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Effects of atrazine, isoproturon and diuron on glutathione metabolism of *Saccharomyces cerevisiae*

Abstract: Triazines and phenylureas, mainly used in agricultural applications for the selective control of germinating grasses and broad-leaved weeds, are often found in contaminated groundwater, surface water and the effluents of wastewater treatment plants. The toxicity of these herbicides in eukaryotic cells is poorly understood. *Saccharomyces cerevisiae* is a promising unicellular organism for the toxicological evaluation of xenobiotics because its metabolism is similar to that of higher-level organisms. Consequently, the aim of this study was to compare the effects of three herbicides on yeast-cell growth and the glutathione cycle. *Saccharomyces cerevisiae* grown in the presence of 5 or 50 μM atrazine, diuron or isoproturon were compared with control cells grown in a rich medium. The results show that the glutathione-dependent buffer capacity decreased significantly in *S. cerevisiae* grown in the presence of both levels of any of the three herbicides, except in cells exposed to 50 μM isoproturon. In addition, chlorine herbicides inhibited cell growth, probably due to a decrease in antioxidant power and glutathione reductase activity. Isoproturon at 50 μM induced yeast-cell growth, increasing the buffer capacity mediated by glutathione as well as glutathione reductase and glutathione peroxidase activities of UE-ME_3 strain. This strain may be useful in studies of isoproturon degradation.

Keywords: oxidative stress; phenylurea; triazine; yeast.

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1 Introduction

Under normoxic conditions, living organisms can very efficiently prevent reactive accumulation of oxygen species (ROS) and attenuate oxidative damage using various defensive strategies such as those involving tripeptide glutathione [1]. Glutathione (gamma-glutamylcysteinylglycine; GSH) is a low-molecular-mass thiol with functions in many cellular processes including protection against xenobiotics, carcinogens and ROS. GSH acts *per se* or, more effectively, when used as a cofactor in enzyme reactions such as those catalyzed by glutathione reductase (GR) in a process that requires reducing equivalents, such as NADPH, generated by glucose-6-phosphate dehydrogenase (G6PD, the key enzyme of the pentose phosphate pathway). In response to the cell occurrence of H_2O_2 or lipid hydroperoxides (ROOH), several genes encoding antioxidant enzymes, such as glutathione peroxidase (GPx), are induced and convert it into H_2O or the corresponding alcohol (ROH) (Figure 1) [2].

The biological importance of GSH is dependent upon the redox-active free sulfhydryl moiety of its cysteine residue [3]. However, sudden exposure of eukaryotic cells to xenobiotics as herbicides may cause a redox imbalance of the cell, causing oxidative stress. The yeast *Saccharomyces cerevisiae* is a promising unicellular eukaryotic organism for the toxicological evaluation of xenobiotics such as herbicides because its cellular structure and functional organization present many similarities to those of higher-level organisms. Furthermore, *S. cerevisiae* is a eukaryotic organism that is generally regarded as safe (GRAS), providing for its safe handling during the tests performed in this study [4]. However, the toxicity of phenylurea and triazine herbicides in yeast is still poorly understood. These photosystem II inhibitor herbicides are mainly used in agricultural applications for the selective control of germinating grass and broad-leaved weeds in many crops (e.g., cereals).

Diuron (DIU) is also used for total weed control on non-cultivated areas in the maintenance of roads, railways and parks. Isoproturon (IPU), diuron and atrazine (ATZ) (Figure 2) are the most widely used herbicides

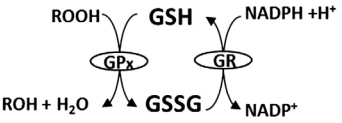

Figure 1: Glutathione cycle: a ROS scavenging pathway in *Saccharomyces cerevisiae*.

