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Comparative Analysis of the Effects of Locally Used Herbicides and Their Active Ingredients on a Wild-Type Wine *Saccharomyces cerevisiae* Strain

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Herbicides are released to the environment with potential ecotoxicological risks for mammals. Yeast is a good model to elucidate toxicity mechanisms. We investigated how three commercial herbicides (Proper Energy, Pointer, and Silglif) and their active ingredients (respectively, fenoxaprop-P-ethyl, tribenuron methyl, and glyphosate) can affect biological activities of an oenological *Saccharomyces cerevisiae* strain, which may be resident on grape vineyards of the same geographical areas where herbicides are used. The use of commercial grade herbicides employed in Italy allowed us to reproduce the same conditions applied in crops; at the same time, assaying pure single active compounds made it possible to compare the effects obtained with commercial formulations. Interestingly, we found that while pure active compounds affect cell growth and metabolism at a lower extent, commercial preparations have a significant major negative influence on yeast biology.

KEYWORDS: Pesticides; herbicides; ecotoxicity; wine yeast

INTRODUCTION

Agricultural pesticides are nowadays a widely used class of compounds, and consequently, they are released into the environment in large amounts. They belong to the more heterogeneous group of "xenobiotics", which are defined as chemicals foreign to biological systems (*1*). Herbicides are among them the most used (and often misused) compounds; thus, both ecotoxicological problems and potential risks for mammals should be considered during application. At the same time, toxicity data for xenobiotics often refer to other species rather than to humans, thus making further evaluations really necessary. Moreover, herbicides and other pesticides can also have deleterious consequences on soil resident microorganisms and, consequently, on soil quality, with a phenomenon dependent on commercial formulations, active ingredients (AIs), climatic conditions, applied doses, and soil type as well (*2*).

In the last years, the yeast *Saccharomyces cerevisiae* has been proposed as a good experimental model to assess xenobiotic toxicity (*3*−*6*). Its use is particularly advantageous since it is extremely easy to manipulate, while at the same time avoiding complexity and accessibility problems found with higher eukaryotes. Yeast growth in a well-defined medium allows one to also continuously check physical and chemical conditions, thus warranting high reproducibility of experiments. Moreover, because it has been extensively studied, *S. cerevisiae* is a well-characterized organism from either a genomic, proteomic, or metabolic point of view, revealing the possibility of studying biological mechanisms common to fungi, plants, and animals. At the same time, generic stress responses are evolutionarily well-conserved; understanding how yeast cells respond to xenobiotics can implement current knowledge of toxicity mechanisms in more complex systems, thus furnishing the bases for wider ecotoxicological considerations.

Current literature provides a discrete number of studies reporting the effects of various agricultural pesticides on yeast biological parameters; in particular, many of them are dedicated to fungicides used for vineyard treatments (*5*, *7*−*14*) while studies on herbicides are more rarely found and almost limited to 2,4-dichlorophenoxyacetic acid (2,4-D) and derivates (*3*, *4*,

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Table 1. Commercial Grade Herbicides Used in This Work and Their Most Important Features (Physical State, Solubility, Chemical Class, AIs, and Mechanism of Action)

trade name	description	mechanism of action
PE	oil/water emulsion containing F (55 g/L, aryloxyphenoxy propionate) and mefenpyr diethyl (30 g/L, pirazoline derivate)	F causes the disruption of fatty acid biosynthesis in grasses by selective inhibition of the enzyme acetyl-CoA carboxylase (ACC); mefenpyr diethyl is the safener that protects treated crop plants from herbicide injuries through GST-mediated reactions
P	water-dispersible granules containing T (750 g/kg, sulfonyl urea)	T inhibits the enzyme acetolactate synthase (ALS) also known as acetohydroxyacidic synthase (AHAS), involved in the biosynthesis of essential amino acids
S	soluble concentrate containing G (304 g/L, organophosphate)	G inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), involved in the biosynthesis of aromatic compounds

6, *15*−*18*). The use of high-purity single AIs of pesticides, rather than the commercially available formulations, is a common feature of these studies. In this context, a good exception is represented by the herbicide glyphosate (G) and some of its formulations. Studies conducted on several organisms, as well as on human cell lines, revealed, as should be expected, how responses obtained with G alone differ from those obtained with commercial preparations. These contain, in fact, various additives that promote the required action of the AI during crop treatments, and these are the substances to whom target and nontarget organisms are thus exposed. Therefore, for toxicity evaluations, these are the most appropriate substances to test.

In this work, we aimed to investigate how three herbicides [Proper Energy (PE), Pointer (P), and Silglif (S)] with AIs belonging to different classes (aryloxyphenoxy-propionates, sulfonylureas, and organophosphates, respectively) can affect some metabolic activities of an oenological *S. cerevisiae* strain, with particular attention to growth, colony-forming ability, and ethanol production. Moreover, we wanted to evaluate the global metabolic profile of yeast cells in response to this type of stress. We decided to use commercial grade herbicides, to reproduce as far as possible the same conditions during application in crops, and to compare the obtained responses with those obtained with single high-purity AI supplementations [fenoxaprop-P-ethyl (F), tribenuron methyl (T), and G, respectively]. We chose these compounds over those that have been authorized by the law 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. 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 more close to physiological ones.

MATERIAL AND METHODS

General Materials. All high-purity reagents were from Oxoid (Garbagnate M.se, Milan, Italy), Sigma (Milan, Italy), and 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, respectively, F, T, and G, were analytical standards from Riedel de Haën (Schweiz, Germany). All water used was Milli-Q (Millipore, Bedford, MA).

