

***Saccharomyces cerevisiae* as a Tool to Evaluate the Effects of Herbicides on Eukaryotic Life**

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1. Introduction

1.1 Herbicides and their toxicity

Agricultural herbicides play undoubted beneficial roles in preserving crop yields, although they may pose serious concerns for the environment and humans because of their widespread and intensive use/misuse (due to careless applications, high and repeated application rate, accidental spillage). It is also evident the potential to injure non-target cultivars and microorganisms, in particular those contributing to soil quality, and to cause adverse side-effects in mammals, including humans (Cabral et al.,2003; Cabral et al.,2004).

Herbicide-related toxicity may be often underestimated because little information is provided on the effects of herbicides as complete formulations. As a matter of fact, acute or chronic toxicity tests are generally carried out using pure active ingredients (AIs). Herbicide-induced damaging effects are proportional to the absorbed substance and to the time of exposure, but also to the inherent characteristics of each compound. Agriculturists are most likely exposed to acute intoxication, by inhalation or direct contact with toxic substances, though each person can be subjected to possible toxic effects due to accumulation of pesticide residues in the body, being the diet the most important source of exposure.

Typically, pure herbicide molecules are of limited value to end users. To give them practical value and make them usable, most herbicides are combined with appropriate solvents or surfactants to obtain a formulation. A given chemical may be formulated in a variety of differing formulations and sold under different trade names. For this reason, pure AIs are mixed with coformulants, also called 'inert' or auxiliary substances, to allow their use in common and convenient vehicles, such as water, and to obtain uniform and effective distributions. Several substances fall within the definition of coformulant, including: carrier substances, solvents, surfactants, dispersing agents, adhesives, absorption-promoting agents, antioxidants, bactericides, dyes, fillers, and perfumes. Coformulants can have various technical and physico/chemical properties in relation to their function in the pesticide formulation. These chemicals can be expected to have various toxicological profiles, some of them harmless (e.g., water) and some with serious toxicological properties (e.g., the organic solvent isophorone, which is a suspected carcinogen). Because

coformulants represent the highest amount in pesticide mixtures, even a minor toxicological concern could become significant in relation to their use (Tobiassen et al., 2003) especially considering the fact that, besides being toxic themselves, they can also increase the toxic effects of AIs (Surgan, 2005; Séralini, 2005). However, for the majority of herbicides that are currently used, no information on the identity of coformulants is publically made available. Consequently, adjuvants can be included in herbicide formulations as a part of the total product which is sold by the manufacturer or as an additive to be mixed with pesticide products in the spray tank. Adjuvants can directly increase the toxicity of pesticide formulations. For instance, they can promote their penetration through clothes and skin and increase their persistence in the environment (Cox & Surgan, 2006). Consequently, different problems might be posed when manipulating different formulations. As an example, in the case of emulsifiable concentrates, problems of dosing and mixing could be encountered and the absorption through skin could be facilitated. Granules, since they usually do not adhere to foliage and are not intended for foliar applications, may have more serious consequences for soil quality and microbial populations commonly associated (<http://www.ext.colostate.edu/pubs/crops/00558.html>).

1.2 Yeast as an eukaryotic model system

The yeast *Saccharomyces cerevisiae* is an optimal eukaryotic model system to study toxic effects and mammalian biological responses upon exposure to exogenous and endogenous perturbations. The high degree of evolutionary conservation of stress pathways between yeast and higher eukaryotes means that yeast can serve as a suitable model system for the characterization of stress responses in more complex organisms (Estruch, 2000). Particularly important advantages deriving from the use of *S. cerevisiae* as a well consolidated eukaryotic model are the non-pathogenicity and the feasibility of such a model, thus avoiding risks for manipulators. Being unicellular, it is a simple and easily accessible system allowing to avoid ethical and safety problems arising from the use of more complex eukaryotic cells, representing a consolidated, appropriate and cost-effective alternative to animal testing. At the same time, the obtained response is easier to decipher and, once extrapolated to humans, it could produce a better understanding of molecular mechanisms of toxicity. Being the first eukaryotic organism whose genome was completely sequenced in 1996 (Goffeau et al., 1996), yeast offers also tremendous opportunities for genome/proteome/metabolome and other 'omic' studies.

Yeast cells have remarkable similarities to mammalian cells at the macromolecular and organelle level, and a number of yeast proteins have been shown to be functionally interchangeable with the highly homologous human proteins. Thus it is not surprising the use of yeast as a model system with relevant contributions to the understanding of molecular mechanisms underlying oxidative stress. The involvement of oxidative stress in ageing, apoptosis, and a significant number of diseases led to the characterization of the antioxidant systems and the elucidation of their functional physiological role. For example, yeast cells have contributed in recent years to clarify the role reactive oxygen species (ROS) in diseases such as amyotrophic lateral sclerosis or Friedreich's ataxia (Costa & Moradas-Ferreira, 2001).

Studies on the evaluation of pesticides still depend extensively on the use of animals, but in the last years several publications have dealt with the development and appropriateness of alternative methods for assessing toxicity, that do not depend on animal utilization but explore the use of rapid and cost-effective alternatives (Ribeiro et al., 2000).

Yeast cells have already been proposed as a tool for assessing toxicity of environmental pollutants (Cabral et al., 2004; Cabral et al., 2003; Simoes et al., 2003), as shown by the few following examples:

- i. *S. cerevisiae* can be a bio-accumulator of metals such as Cu, Cr, Cd, Ni, Zn, and Pb (Malik, 2004).
- ii. *S. cerevisiae* shows affinity for different pesticides, and in particular can partially degrade and significantly adsorb the fungicides quinoxifen (Cabras et al., 2000) and fenhexamid (Cabras et al., 2003). Moreover, yeast can degrade and adsorb several pyrethroid pesticides (Cabras et al., 1995).
- iii. Wild-type yeasts can be used as biosensors and a valid tool for preliminary evaluations of xenobiotic toxicity (Baronian, 2004) (Campanella et al., 1995).

