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# WILD-TYPE WINE *SACCHAROMYCES CEREVISIAE* AS A TOOL TO EVALUATE THE EFFECTS ON EUKARYOTIC LIFE OF LOCALLY USED HERBICIDES

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ABSTRACT

Nowadays, agricultural pesticides are frequently used to avoid attacks by weeds, moulds, bacteria, insects and mice that can damage cultivars. They belong to the heterogeneous class of xenobiotics. The toxicity of these compounds on mammals is often unknown. Thanks to the high degree in the evolutionary context of generic stress responses, understanding how yeast cells respond to pesticides can contribute in elucidating toxicity mechanisms in more complex and less accessible eukaryotes. In this work, we aimed to investigate how three herbicides belonging to different classes, never tested before on eukaryotes, can affect the biological activity of enological *Saccharomyces cerevisiae* strains, with particular attention to cell growth, viability and ethanol production. We also evaluated the global metabolic profile of yeast cells by testing several different carbon sources and how metabolic patterns could be affected by xenobiotics. Differing from all the previously reported works, we used commercial grade herbicides, authorized for use in the central regions of Italy, in order to reproduce as far as possible the same conditions applied in the fields. At the same time, we chose, instead of a commercial strain, a wild-type *S. cerevisiae* strain adopted for enological applications, existing on vineyards of the same geographical areas where herbicides are used, in order to get important information also on the safety of locally produced wines.

*Keywords: active ingredients, biological system, enology, eukaryotes, herbicides, pesticides, Saccharomyces cerevisiae, toxicity, vinification, xenobiotics.*

## 1 INTRODUCTION

Nowadays, agricultural pesticides are frequently used to avoid attacks by weeds, moulds, bacteria, insects and mice that can potentially damage cultivars. They belong to the high heterogeneous group of compounds called xenobiotics, which have been defined as chemicals foreign to biological systems and include industrial chemicals and environmental pollutants [1].

The widespread and intensive use, but often misuse due to careless applications or wrong spillage, of fungicides, insecticides and herbicides has resulted in a large number of environmental and toxicological problems, with a consequent growing concern for human health. The toxicity of these compounds on mammals is often unknown and available data refer to other species rather than human beings [2, 3].

Moreover, pesticide applications can seriously affect soil quality and have a deleterious impact on resident microorganisms. The latter is dependent on active ingredients, commercial formulations, applied doses, soil type and climatic conditions too [4].

Considering the large amounts of agricultural pesticides released into the environment, a further toxicity evaluation appears necessary. To date, toxicological risk assessments of pesticides have been conducted using mammals (e.g. rats, mice, guinea pigs and so on) to study both acute and chronic effects. In order to avoid animal utilization, and due to the large demand for the development and validation of simple and rapid screening procedures, in the last few years a number of publications have dealt with some alternative methods that do not raise ethical problems and are at the same

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time appropriate and cost-effective [5]. Short-term toxicity assays based on the evaluation of the xenobiotic effects on biological functions or enzyme activities of several biological systems are a continuously growing field of investigation. Particularly, soil microorganisms, aquatic organisms, insects and birds have been chosen to determine the pesticide ecotoxicological impact [6]. Among the alternatives to animal experimentation, the yeast *Saccharomyces cerevisiae* has emerged as an optimal eukaryotic model system, and we chose it to investigate the effects of three locally used herbicides. The intriguing features that yeast possesses motivated our choice. In fact, yeast is a more complex organism with respect to bacteria and prokaryotes in general, whose metabolic mechanisms are too simple to be extrapolated to eukaryotes. At the same time, it is a simple biological system to work on. It is a unicellular, non-pathogenic and easy to manipulate organism that can grow in a synthetic culture medium, thus allowing experimental reproducibility and continuous checking of its physical and chemical conditions. *S. cerevisiae* has been well characterized too, from the genomic, metabolic and proteomic points of view. It was the first eukaryote whose genome was completely sequenced in 1996 [7], and a huge amount of data is now available about its metabolic pathways and its protein repertoires. Moreover, it is well known that using *S. cerevisiae*, it is possible to study biological mechanisms common to fungi, plants, animals and humans. Particularly, thanks to the high degree in the evolutionary context of generic stress responses, understanding how yeast cells respond to pesticides can contribute in elucidating the toxicity mechanisms in more complex and less accessible eukaryotes [8]. As reported, yeast has already been proposed as a tool for assessing the toxicity of environmental pollutants and the alterations caused by environmental insults [2, 3, 9].

We decided to conduct our study on an enological and wild-type yeast strain, and this allowed us to investigate if the fermentation process could be affected by herbicides. Literature can provide a number of studies assessing the impact of fungicides, insecticides and herbicides on yeast growth, viability, biomass production and fermentation ability. In particular, most of the efforts have been dedicated to fungicides used for treatments in vineyards, either for establishing the fate of the compounds during the vinification steps or for elucidating the effects on the fermentation process [5, 10–17]. On the contrary, studies concerning the effects of herbicides on *S. cerevisiae* are more rarely found and are almost limited to 2,4-dichlorophenoxyacetic acid and its derivates [2, 3, 9, 18–21].

In this work, we aimed to investigate how three herbicides (Proper Energy®, Pointer® and Silglif®) with active ingredient (a.i.), belonging to different classes (aryloxyphenoxy-propionates, sulfonylureas and organophosphates respectively) can affect the biological activity of an enological *S. cerevisiae* strain, with particular attention to growth, viability and ethanol production. Moreover, we wanted to evaluate the global metabolic profile of yeast cells in response to such stress. We decided to use commercial grade herbicides in order to reproduce as far as possible the same conditions during application in fields. We chose compounds that have been authorized by the law and used in the central regions of Italy during the last few years. At the same time, we chose, as the eukaryotic cell model, an *S. cerevisiae* strain adopted for enological applications instead of a conventional commercial or laboratory adapted/mutated baker's yeast strain. This type of yeast exists on grapes in vineyards of the same geographical areas where herbicides are used. Thus, the use of a locally isolated wine wild-type *S. cerevisiae* strain is advantageous over other previous approaches for several reasons. First of all, an unmanipulated strain can reveal biological effects closer to physiological ones. Moreover, the effects of herbicides on yeast strains utilized for local wine production may also give vital information on the safety of the wine produced.

