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Oxidative Damage Mediated by Herbicides on Yeast Cells

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Agricultural herbicides are among the most commonly used pesticides worldwide, posing serious concerns for both humans, exposed to these chemicals through many routes, and the environment. To clarify the effects of three herbicides as commercial formulations (namely, Pointer, Silglif, and Proper Energy), parameters related to oxidative issues were investigated on an autochthonous wine yeast strain. It was demonstrated that herbicides were able to affect the enzymatic activities of catalase and superoxide dismutase, as well as to induce carbonylation and thiol oxidation as post-translational modifications of proteins. *Saccharomyces cerevisiae* is an optimal model system to study responses to xenobiotics and oxidative stress. Thus, the results obtained could further the understanding of mechanisms underlying the toxicity of herbicides.

KEYWORDS: Herbicides; oxidative stress; protein carbonylation; thiol oxidation; wine yeast

INTRODUCTION

Pesticides have undoubted beneficial effects for preserving crop yield; however, they are extensively used and, sometimes, abused, posing serious health concerns. Among them, herbicides play a crucial role and must deserve particular attention because the general population is potentially exposed to such chemicals through many routes. Typically, pure herbicidal molecules are of limited relevance for users, and for this reason they are always applied in combination with the so-called “inert ingredients”, including within this definition many substances that have to guarantee and promote the desired action of active compounds. One of the most important reasons for formulating herbicides is to allow their use in common and convenient vehicles, such as water, and to obtain uniform and effective distributions. Nevertheless, the definition of “inert” can be misleading, because adjuvants can be toxic themselves or supplement or even increase toxic effects of pesticidal ingredients (*1, 2*). Recent efforts have been dedicated to better understand the toxicity of the herbicide glyphosate and some of its formulations (*3–6*). Unfortunately, this is not the case of the majority of herbicides that are currently and constantly used; for many of them, little or no information about the identity of adjuvants is publicly made available. Indeed, acute or chronic toxicity tests are often conducted by using the active molecules alone. Instead, adjuvants can directly increase the toxicity of pesticide formulations, make their penetration easier through clothes and skin, increase their persistence in the environment, and sustain a variety of damaging effects to nontarget organisms (*7*). In the case of emulsifiable concentrates, problems of dosing and mixing could be encountered and absorption through the skin could be facilitated. Granules, because 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 (*8*).

In a previous work (*9*) we performed a comparative analysis on an autochthonous *Saccharomyces cerevisiae* strain, isolated during the spontaneous fermentation of grapes and selected as potential “starter” for the production of high-quality wines (*10, 11*), testing in parallel three herbicides and their corresponding active ingredients alone. These herbicides were chosen among those authorized by the law and employed in the same geographical areas form which the yeast strain derived. They were Pointer (P), water dispersible granules containing tribenuron methyl; Silglif (S), a soluble concentrate containing glyphosate; and Proper Energy (PE), an oil/water emulsion containing fenoxaprop-P-ethyl. We clearly demonstrated that when active ingredients are mixed with the adjuvants, they exert more intense damaging effects on yeast cells (*8*).

To clarify the mechanisms underlying the toxicity of these herbicides, we decided to investigate if such effects could be ascribed to oxidative stress. Aiming at pointing out the most intense responses, in the present work we limited our research to only the highest concentration previously tested. We observed that herbicide treatments were able to affect the enzymatic activity of catalase and superoxide dismutase (SOD), as well as to induce oxidative modifications of proteins.

MATERIALS AND METHODS

General Materials. All high-quality reagents were from Oxoid (Garbagnate Milanese, Milan, Italy), Sigma-Aldrich (Milan, Italy), and

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J. T. Baker (Deventer, Holland). All water used was Milli-Q (Millipore, Bedford, MA).

Commercial grade herbicides were Proper Energy and Pointer (Bayer CropScience, Milan, Italy) and Silglif (Siapa, Milan, Italy).

Yeast Strain and Culture Conditions. The yeast strain used in this work was *S. cerevisiae* K310 that had been isolated from naturally fermenting must during the vinification of a high-quality wine (*10, 11*). K310 was precultured in YPD medium at 30 °C with rotary shaking (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 (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 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.

