BINARY COMBINATIONS OF ATRAZINE AND ISOPROTURON INFLUENCE CELL GROWTH AND THE GLUTATHIONE CYCLE IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT. The widely use of herbicides such as atrazine (ATZ) and Isoproturon (IPU) in agriculture has increased their environmental circulation, leading to their accumulation in water and soil. The consumption of water and food from these ecosystems can drastically compromise human health and well-being. Although ATZ and IPU can be found in numerous combinations in nature, most research involving these pollutants only analyses the toxicity of each molecule in non-target organisms. Thus, the aim of this work was to evaluate if and how binary combinations of ATZ and IPU µM (25:5; 50:5) significantly affect the growth and antioxidant mechanisms of *Saccharomyces cerevisiae*, a Crabtree-positive yeast. The findings show that exposure to those binary combinations of ATZ and IPU favored the Crabtree effect with an increase in cell proliferation, in terms of viability and specific growth rate, and a slowdown in antioxidant activity mediated by the glutathione cycle.

Keywords: *Yeast; herbicides; Crabtree effect; peroxidases enzymes*

Introduction

The environmental circulation of plant protection products has increased in several places around the globe in the last seven decades, due to their increasing use in agriculture. Their accumulation in water and soil is a serious environmental health problem that needs to be detected, characterized, and corrected [1,2].

Atrazine (ATZ), a triazine, widely used in weed control, has been associated with a high risk of environmental contamination, because its chemical and biological derivatives have high toxicity and high dispersion capacity, affecting the quality of soils, crops, surface, and groundwater [1,2].

Isoproturon (IPU), a phenylurea herbicide commonly used in pre- and post-emergence weed management, is another contaminant of aquatic systems that, as a potent inhibitor of photosynthesis, is a generator of reactive oxygen species (ROS) that, in excess, are harmful to life [2,3,4]. Consequently, human health and well-being may be threatened if these ecosystems' water and food are consumed.

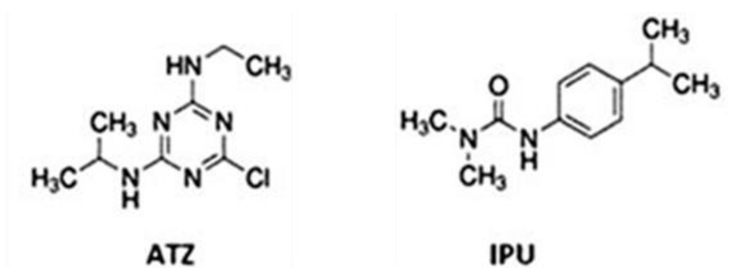

Fig.1- Chemical structure of atrazine (ATZ) and isoproturon (IPU) [8].

Aerobic respiration generates reactive oxygen species such as the superoxide anion radicals ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), and lipid hydroperoxides, which are the main sources of oxidative stress in eukaryotic organisms [6].

Antioxidants (AOX) are chemical structures that can scavenge reactive oxygen species (ROS) and prevent or reduce oxidative stress. They have been divided into two categories: *i)* enzymes from the glutathione-

cycle, such as glutathione peroxidase and glutathione reductase, or *ii)* non-enzymatic, tiny sacrificial hydrophilic agents, such as the tripeptide glutathione [7].

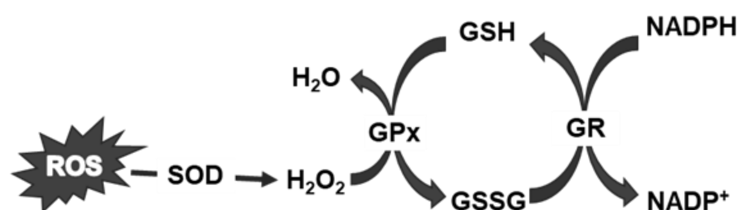

Fig.2- Schematic diagram of glutathione cycle. Abbreviation in the figure: GPx-glutathione peroxidase, GR-glutathione reductase, GSH-glutathione, GSSG-glutathione disulfide, ROS-reactive oxygen species, $NADP^+$-nicotinamide adenine dinucleotide phosphate (oxidized), and NADPH-nicotinamide adenine dinucleotide phosphate (reduced) [8].

Although pollutants such as ATZ and IPU herbicides can be found in numerous combinations in nature, much research on them focus on the toxicity of each molecule in non-target organisms, such as eukaryotic unicellular model *Saccharomyces cerevisiae* selected for this work [9].