Yeast Strain, Culture Conditions, and Colony-Forming Ability Assays. The yeast strain used in this work was *S. cerevisiae* K310; it was isolated from naturally fermenting must during vinification of a high-quality wine, in the geographical area of "Brunello di Montalcino" (*19*), and is well-characterized physiologically, for its protein repertoire and stress response (*20*−*24*). K310 was precultured in yeast peptone dextrose (YPD) medium at 30 °C with rotary shaking up (120 rpm) for 10 h. At this time, an appropriate aliquot of the cell culture was inoculated in 150 mL of a modified YPD medium and adjusted to a final pH of 4.5 by adding 0.2 M citrate/phosphate buffer containing 100 g/L glucose, to obtain an initial cell concentration of 1×10^4 cells/mL. The cell suspension was then incubated at 28 °C in the dark without shaking, allowing semianaerobic growth.

Cell growth was monitored by measuring the culture absorbance at 660 nm, and the maximal growth rate (μ_{max}) was calculated from the nonlinear Gompertz equation fitted to yeast growth curves (*25*). Colony-forming ability assays were conducted in triplicate 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 3 days. At times chosen for determining colony-forming ability, samples were collected and the pH was checked.

Herbicide Supplementations. Five different concentrations of commercial grade herbicides and their corresponding AIs were tested on *S. cerevisiae* K310; they were calculated referring to the maximum residue limits (MRLs), calculated for the corresponding AI, allowed in Italy in several foodstuffs (*26*). In particular, the lowest concentration corresponded to an AI concentration similar to the one allowed by the law, while others were chosen in order to mimic various conditions from crop application during residue formation. Commercial grade 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 culture medium at the beginning of the exponential growth phase (16th hour of cell culture, about 3×10^6 cells/mL) when yeast cells are most susceptible to applied stresses, by adding appropriate aliquots of the dispersion/emulsion/solution of commercial compounds prepared with water just prior to use. In parallel, single AIs were assayed as well. They were added to yeast cultures at the same time, and the same concentration was obtained with their commercial formulations. In particular, because G was a water-soluble compound, it was directly added to the culture media; on the contrary, F and T were previously dissolved in dimethyl sulfoxide (DMSO), respectively, 1 and 4 mg/mL. In these cases, for all treated cultures, the DMSO concentration conformed to the value of 2.62% v/v for F and 1.87% v/v for T. For them, in addition to a control culture with no supplementations, cultures with only DMSO supplementation were prepared. Tested concentrations of herbicides and AIs are summarized in **Table 2**.

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. (*27*, *28*) 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 determination of ethanol concentrations was performed spectrophotometrically (Agilent 8453 UV−visible spectroscopy system, Waldbronn, Germany) on the obtained filtrate, properly diluted in accordance to manufacturer's instructions.

Metabolic Profiling. 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) for both oxidation and assimilation tests on 65 different substrates were used. On the basis of the results obtained from growth, colony-forming ability, and fermentation analyses, two different concentrations of herbicide/

Table 2. Concentrations of Commercial Herbicides and Corresponding AIs that Were Tested on *S. cerevisiae* K310 Strain

PE	500 mg/L	F (mw, 361.8)	26.20 mg/L (72.42 mM)
	250 mg/L		13.10 mg/L (36.21 mM)
	100 mg/L		5.24 mg/L (14.48 mM)
	10 mg/L		524 μg/L (1.45 mM)
	1 mg/L		52.40 μg/L (0.15 mM)
P	100 mg/L	T (mw, 395.4)	75 mg/L (189.68 mM)
	10 mg/L		7.50 mg/L (18.97 mM)
	1 mg/L		750 μg/L (1.90 mM)
	100 μg/L		75 μg/L (0.19 mM)
	10 μg/L		7.50 μg/L (0.02 mM)
S	1 g/L	G (mw, 169.1)	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 mM)
	10 mg/L		3.04 mg/L (17.98 mM)

AI were chosen in order to test their influence on the yeast metabolic profile. Cells grown for 48 h on BUY agar (Biolog Inc.) were collected and processed according to the manufacturer's instructions with minor modifications. The cell concentration was adjusted to 47% of transmittance (10^8 cells), and aliquots were added to the plate (100 μL/well) with or without the chosen amount of herbicide or AI. 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 or AI without cells were prepared in order to assess the lack of chemical reactions with herbicides and tetrazolium dye and/or the substrate. Results were recorded by the Microplate Reader (change in optical density, OD_{590}) after 24, 48, and 72 h of incubation. Data were stored using Biolog software. All of 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 AWCD (average well color development), was calculated as the arithmetic mean of the OD values of all of the wells in the plate after 72 h of incubation (AWCD). The second one, the maximal rate of color development (μ, expressed in OD h^{-1}), represented the slope of the regression line of the nonlinear curve calculated by plotting the OD value in each well against the incubation time.

Statistical Analysis. All of the experiments were conducted in triplicate; presented data are mean values ± standard deviation. Analysis of variance was performed, and significant differences between the control cultures and the treated ones were evaluated by Dunnett's test. At least a P value < 0.05 was considered significant.

RESULTS

Growth, Colony-Forming Ability, and Ethanol Assay. *PE and F.* When *S. cerevisiae* K310 was cultivated in the presence of PE 500, 250, and 100 mg/L (**Figure 1A**), a drastic growth inhibition occurred. Nevertheless, we observed in all cases a growth resumption, with an increasing dose-dependent period of latency: 32 h for PE 100 mg/L, 52 h for PE 250 mg/L, and 78 h for PE 500 mg/L. For the two lowest herbicide concentrations, no relevant differences in comparison to control were found. A similar pattern was also evident on μ_{max} values (**Table 3**) and ethanol production (**Figure 1B**). For control and cell cultures treated with 1 and 10 mg/L PE, the highest ethanol concentration was reached around the 68th hour; for the culture treated with 100 mg/L PE, this peak was shifted to the 112th hour; with 250 mg/L PE, this was shifted to the 130th hour, and with 500 mg/L PE, this was shifted to the 164th hour. Cell stress was also reflected on colony-forming ability (**Figure 1C**) with a dramatic reduction of CFU/mL following herbicide application at the three highest doses. At the 22nd hour of culture, the comparison with control revealed a reduction of about four log units for 500 and 250 mg/L PE and two log units for 100 mg/L PE; moreover, differences were significant during the entire period of observation.