2. Aim of the work

In the scientific literature, little attention has been paid to the understanding of toxic effects of pesticides as commercial formulations. In this context, the most important exception is represented by the herbicide glyphosate, for which recent toxicity studies have been carried out using some of its formulations (Peixoto, 2005; Mann & Bidwell, 1999; Chan et al., 2007; Pieniazek et al., 2004). On the contrary, for many other herbicides (and pesticides in general) little information is currently available.

In order to fill this gap in the knowledge of herbicide-related toxicity, we undertook a comparative analysis on an autochthonous *S. cerevisiae* strain, isolated during the spontaneous fermentation of grapes and selected as a potential 'starter' for the production of high quality wines (Vagnoli et al., 1993; Trabalzini et al., 2003a; Trabalzini et al., 2003b) testing in parallel three herbicides and their corresponding pure AIs. The three tested herbicides were chosen among those used in the same geographical areas where the yeast strain was isolated. They were: Pointer (P), water dispersible granules containing tribenuron methyl (T); Silglif (S), a soluble concentrate containing glyphosate (G), and Proper Energy (PE), an oil/water emulsion containing fenoxaprop-P-ethyl (F). In order to clarify the mechanisms underlying the toxicity of herbicides as commercial formulations, we moved towards the analysis of parameters related to oxidative stress and undertook a proteomic and redox-proteomic study on the effects of the tested compound on yeast protein repertoires.

Our analyses might contribute to elucidate response mechanisms in more complex and experimentally less accessible eukaryotes while avoiding the complexity of higher eukaryotic cells and consequent ethical problems. Importantly, the use of a *S. cerevisiae* strain used in the wine-making industry provided us with an improved understanding of the perturbing effects of herbicides on fermentation, a process making yeast a milestone in the production of wine, bread, beer and many other foods and beverages.

3. Materials and methods

3.1 General materials

All high-purity reagents were from Oxoid (Garbagnate Milanese, Milan, Italy), Sigma-Aldrich (Milan, Italy) or J. T. Baker (Deventer, Holland).

Commercial grade herbicides, namely PE and P (Aventis CropScience, Milan, Italy) and S (Siapa, Milan, Italy) were from commercial sources. High purity AIs (F, T and G) were analytical standards from Riedel de Haën (Schweiz, Germany).

All of the experiments were performed using Milli-Q (Millipore, Bedford, MA, U.S.A.) water.

3.2 Yeast cell cultures

The yeast strain used in this work was *S. cerevisiae* K310. Yeast cells were pre-cultured in yeast peptone dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] at 30°C with rotary shaking for 10 hours. Then, an aliquot of the cell culture was inoculated in 150 mL of a modified YPD medium (YPDm) adjusted to a final pH of 4.5 by adding 0.2 M citrate/phosphate buffer and containing 100 g/L glucose, to obtain an initial concentration of 1×10^4 cells/mL. The new cell suspension was incubated at 28 °C in the dark without shaking and allowing semi-anaerobic growth.

Commercial grade herbicides were singularly added to the culture medium at the beginning of exponential growth phase (16th hour of cell culture, about 3×10^6 cells/mL). P was added as a water dispersion to a final concentration of 100 mg/L; S was added as a water solution to a final concentration of 1 g/L; PE was added as a water emulsion to a final concentration of 500 mg/L.

In parallel, single AIs were assayed and added to yeast cultures at the same time and the same concentration obtained with commercial formulations. Whereas G was prior dissolved in water, F and T were dissolved in DMSO (1 mg/mL and 4 mg/mL, respectively). Consequently, vehicle controls were prepared for F [2.62% (v/v) DMSO] and T [1.87% (v/v) DMSO]. Final concentrations of AIs in culture medium were as follows: 75 mg/L T, 304 mg/L G and 26.2 mg/L F).

At various moment during growth, cells were harvested by centrifugation, washed and cell-free protein extracts prepared as already described (Trabalzini et al., 2003a) for further analyses. Protein concentration was determined according to the Bradford's method (Bradford, 1976).

3.3 Monitoring of yeast colony forming ability and fermentative performance

K310 colony forming ability was monitored by plating on YPD-agar proper dilutions (ranging from 1:10 up to 1:100000) of the cell suspension; plates were then incubated at 28°C for three days before counting colony forming units (CFU).

Ethanol levels in culture medium were determined using an enzymatic assay (kit code 10 176 290 Boehringer Mannheim, Germany) as already described (Braconi et al., 2006b). Briefly, samples taken from cell suspensions were rapidly cooled and centrifuged (centrifuge 1515R, Eppendorf, Hamburg, Germany). The supernatants were then filtered through a 0.2 µm pore size membrane and determination of ethanol concentrations was performed spectrophotometrically (Agilent 8453 UV-visible spectroscopy system, Waldbronn, Germany) on the obtained filtered, properly diluted in accordance to manufacturer's instructions.

3.4 HPLC analysis of K310 nucleotide patterns

HPLC analysis of nucleotides in K310 cells was carried out on protein extracts with a System Gold Beckman (San Ramon, CA, USA) with a programmable Solvent Module 126 and Dual Channel Scanning Detector Module (mod. 168), equipped with Gold.8 software.

All separations were performed on a ODS Phenomenex Luna C18 column (7.5 cm × 4.6 mm, 3 µm particle size) equipped with a Phenomenex Security Guard pre-column (4 mm L × 3

mm ID) and filter. Analyses were carried out by gradient elution with 0.1 M KH₂PO₄, containing 6 mM pH 5.5 tetrabutylammonium hydrogen phosphate (TBA) (eluent A) and methanol (eluent B).

The chromatographic conditions (total run time 22 min) were set as follows:

initial condition: 95% A and 5% B;

- i. 5 minutes isocratic in the initial conditions;
- ii. 2 minutes gradient to reach 80% A and 20% B;
- iii. 5 minutes isocratic in the new conditions;
- iv. sudden increase to 80% A and 30% B;
- v. 2 minutes isocratic in these conditions;
- vi. restoring of the initial conditions in 8 minutes.

