, filtered through a Millipore membrane (0.45 µm pore size) and injected directly in the apparatus. HPLC was performed with a liquid chromatograph (Shimadzu) equipped with a LC 6A pump, a SIL-9A automatic injector, a SPD 6A UV detector and an integrator (Shimadzu C-R6A Chromatopac). The separation column was 4.0 mm inside diameter x 300 mm long filled with µ-Bondapak C<sub>18</sub>. The mobile phase was methanol/water (65/35, v/v), the flow rate 1 mL min<sup>-1</sup> and detection was recorded at 243 and 240 nm, for chlortoluron and isoproturon, respectively. Each sample was injected, at least, three times and the mean taken.

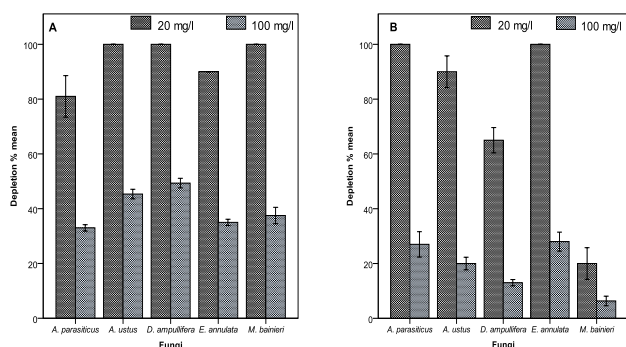
## 2.5 Data Analysis

The Z test, at 0.05 level, was used for pairwise multiple comparison of the different depletion percentages.

## 3. Results and Discussion

### 3.1 Effect of Initial Test Concentration

Two initial concentrations (20 and 100 mgL<sup>-1</sup>) of each of the herbicides were utilized in GS medium. The results obtained, for each chemical and expressed as a percentage of depletion after five days of cultivation, are depicted in Fig. 1 (A: chlortoluron and B: isoproturon). Chlortoluron degradation at 20 mgL<sup>-1</sup>, for the five tested strains, was more significant than that at 100 mgL<sup>-1</sup> ( $|Z| > 1.96$ ). The fungi considerably improved their yield of depletion, notably *A. ustus* (100% at 20 mgL<sup>-1</sup> compared to 46%, at 100 mgL<sup>-1</sup>) and *M. bainieri* (100% at 20 mgL<sup>-1</sup> compared to 38%, at 100 mgL<sup>-1</sup>). Thus, for both strains, the lower the concentration, the higher was the degradation efficiency. The difference was more noticeable, in the case of isoproturon: two strains, *A. parasiticus* and *E. annulata* depleted it at 100% at 20 mgL<sup>-1</sup>, while the depletion by the same fungi were only, respectively of 27 and 28%, at 100 mgL<sup>-1</sup>.



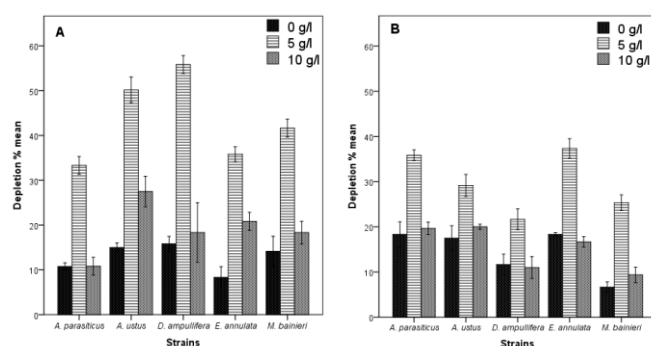
**Fig. 1** Degradation of chlortoluron (A) and isoproturon (B) at 20 and 100 mgL<sup>-1</sup> by *Aspergillus parasiticus*, *Aspergillus ustus*, *Dicyna ampullifera*, *Embellisia annulata* and *Mortierella bainieri* in GS medium

The same profile of microorganisms responses to varied pollutants concentrations was reported by Yadav and Reddy [14], with 2,4-D and 2,4,5-T in a *Phanerochaete chrysosporium* degradation culture media, Donnelly et al. [15], with atrazine and 2,4-D and nine fungi strains and Hadibarata and Kristanti [16] who, studying the potential of the white-rot fungus *Pleurotus eryngii* F032 for degradation and transformation of fluorene, found that the degradation of this product decreased with increasing concentration: from the value of 100%, at initial concentration of 10 mgL<sup>-1</sup>, this degradation fell down to 86.5 and 65.9 % at 20 and 30 mgL<sup>-1</sup>, respectively. In the same way, Sannino et al. [17] had proved that the degradation capacity of two autochthonous microbial strains, (*Methylobacterium populi* VP2 and *Aspergillus sydowii* VP4), isolated from a soil of a highly contaminated industrial site and used to degrade the aqueous extract of contaminants obtained from the same polluted soil, decreased with increasing extract concentration in the minimal selective liquid medium they utilized. Elsewhere, Alleman et al. [18], investigating the effects of the quantity of biomass on the toxicity of pentachlorophenol to six species of white rot fungi, concluded that the quantity of biomass