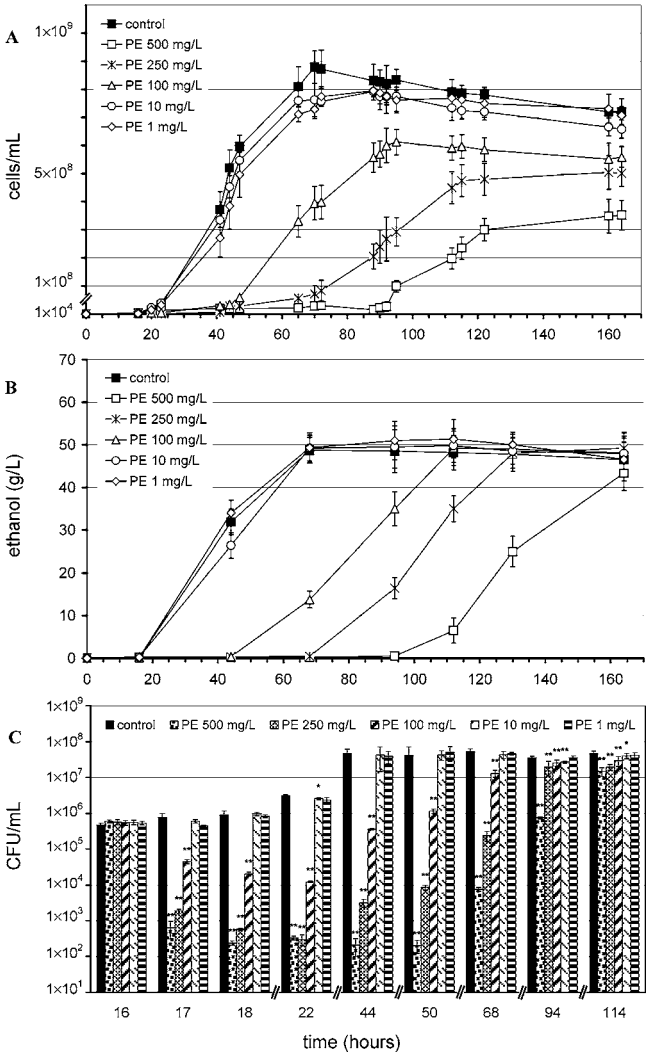

Figure 1. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of PE at the beginning of the exponential phase or in the absence of herbicide (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with control culture.

On the contrary, when yeast cultures were supplemented with the AI F alone, at the same concentrations obtained with its commercial formulation, no effects were observed for all of the considered parameters, and growth, colony-forming ability, and ethanol production did not significantly differ from controls during the observed period of incubation (**Figure 2A−C** and **Table 3**).

P and T. Only the highest concentration of P (100 mg/L) caused a slight but significant reduction of *S. cerevisiae* K310 growth and ethanol production (**Figure 3A**,**B**). However, considering colony-forming ability, all tested concentrations of this herbicide resulted in an immediate and significant reduction of CFU/mL, which continued to be relevant at least up to the 22nd hour of culture for 100, 10, and 1 mg/L P. Nevertheless, μ_{max} was never significantly affected (**Table 3**).

A similar pattern was also observed when yeast cells were exposed to T. In particular, in the presence of 75 mg/L T, we

Table 3. Maximal Growth Rate Values Calculated from the Nonlinear Gompertz Equation Fitted to K310 Growth Curves Obtained in Presence of Commercial Herbicides or AIs

commercial herbicides		μmax	AIs		μmax
PE	500 mg/L	0.11	F	26.20 mg/L	0.61
	250 mg/L	0.21		13.10 mg/L	0.65
	100 mg/L	0.38		5.24 mg/L	0.68
	10 mg/L	0.43		524 μg/L	0.63
	1 mg/L	0.53		52.40 μg/L	0.71
	control	0.59		DMSO (2.62% v/v)	0.63
				control	0.67
P	100 mg/L	0.76	T	75 mg/L	0.61
	10 mg/L	0.75		7.50 mg/L	0.67
	1 mg/L	0.76		750 μg/L	0.65
	100 μg/L	0.76		75 μg/L	0.66
	10 μg/L	0.71		7.50 μg/L	0.64
	control	0.77		DMSO (1.87% v/v)	0.66
				control	0.73
S	1000 mg/L	0.38	G	304 mg/L	0.69
	500 mg/L	0.49		152 mg/L	0.66
	250 mg/L	0.55		76 mg/L	0.66
	100 mg/L	0.61		30.40 mg/L	0.64
	10 mg/L	0.64		3.04 mg/L	0.67
	control	0.69		control	0.67

observed for growth an initial lag period (about 14 h long) and then a resumption, which resulted in absorbance values similar to those of the controls only around the 80th hour of culture (**Figure 4A**). Moreover, the highest concentration of T resulted in an initial slower ethanol production, even if then the fermentation pattern was no longer affected, and around the 100th hour of culture, no more differences with controls were found (**Figure 4B**). Considering colony-forming ability, as seen for the corresponding commercial formulation, all tested concentrations of T caused, just after the supplementation, a significant loss of CFU/mL, which was evident at least up to the 22nd hour of culture (**Figure 4C**). For μ_{max}, no differences were found between treated cultures and controls (**Table 3**).