Flow rate was 1 mL/minute at room temperature. Both UV absorbances at 260 and 280 nm were monitored and used to record sample chromatographic spectra. On individual peaks, absorbance spectra were recorded from 194 to 354 nm. Compound of interest were identified on the basis of their retention time or with the co-elution with proper internal standards. Single compounds were quantified according to calibration curves obtained with standard solutions.

3.5 Catalase and superoxide dismutase (SOD) activity assays

For enzyme activity determination, 50 µg of proteins were resolved by 10% discontinuous native PAGE according to Ornstein (Ornstein, 1964) and stained as follows, performing all operations in the dark.

For catalase activity, gels were incubated 5 minutes in 5% (v/v) methanol, briefly washed three times with water and incubated 10 minutes in 10 mM H₂O₂. Gels were again rinsed with water and soaked in a 1:1 mixture of freshly prepared 2% (w/v) potassium ferric cyanide and 2% (w/v) ferric chloride. Gels turned to blue except in the zones where H₂O₂ was decomposed by active catalase. Colour development was blocked soaking the gels in 10% (v/v) acetic acid and 5% (v/v) methanol (Agarwal et al., 2005).

For SOD activity, gels were first soaked in 2.5 mM NBT¹ for 20 minutes, then briefly washed with water and soaked in 500 mM PBS² containing 2.8 mM TEMED³ and 28 µM riboflavin. For revelation, gels were illuminated on a light box for 20 minutes until the appearance of white bands on a dark background (Teixeira et al., 2004).

Images of gels were acquired (Image Scanner, Amersham Biosciences) and analysed with Image Master™ Platinum (Amersham Biosciences) choosing as a reference parameter the intensity of bands, which is automatically normalized against the surrounding background.

3.6 Proteomics and redox-proteomics

3.6.1 Two-dimensional gel electrophoresis (2D-PAGE)

K310 proteins were first mixed with a buffer containing 8 M urea, 35 mM CHAPS⁴, 10 mM DTE⁵, and a trace of Bromophenol Blue. Proteins were adsorbed onto Immobiline Dry Strips (IPG 18 cm, non linear 3-10 pH range, Bio-Rad, Milan - Italy) and allowed to stand at room

¹ Nitro blue tetrazolium

² Phosphate buffered saline

³ N,N,N',N'-Tetramethylethylenediamine

⁴ 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

⁵ Dithioerythritol

temperature for 10 hours. 100 µg (2D gels to be transferred onto nitrocellulose (NC) membranes or 1000 µg (preparative 2D gels) of proteins were used, respectively. Isoelectric focusing (IEF) was carried out with a Protean IEF cell (Bio-Rad) according to manufacturer's instruction and then IPG strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS⁶, 0.05 M Tris-HCl pH 6.8 containing first 2% (w/v) DTE and later 2.5% (w/v) IAA⁷. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was carried out applying 40 V per gel until the dye front reached the bottom of gels. Preparative gels were stained with Coomassie Brilliant Blue as described (Candiano et al., 2004).

3.6.2 Western blot analysis of carbonylated proteins

After the IEF, strips for the Western blot detection of carbonylated proteins were briefly rinsed with water and incubated at room temperature for 20 minutes with 10 mM DNPH⁸ in 5% (w/v) trifluoroacetic acid (Reinheckel et al., 2000). Subsequently, strips were rapidly washed twice with a solution containing 8M urea, 20% (v/v) glycerol, 9 M SDS and 150 mM Tris-HCl pH 6.8 and then subjected to equilibration procedures and SDS-PAGE as described previously. The obtained 2D-gels were washed and equilibrated in a transfer buffer [50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20% (v/v) methanol] and protein transfer from 2D gels onto NC membranes was carried out using a semidry Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm² for 75 minutes. Protein transfer was checked with staining of membranes with 0.2% (w/v) Ponceau S in 3% (v/v) trichloroacetic acid. Carbonylated proteins were detected with anti-DNP antibodies (Sigma-Aldrich) (at the dilution of 1:10000) and secondary horseradish peroxidase-linked antibodies (at the dilution of 1:7000), followed by a chemiluminescence reaction using the Immun-starTM HRP kit (Bio-Rad).

3.6.3 Western blot detection of protein thiols labelled with biotinylated iodoacetamide (BIAM)

Before the IEF, K310 proteins were derivatized in MES-Tris buffer (pH 6.5) containing 200 µM BIAM for 15 minutes in the dark. The labelling reaction was quenched by the addition of 2 mM β-mercaptoethanol (final concentration) (Kim et al., 2000). Then proteins were resolved through 2D-PAGE and transferred onto NC membranes as already described (see 3.6.2). BIAM-labelled proteins were detected with horseradish peroxidase (HRP)-linked streptavidin (dilution 1:10000), followed by a chemiluminescence reaction using the Immun-starTM HRP kit (Bio-Rad).

3.6.4 Image analysis

Digitalized images of Coomassie stained gels and Western Blot films were acquired (Image Scanner, Amersham Biosciences) and analyzed with Image MasterTM Platinum (Amersham Biosciences). For comparative proteomic analysis, spot % relative abundance was used to compare herbicide-treated samples with the untreated control. In case of multiple spots identified as different molecular species of a same protein, the average % relative abundance was calculated.

⁶ Sodium dodecyl sulfate

⁷ Iodoacetamide

⁸ 2,4-Dinitrophenylhydrazine

3.6.5 Protein identification

Protein spots were identified by gel matching with previously produced and characterized proteomic maps of K310 strain (Trabalzini et al., 2003a; Trabalzini et al., 2003b). Alternatively, spots from 2D gels were excised, minced, washed with water, in-gel reduced, S-alkylated, digested with trypsin and identified by MALDI-ToF⁹ mass spectrometry as described previously (Braconi et al., 2009).

4. Results and discussion

4.1 Yeast colony forming ability and fermentative performance

In a first phase of work, we aimed at investigating how the herbicides P, S and P E, with AIs belonging to different classes (T, a sulfonylurea; G, an organophosphate, and F, an aryloxyphenoxy-propionate, respectively) can affect colony-forming ability and ethanol production in a wild-type wine yeast strain. We decided to use commercial grade herbicides in order to reproduce, as far as possible, the same conditions during application in crops, and to compare results with those obtained in parallel with single high-purity AIs.