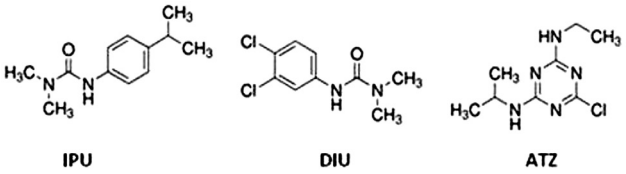

Figure 2: Chemical structure of isoproturon (IPU), diuron (DIU) and atrazine (ATZ).

commonly found in contaminated groundwater, surface water and the effluents of wastewater treatment plants in Europe. Several of these herbicides (DIU, ATZ) are classified as persistent organic pollutants (POPs) because chemical and biological degradation at ground level is very low [5–7]. However, the continued presence in soils and natural waters of herbicides undergoing moderate chemical/biological degradation, such as IPU, depends on weather conditions due to its poor leaching in regions with long summers [8, 9]. Thus, the removal of these herbicides from the environment has received considerable attention. Microorganisms play an important role in removing xenobiotics from the environment, and microbial bioremediation is considered to be a cost-effective tool for the detoxification of these substances [10]. Consequently, the main purpose of this study was to compare the effects of IPU, DIU and ATZ on yeast-cell growth and their antioxidant enzymes of the glutathione cycle.

2 Materials and methods

Saccharomyces cerevisiae UE-ME$_3$, a wild-type yeast isolated from the musts of Alentejo and deposited in the collection of the oenology laboratory of the University of Évora, were inoculated at mid-exponential phase with yeast extract peptone dextrose (YEPD) liquid medium and 2% (w/v) glucose at 28°C, shaken at 150 rpm in the presence of the herbicides ATZ, DIU or IPU in different concentrations (5 or 50 µM) and compared with control. The samples were incubated for 72 h until they reached the stationary phase of growth, which was determined by reading the optical density ($OD_{640\,nm}$). Yeasts were harvested and centrifuged at 3000 *g* for 10 min and washed with ultra-pure sterile water to remove all the culture medium. The obtained cells were suspended in 10 ml of 10 mM phosphate buffer pH 7.0 (lysis buffer) and disrupted by sonication using a Branson Sonifier 450 (three times, 5 min). The samples were centrifuged 15 min at 12,000 *g* in refrigerated super centrifuge, Hermle Z323 K [11]. Post-12,000 *g* supernatants were used for the determination of content in non-protein thiols and enzyme activities. For the determination of GSH and GSSG contents, aliquots of supernatants reacted in a mixture containing commercial *o*-phthalaldehyde (OPT), 5 mM ethylenedinitrilotetraacetic acid (EDTA) in 0.1 M sodium phosphate buffer pH 8.0, in the first case or in a mixture containing commercial OPT, 0.04 M *N*-ethylmaleimide in 0.1 M NaOH in the second case, using GSH or GSSG as standard, respectively. The luminescence was read at λ_{exc} 350 nm, and λ_{em} 420 nm, using a single-beam spectrofluorophotometer (Shimadzu RF-5001PC) [12]. GR (E.C 1.6.4.2) activity was determined using a reaction mixture containing 15 mM EDTA, 63.5 mM GSSG in 0.12 M phosphate buffer pH 7.2, incubated at 37°C for 300 s. The reaction was started after the addition of 9.6 mM NADPH and followed reading the absorbance at 340 nm for 180 s [13]. GPx (E.C 1.11.1.7) activity was determined in a reaction mixture containing 5 mM GSH, 0.24 U/ml GR in 0.12 M phosphate buffer pH 7.2, incubated at 37°C for 30 s using a double-beam spectrophotometer (Hitachi-U2001) [14]. All reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA. All values were presented as a mean of five independent experiments ± standard error of mean (SEM). The normality and homogeneity of variance were assessed by the P-plot and Levene's test, respectively. The statistical analyses of results were performed by ANOVA I and the Duncan test to determine significant differences ($p<0.01$) between treatments, using SPSS® statistical software, version 22.0 (SPSS Inc., Chicago, IL, USA) for Windows®, licensed to University of Évora [15].