S and G. All tested concentrations of S resulted in an impairment of *S. cerevisiae* K310 growth; in particular, we observed a dose-dependent slackening and slight alterations of growth kinetics (**Figure 5A**) and μ_{max} (**Table 3**). Considering colony-forming ability (**Figure 5C**), the supplementation of yeast cultures with 1000, 500, and 250 mg/L S resulted, after 1 h, in a slight but significant reduction of CFU/mL; after this moment, no differences in comparison to control were found, except for the highest herbicide concentration that resulted in a significant reduction of the maximum reached number of CFU/mL at the 50th and 68th hours of culture. Also, fermentation was affected by herbicide supplementation (**Figure 5B**) and four out of five concentrations resulted in a lower ethanol production with respect to control until the 90th hour of culture. After this moment, differences were no more significant.

In evident contrast with these results, G alone did not influence in a negative manner any of the considered parameters, and all tested concentrations of G did not cause any impairment in yeast growth, fermentation ability, colony-forming ability (**Figure 6A−C**), or μ_{max} (**Table 3**).

Metabolic Profiling. The metabolic profile of *S. cerevisiae* K310, as ability to use YT microplate substrates as carbon sources, was very slightly influenced by the treatments with commercial herbicides. Some more significant changes were observed in quantitative metabolic reactions, i.e., AWCD and μ. These statements are true also for single AI supplementation but with the important exception observed when K310 was exposed to 304 mg/L G. Results obtained after 72 h of

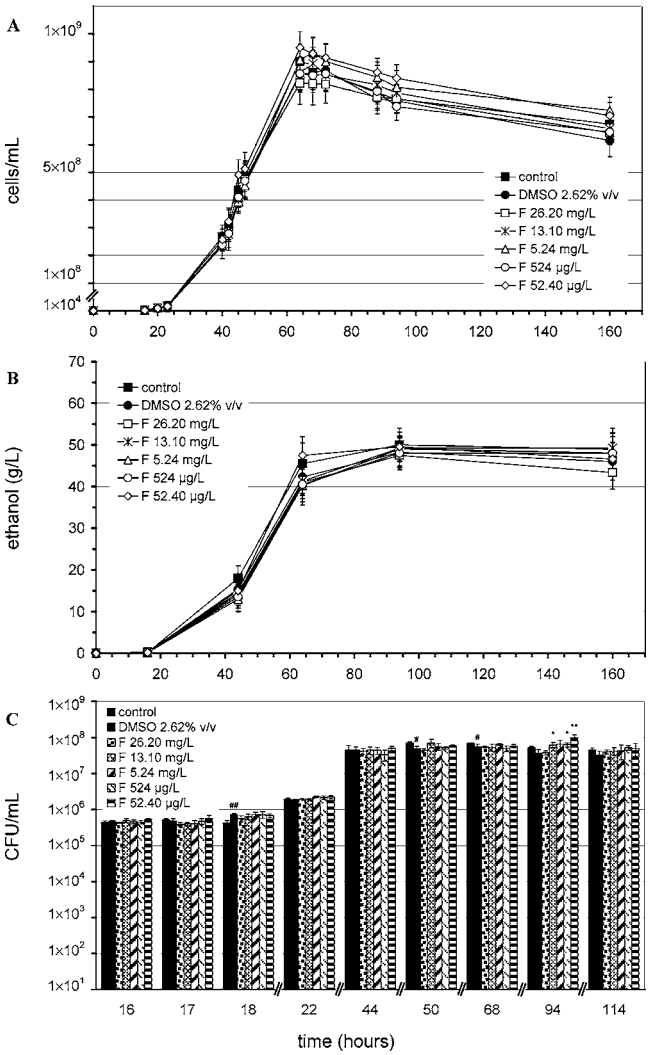

Figure 2. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of F or DMSO alone (2.62% v/v) at the beginning of the 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 are indicated with vertical bars. $^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ as compared with control culture; $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with culture + DMSO (2.62% v/v).

incubation are reported in **Figures 7** and **8**; they can be described in detail as follows.

PE and F. When compared to the control, both tested concentrations of PE (1 and 100 mg/L) were shown to induce a positive reaction for the coassimilation of methyl succinate + D-xylose; at the same time, the average value of the overall metabolic activity was not significantly affected. Differently from the commercial formulation, the AI F, at the concentration of 52.4 μg/L, inhibited the assimilation reactions for trehalose and amygdalin, while when tested at the concentration of 5.24 mg/L, it was able to inhibit three oxidation reactions (raffinose, stachyose, and trehalose) and four assimilation reactions (stachyose, trehalose, amygdalin, and ribose). Moreover, the two tested concentrations inhibited the coassimilation of methyl-succinate + D-xylose. The overall metabolic activity was proportionally reduced: 20 and 45%, respectively, in comparison to the control.

P and T. When compared to the control, the use of 1 mg/L P induced the coassimilation of methyl succinate + D-xylose