The tested herbicides were chosen among those authorized and employed in central regions of Italy during the last years. At the same time, we chose, as the eukaryotic cell model, a *S. cerevisiae* strain adopted for oenological applications instead of a conventional commercial or laboratory-adapted/mutated baker's yeast strain. This type of yeast may reside on grapes in vineyards of the same geographical areas where herbicides are used (Vagnoli et al., 1993). Therefore, the use of a locally isolated wine wild-type *S. cerevisiae* strain is advantageous with respect to other previous approaches since this unmanipulated strain can reveal biological effects closer to physiological ones. Additionally, K310 strain is well-characterized physiologically, for its protein repertoire and stress response (Trabalzini et al., 2003a; Trabalzini et al., 2003b; Santucci et al., 2000; Martini et al., 2006; Martini et al., 2004; Ricci, et al., 2004).

In Fig. 1, results obtained for colony forming ability assays are reported. Only in the case of 100 mg/L P and 75mg/L T a good homology was found. As a matter of fact, both the tested compound were found to negatively affect yeast colony forming ability up to the mid-log phase of growth (Fig. 1 A,B). On the contrary, the commercial herbicides S and PE (Fig. 1 A) were found to be more cytotoxic than their pure AIs since they had negative effects in colony forming ability whereas no significant differences were found for G- and F- treated cells with respect to controls (Fig. 1 B).

Fermentative performances of herbicide- treated yeast cells were evaluated measuring ethanol levels in culture medium (Fig. 2). As observed for colony forming ability, only in the comparison between the effects of P and its AI T a good homology was found, since we detected in both the samples a similar delay in ethanol production with respect to the untreated control (Fig. 2 A, D). On the contrary, whereas G- and F- treated cells showed no differences with respect to their controls in ethanol levels (Fig. 2B, C), their commercial formulations S and PE had significant negative effects for K310 fermentative performance (Fig. 2 E, F). Importantly, in the case of PE- treated cells, fermentation showed a 100 hours delay *versus* the untreated control (Fig. 2 C).

The finding that the herbicide S, differently from its pure AI G, had clear negative effects on K310 was in accordance with findings by Low and colleagues. They reported that bakers'

⁹ Matrix-assisted laser desorption/ionization - time of flight

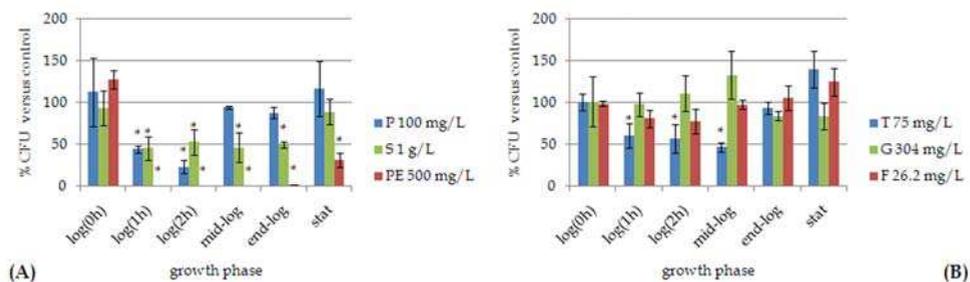


Fig. 1. Colony forming ability assay. K310 cells were treated with the tested herbicides as commercial formulations (A) or their pure AIs (B) and colony forming ability was evaluated by plate assay. Results are reported as % CFU *versus* control (cells grown in standard medium for P-, S-, PE, and G- treated cells; DMSO vehicle for T- and F- treated cells, as detailed under Material and Methods). Data are presented as average values of three independent experiments carried out under identical conditions; standard deviation is indicated with vertical bars. * $P < 0.05$ compared with control.

yeast can metabolize up to 20% of G during the bread making process, probably with detoxification mechanisms which can result in the production of new degradation compounds, whose action is still unknown (Low et al., 2005).

Additionally, our finding is also in good agreement with a consistent number of reports indicating that commercial formulations containing G are much more toxic than G alone in several organisms/cells (dos Santos et al., 2005; Tsui & Chu, 2003; Peixoto, 2005; Benedetti et al., 2004).

Treatment of yeast cells with the herbicide PE induced a period of latency with a significant loss of colony-forming ability, followed by a restoration of the exponential phase of growth, presumably attributable to a cell population adapted to the chemical stress. This finding is in accordance with previously reported works on 2,4-D herbicide, to which yeast cells were proven to adapt before restoring growth (Viegas et al., 2005).

In the overall, our data strongly indicated how the effects produced by commercial herbicide formulations should be distinguished by those produced by pure AIs. This pointed out that coformulants can substantially contribute to cell damage. Such a damage cannot be revealed if, as usual, single AIs are tested. Because AIs are never applied alone in crops but always with several coformulants, our data reinforced the hypothesis that, for ecotoxicological considerations, commercial compounds are the most appropriate ones to be tested. Additionally, our data for S are particularly important considering that the introduction in the market of crops genetically tolerant to G will probably result in an increased use of this herbicide.

Our results should be read considering yeast not only as an eukaryotic model, but also and especially as a microorganism used for the production of wine, nowadays considered in all respects as a food. We found that significant amounts of herbicides can inhibit initial wine-making steps. Additionally, considering yeast ability to metabolize several compounds during alcoholic fermentation for the production of H_2S and other sulphur compounds (for example, in physiological conditions, intermediates of methionine biosynthesis), the possibility that yeast strains could utilize sulphur compounds contained in commercial formulations should not be underestimated. This could have important consequences on

productivity of wine-making processes, from either a quantitative (fermentation yield and final ethanol production) or a qualitative point of view (organoleptic and sanitary properties of produced wines), thus with relapses for both the economic profile and the consumers' health. Altogether, results of this comparative analysis strongly encouraged us to deepen our studies on the effects of commercial herbicides on yeast cells.