Thus, the aim of this study was to evaluate if and how binary combinations of ATZ and IPU affect the cell growth and antioxidant processes of *Saccharomyces cerevisiae* UE-ME_3, a Crabtree-positive yeast, with catabolic repression of respiratory metabolism by glucose, a phenomenon like aerobic glycolysis seen in cancer cells [10].

Material and Methods

Microorganisms and growth conditions

Culture flasks containing 100 mL of YPD medium (1% w/v yeast extract, 2% w/v peptone and 2% w/v glucose) or YPD in the presence of the herbicides ATZ or IPU in different concentrations (µM 25, ATZ25; µM50, ATZ50 or 5 µM IPU; IPU5) or both herbicides (ATZ25 IPU5; ATZ50 IPU5) were inoculated using a fresh culture of *Saccharomyces cerevisiae* UE-ME_3, a wine wild-type yeast from Alentejo, Portugal, belonging to the Enology laboratory collection of University of Évora, and shaken at 150 rpm at 28°C in a shaking water bath with controlled temperature.

Determination of specific growth rate

The determination of the OD of a microbial culture using spectrophotometry is one of the methods used for monitoring cell growth. Thus, to determine specific growth rate (µ) were taken from the culture media aliquots at times t48h and t72h for a reading OD_{640nm}. OD_{640nm} enabled the determination of the parameter µ (h^{-1}) in the five experiments in accordance with the following mathematical expression [11]:

$$\mu=(\ln(Gc)-\ln(Gt))/(t72h-t48h)$$

(Gc - OD_{640nm} in t48h; Gt - OD_{640nm} in t72h; t48h - initial time (h); t72h - final time (h))

Cell homogenization and subcellular fractionation

The cells from the five experiments were harvested by centrifugation at 5,000 *g* for 10 min, rinsed in sterile double-distilled water, suspended in 10 mM phosphate buffer pH 7.0, and disintegrated in an ultrasonic homogenizing (10s, 3x5 min, 0.1 kHz pulse) to obtain post-12,000 g supernatant at the end of the exposure test (t72h). The homogenates were subjected to differential centrifugation at 12,000 *g* for 20 min using a refrigerated supercentrifuge and post-12,000 *g* supernatant was obtained in accordance with [12].

Measurements

Aliquots of Post-12,000 *g* supernatants were stored at -20°C for the subsequent determination of reactive oxygen species (ROS), glutathione (GSH), glutathione disulfide (GSSG) and protein contents as well as glutathione reductase (GR), glutathione peroxidase (GPx) and ascorbate peroxidase (APx) enzyme activities. ROS content was determined in accordance with [13], reading the fluorescence of DCF at λ_{exc} 488 nm and λ_{em} 525 nm. GSH and GSSG levels were determined in accordance with [14], reading fluorescense of o-phthalaldehyde GSH product at pH 8.0, or o-phthalaldehyde GSSG product at pH 12, at room temperature, selecting λ_{exc} 350 nm and λ_{em} 420 nm. All fluorometric determinations were realized in a spectrofluorphotometer Shimadzu RF-5002 PC. GR enzyme activity was determined in accordance with [15] accompanying the oxidation of NADPH, reading the absorbance at 340 nm for 120s, at 37°C and using $\varepsilon = 6.22$ mM^{-1} cm^{-1} for NADPH. GPX (PhGPx) enzyme activity was determined by the method of [16] accompanying the oxidation of NADPH, by reading the absorbance at 340 nm for 180 s at 37°C and using $\varepsilon = 6.22$ mM^{-1} cm^{-1} for NADPH. APX enzyme activity was determined in accordance with [17]. The ascorbate consumption was followed by reading the absorbance at 290 nm, for 180 s at 37°C and using $\varepsilon = 2.8$ mM^{-1}.cm^{-1} for ascorbate. Protein content was determined in accordance with [18]. All spectrophotometric determinations were realized in a double-beam spectrophotometer Hitachi U2001 under temperature control by Grant water-circulating bath.

Statistical analysis of results

Data were processed via analysis of variance ANOVA I, using SPSS Statistics 25 software (Chicago, IL, USA), licensed to the University of Évora. Means were separated at the 5% level using Duncan's new multiple range test.

Results and Discussion

Cell viability and specific growth rate

Cell viability (%) of *S. cerevisiae* UE-ME$_3$ at 72 h of growth and exposure alone to ATZ was lower than that of control assay cells (YPD). However, the viability of cells exposed either to IPU or binary ATZ-IPU combinations was shown to be higher than the control (Figure 3A).