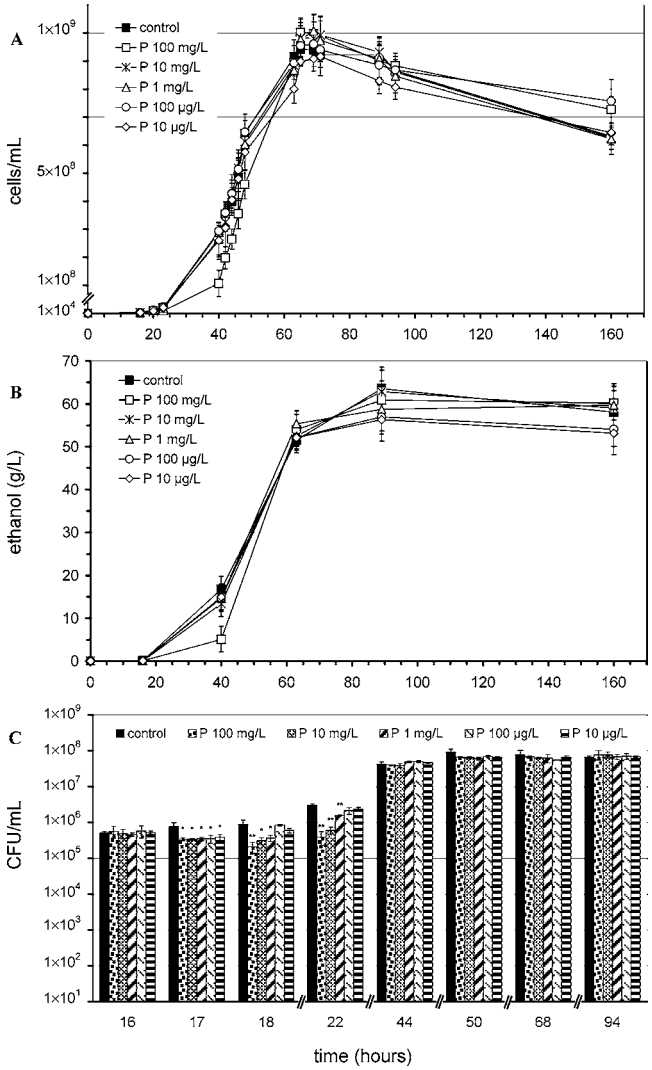

Figure 3. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of P at the beginning of the exponential phase or in the absence of herbicide (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with control culture.

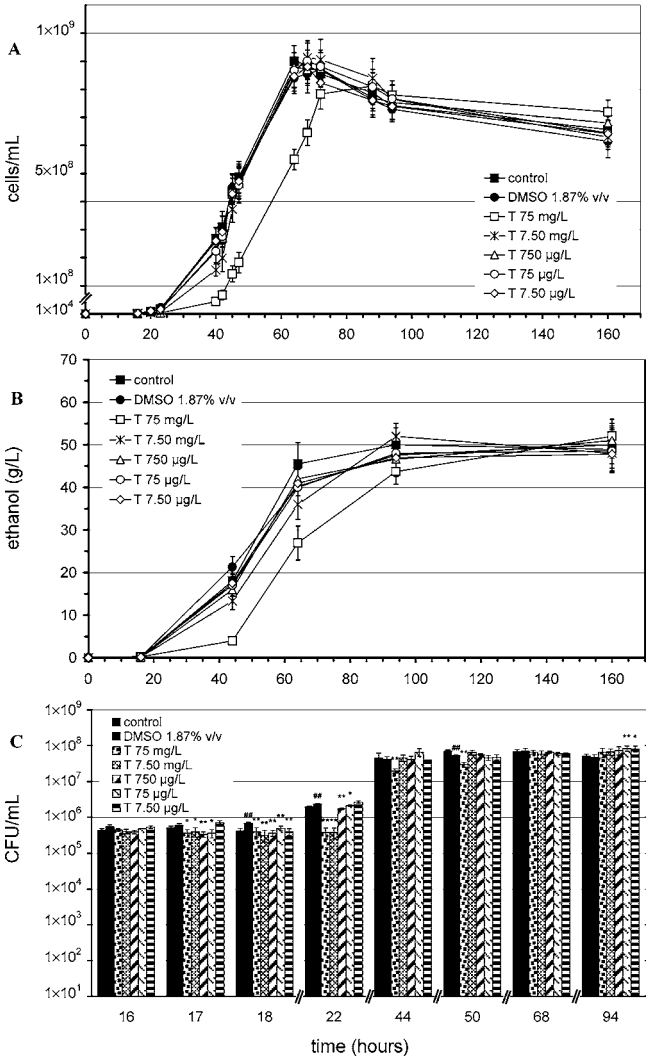

Figure 4. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of T or DMSO alone (1.87% v/v) at the beginning of the 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 are indicated with vertical bars. $^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ as compared with control culture; $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with culture + DMSO (1.87% v/v).

and did not affect the AWCD. P (100 mg/L) inhibited the assimilation of two substrates (D-ribose and amygdalin) and strongly reduced the level of the overall metabolic activity (−33%). With respect to the results obtained with the commercial formulation, the use of 750 μg/L T induced the assimilation of sorbitol and inhibited the oxidation of trehalose and the assimilation of ribose. T (75 mg/L) inhibited the oxidation of trehalose and the assimilation of ribose. The overall metabolic activity was proportionally reduced in comparison to the control: 17 and 25% for the two tested concentrations.

S and G. The comparison with control showed that 10 mg/L S inhibited the assimilation of D-ribose but increased the overall metabolic activity up to 20%. The highest concentration of S (1000 mg/L) inhibited the assimilation of D-ribose and amygdalin and did not significantly affect the metabolic profile from a quantitative point of view. Comparing the results with those obtained with the corresponding concentrations of commercial herbicide showed how the use of G at 3.04 mg/L inhibited the assimilation of turanose and did not affect the overall metabolic activity, while G 304 mg/L showed the most important metabolic alterations. In particular, it was able to induce the oxidation of L-proline and the assimilation of several carbon sources (cellobiose, gentiobiose, maltotriose, melezitose, melibiose, psicose, salicin, maltitol, mannitol, sorbitol, adonitol, arabitol, xylose, and melibiose + D-xylose) and to inhibit the assimilation of D-ribose and turanose. It was also able to greatly increase the overall metabolic activity up to 80% in comparison with the control culture.

DISCUSSION

In this work, we used an autochthonous oenological *S. cerevisiae* strain, namely, K310, to evaluate the impact on its biological activity of three different herbicides (PE, P, and S) and their AIs (F, T, and G, respectively). These compounds have been selected among those authorized by the law for crop treatments in central regions of Italy.