3 Results and discussion

Results show that 50 µM IPU exposure induced *Saccharomyces cerevisiae* UE-ME$_3$ growth, although cell growth was not affected by 5 µM IPU (Figure 3). Although the response to IPU appears to be contradictory, similar responses may be found in the literature. For example, rats treated with an 800 mg/kg dose of IPU exhibit an increase in the weight of the liver, kidney and heart [16]. In addition, the growth rates of the ciliate (*Tetrahymena pyriformis*) and common earthworm (*Lumbricus terrestris*) were inhibited at 700 g/L or 1.4 g IPU/kg soil, respectively, whereas other

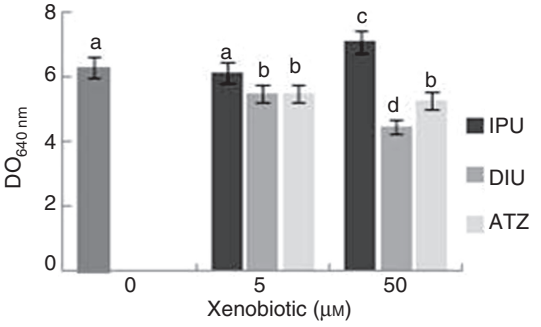

Figure 3: Optical density in the presence or absence of IPU, DIU and ATZ in *Saccharomyces cerevisiae* UE-ME$_3$.
The results represent the mean of five independent experiments ± SEM. The bars marked with different letters are significantly different ($p<0.01$).

hydrotrophic organisms (*Daphnia magna*) and the nematode (*Caenorhabditis elegans*) were not affected by IPU up to concentrations of 1000 g/L [17, 18]. However, a significant decrease in yeast growth in cultures containing DIU or ATZ was observed for both levels studied (Figure 3), a response that may be related to the disruption of mitosis in plants, disturbances in survival and growth in amphibian embryos and tadpoles' cladocerans, amphipods, midges, minnows, worms and snails described for DIU and ATZ [19–22].

Previous studies have suggested that ATZ and DIU present slight toxicity to mammals and birds as well as moderate toxicity to aquatic invertebrates, inducing antioxidant depletion such as tripeptide glutathione in the liver of rainbow trout (*Oncorhynchus mykiss*), sand trout (*Pseudaphritis urvillii*) and crucian carp (*Carassius carassius*) exposed to sublethal concentrations of the herbicides [20–26]. In this study, the buffer capacity mediated by glutathione (GSH/GSSG ratio) decreased significantly in *S. cerevisiae* grown in the presence of both levels of any of the three herbicides, except in yeast cells exposed to 50 μM IPU, where there occurred an unaccountable increase that positively correlated with the increase in cell growth previously mentioned ($r=0.786$, $p<0.01$) (Figure 4). This response, which is identical to that observed in liver rats treated with 675 mg/kg, 1/6th LD50 [27], may be explained by a higher turnover mediated by the gluthione cycle [26].

Moreover, the results also show a significant decrease in GR activity in *S. cerevisiae* grown in the presence of 5 or 50 μM IPU ($p<0.01$) (Figure 5). The GR activity of *S. cerevisiae* is probably involved in the detoxification of IPU, ensuring the efficient recycling of glutathione to protect cells against phenylurea herbicide, as described in wheat leaves (*Triticum aestivum*) [28].

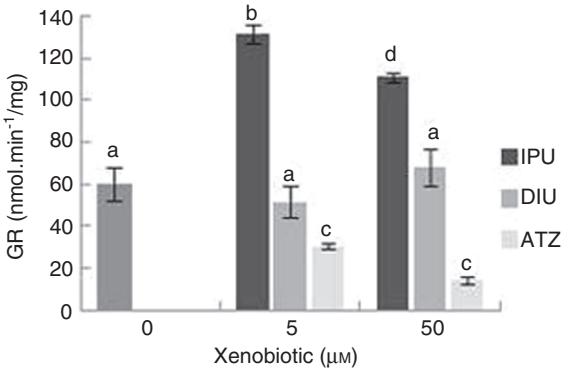

Figure 5: GR activity of post-12,000 *g* supernatant of *Saccharomyces cerevisiae* UE-ME$_3$ grown in YEPD medium, in the absence or presence of IPU, DIU and ATZ.
The results represent the mean of five independent experiments ± SEM. The bars marked with different letters are significantly different ($p<0.01$).