The specific growth rate (μ) of cells grown only in YPD medium (control, respiratory-fermentative cells) determined between 48h and 72h was 0.0025 h^{-1}. However, cells exposed alone to ATZ or IPU herbicides or to ATZ-IPU combinations showed μ equal to or greater than 0.0081h^{-1}. The significant increase in μ could be due to IPU and/or ATZ exposure may have slowed down *S. cerevisiae*'s energy metabolism shift from fermentative to respiratory (Figure 3B) [19,20].

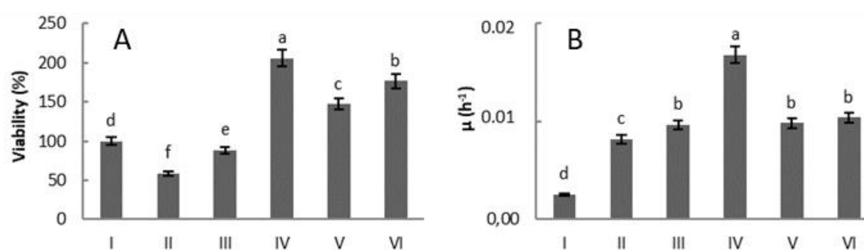

Fig. 3 - Cell viability (%72h) (A) and specific growth rate (B) (OD t48 t72) of *S. cerevisiae* UE-ME$_3$ grown in YPD (I) or YPD medium in the presence of 25 µM (II) or 50 µM ATZ (III) or 5 µM (IV) IPU or binary mixtures of both herbicides (25 µM ATZ, 5 µM IPU (V); 50 µM ATZ; 5 µM IPU (VI)). Results represent the mean of five independent experiments ± standard deviation. Bars marked with different letters are significantly different ($p < 0.01$).

Glutathione and ROS content

Glutathione tripeptide is an essential metabolite used in yeast cells as a reducing agent under normal growth conditions. Although reactive oxygen species convert glutathione to glutathione disulfide, GSH is the most abundant form in eukaryotic cells due to the role of the glutathione reductase enzyme in regenerating GSH from GSSG [21,22]. GSH is also essential because it functions as a cofactor for antioxidant enzymes such

as glutathione peroxidases. GSH content was not affected by alone exposure to 25 µM ATZ or 5 µM IPU. However, *S. cerevisiae* exposed to 50 µM ATZ or binary mixtures of both herbicides 25 µM ATZ, 5 µM IPU or 50 µM ATZ, 5 µM IPU had significantly lower GSH levels than control (Fig. 4A). Cells grown only in the presence of 5 µM IPU or exposed to the binary combination 50 µM ATZ; 5 µM IPU had increased GSSG content (Fig. 4B). Except in the 25 µM ATZ exposure assay, the GSH/GSSG ratio assumed lower values than the control (YPD) in any of the ATZ or/and IPU exposure experiments (Fig. 4D). The lowest values of GSH/GSSG ratio were found in both herbicide's binary exposure assays. This response points to a marked increase in the yeast cell oxidative stress due to the binary's atrazine-isoproturon combinations. Figure 4C shows that ROS levels in *S. cerevisiae* grown in herbicide exposure experiments were consistently higher than those measured in control cells (YPD). The highest ROS levels found in cells grown in the presence of 5 µM IPU or 50 µM ATZ + 5 µM IPU confirms an increase in oxidative stress conditions linked to a drop in GSH or an increase in GSSG, as previously discussed in Figures 4 A, B, and D.