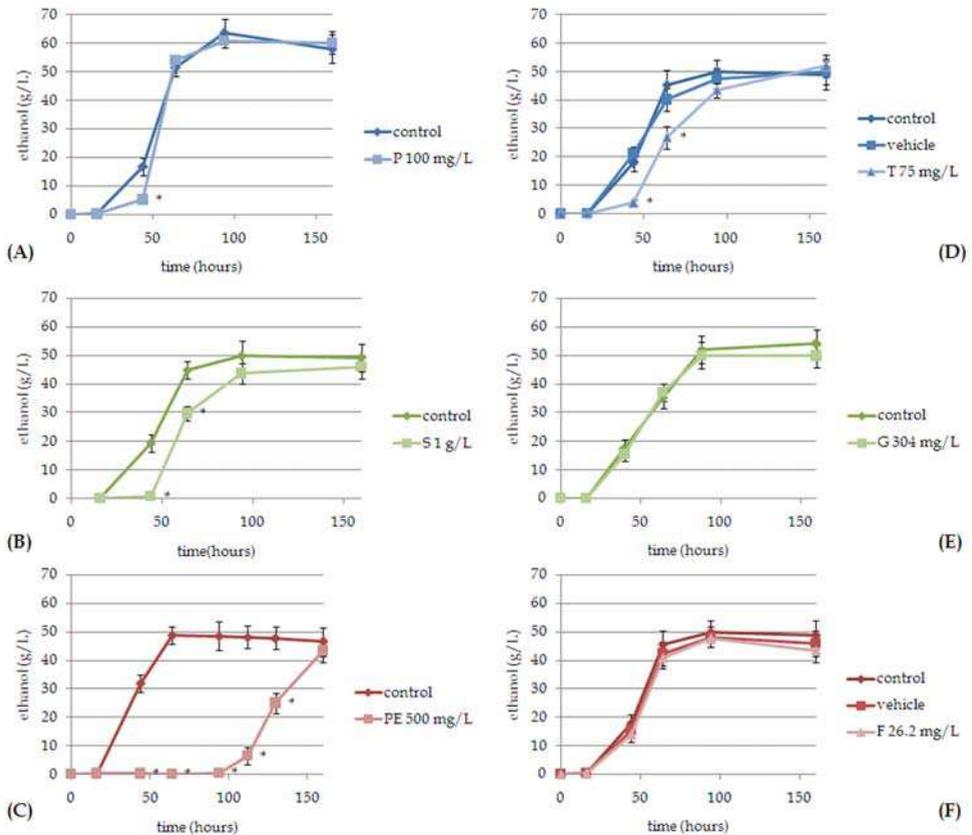


Fig. 2. Fermentative performance assay. K310 cells were treated with the tested herbicides as commercial formulations (A-C) or their pure AIs (D-F) and fermentative performance was evaluated by measuring ethanol levels in culture medium. Results are reported as g/L ethanol. Cells grown in standard medium were used as a control for P-, S-, PE, and G-treated cells; a DMSO vehicle for T- and F- treated cells, as detailed under Material and Methods, was used as well. Data are presented as average values of three independent experiments carried out under identical conditions; standard deviation is indicated with vertical bars. * $P < 0.05$ compared with control.

4.2 Analysis of nucleotide patterns

On the basis of our preliminary comparative investigations (Braconi et al., 2006a; Braconi et al., 2006b), from this point on we carried out our analyses only in cells treated with the

commercial herbicides P, S and PE. Since we found that significant negative effects could be induced in a very short time after the addition of herbicides to yeast culture, our analyses were limited to 2 hours of treatment with the tested compounds. First, we investigated through an HPLC analysis if the tested herbicides could impair the K310 intracellular pools of some triphosphate nucleotides, their hydrolysis products, as well as NAD e NADP (Fig. 3).

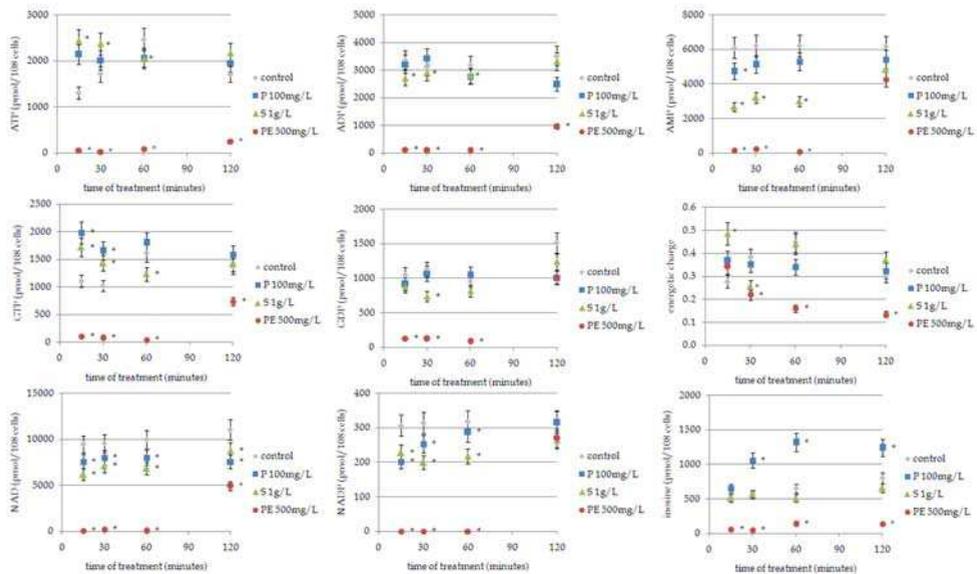


Fig. 3. HPLC analysis of nucleotide patterns in K310 cells treated with commercial herbicide formulations (P 100 mg/L, S 1 g/L or PE 500 mg/L) or in the absence of herbicides (control). Data are presented as average values of three independent experiments carried out under identical conditions; standard deviation is indicated with vertical bars. * $P < 0.05$ compared with control.