The results obtained showed that Proper Energy® was the most toxic herbicide formulation in regard to growth, viability and fermentation ability, while the impact of the other two herbicides was much less pronounced. Interestingly, considering the metabolic profiles, Proper Energy® was also the herbicide with the most important effects from a qualitative point of view, while Pointer® gave an important alteration from a quantitative point of view.

## 2 MATERIALS AND METHODS

### 2.1 General materials

All high-purity reagents were from Oxoid (Garbagnate M.se, Milan, Italy) and J. T. Baker (Deventer, Holland).

Herbicides used (commercial grade) were: Proper Energy® (PE) and Pointer® (P) (Aventis CropScience, Milan, Italy), Silglif® (S) (Siapa, Milan, Italy).

All water used was from Milli-Q (Millipore, Bedford, MA, U.S.A.).

### 2.2 Yeast strain, culture conditions and viability assays

The yeast strain used in this work was *S. cerevisiae* (namely K310), which was isolated from naturally fermenting must during the vinification of high-quality wine, in the geographical area of Brunello di Montalcino [22]; it has been well characterized physiologically, for its protein repertoire and stress response [23–27]. K310 was pre-cultured in yeast peptone dextrose (YPD) medium at 30°C with rotary shaking up (120 rpm) for 10 hours. At this time, an appropriate aliquot of the cell culture was inoculated in the YPD, 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 cell concentration of $1 \times 10^4$ cells/mL. The cell suspension was then incubated at 28°C without shaking and allowing semi-anaerobic growth. Overall, the culture conditions adopted were similar to those of vinification, although with control and reproducibility of single experiments in the laboratory.

The cell growth was monitored by measuring the culture absorbance at 660 nm; at chosen periods, samples were collected and the pH checked. Viability assays were conducted in triplicate by plating on YPD–agar proper dilutions (ranging from 1 : 10 up to 1 : 100000) of the cell suspension; the plates were then incubated at 28°C for three days.

To assess the reproducibility, all the experiments were conducted in triplicate; the data presented here are average values with standard deviations.

### 2.3 Herbicide supplementations

Herbicides that were used in this work and their most relevant features are listed in Table 1. For the experiments, they were singularly added to the culture medium at the beginning of the exponential growth phase (16th hour of cell culture) by adding appropriate aliquots of the dispersion/emulsion/solution of commercial compounds prepared with water just prior to use. Concentrations that were used and the relative amount of active ingredients for each compound are listed in Table 2a, b and c.

For each experiment, five different concentrations of commercial grade herbicides were tested on *S. cerevisiae* K310; in parallel, a control culture with no supplementations was prepared too.

### 2.4 Determination of ethanol concentration

The levels of ethanol were determined using an enzymatic assay (kit code 10 176 290 Boehringer Mannheim, Germany) following the protocol proposed by Mashego *et al*. [28, 29] with minor modifications. 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 the determination of the ethanol concentrations was performed spectrophotometrically (Agilent 8453 UV-visible spectroscopy system, Waldbronn, Germany) on the obtained filtrate, properly diluted in accordance with the manufacturer's instructions.

Table 1: Commercial grade herbicides used in this work and their most important features: physical state and solubility, active ingredients and mechanism of action.

| Trade name (short name) | Physical state and solubility | Active ingredients (concentration) | Chemical class | Mechanism of action |
|---|---|---|---|---|
| Proper Energy® (PE) | Oil/water emulsion | Fenoxaprop-P-ethyl (55 g/L) | Aryloxyphenoxy propionate | Disruption of fatty acid biosynthesis in grasses by selective inhibition of the enzyme acetyl-CoA carboxylase |
| | | Mefenpyr-diethyl (30 g/L) | Pirazoline derivate | Safener that protects treated crop plants from herbicide injuries through glutathione S-transferase-mediated reactions |
| Pointer® (P) | Water dispersible granules | Tribenuron methyl (750 g/L) | Sulfonyl urea | Inhibition of the enzyme acetolactate synthase also known as acetohydroxyacidic synthase, involved in essential amino acid biosynthesis |
| Silglif® (S) | Soluble concentrate | Glyphosate (304 g/L) | Organophosphate | Inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, involved in the biosynthesis of aromatic compounds |

Table 2: (a–c) Concentrations of commercial herbicides that were tested on *S. cerevisiae* K310 strain and relative concentrations of active ingredients.

(a)

| Proper Energy® | Fenoxaprop-p-ethyl (MW 361.8) | Mefenpyr-diethyl (MW 373.2) |
|---|---|---|
| 500 mg/L | 26.20 mg/L (72.42 μM) | 14.30 mg/L (38.32 μM) |
| 250 mg/L | 13.10 mg/L (36.21 μM) | 7.15 mg/L (19.16 μM) |
| 100 mg/L | 5.24 mg/L (14.48 μM) | 2.86 mg/L (7.66 μM) |
| 10 mg/L | 524 μg/L (1.45 μM) | 286 μg/L (0.77 μM) |
| 1 mg/L | 52.40 μg/L (0.15 μM) | 28.60 μg/L (0.08 μM) |

(b)

| Pointer® | Tribenuron methyl (MW 395.4) |
|---|---|
| 100 mg/L | 75 mg/L (189.68 μM) |
| 10 mg/L | 7.50 mg/L (18.97 μM) |
| 1 mg/L | 750 μg/L (1.90 μM) |
| 100 μg/L | 75 μg/L (0.19 μM) |
| 10 μg/L | 7.50 μg/L (0.02 μM) |

(c)

| Silglif® | Glyphosate (MW 169.1) |
|---|---|
| 1 g/L | 304 mg/L (1.80 mM) |
| 500 mg/L | 152 mg/L (0.90 mM) |
| 250 mg/L | 76 mg/L (0.45 mM) |
| 100 mg/L | 30.40 mg/L (179.78 μM) |
| 10 mg/L | 3.04 mg/L (17.98 μM) |

### 2.5 Metabolic profiling

The qualitative metabolic profiles of *S. cerevisiae* K310 were generated by means of the Biolog Microstation System 4.2 (Biolog Inc., Hayward, CA). Yeast-designed plates (YT) were used. Each YT plate is divided into two sections: the top section contains 35 different substrates as single carbon sources and the tetrazolium redox dye, which is irreversibly reduced to an insoluble purple product when cells oxidize the carbon source present in the well. The bottom of the plate consists of 60 wells without the tetrazolium redox dye. The last row of the panel has wells containing two carbon sources: these wells test for the co-utilization of various carbon sources with D-xylose. Some yeast species are inhibited by the tetrazolium violet redox dye, so the YT MicroPlate is configured with both oxidation tests and assimilation tests.