Commercial grade herbicides were singularly added to culture medium at the beginning of exponential growth phase (16th hour of cell culture, about 3×10^6 cells/mL) when yeast cells are most susceptible to applied stresses. Pointer was added as a water dispersion to a final concentration of 100 mg/L, Silglif was added as a water solution to a final concentration of 1 g/L, and Proper Energy was added as a water emulsion to a final concentration of 500 mg/L.

Growth, Colony-Forming Ability, and Fermentation Assays. Cell growth was monitored by measuring culture absorbance at 660 nm. This allowed us to choose different sampling times for further analyses, as follows: (i) four samplings in the log phase (at 15, 30, 60, and 120 min after the addition of herbicides); (ii) one sampling in the midlog phase (40th hour of growth for control, P, and S; 116th hour for PE); (iii) one at the end of log phase (64th hour of growth for control and P; 90th hour for S; 124th hour for PE); and (iv) one after the entry into the stationary phase (90th hour of growth for control and P; 116th hour for S and 164th hour for PE).

At the above indicated times, colony-forming ability assays were carried out in triplicate by plating cell suspensions (from undiluted to 1:100000 dilutions) on YPD agar. Plates were incubated at 30 °C and colonies counted after 3 days.

Levels of ethanol in the culture medium were determined using an enzymatic assay (kit code 10 176 290, Boehringer Mannheim, Germany). 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 with the manufacturer's instructions (*12, 13*).

Preparation of Yeast Cell Extracts. *S. cerevisiae* K310 cell suspension was centrifuged for 5 min at 4 °C at 3000*g* in a Beckman model J2-21 centrifuge equipped with a JA10 rotor. The supernatant was discarded and the pellet washed with distilled water and then with yeast medium buffer, pH 6.0 (YMB; 50 mM MES, 10 mM $MgCl_2 \cdot 6H_2O$, 10 mM EDTA $\cdot 2H_2O$). The pellet was then suspended in 2 volumes of YMB containing a cocktail of protease inhibitors (Sigma-Aldrich) and 0.1 M phenylmethanesulfonyl fluoride (PMSF); 1 volume of glass beads 425−600 μm (Sigma-Aldrich) was added, too. Disruption of the cells was achieved by vortexing samples for 8 min, alternating 30 s of vortexing with 30 s of rest in an ice bath. Cells were then centrifuged for 5 min at 4 °C at 300*g* in a Beckman model J2-21 centrifuge, using a JA20 rotor. The supernatant was collected and its protein concentration determined according to Bradford's method (*14*). Yeast extracts were then subjected to native polyacrylamide gel electrophoresis (PAGE) for enzyme activity assays or to derivatization, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting procedures for the analysis of oxidative protein post-translational modifications (PTMs) as subsequently described.

Activity of Catalase and Superoxide Dismutase (SOD) Enzymes. For enzyme activity determination, 50 μg of proteins was resolved through a 10% discontinuous native PAGE according to Ornstein's method (*15*) and stained as described, performing all operations in the dark.

For catalase activity, gels were incubated for 5 min in 5% (v/v) methanol, briefly washed three times with water, and incubated for 10 min in 10 mM H_2O_2. 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 blue except in the zones where H_2O_2 was decomposed by active catalase. Color development was blocked by soaking the gels in 10% (v/v) acetic acid and 5% (v/v) methanol (*16*).

For SOD activity, gels were first soaked in 2.5 mM nitro blue tetrazolium (NBT) for 20 min, then briefly washed with water, and soaked in 500 mM PBS containing 2.8 mM TEMED and 28 μM riboflavin. For revelation, gels were illuminated on a light box for 20 min until the appearance of white bands on a dark background (*17*).

Analysis of Protein Carbonylation. Ten micrograms of proteins was first incubated in the dark in 6% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) trifluoroacetic acid (TFA), and 5 mM 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyls; then the buffer reaction was neutralized with 2 M Tris base containing 30% (v/v) glycerol and 2% (v/v) β-mercaptoethanol (*18*). All samples were subsequently subjected to SDS-PAGE (12% polyacrylamide) (*19*). Gels obtained were stained with Coomassie brilliant blue (*20*) or, alternatively, transferred to nitrocellulose sheets with the semidry Novablot transblot cell (Bio-Rad), applying 1.5 mA/cm^2 for a total time of 45 min. For the immunorevelation of protein carbonyls, NC sheets were subsequently incubated with rabbit anti-dinitrophenyl antibodies (Sigma-Aldrich) 1:10000, followed by peroxidase-conjugated anti-rabbit antibodies (Sigma-Aldrich) 1:7000, and revelation was achieved through chemiluminescence (Immun-Star HRP Chemiluminescent Kit, Bio-Rad).