was an important factor in determining whether fungi could grow and degrade this product. It has been shown that it is the pollutant dose (µg of pollutant per mg of mycelia) and not the concentration (µgL<sup>-1</sup>) that determines if fungi can grow and degrade the pollutant. The rate of contaminant degradation is often dependent on the concentration of the contaminant and the number of organisms able to metabolize the contaminant, as well as the amount of enzymes produced by each cell [19]. So, an appropriate pollutant/biomass ratio is required to obtain noticeable biodegradation activity. According to our results, as the quantity of biomass remained the same, while the initial concentration of the two xenobiotics were varied, it seemed that the fungi better withstood the toxicity of the two phenylureas at 20 mgL<sup>-1</sup> than at 100 mgL<sup>-1</sup> and that at 20 mgL<sup>-1</sup>, the notional pollutant/biomass ratio needed for optimum biodegradation activity, was approached.

### 3.2 Effect of Glucose Amount

In this part of the study, a medium (ATM) reconstituted from media generally utilized in xenobiotics degradation by the fungus *P. chrysosporium* [13] was used. Three concentrations of glucose (0, 5 and 10 mgL<sup>-1</sup>, concentrations before inoculation) were utilized, whereas the nitrogen amount was maintained at 2.4 mM and the initial concentration of the herbicides at 100 mgL<sup>-1</sup>. The best yields of chlortoluron and isoproturon degradation were obtained at 5 mgL<sup>-1</sup> of glucose (Figs. 2A and B), while at 0 and 10 mgL<sup>-1</sup>, the extent decreased, for the five strains ( $|Z| > 1.96$ ). The most interesting examples are given by *D. Ampullifera* for chlortoluron (mean degradation of 56%, at 5 mgL<sup>-1</sup> of glucose vs. 16% and 18%, at 0 and 10 mgL<sup>-1</sup>, respectively) and by *E. annulata* for isoproturon (mean degradation of 37%, at 5 mgL<sup>-1</sup> of glucose vs. 18% and 16%, at 0 and 10 mgL<sup>-1</sup>, respectively).



**Fig. 2** Effect of glucose amount on chlortoluron (A) and isoproturon (B) degradation by *Aspergillus parasiticus*, *Aspergillus ustus*, *Dicyna ampullifera*, *Embellisia annulata* and *Mortierella bainieri*, at 100 mgL<sup>-1</sup>, each, in ATM medium

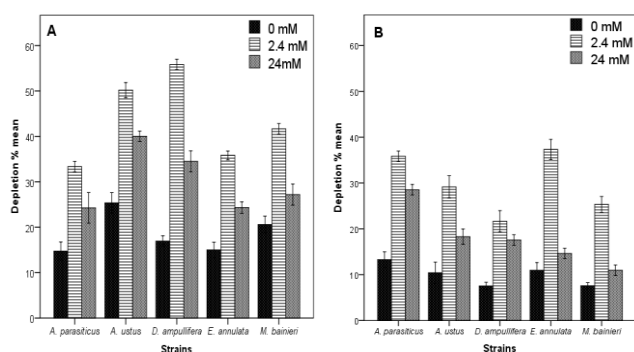
When a culture medium contains two sources of carbon, for preference, the microorganisms utilize the most assimilable and the most concentrated, until its concentration counterbalances the one that is more diluted, at which time both sources are then simultaneously utilized [20]. This simultaneous utilization, according to some authors, led to an increase in degradation activity or in the tolerance of the organisms to high xenobiotics concentrations, by providing a good carbon source readily metabolisable to support cell growth [21, 22]. Nevertheless, the effect of glucose on xenobiotics degradation by microorganisms is not in line with this last observation. Although investigations such as that of Yadav and Reddy [14] and that of Zhang et al. [23] reported, respectively, that 2,4-D and 2,4,5-trichlorophenoxyacetic acid degradation by *P. chrysosporium* were more important in a glucose-supplemented-medium than in one that was not and that degradation of nicosulfuron by *Klebsiella* sp. increased with increasing glucose concentration from 0 to 5 gL<sup>-1</sup>, authors such as Chakraborty et al. [24] found that phenol degradation efficiency by native microorganisms, isolated from coke processing wastewater, decreased from 97.88%, at 0.25% of glucose concentration to 55.36%, with increasing glucose concentration to 0.5%. These authors suggested that this might be due to the fact that glucose acts as a growth substrate in presence of phenol in the wastewater, due to its simple structure as compared to phenol. Analysis of our findings seems to indicate that high levels of glucose affect fungi degradation of chlortoluron and isoproturon. Unlike the case of many other organic compounds [21, 25, 26], the degradation of these two molecules seems not to be related to the bioavailability of another source of carbon used as a source of growth: there is not a cometabolism phenomenon. Besides, degradation was observed even without glucose in the medium (Figs. 2A and B), leading us to think that the biomasses acclimatization to the medium was sufficient, after two days, to allow the start of degradation. The same trend of fungal