Cells grown for 48 hours on BUY agar (Biolog Inc., Hayward, CA) were collected and processed according to the manufacturer's instructions with minor modifications. The cell concentration was adjusted to 47% of transmittance and aliquots were added to the plate (100 μL/well) with or without the chosen amount of herbicide. Then plates were incubated at 28°C in the dark and each experiment was run in triplicate. Additional control plates containing the proper dilution of herbicides without cells were prepared in order to assess the lack of chemical reactions with herbicides and tetrazolium dye and/or the substrate. The results were recorded by the Microplate Reader (change in optical density, $OD_{590}$) after 24, 48 and 72 hours of incubation. The data were stored using Biolog software. All the replica plates showed very high reproducibility.

Apart from the usual qualitative profiles, quantitative metabolic profiles of *S. cerevisiae* K310 were obtained by calculating two indices of metabolic activity. The first one, the average well color development (AWCD) is calculated as the arithmetic mean of the OD values of all the wells in the plate after 72 hours of incubation. The second one, the maximal rate of color development ($\mu$, expressed in OD/h) represents the slope of the regression line of the non-linear curve calculated plotting the OD value in each well against the incubation time.

## 3 RESULTS AND DISCUSSION

In this work, we used an autochthonous enological *S. cerevisiae* strain, namely K310, to evaluate the impact on its biological activity of three different herbicides (Proper Energy®, Pointer® and Silglif®, active ingredients fenoxaprop-P-ethyl, tribenuron methyl and glyphosate respectively), which have been selected among compounds authorized by the law for crop treatments in the central regions of Italy. K310 is a wild-type *S. cerevisiae* strain isolated during spontaneous fermentation in natural musts and selected as a potential 'starter' for guided fermentations in the production of high-quality wines. This feature is attributed to the ability of K310 to overcome fermentation 'stresses' and to carry out the fermentation process. In this work, we utilized this enological and autochthonous *S. cerevisiae* strain with the aim to evaluate the impact of three different herbicides, as commercial formulations, on its biological activity. Cells were grown in a synthetic medium, in order to obtain experimental reproducibility, modified in order to reproduce, as far as possible, conditions of natural fermentation; with the same purpose, we decided to adopt semi-anaerobiosis conditions and an initial glucose concentration equal to 100 g/L, corresponding to its concentration in natural musts. Monitoring of several parameters during growth confirmed that the responses obtained were attributable only to the stressing agent that was applied. In fact, since cell cultures were maintained at a constant temperature of 28°C and in semi-anaerobiosis, we can exclude the occurrence of heat/cold shocks, oxidative stress and aerobiosis stress. The pH values of the culture media did not differ significantly from the initial 4.5 value (data not shown), thus

excluding the occurrence of changes in the ionic composition that might cause cell stress. Moreover, *S. cerevisiae* being a well-consolidated model in the study of responses to xenobiotics, and K310 being an unmanipulated enological strain grown in conditions similar to the natural ones, responses obtained should be considered more physiological and more directly correlated to higher eukaryotes.

For each commercial herbicide formulation, five different concentrations were tested on *S. cerevisiae* K310; they have been calculated referring to the maximum residue limits, calculated for the relative active ingredient allowed in Italy for several foodstuffs [30]. In particular, the lowest concentration corresponded to an active ingredient concentration similar to that allowed by the law, while others were chosen in order to mimic various conditions of field application during residue formation.

Single herbicide supplementation was done at the beginning of the exponential growth phase, when cells are most susceptible to applied stresses. Then, we evaluated at various times the effects on cell growth kinetics, viability and fermentation ability. On the bases of the obtained results, two different concentrations of herbicides were chosen in order to test their influence on the metabolic profile of *S. cerevisiae* K310. The metabolic profile was obtained by testing the capability to utilize 60 different carbon sources on YT Biolog™ Microplates configured with both oxidation and assimilation tests. The resulting metabolic profiles were analyzed both for qualitative and kinetics aspects. All experiments were carried out in triplicate.

### 3.1 Growth, viability and ethanol assay

#### 3.1.1 Proper Energy®

When *S. cerevisiae* K310 was cultivated in the presence of Proper Energy® 500, 250 and 100 mg/L (Fig. 1, upper panel), a drastic growth inhibition occurred. Nevertheless, we observed in all cases a growth resumption, with an increasing dose-dependent period of latency: 32 hours for PE 100 mg/L, 52 hours for PE 250 mg/L and 78 hours for PE 500 mg/L. For the two lowest herbicide concentrations, no relevant differences in comparison to control were found, excepting a brief period of latency just after herbicide supplementation (about 8 hours long) that however did not affect growth kinetics.

Cell stress was also reflected on cell viability (Fig. 1, central panel) with dramatic cell death following herbicide application at the three highest doses. However, as observed for growth, the cells were able to regain control values: around the 94th hour of cell culture for PE 100 and 250 mg/L, and around the 114th for PE 500 mg/L.

The same pattern was also evident on ethanol production (Fig. 1, lower panel). For control and cell cultures treated with PE 1 and 10 mg/L, the highest ethanol concentration was reached around the 68th hour; for culture treated with PE 100 mg/L this peak was shifted to the 112th hour, with PE 250 mg/L to the 130th hour and with PE 500 mg/L to the 164th hour.

#### 3.1.2 Pointer®

Only the highest concentration of Pointer® (100 mg/L) caused a slight reduction of *S. cerevisiae* K310 growth, viability and ethanol production (Fig. 2). Particularly, while cell viability reached control values after 44 hours of culture, growth and ethanol concentration were equivalent to control only around the 60th hour. For each one of the other concentrations tested, no significant differences with control were observed.