Interestingly, a similar response profile was determined for GPx activity in cells exposed to IPU and ATZ, less evident for ATZ. In the case of DIU, a decrease in GPx activity in cells exposed to 5 μM of this herbicide was detected, whereas in cells exposed to 50 μM DIU, the opposite was observed (Figure 6).

Therefore, it seems that for higher levels of DIU, cell machinery seeks to eliminate the oxidative stress induced by the herbicide by consuming peroxides or lipid hydroperoxides that were generated. However, the lack of an

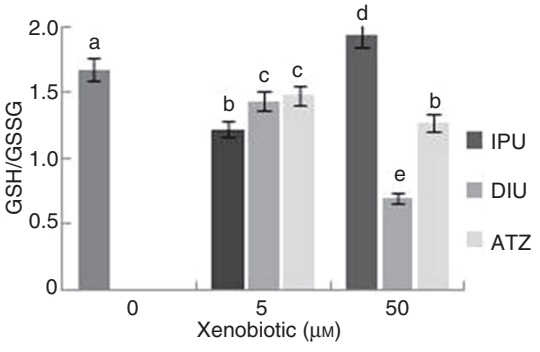

Figure 4: GSH/GSSG ratio determined in post-12,000 *g* supernatant of *Saccharomyces cerevisiae* UE-ME$_3$ grown in the absence or presence of IPU, DIU and ATZ, in YEPD medium.
The results represent the mean of five independent experiments ± SEM. The bars marked with different letters are significantly different ($p<0.01$).

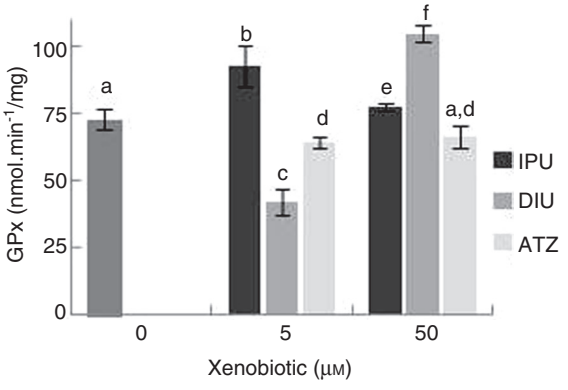

Figure 6: GPx activity of post-12,000 *g* supernatant of *Saccharomyces cerevisiae* UE-ME$_3$ grown in YEPD medium, in the absence or presence of IPU, DIU and ATZ.
The results represent the mean of five independent experiments ± SEM. The bars marked with different letters are significantly different ($p<0.01$).

adequate response by GR activity seems to hinder the growth of *S. cerevisiae* UE-ME$_3$ due to a decrease in intracellular redox power.

4 Conclusions

Chlorine herbicides such as DIU and ATZ inhibited cell growth, decreasing the GSH/GSSG ratio and antioxidant GR activity. Moreover, it was observed that 50 μM IPU was able to induce cell growth of S. *cerevisiae* UE-ME$_3$, increasing the buffering capacity mediated by glutathione as well as GR and GPx activities, properties that indicate a UE-ME$_3$ strain that is tolerant to 50 μM IPU. As only a few microorganisms that are able to metabolize phenylurea herbicides have been isolated at present, this wild-type yeast may be useful in future studies of IPU degradation, bearing in mind its possible application in bioremediation, in accordance with Nwachukwu [29].

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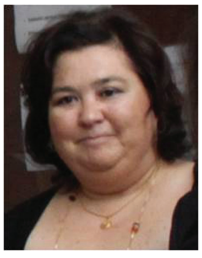

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