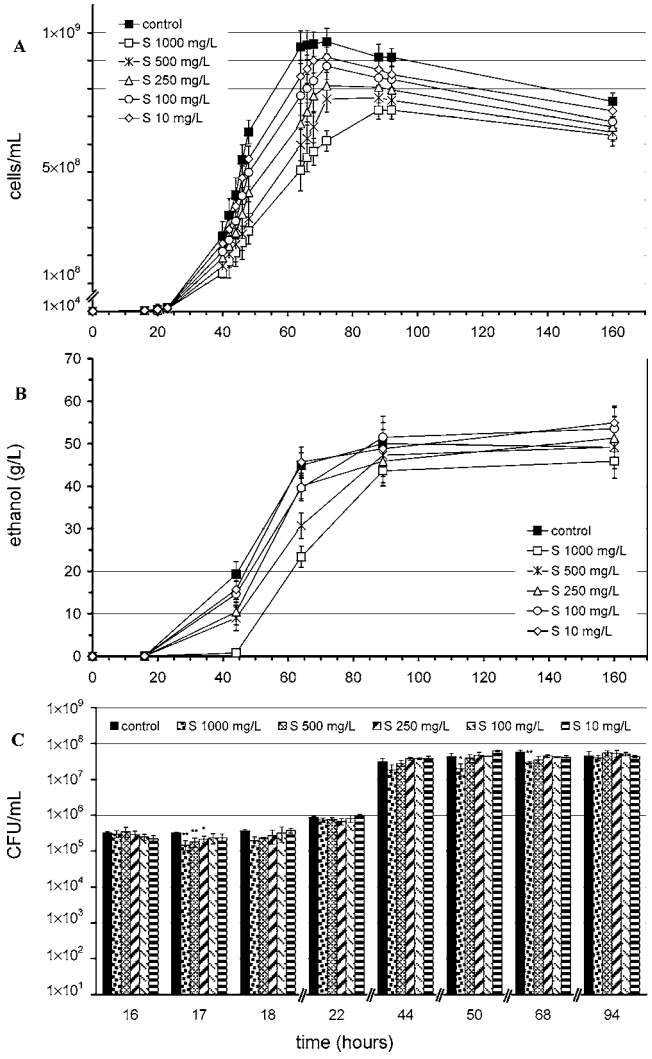

Figure 5. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of S at the beginning of the exponential phase or in the absence of herbicide (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with control culture.

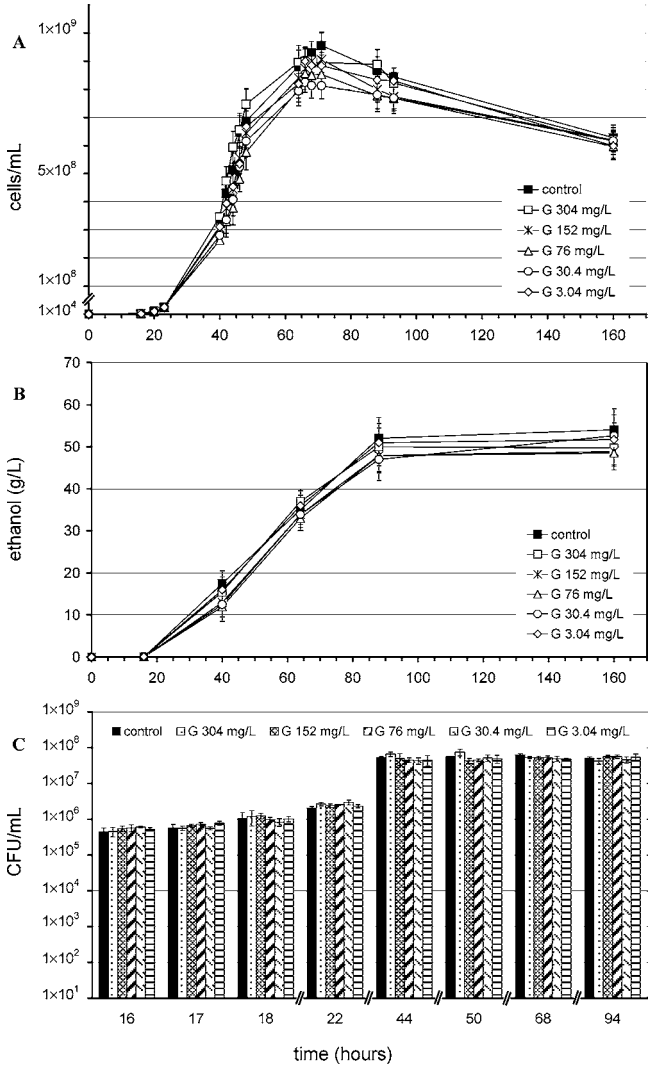

Figure 6. Growth (**A**), fermentation ability (**B**), and colony-forming ability (**C**) of *S. cerevisiae* K310 grown in YPD medium (100 g/L glucose, pH 4.5, incubation at 28 °C in semiaerobiosis) supplemented with five different concentrations of G at the beginning of the exponential phase or in the absence of herbicide (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared with control culture.

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 due to the ability of K310 to show good fermentation performances. Moreover, because *S. cerevisiae* is a well-consolidated model in the study of responses to xenobiotics and because K310 is an unmanipulated oenological strain grown in conditions similar to the natural ones, responses obtained should be considered more physiological.

Our type of approach, based on the comparison of the effects of herbicides and their AIs, proved to be particularly interesting. In fact, taken together, our data strongly suggest how the effects produced by commercial herbicide formulations should be distinguished by those produced by single AIs. This indicates how all of the coformulants present in the commercially available formulations, usually employed to promote the penetration, solubility, activity, stability, and application of the AIs, can act on the cell, alone, or synergistically and cause damaging effects. These are clearly not revealable if, as usual, single AIs are tested. Thus, because AIs are never applied alone in crops but always with several additives, for ecotoxicological considerations, the formulated compounds are the most appropriate ones to test. In particular, in the cases of PE and S, the commercial product seemed to be more cytotoxic than the AI alone or, at least, with opposite effects on cell activity parameters. Only in the cases of P/T, there was a good homology between the results obtained.