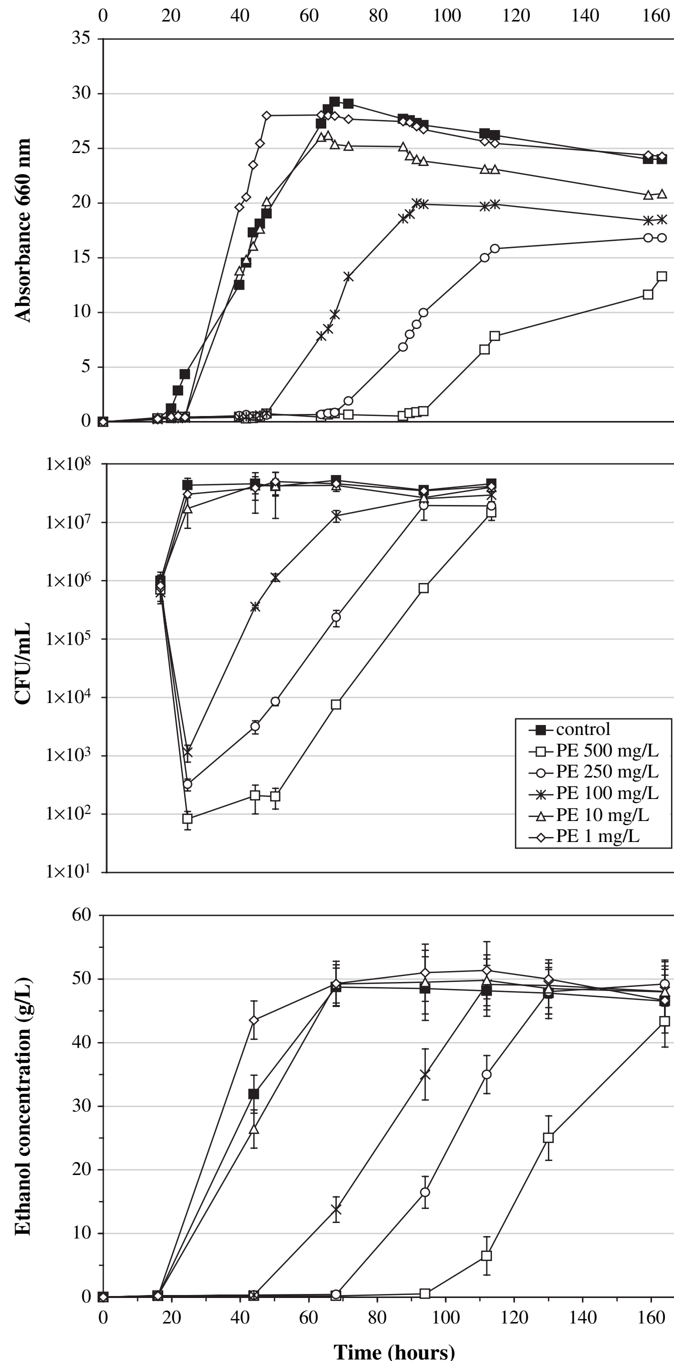


Figure 1: Growth curve (upper panel), viability curve (central panel) and fermentation ability (lower panel) of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of PE at the beginning of the exponential phase or in the absence of herbicide (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations observed for growth are listed in Table 3, while those observed for viability and fermentation ability are indicated with vertical bars.

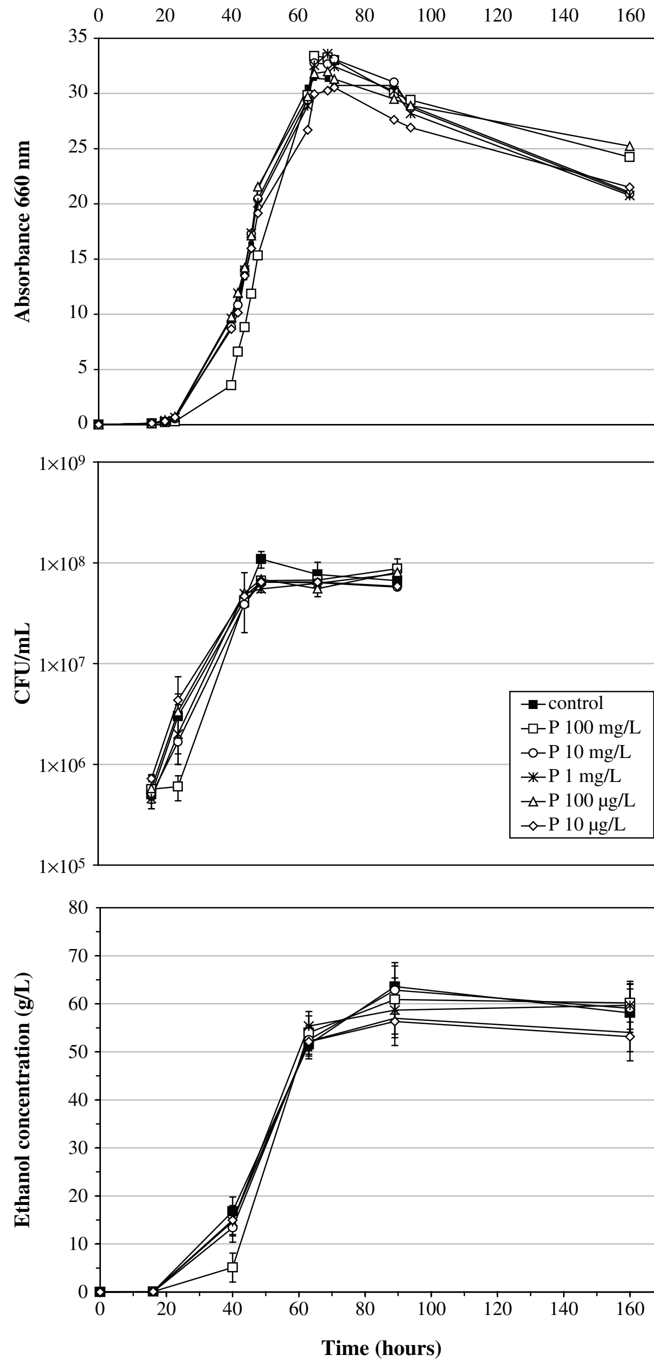


Figure 2: The growth curve (upper panel), viability curve (central panel) and fermentation ability (lower panel) of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of P at the beginning of the exponential phase or in the absence of herbicide (control). The data reported are average values of three independent experiments carried out under identical conditions; standard deviations observed for growth are listed in Table 4, while those observed for viability and fermentation ability are indicated with vertical bars.