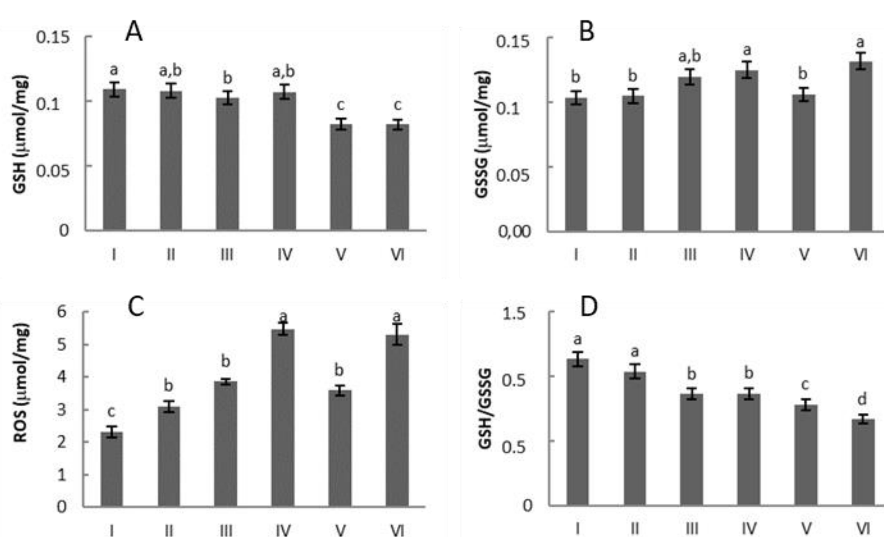

Fig. 4 - GSH (**A**), GSSG (**B**) and ROS (**C**) content and GSH/GSSG ratio (**D**) of *S. cerevisiae* UE-ME$_3$ grown in YPD (I) or YPD medium in the presence of 25 µM (II) or 50 µM ATZ (III) or 5 µM (IV) IPU or binary mixtures of both herbicides (25 µM ATZ, 5 µM IPU (V); 50 µM ATZ; 5 µM IPU (VI)). Results represent the mean of five independent experiments ± standard deviation. Bars marked with different letters are significantly different ($p < 0.01$).

Antioxidant enzymes related to the glutathione cycle

The main enzyme response to oxidative stress due to the accumulation of lipid hydroperoxides and hydrogen peroxide is glutathione peroxidase [23] and catalase-ascorbate peroxidase [24]. Rather of glutathione peroxidase, yeast cells express three isomorphic phospho-glutathione-peroxidases (PhGPx), which recognize lipid hydroperoxides as substrates and protect membrane lipids. Glutathione reductase, the other member of the glutathione cycle, is an oxidoreductase that regenerates GSH by using reducing equivalents in the form of NADPH supplied by the pentose phosphate pathway, completing the GPx (PhGPx) role [21,22].

Figure 5A shows that the GR activity of *S. cerevisiae* UE-ME$_3$ was unaffected by either amount of ATZ exposure (25 µM or 50 µM), a response that differs from that seen in the 5 µM IPU exposure assay, where this catalytic activity increased dramatically. However, exposure to any of the binary ATZ-IPU combinations caused a highly significant decrease in GR. The loss of cell ability to regenerate GSH when ATZ combines with IPU appears to partly explain the elevated levels of ROS and oxidative stress evidenced by the reported drop in the GSH/GSSG ratio previously discussed. When *S. cerevisiae* UE-ME$_3$ was cultivated exclusively in the presence of ATZ or IPU, the activity of glutathione peroxidase (PhGPx) and catalase-ascorbate

peroxidase (APx) assumed higher values than in the control cells. However, APx activity was significantly decreased in *S. cerevisiae* exposed to any of the binary ATZ-IPU combinations selected for this study.

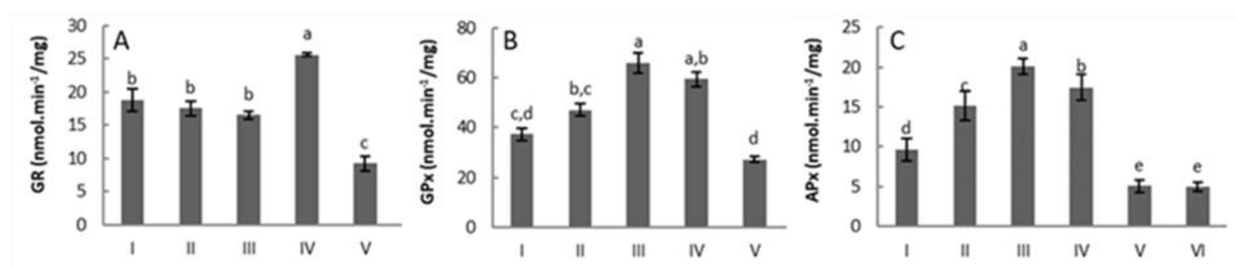

Fig. 5 - GR (**A**) GPx (**B**) and APx (**C**) enzyme activity of *S. cerevisiae* UE-ME$_3$ grown in YPD (I) or YPD medium in the presence of 25 μM (II) or 50 μM ATZ (III) or 5 μM (IV) IPU or binary mixtures of both herbicides (25 μM ATZ, 5 μM IPU (V); 50 μM ATZ; 5 μM IPU (VI)). Results represent the mean of five independent experiments ± standard deviation. Bars marked with different letters are significantly different ($p < 0.01$).

Increased intracellular ROS levels may have also been caused by the cell loss of ability to remove lipid hydroperoxides and/or hydrogen peroxide caused by binary ATZ-IPU exposure.

Conclusions

Exposure to binary combinations of atrazine-isoproturon favors the Crabtree effect with an increase in cell proliferation, eventually ensured by the fermentative metabolism with a consequent decrease in antioxidant activity mediated by the glutathione enzymes, a response which resembles the aerobic activity of cancer cells.

Acknowledgement

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