Analysis of Protein Thiol Oxidation. Ten micrograms of proteins was incubated for 15 min in the dark in YMB buffer at pH 6.5 or 8.5 containing 20 μM biotinylated iodoacetamide (BIAM) (Sigma-Aldrich) to alkylate nonoxidized protein thiols (*21*). Reaction was quenched by adding 20 mM β-mercapoethanol (final concentration), and samples were subjected to SDS-PAGE (12% polyacrylamide) (*19*). Gels were stained with Coomassie brilliant blue (*20*) or, alternatively, transferred to nitrocellulose sheets with the semidry Novablot transblot cell (Bio-Rad), applying 1.5 mA/cm^2 for a total time of 45 min. For the immunorevelation of nonoxidized protein thiols, NC sheets were incubated with peroxidase-conjugated streptavidin (Sigma-Aldrich) 1:100000, and revelation was achieved through chemiluminescence.

Image Analysis. Images of gels and films were acquired by a laser densitometer (Image Scanner, Amersham Biosciences) and analyzed with the Image Master Platinum software (Amersham Biosciences), choosing as a reference parameter the intensity of bands, which is automatically normalized by the software against the surrounding background.

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

RESULTS

Growth, Colony-Forming Ability, and Fermentation Patterns. In **Figure 1** is reported the growth kinetic of K310 obtained in control conditions (YPDm with no supplementations) and in the presence of P, 100 mg/L; S, 1 g/L; or PE, 500 mg/L. Herbicides were added at the beginning of exponential growth (16th hour), because this is the moment of the highest susceptibility to cell stress, and growth was then monitored. As can be observed, P altered slightly the growth of yeast, reducing the total number of cells per milliliter during the early log phase without affecting the growth rate. S had more important consequences; it reduced the number of cells per milliliter during the entire period of observation and slackened growth more importantly, shifting the attainment of stationary phase from the 64th to the 90th hour of culture. However, PE exerted the most deleterious effects on K310 growth, because it was able to protract the lag phase until the 90th hour, dramatically altering kinetics and cell biomass production once

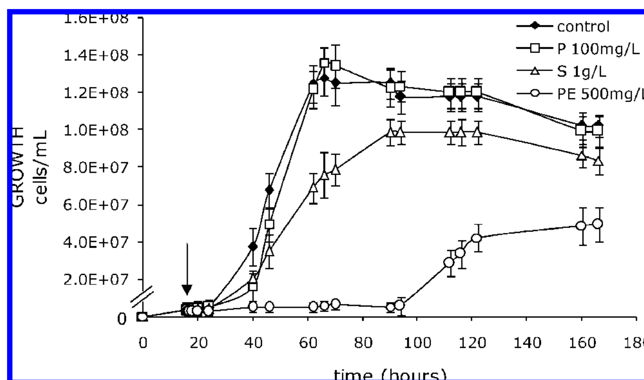

Figure 1. Growth of K310 in YPDm medium (glucose, 100 g/L; pH 4.5; incubation at 28 °C in semianaerobiosis) in the presence of herbicide formulations (P, 100 mg/L; S, 1 g/L; or PE, 500 mg/L) or in the absence of herbicides (control). The arrow indicates the moment of herbicide supplementation (16th hour, beginning of the log phase). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars.

growth resumed. These data suggested that the herbicides S and PE may negatively affect the growth K310 strain, probably by altering yeast metabolic pathways.

We also evaluated the colony-forming ability and fermentative ability of yeast cells. We chose different sampling times on the basis of what we had observed for growth patterns; in particular, four samples were collected after a brief exposure to herbicides (here indicated as log 15, 30, 60, and 120 min), whereas subsequent samples were collected at different growth phases (one at midlog, one at the end of log phase, and one after the entry into stationary phase). **Figure 2** reports the ability of herbicides to alter both yeast cells' colony-forming ability (**A**) and fermentation (**B**). In particular, P significantly reduced the number of colony-forming units per milliliter only after 60 and 120 min of treatment and no longer affected colony-forming ability; however, it negatively affected yeast fermentative ability up to the midlog phase. As observed for growth, S had more important effects, being able to significantly reduce colony-forming ability until the entry into the stationary phase and to lower ethanol production during the entire period of observation. The herbicide PE dramatically reduced in a short time the number of colony-forming units per milliliter (2.5 log units during the first 2 h of treatment), even if differences with the control culture were no more significant at midlog and stationary phases. With regard to the fermentative power, PE suddenly blocked ethanol production just after the supplementation and resulted in lower ethanol production during the entire observation period.