When grown in the presence of 100 mg/L P, K310 cells exhibited a sudden raise in ATP and GTP levels; conversely, ADP, GDP and total energetic charge were quite similar between P-treated and control cells. Inosine levels were higher in the presence of P, whereas AMP, NAD and NADP levels were lower in P-treated cells *versus* the control.

Responses obtained treating K310 with 1 g/L S were partially superimposable with those obtained with P. The treatment with S made ATP and GTP levels raise, concomitantly with the decrease in AMP, NAD and NADP levels. The total energetic charge was significantly lowered after 30 minutes treatment in S-treated cells, while similar to the control at the other samplings.

When exposed to 500 mg/L PE, K310 cells showed the most important alterations in nucleotide patterns. All the parameters under evaluation were decreased by PE treatment during the whole observed period, though values registered after 120 minutes indicated a raise in AMP, GDP and NADP levels.

Stress responses involve many adaptive mechanisms in a number of metabolic processes to allow cell homeostasis and maintenance of the integrity of those structures necessary to survive. Yeast cells acquired, through the evolution, the ability to cope and survive to

sudden environmental changes, and developed a peculiar ability in changing the internal *milieu* to adapt to new growth conditions. One of the most relevant aspects of this response is cell energetic balance, and for this reason we decided to investigate, through an HPLC analysis, if P, S and PE could alter nucleotide patterns in K310 cells.

Globally, our results suggested that both P- and S-treated cells had to face an increased energy demand to cope with the herbicide-induced stress, phenomenon that had been already reported for yeast cells exposed to the herbicide 2,4-D (Teixeira et al., 2005). On the other hand, they seemed to indicate a sudden impairment in the redox balance, as indicated by the lower levels of NAD and NADP, which are indirect indexes of a decreased reducing power generated by oxidative stress. Additionally, the increased levels of inosine represent an indirect index that cytotoxic effects of the tested herbicides might be exerted through oxidative mechanisms (Carvalho et al., 2003).

Our results also showed that PE-mediated cytotoxicity in K310 cells could be the result of important alterations of the cell redox homeostasis and/or imbalances of vital cell metabolic processes that cells could not counteract. Nevertheless, the trend showed by various parameters after 120 minutes of treatment with PE suggested the existence of a population of yeast cells which can adapt and survive to such stressing growth conditions, like already indicated by our previous experiments (Braconi et al., 2006a) (Braconi et al., 2006b).

4.3 Monitoring of herbicide-induced oxidative stress

Possible oxidative damage induced by G-containing herbicides has been already postulated (Beuret et al., 2005; Gehin et al., 2005; Pieniazek et al., 2004; Richard et al., 2005). Considering these findings in the light of our previous investigations on nucleotide patterns, which suggested a redox imbalance in herbicide treated cells, we decided to investigate if herbicide treatments could affect the enzymatic activity of catalase and superoxide dismutase (SOD), two key enzymes in the cell response to oxidative stress. In order to avoid the production of interfering substances during a spectrophotometric assay due to the presence of herbicide residues, we chose to evaluate catalase and SOD enzymatic activities by specific staining after native PAGE electrophoresis of K310 proteins (Braconi et al., 2008). Images of gels were acquired and quantitatively evaluated selecting the intensity of unstained active protein bands, and reported these values as arbitrary units in Fig. 4.

During a 2 hours treatment, only S was able to promptly induce catalase activity even when such an enzymatic activity was not detectable under control conditions or in the presence of P or PE (Fig. 4A). It is known, in fact, that a high glucose concentration in culture medium (such as it can be found in the conditions we tested) can exert a negative pressure on antioxidant systems, catalase included (Martínez-Pastor et al., 1996). The activation of catalase is one of the most common cellular responses to redox alterations, since this enzyme is easily induced by a wide range of *stimuli* often related to the energetic status of the cell. In turn, we could hypothesize that catalase activation in S-treated cells might represent an adaptation mechanism to the herbicide-induced oxidative stress.

Results obtained for SOD activity (Fig. 4B) showed quite a different situation. In the case of S, SOD activity was initially lowered (30 minutes treatment) then restored (60 minutes) and increased (120 minutes) in respect to control. In the presence of P and PE, SOD activity was suddenly decreased by both herbicides with effects that were observable throughout the whole observed period.

The general inactivation of SOD observed after short treatments with herbicides could be the result of oxidative damage of this enzyme, which is known to be inactivated by various

peroxides (Pigeolet et al., 1990). This can probably reflect the decreased ability of yeast cells to adapt efficiently to the oxidative stress encountered. A sudden decrease in SOD activity had been already associated to brief treatments of human erythrocytes with the herbicide 2,4,5-trichlorophenoxyacetic acid and to one of its metabolites (2,4,5-trichlorophenol) (Bukowska, 2004). Whereas SOD is considered an essential anti-oxidant enzyme, at the same time it can have pro-oxidant effects *in vivo* (Lushchak et al., 2005), and thus SOD inactivation can be read as a defence mechanism as well.