Table 3: Standard deviation values referred to growth curve of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of PE at the beginning of the exponential phase or in the absence of herbicide (control) (Fig. 1). Data reported are from three independent experiments carried out under identical conditions.

| Culture (h) | Standard deviation values | | | | | |
|---|---|---|---|---|---|---|
| | Control | PE 500 mg/L | PE 250 mg/L | PE 100 mg/L | PE 10 mg/L | PE 1 mg/L |
| 16 | ± 0.117 | ± 0.088 | ± 0.009 | ± 0.026 | ± 0.059 | ± 0.004 |
| 20 | ± 0.553 | ± 0.070 | ± 0.046 | ± 0.088 | ± 0.020 | ± 0.077 |
| 22 | ± 0.178 | ± 0.066 | ± 0.087 | ± 0.099 | ± 0.054 | ± 0.091 |
| 24 | ± 1.059 | ± 0.042 | ± 0.088 | ± 0.393 | ± 0.066 | ± 0.348 |
| 40 | ± 2.100 | ± 0.097 | ± 0.076 | ± 0.401 | ± 1.764 | ± 2.284 |
| 42 | ± 1.693 | ± 0.014 | ± 0.024 | ± 0.656 | ± 1.562 | ± 2.879 |
| 44 | ± 2.147 | ± 0.274 | ± 0.167 | ± 0.990 | ± 1.328 | ± 2.735 |
| 46 | ± 1.031 | ± 0.165 | ± 0.099 | ± 1.059 | ± 1.005 | ± 1.933 |
| 48 | ± 1.371 | ± 0.031 | ± 0.162 | ± 1.059 | ± 1.422 | ± 2.668 |
| 64 | ± 3.093 | ± 0.078 | ± 0.198 | ± 1.059 | ± 1.304 | ± 2.029 |
| 66 | ± 2.350 | ± 0.024 | ± 0.335 | ± 1.900 | ± 1.880 | ± 0.872 |
| 68 | ± 1.937 | ± 0.099 | ± 0.976 | ± 2.009 | ± 1.959 | ± 1.119 |
| 72 | ± 2.266 | ± 0.065 | ± 1.223 | ± 1.987 | ± 0.484 | ± 1.140 |
| 88 | ± 1.909 | ± 0.005 | ± 1.477 | ± 1.765 | ± 1.009 | ± 1.096 |
| 90 | ± 1.446 | ± 0.154 | ± 1.996 | ± 1.554 | ± 1.604 | ± 1.287 |
| 92 | ± 2.185 | ± 0.556 | ± 2.610 | ± 2.039 | ± 0.996 | ± 1.995 |
| 94 | ± 1.230 | ± 0.678 | ± 1.699 | ± 1.501 | ± 1.810 | ± 1.546 |
| 112 | ± 1.504 | ± 1.259 | ± 1.898 | ± 1.440 | ± 1.502 | ± 1.110 |
| 115 | ± 0.931 | ± 1.336 | ± 1.900 | ± 1.399 | ± 1.001 | ± 1.671 |
| 160 | ± 2.100 | ± 1.985 | ± 2.001 | ± 1.870 | ± 1.047 | ± 1.692 |
| 164 | ± 1.540 | ± 1.775 | ± 1.566 | ± 1.212 | ± 1.059 | ± 1.059 |

#### 3.1.3 Silglif®

All tested concentrations of Silglif® resulted in an impairment of *S. cerevisiae* K310 growth; in particular, we observed dose-dependent slackening and slight alterations of growth kinetics (Fig. 3, upper panel).

During culture, no great differences in comparison to control were found for cell viability (Fig. 3, central panel), except for the highest herbicide concentration that resulted in a slight reduction in the maximum number of colony forming units/mL. However, based on previous observations [31] indicating that a commercial formulation of glyphosate was able to affect cell viability immediately after addition to the yeast cell suspension, only for Silglif® this parameter was observed also just after herbicide supplementation. The data obtained indicate that at this time there was a noticeable cell death for all five herbicide concentrations, but also that the yeast cells were able to rapidly overcome such stress (Fig. 4).

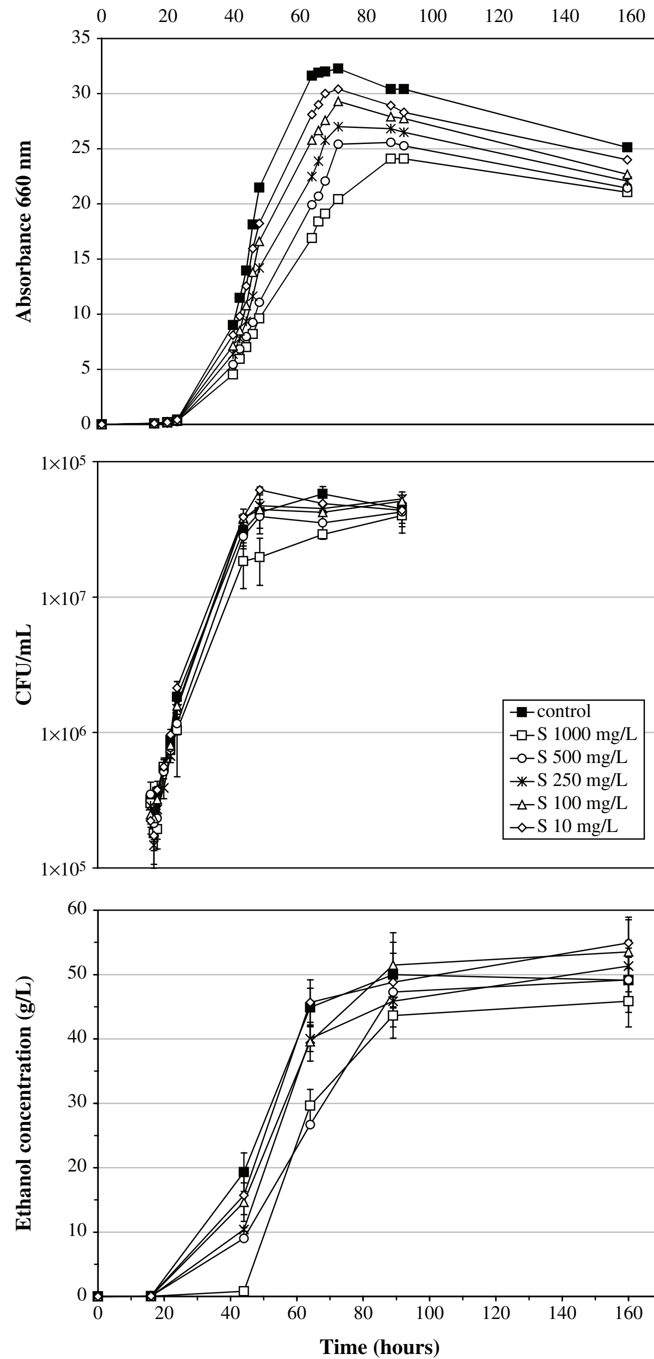


Figure 3: Growth curve (upper panel), viability curve (central panel) and fermentation ability (lower panel) of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of S at the beginning of the exponential phase or in the absence of herbicide (control). The data reported are average values of three independent experiments carried out under identical conditions; standard deviations observed for growth are listed in Table 5, while those observed for viability and fermentation ability are indicated with vertical bars.