activity to increasing glucose amounts in the culture medium has already been observed by Seigle-Murandi et al. [27]. In fact, these authors found that variation of glucose levels, from 5 to 10 gL<sup>-1</sup>, repressed PCP degradation by a series of *Micromycetes* strains.

### 3.3 Effect of Nitrogen Amount

Three concentrations of nitrogen (0, 2.4 and 24 mM, before inoculation), as ammonium tartrate, were utilized, while the glucose amount was maintained at 5 gL<sup>-1</sup> and the initial concentration of chlortoluron and isoproturon at 100 mgL<sup>-1</sup>, each. The best yields of chlortoluron depletion were obtained at 2.4 mM of nitrogen (Fig. 3A). At 0 and 24 mM, the extent decreased (there is a statically significant difference between the level of degradation at 2.4 mM and the levels at 0 and 24 mM,  $|Z| > 1.96$ ). The same profile was found with isoproturon (Fig. 3B) and the most interesting example is given by *E. annulata* (37% mean degradation at 2.4 mM vs. 11% and 15%, at 0 and 24 mM, respectively).

The effects of the amount of nitrogen on xenobiotics biodegradation have been studied for a long time, but the results are not at all convergent. A first group of studies seems to prove that both yield and rate of pollutants degradation by microorganisms decrease with increasing amounts of nitrogen. For instance, Mileski et al. [13] found that PCP mineralization by *P. chrysosporium* was more important in a 1.2 mM-nitrogen-amended medium (50.5%) than in a 12 mM-nitrogen-amended one (10.2%). The same behaviour was observed with 2,4-DCP [28], 2,4,5-trichlorophenol [29] and atrazine [30]. Contrary to this, there are studies that demonstrate the opposite trend: degradation and mineralization of pollutants are enhanced by increasing amounts of nitrogen [14, 31, 32]. According to our findings, it appears that the two phenylureas degradation by the five fungi requires a medium level of nitrogen (2.4 mM) and that a high quantity or a lack of this element in the culture medium inhibits the degradation process. Such behaviour was also found with pentachloronitrobenzene and *Spororhrix cyanescens* [33]. This fungus degraded PCNB at levels of 5.7, 19.4 and 13.5 µg per mg of biomass, at 0, 0.5 and 2 gL<sup>-1</sup> of nitrogen.



**Fig. 3** Effect of nitrogen amount on chlortoluron (A) and isoproturon (B) degradation by *Aspergillus parasiticus*, *Aspergillus ustus*, *Dicyma ampullifera*, *Embellisia annulata* and *Mortierella bainieri*, at 100 mgL<sup>-1</sup>, each, in ATM medium

Another kind of investigation points out the relationship between carbon/nitrogen (C/N) ratio-degradation parameters (lag time, rate and yield). It was demonstrated that a suitable value of this ratio, for an optimum level of biodegradation, varies with the effective microorganisms. A very high or low value of this ratio can inhibit or stop the biodissimilation phenomenon [34, 35]. Our results concerning chlortoluron and isoproturon degradation by the five strains seem to follow the same logic: the two substrates are best degraded at the C/N value of 12. As this value goes down (0 or 1.2) or up (24), the level of degradation decreases. These findings may indicate that a suitable value for this ratio for chlortoluron and isoproturon degradation by fungi, under our experimental conditions, is around 12.

## 4. Conclusion

In this study, we have proven that the efficiency of two phenylurea herbicides (chlortoluron and isoproturon) degradation by five fungi strains (*Aspergillus parasiticus*, *Aspergillus ustus*, *Dicyma ampullifera*, *Embellisia annulata* and *Mortierella bainieri*) was regulated by culture parameters:

- The level of depletion percentages is under the initial chemical concentration influence, the lower the initial concentration, the higher the extent of degradation;

- Glucose and nitrogen amounts in the medium regulate the level of biodissimilation process, under medium carbon as well as nitrogen concentration conditions, a significant increase of consumption level was observed.

Thus, optimization of the degradation phenomenon of chlortoluron and isoproturon is possible and further investigations will be conducted to assess the degradation of mixtures of these two compounds by pure cultures or a consortium of fungi strains.

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