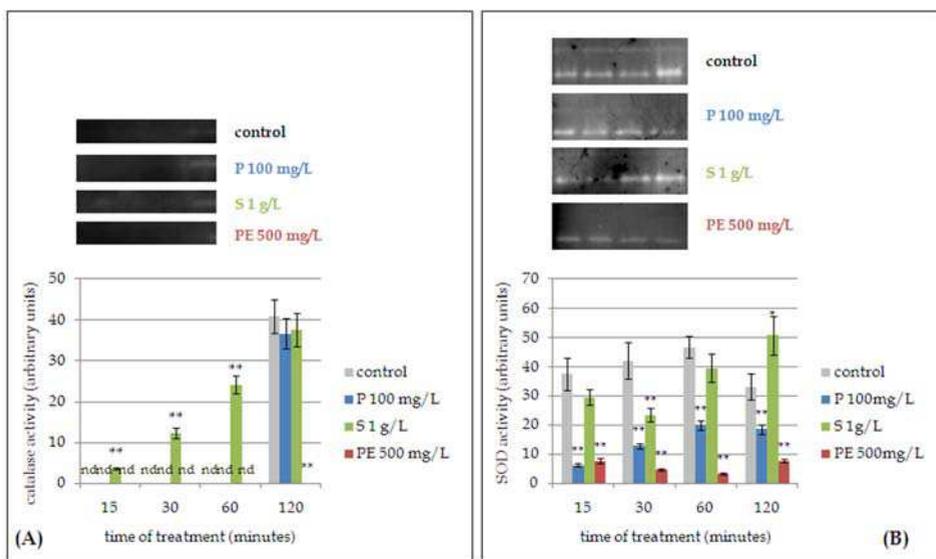


Fig. 4. Native PAGE and staining of active catalase (A) and SOD (B) in K310 cells treated with commercial herbicide formulations (P 100 mg/L, S 1 g/L or PE 500 mg/L) or in the absence of herbicides (control). Quantitative evaluation was performed on band intensities, as detailed under Material and Methods. Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. * $P < 0.05$ compared with the control culture.; nd: not detectable

Literature reports about the activity of antioxidant enzymes after herbicide treatments are quite heterogeneous, probably reflecting different experimental conditions and the specificity of stress responses in different systems. Other factors that must be taken into account are the different ROS types that can be generated, their toxicity for the cells, their detoxification rate as well as the involvement of enzymatic and non-enzymatic defense mechanisms. Only as a few examples, pure G did not alter catalase and SOD activity in the liver of rats (Beuret et al., 2005) while SOD activity in roots and leaves of pea plants was not affected by the treatment with imazethapyr, an imidazolinone herbicide (Zabalza et al., 2007). Moreover, while catalase activation in yeast had been already associated to the herbicide 2,4-D (Teixeira et al., 2004; Teixeira et al., 2007), either a commercial formulation containing 2,4-D or pure G did not alter catalase activity in mice (Dinamarca et al., 2007). Nevertheless, our data for S are in good agreement with a recent report for another G-based formulation (Roundup Ultra 360 SL) that induced catalase activity in human erythrocytes after very short treatments (Pieniazek et al., 2004).

4.3 Proteomics and redox-proteomics

4.3.1 Comparative proteomic analysis of herbicide-induced effects

In order to gain a deeper insight into the effects of P, S and PE on K310 protein profile, we carried out a comparative proteomic analysis combining 2D-PAGE and protein identification by gel-matching or, alternatively, MALDI-ToF mass spectrometry.

Proteome analysis is conceptually attractive because it fits with the concept that determination of protein rather than mRNA levels has major advantages as it is proteins that carry out functions within the cells (Jamnik & Raspor, 2005). In our work, we found that the herbicides P, S and PE could affect the expression of several yeast proteins, which were identified, classified according to their cell function, and reported in detail in a previous paper of ours (Braconi et al., 2009). Among the differently expressed proteins, many were found to be involved in cell rescue and defence (Fig. 5A). This group includes a heterogeneous set of molecular chaperones, such as heat shock proteins (HSPs), devoted to guarantee the correct protein folding and to avoid protein aggregation (Young, 2004). HSPs are induced in response to a wide variety of *stimuli* and many of them may help in regulating cell metabolism under stressing conditions.

Additionally, the comparative proteomic analysis showed very clearly the under-expression of several enzymes belonging to the carbohydrate metabolism group in herbicide-treated cells (Braconi et al., 2009). Our data were, again, in accordance with works studying cellular responses to oxidative stress (Godon et al., 1998; Magherini et al., 2007; Perzov et al., 2000; Sales et al. 2000; Weeks et al., 2006) and underlined the susceptibility of glycolysis to oxidative insults. A decrease in the activity of glycolytic enzymes is thought to be beneficial during exposure to oxidants (Costa et al., 2002; Shanmuganathan et al., 2004). This finding suggested, in turn, the need of cells to save energy to face the herbicide-induced

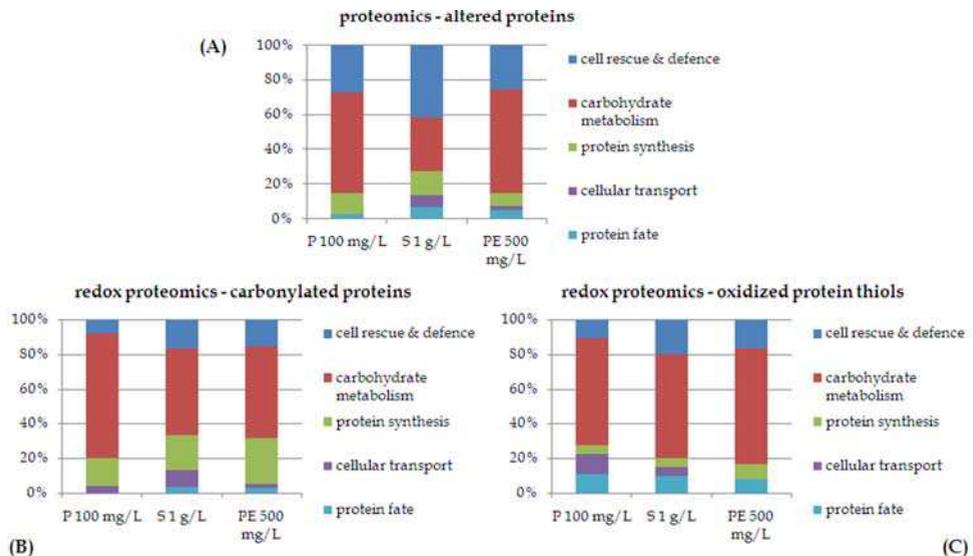


Fig. 5. Functional classification of: proteins differently expressed in K310 cells treated with the herbicides P, S or PE *versus* the untreated control (A), and proteins carbonylated (B) or with oxidized thiols following herbicide treatment (C).

stress as well as the redirection of cell metabolic fluxes to set up appropriate defence mechanisms. Alterations of carbohydrate metabolism for the generation of NADPH is a fundamental cellular response to the oxidative stress, since this factor is required for many defence mechanisms such as glutathione reductase and thioredoxine reductase, which have a critical role in maintaining the proper redox-balance of thiol groups. At the same time, the inhibition of the glycolytic route could support the production of protective substances, such as trehalose.