It can be summarized that P and T showed a discrete effect only for the highest tested concentrations (100 mg/L P and 75 mg/L T). PE, in the concentration range from 100 to 500 mg/L, had the most evident deleterious impact on cell growth, colony-forming ability, and ethanol production, with a clear dose−response effect; on the contrary, when applied alone, the AI F had no effects on these parameters. In fact, a sudden exposure of yeast cells to the herbicide PE induced a period of latency with a consistent loss of colony-forming ability, followed by a restoration of the exponential phase of growth, presumably attributed to a cell population adapted to the chemical stress.

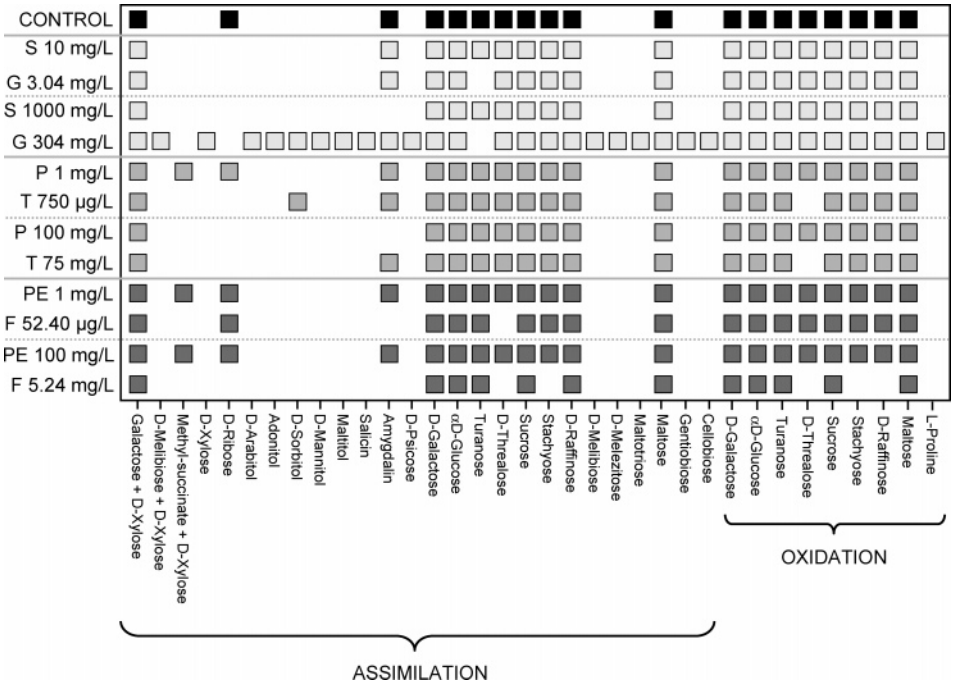

Figure 7. Metabolic profiles developed by *S. cerevisiae* K310 strain in YT Biolog microplates, in standard conditions (control) and in the presence of different concentrations of herbicides—PE (1 and 100 mg/L), P (1 and 100 mg/L), and S (10 and 1000 mg/L)—or AI—F (52.40 μg/L and 5.24 mg/L), T (750 μg/L and 75 mg/L), and G (304 and 3.04 mg/L). Squares indicate positive reactions.

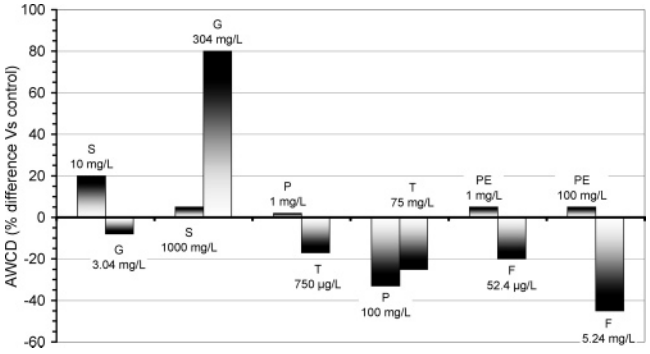

Figure 8. AWCD percent differences in respect of control (standard conditions set at 0%) in YT microplates, by *S. cerevisiae* K310 strain in the presence of different concentrations of herbicides—PE (1 and 100 mg/L), P (1 and 100 mg/L), and S (10 and 1000 mg/L)—or AI—F (52.40 μg/L and 5.24 mg/L), T (750 μg/L and 75 mg/L), and G (3.04 and 304 mg/L).

In this case, the specific cellular damage induced by the herbicide, the lag period length, and the cell growth seemed to be a function 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 how there was no inhibition for 10 and 1 mg/L PE. At the same time, it should be pointed out how this recovery pattern was observable only for the commercial formulation and not for the corresponding AI alone. 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 recovery of the exponential growth phase (*18*).

Moreover, while S showed for all concentrations (considering growth) or in the range from 250 to 1000 mg/L (considering cell colony-forming ability) a clear dose−effect negative response, G even at the highest concentration showed no significant effects. This can be explained on the basis of the only work present in the literature reporting the effects of this compound on yeast metabolism; in particular, it is reported how bakers' yeast can metabolize up to 20% of G during the breadmaking process, probably with detoxification mechanisms that can result in the production of new degradation compounds, whose action is still unknown (*29*).