Table 4: Standard deviation values referred to growth curve of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of P at the beginning of the exponential phase or in the absence of herbicide (control) (Fig. 2). The data reported are from three independent experiments carried out under identical conditions.

| Culture (h) | Standard deviation values | | | | | |
|---|---|---|---|---|---|---|
| | Control | P 100 mg/L | P 10 mg/L | P 1 mg/L | P 100 μg/L | P 10 μg/L |
| 16 | ± 0.075 | ± 0.055 | ± 0.034 | ± 0.002 | ± 0.059 | ± 0.004 |
| 20 | ± 0.412 | ± 0.154 | ± 0.003 | ± 0.080 | ± 0.010 | ± 0.045 |
| 23 | ± 0.562 | ± 0.410 | ± 0.099 | ± 0.241 | ± 0.333 | ± 0.250 |
| 40 | ± 1.985 | ± 1.560 | ± 2.937 | ± 0.103 | ± 0.645 | ± 2.213 |
| 42 | ± 1.413 | ± 1.856 | ± 1.119 | ± 0.834 | ± 0.585 | ± 2.823 |
| 44 | ± 1.654 | ± 1.878 | ± 1.819 | ± 1.464 | ± 2.271 | ± 1.297 |
| 46 | ± 1.871 | ± 2.506 | ± 1.802 | ± 1.073 | ± 2.280 | ± 2.563 |
| 48 | ± 1.564 | ± 1.763 | ± 0.940 | ± 1.088 | ± 2.123 | ± 2.061 |
| 63 | ± 2.016 | ± 2.001 | ± 0.663 | ± 0.663 | ± 0.238 | ± 1.706 |
| 65 | ± 1.857 | ± 1.546 | ± 2.369 | ± 2.002 | ± 2.367 | ± 0.574 |
| 69 | ± 1.944 | ± 2.358 | ± 2.001 | ± 1.879 | ± 2.631 | ± 1.474 |
| 71 | ± 2.489 | ± 2.365 | ± 2.161 | ± 2.327 | ± 2.085 | ± 0.621 |
| 89 | ± 1.567 | ± 2.000 | ± 1.918 | ± 2.511 | ± 2.322 | ± 1.483 |
| 94 | ± 1.678 | ± 1.568 | ± 1.870 | ± 1.996 | ± 2.015 | ± 1.452 |
| 160 | ± 1.014 | ± 1.565 | ± 1.387 | ± 1.872 | ± 2.587 | ± 2.605 |

Also fermentation was affected by herbicide supplementation (Fig. 3, lower panel) and all tested concentrations resulted in a lower ethanol production in respect to control until the 90th hour of culture. After this moment, differences were no more significant.

Results obtained showed, in the case of Proper Energy®, a clear dose-response effect for all parameters of cell functionality. In fact, a sudden exposure of yeast cells to this herbicide induces a period of latency with a consistent loss of viability, followed by a restoration of the exponential phase of growth, presumably attributed to a cell population adapted to the chemical stress. In this case, the specific cell death entity induced by the herbicide, the length of the lag period and the cell growth seemed to be functions of the applied herbicide concentration, at least in the concentration range from 100 to 500 mg/L. The recovery–adaptation period was observable only for high concentrations of PE (100, 200 and 500 mg/L), suggesting the idea of a threshold concentration; in this sense, it should be noted that there was no recovery for PE 10 and 1 mg/L. Our results are in accordance with previously reported works on 2,4-D herbicide, indicating that 2,4-D induces on yeast cells an initial period of herbicide-induced cell death, during which the vital population adapts itself to recover the exponential growth phase [21].

### 3.2 Metabolic profiling

The metabolic profile of *S. cerevisiae* K310, as its ability to use YT microplate substrates as carbon sources, was very slightly influenced by the treatments with herbicides (Fig. 5). Some more significant

Table 5: Standard deviation values referred to growth curve of *S. cerevisiae* K310 grown in YPD medium (glucose 100 g/L, pH of 4.5, incubation at 28°C in semi-aerobiosis) supplemented with five different concentrations of S at the beginning of the exponential phase or in the absence of herbicide (control) (Fig. 3). The data reported are from three independent experiments carried out under identical conditions.

| Culture (h) | Standard deviation values | | | | | |
|---|---|---|---|---|---|---|
| | Control | S 1000 mg/L | S 500 mg/L | S 250 mg/L | S 100 mg/L | S 10 mg/L |
| 16 | ± 0.055 | ± 0.088 | ± 0.123 | ± 0.049 | ± 0.047 | ± 0.098 |
| 20 | ± 0.682 | ± 0.084 | ± 0.032 | ± 0.158 | ± 0.155 | ± 0.017 |
| 23 | ± 0.723 | ± 0.132 | ± 0.150 | ± 0.236 | ± 0.028 | ± 0.059 |
| 40 | ± 1.760 | ± 0.568 | ± 0.789 | ± 0.668 | ± 0.791 | ± 0.103 |
| 42 | ± 2.054 | ± 1.965 | ± 1.584 | ± 1.854 | ± 1.603 | ± 1.278 |
| 44 | ± 2.043 | ± 1.651 | ± 2.003 | ± 2.510 | ± 1.991 | ± 1.651 |
| 46 | ± 1.843 | ± 2.004 | ± 2.001 | ± 1.852 | ± 1.004 | ± 1.431 |
| 48 | ± 1.420 | ± 1.586 | ± 1.999 | ± 2.510 | ± 1.682 | ± 1.538 |
| 64 | ± 1.956 | ± 2.507 | ± 1.876 | ± 2.410 | ± 2.300 | ± 1.968 |
| 66 | ± 1.743 | ± 1.685 | ± 1.998 | ± 1.560 | ± 1.706 | ± 1.684 |
| 68 | ± 1.444 | ± 1.604 | ± 1.873 | ± 2.015 | ± 2.103 | ± 1.482 |
| 72 | ± 1.580 | ± 1.232 | ± 1.545 | ± 1.600 | ± 1.540 | ± 1.231 |
| 88 | ± 1.574 | ± 1.055 | ± 1.510 | ± 1.871 | ± 2.636 | ± 1.065 |
| 92 | ± 1.046 | ± 1.058 | ± 1.444 | ± 1.822 | ± 1.505 | ± 1.552 |
| 160 | ± 1.023 | ± 1.255 | ± 1.055 | ± 1.200 | ± 1.420 | ± 1.332 |

changes were observed in quantitative metabolic reactions: i.e. AWCD and maximal rate of color development (Fig. 6). Results obtained after 72 hours of incubation can be described in detail as follows:

### 3.2.1 Proper Energy®

PE, at both concentrations tested (1 and 100 mg/L), induce a new positive reaction of co-assimilation of methyl succinate + D-xylose, while the average value of the overall metabolic activity was not significantly affected in respect to control.

### 3.2.2 Pointer®

The use of P, at a concentration of 1 mg/L, induced the co-assimilation of methyl succinate + D-xylose and did not affect the AWCD. P at 100 mg/L inhibited the assimilation of two substrates: D-ribose and amygdalin and strongly reduced the level of the overall metabolic activity in respect to control (–33%).

### 3.2.3 Silglif®

The use of S 10 mg/L inhibited the assimilation of D-ribose. The highest concentration of S (1000 mg/L) inhibited the assimilation of D-ribose and amygdalin, but increased the overall metabolic activity up to 20%.

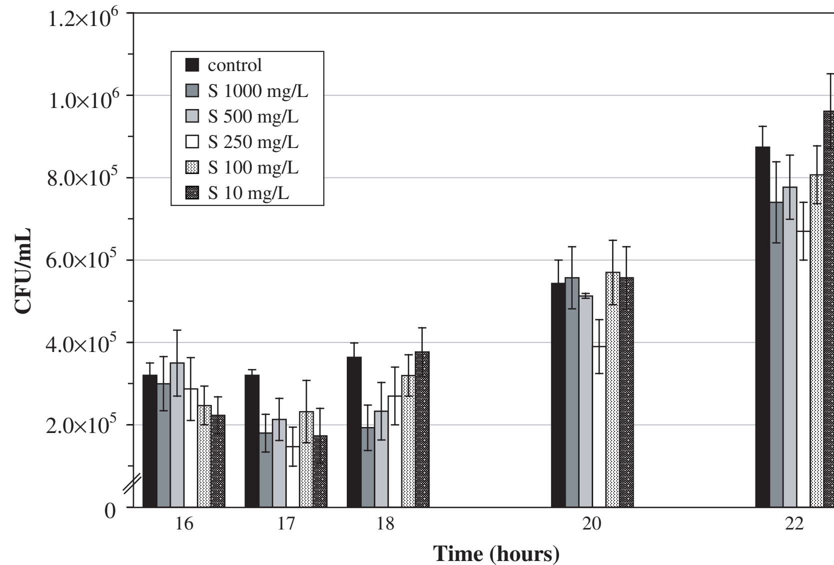


Figure 4: Viability of *S. cerevisiae* K310 just after herbicide supplementation with five different concentrations of S at the 16th hour of cell culture or in the absence of herbicide (control). The data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars.

Summarizing the results, the influence of the three herbicides tested at different concentrations on the metabolic profile of *S. cerevisiae* K310 lead to the following conclusions:

- The qualitative overall metabolic profiles were slightly or not affected by herbicides. The most sensitive reactions to herbicides were with the substrates, D-ribose and amygdalin. In particular, the assimilation of D-ribose was completely inhibited at all the S concentrations and at the highest P concentration. Amygdalin metabolism was completely inhibited by S and P at the highest concentrations. On the other hand, P 1 mg/L and PE at both concentrations induced the co-assimilation of methyl-succinate + D-xylose.
- The quantitative metabolic profiles were on the whole negatively affected by P 100 mg/L, while positively influenced by S 10 mg/L. In particular, P 100 mg/L decreased the AWCD by 33% compared to control, while S 10 mg/L increased the AWCD by 20%. A few substrates reached a noticeable increase of the maximal rate of color development, also if the overall AWCD was decreased. In the presence of S 10 mg/L, the assimilation rates increased as follows: maltose (+85%), stachyose (+73%) and trehalose (+50%). In the presence of PE 100 mg/L the assimilation rate of D-ribose was strongly stimulated (+106%). In the presence of P 100 mg/L, while the strongest inhibition among all the considered herbicides was observed (33% overall) the assimilation rate of turanose was stimulated (+55%).

In yeast research, the Biolog system has been mainly employed for identification purposes. However, we have used it to study the effects of chemical stress on the metabolism of *S. cerevisiae.* The highlights of the results obtained in this study are as follows:

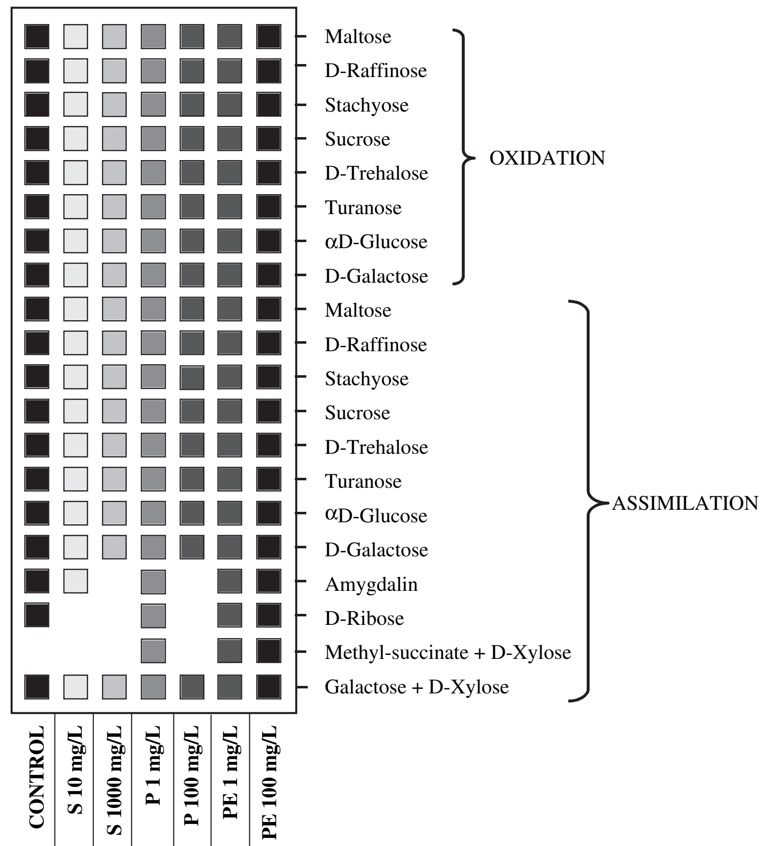


Figure 5: Metabolic profiles developed by *S. cerevisiae* K310 strain in Biolog microplates YT, in standard conditions (control) and in the presence of different concentrations of herbicides: PE (1 and 100 mg/L); P (1 and 100 mg/L); S (10 and 1000 mg/L). Squares indicate positive reactions.

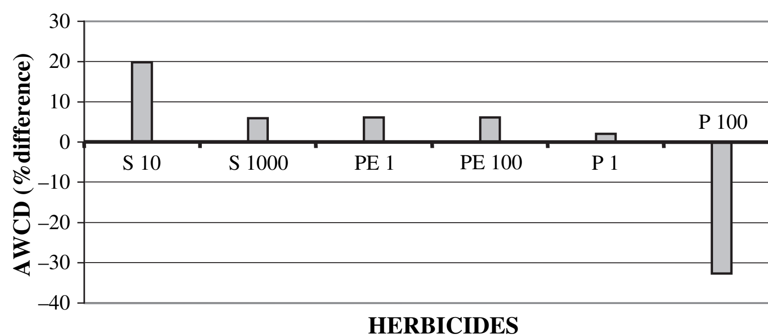


Figure 6: AWCD percent differences with respect to AWCD of control (standard conditions set at 0%) in YT microplates, by *S. cerevisiae* K310 strain in the presence of different concentrations of herbicides: PE (1 and 100 mg/L); P (1 and 100 mg/L); S (10 and 1000 mg/L).

- Though the metabolic profile is not significantly altered by the tested herbicides, as pattern of substrates used, the possibility to test simultaneously a high number of substrates permitted to identify three substrates whose assimilation is apparently more sensitive to herbicides. In fact, the assimilation of D-ribose and amygdalin was totally inhibited, while the co-assimilation of methyl-succinate + D-xylose was induced.
- The quantitative analysis of metabolic profiles has been more effective in emphasizing herbicide effects. In particular, it allowed in identifying critical concentrations of herbicides that altered either negatively or positively the considered indices of metabolic activity, i.e. the AWCD and the maximal rate of color development.

Thus, the use of the Biolog system to study the response of the metabolic activity of yeasts to chemically induced stress seems to be a useful tool both for a general evaluation of the effect of the herbicides on the metabolic potential and for the discovery of specific targets of stress, which can indicate further studies to investigate thoroughly the yeast metabolomics.

It can be thus summarized that Proper Energy®, in the concentration range from 100 to 500 mg/L, had the most evident deleterious impact on cell growth, viability and ethanol production. For these parameters, Pointer® showed a distinct effect only for the highest concentration (100 mg/L), while Silglif® for all tested concentrations (from 10 to 1000 mg/L), with a clear dose-effect response. Moreover, from a metabolic point of view, Proper Energy® (1 and 100 mg/L) had the most important qualitative effect, together with Pointer® 1 mg/L. However, it was Pointer® 100 mg/L that had the most pronounced effect in the quantitative metabolic analysis.

Particularly, the most relevant feature of our study is the use of commercial preparations of herbicide rather than the pure active ingredients. Since active ingredients are never applied alone but always with several additives, for ecotoxicological considerations the formulated compounds are thus the most appropriate ones to test. However, it should be underlined how innovative our results are. In fact, to the best of our knowledge, this is the first study to assess the toxicity of herbicides Proper Energy®, Pointer® and Silglif® as commercial formulations. Moreover, taking into account their active ingredients, it should be noted that the literature can provide some results only for glyphosate, while for fenoxaprop-P-ethyl and tribenuron methyl, no data were previously reported. In addition, our results for Silglif® are in contrast with a recent observation demonstrating the harmlessness of its active ingredient by itself and in the commercial formulation Roundup® [32]; however, they are in partial agreement with a consistent number of reports indicating that the commercial formulation is much more toxic than the active ingredient alone. In particular, even if glyphosate has been found to be nontoxic for mammals, birds, fish, insects and most bacteria, and also does not bioaccumulate in animal tissues [33], some authors reported a positive association between exposure to glyphosate formulations and multiple myeloma [34] and non-Hodgkin lymphoma [35], as well as inhibition of transcription during the hatching of sea urchins [36], mutagenesis effects [37] and major changes in the function of human erythrocytes when compared to glyphosate alone [33]. These data are very important considering that the introduction in the market of crops genetically tolerant to glyphosate will probably result in an increase in the use of this herbicide. In particular, a recent Italian study reports, only for the period 2000–2001, 53 cases of human poisoning by glyphosate formulations [38].

Also, since environmental factors such as pH and temperature are known to modify pesticide toxicity [39], it is important to test such products using conditions more similar to the physiological ones. In this sense, our study on an enological yeast strain, isolated from natural fermenting musts and grown in laboratory in conditions close to those of cellar vinification, ensures that the observed herbicide effects could be better extrapolated to what could really happen in nature. This was done by considering yeast as a eukaryotic model and, especially, as a microorganism utilized for the production of wine, nowadays considered in all respects as a food.

Moreover, considering the fermentation ability and ethanol production of *S. cerevisiae* K310, it should be underlined how significant amounts of pesticides on grapes can inhibit the initial vinification steps, thus altering, as seen, the yeast cell functionality. Also, considering the ability of yeast to metabolize various compounds during alcoholic fermentation for the production of $H_2S$ and other sulfur compounds (for example, in physiological conditions, intermediates of methionine biosynthesis), the possibility that these strains could utilize sulfur compounds contained in commercial formulations should not be underestimated. This could have important consequences on the productivity of vinification processes, either from 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 consumer health.

The information we obtained encourage us to deepen our studies on wild-type enological yeast strains using herbicide/pesticide products in their commercial formulations, in parallel with studies on single active ingredients.

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