4.3.2 Redox-proteomic analysis of herbicide-induced oxidation of proteins

Oxidative stress occurs when the rate at which ROS are generated exceeds the capacity of the cell to remove them by anti-oxidants. When an increased ROS production is concomitant with a reduction of anti-oxidant systems, cells have to cope with oxidative damage of proteins, DNA and lipids. A large body of evidence suggests that herbicides may promote toxic events via the intermediate release of ROS which, because of their extreme reactivity, lead to the formation of lesions in target molecules. Although this notion was established for an array of herbicides, including some of those employed in the present study (Soltaninejad. & Abdollahi, 2009; Muniz et al., 2008), very little information is available on the specific molecular targets of such a damage.

Since proteins are considered the most important targets of oxidative stress (Sheehan, 2006), we evaluated through redox-proteomic techniques two commonly investigated oxidative post-translational modifications (PTMs) of proteins: carbonylation and thiol oxidation.

The introduction of carbonyls groups into proteins is an irreversible, easy detectable and non-enzymatic PTM often accompanied by loss of function. For these reasons, it is universally accepted as a good indicator of oxidative stress (Dalle-Donne et al., 2003a; Dalle-Donne et al., 2003b; Dalle-Donne et al., 2006; Dalle-Donne et al., 2005). Carbonylation of mistranslated or otherwise aberrant proteins points to an important physiological role of carbonylation in the control of protein quality. Being irreversible, carbonylation can be a signal for a protein degradation pathway rather than the chaperone-assisted repair. This can in turn act as a mechanism providing amino acids for *de novo* protein synthesis by targeting proteins that are no longer required or have become damaged to the degradation (Nyström, 2005).

In our work we found that carbonylation is a rapid and dynamic event, mainly affecting proteins involved in the cell rescue and defence or in the carbohydrate metabolism (Braconi et al., 2009) (Fig. 5B), with good homology with previous studies on yeast cells treated with oxidizing agents (Cabisco et al., 2002; Shanmuganathan et al., 2004; Dirmeier et al., 2002; Reverter-Branchat et al., 2004; Sumner et al., 2005). Nonetheless, if the herbicide P and S induced the carbonylation of specific patterns of K310 proteins, in the case of PE protein carbonylation was nearly random (Braconi et al., 2009).

Since carbonylation often induces alterations in protein structure and function, the carbonylation of proteins with fundamental cellular roles might explain the negative effects observed in the analysis of herbicide-induced toxicity in K310 cells. Hence, the carbonylation of HSPs may counteract their beneficial effect in protecting cells and provide at the same time a pivotal decisional checkpoint to determine the fate of cells subjected to oxidative damage. Additionally, carbonylation of glycolytic enzymes might account for herbicide-induced alteration of yeast fermentative performances and have negative relapses in the wine-making process.

Thiol oxidation has a pivotal role during oxidative stress, since it has an increasingly recognized role in protein structure/function and redox signalling (Biswas et al., 2006). If on

the one hand thiol oxidation can activate specific protein functions or protect critical residues from irreversible oxidation, on the other it can generate a wide range of chemically reactive sulphur species propagating the oxidative imbalance in the form of pro-oxidant redox cascades (Jacob et al., 2006).

Either in the case of treatment of K310 cells with P, S or PE, we were able to point out herbicide-mediated oxidative damage towards yeast protein thiols (Braconi et al., 2010). Once classified according to their functions, we found that many of the proteins containing oxidized thiols were involved in carbohydrate metabolism (Braconi et al., 2010) (Fig. 5C), confirming what recently observed by other authors (Le Moan et al., 2006; Magherini et al., 2009; McDonagh et al., 2009). Our results globally indicated that thiol oxidation could coordinate the metabolic response to herbicide-induced oxidative stress and regulate fluxes of carbohydrates (McDonagh et al., 2009); this could eventually have, in turn, negative relapses on the fermentative performance of yeasts. Thiol specific oxidation was observed also for some proteins involved in the stress response or covering specific important functions in yeast cells (Braconi et al., 2010) (Fig. 5C), in good agreement with results from Le Moan (Le Moan et al., 2006).

5. Conclusions and future perspectives

The importance of evaluating the potential toxicity of complete pesticide formulations, rather than just testing their AIs, began to be appreciated only recently. In this context, the yeast *S. cerevisiae* is an optimal model system to study herbicide-induced toxicity and, especially, herbicide-induced oxidative stress. Cell stress studies are usually carried out in baker's yeast strains, often genetically manipulated, under extreme environmental conditions very unlike the physiological ones. Whereas baker's *S. cerevisiae* strains have been widely studied both by genome sequencing and other types of characterization, brewing or wine yeasts are still more neglected. Nevertheless, oenological *S. cerevisiae* strains are much more interesting cell models for eukaryote stress studies, since they are constitutively stress-resistant, adapted to grow under the stressful conditions of grape must (glucose excess and then rapid exhaustion, low assimilable nitrogen, low pH, low content of oxygen, high concentration of ethanol) and thus can represent a good example of tolerance/adaptation response.

In our work, we used a wild-type wine yeast strain, namely K310, for the evaluation of toxic effects induced by three herbicides. Hence, K310 strain should be considered not only a good model cell, but also a microorganism with important biotechnological applications in the wine making process. Globally our findings reinforced the hypothesis that, for eco-toxicological considerations, commercial pesticides should be tested in parallel with their AIs. Our investigations allowed us to highlight how significant amounts of herbicides can inhibit initial wine-making steps and have negative impacts on yeast fermentative ability, having in turn negative consequences for the biological processes that oenological *S. cerevisiae* strains are supposed to properly carry out. Protein oxidation, and specifically carbonylation and thiol oxidation of enzymes involved in the fermentative processes as well as in the cell rescue and defence, was suggested to play a key role in herbicide-induced toxicity.

6. References

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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