In yeast research, the Biolog system has mainly been employed for identification purposes. Here, we suggest its use for studying the effects of chemical stress on the metabolism of *S. cerevisiae*. The most important alteration of the qualitative and quantitative metabolic profile was that obtained when yeast cells were exposed to the highest concentration of G; however, these effects were not observable with the corresponding S concentration. In the case of P/T, results were quite similar either from a qualitative or a quantitative point of view. For PE and F, while there were small qualitative differences, results of quantitative analysis were opposite for commercial formulation and AIs. Results obtained with the analysis of the K310 metabolic profile lead to the conclusion that qualitative overall metabolic profiles were slightly or not affected by herbicides, with the important exception observed for the highest concentration of G, able to induce the oxidation and assimilation of several carbon sources. However, even when the metabolic profile was not significantly altered by the herbicides, in terms of substrates used, the possibility to test simultaneously a high number of substrates permitted to identify those substrates whose assimilation was apparently more sensitive to xenobiotics. In general, the quantitative metabolic analysis proved to be more effective in emphasizing herbicide/AI effects, allowing us to identify critical concentrations that altered yeast metabolic activity parameters. In particular, the homology of the results obtained for P/T, as seen for growth and fermentation patterns, can be underlined. On the contrary, in the other two cases, responses obtained with commercial herbicides were clearly distinguishable from those obtained with AIs, and also with this approach,

the highest tested concentration of G provided the most intense alteration of the overall metabolic activity. Thus, the use of the Biolog system to study the response of the metabolic activity of yeasts to chemically induced stress seems to be an useful tool for both a general evaluation of the effect of the herbicides and their AIs on the metabolic potential as for the discovery of specific targets of stress, which can indicate further studies to investigate thoroughly the yeast metabolics.

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 PE, P, and S as herbicide commercial formulations. Moreover, considering their AIs, it should be noted how literature can provide some results only for G, while for F and T, no data were previously reported. In addition, our results for S are in contrast with a recent observation demonstrating the harmlessness of its AI alone and in the commercial formulation Roundup (*30*). However, as far as regards K310 growth and colony-forming ability, our data for S are in partial agreement with a consistent number of reports indicating that various commercial formulations containing G are much more toxic than the AI alone. In particular, this latter has been found to inhibit bacterial growth (*2*) and to be toxic for several aquatic organisms (*31*); moreover, studies conducted on rats revealed for them an impairment of bioenergetic reactions and metabolism in liver (*32*, *33*) as well as oxidative damage (*34*). In addition, adverse effects on transcription and cell cycle regulation have been highlighted (*35*, *36*). Oxidative damage on human epidermal cells (*37*), on human erythrocytes (*38*), and on human placental cells has also been proved (*39*). Moreover, for humans, some authors reported a positive association between exposition to G formulations and multiple myeloma (*40*) and non-Hodgkin's lymphoma (*41*). These data are very important considering that the introduction in the market of crops genetically tolerant to G will probably result in an increase of the use of this herbicide. In particular, a recent Italian study reports, only for the years 2000−2001, 53 human poisoning cases by G formulations (*42*).

Also, because environmental factors as pH and temperature are known to modify pesticide toxicity, it is important to test such products using conditions the most close to the physiological ones. In this sense, our study on an oenological yeast strain, isolated from natural fermenting musts and grown in a laboratory in a modified medium, made it possible to observe effects for herbicide or AIs that are probably more similar to what could really happen in nature. This is not only considering yeast as an eukaryotic model, but also and especially as a microorganism utilized for the production of wine, nowadays considered in all respects as a food. In addition, the use of a buffered medium allowed us to eliminate the pH-associated toxicity attributable to G acid (*31*). Moreover, because F stability is known to be affected by buffer pH, it should also be underlined that, at a pH value of 4.6, Zablotowicz and colleagues reported the absence of nonenzymatic transformations for this AI (*43*).

Considering the fermentation ability and ethanol production of *S. cerevisiae* K310, it should be pointed out how significant amounts of pesticides on grapes can inhibit initial vinification steps, thus altering, as seen, yeast cell functionality. Also, considering the yeast ability 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 productivity of vinification 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 consumer health.

On the basis of the results obtained, we feel strongly encouraged to deepen our studies on wild-type oenological yeast strains using herbicide/pesticide products in their commercial formulations, in parallel with studies on single AIs.

Concluding, we utilized a wild-type wine *S. cerevisiae* strain with the aim to evaluate the impact of three different herbicides, as commercial formulations (PE, P, and S) and their high-purity AIs (F, T, and G, respectively) on growth, fermentation, and metabolic patterns. We found that while pure active compounds affected at a lower extent cell growth and metabolism, commercial preparations, containing various additives, had a significant major negative influence on yeast biological parameters. In particular, only one case out of three responses obtained with commercial herbicides was similar to those obtained with the corresponding AI (P and T). In the two remaining cases, a negative impact on yeast growth, fermentation, and colony-forming ability was exclusively evident for herbicide formulation (S and PE) and not detectable for AIs alone (G and F, respectively).

The innovative use of the Biolog system to study responses to xenobiotics proved to be particularly useful both for a general evaluation of their effects on yeast metabolism and for the discovery of the specific target of stress. The analysis of the obtained metabolic profiles revealed, either in qualitative or in quantitative terms, that they were in general slightly or moderately affected by herbicides or AIs; however, the supplementation with the highest concentration of G was able to induce the assimilation and oxidation of several carbon sources and to greatly increase the overall metabolic activity.

ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; AI, active ingredient; AWCD, average well color development; DMSO, dimethyl sulfoxide; F, fenoxaprop-P-ethyl; G, glyphosate; GST, glutathione S-transferase; MRL, maximum residue limit; mw, molecular weight; P, Pointer; PE, Proper Energy; S, Silglif; T, tribenuron methyl; YPD, yeast peptone dextrose; YT, yeast-designed plates; μmax, maximal growth rate.

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