Activity of Antioxidant Enzymes. To avoid the production of interfering substances during a spectrophotometric assay due to the presence of residues of herbicides, we chose to evaluate the enzymatic activities of catalase and SOD by performing a native PAGE electrophoresis of cell extracts and staining, as subsequently described. Images of gels were then acquired and quantitatively evaluated. We selected as a reference parameter the intensity of bands, which was automatically normalized by the software against the background, and reported these values as arbitrary units in **Figure 2C**,**D**.

All of the tested herbicides were able to significantly increase the activity of catalase (**Figure 2C**) at various moments of growth. However, particular attention should be paid to the responses observed in the presence of S, which was able to promptly induce catalase activity even when it was not detectable in control conditions or in the presence of P and PE during the early log phase. In the case of P supplementation, catalase activity was comparable to the control except for the sampling at midlog phase; for PE supplementation, we observed an increased catalase activity during the late log phase and stationary phases.

The results obtained for SOD activity (**Figure 2D**) showed quite a different situation. In the case of S, during the early log phase the activity of SOD was initially lowered (after 30 min of treatment), then restored (60 min) and increased (120 min) with respect to control. Afterward, it was comparable to control at midlog phase and significantly increased again during the late log and stationary phases. In the presence of P and PE, SOD activity was suddenly decreased by both herbicides and enzyme inactivation was observed also for the subsequent samplings: at midlog phase for PE and at end log for P and PE. During the stationary phase, SOD activity was comparable to that of the control.

Oxidative Post-translational Modifications of Proteins. Assessment of the oxidative damage due to herbicides was also evaluated through the observation of carbonylation and thiol oxidation of K310 proteins.

The evaluation of carbonylated proteins following herbicide treatments is reported in **Figures 3** and **4**. From a quantitative point of view, P caused a more extensive degree of carbonylation during the exponential growth phase after 30, 60, and 120 min of treatment. In the presence of S, quite a dynamic pattern of oxidation was observable with levels of carbonylated proteins similar or slightly lower with respect to the control during the entire observation period. PE induced a sudden increase of carbonylated proteins during the log phase, particularly at 30 and 120 min of treatment, as well as during the stationary phase. However, the qualitative analysis (**Figure 4**) showed the most intriguing results. Indeed, by grouping and summing the intensities of immunoreactive bands in various ranges of molecular weight we were able to observe: (i) the carbonylation of proteins with molecular weight lower than 25 kDa only after treatment with P and PE during the log phase; (ii) the carbonylation of proteins with molecular weights higher than 116 kDa mainly following the treatment with P (during the log phase), S (during the end log and stationary phase), and PE (during the entire period of observation); (iii) a more important carbonylation of proteins between 45 and 62 kDa (2-fold vs control) in the case of P treatment during the early log phase; and (iv) a higher presence in the SDS-PAGE stacking gel of carbonylated protein aggregates in cells treated with P (5-fold at 15 min, 2-fold at 30 and 60 min vs control) and PE (5-fold at 15 min, 2-fold at 30, 60, and 120 min vs control).

We also performed a time course analysis of thiol residues that can be oxidized by herbicide treatment. Thiol oxidation was indirectly evaluated by derivatizing free (nonoxidized) protein sulfhydryl groups. The assay relies on the selective labeling of nonoxidized thiols with BIAM followed by streptavidin immunostaining. As a consequence, oxidation can be indirectly evaluated as a lower signal in the immunoblots. Labeling was performed at two distinct pH values, 6.5 and 8.5, because the majority of protein thiols have a pK_a near 8.5; nevertheless, some redox-sensitive proteins have a pK_a lowered by charge interactions with surrounding amino acid residues and can exist as thiolate anions at pH values near neutrality (*21–23*). Among these proteins, many have a protective role in the oxidative stress response, as in the case of disulfur isomerase, Tyr phosphatase, glyceraldehyde-6-phosphate dehydrogenase, and peroxiredoxins. In **Figure 5** the immunoblots of nonoxidized

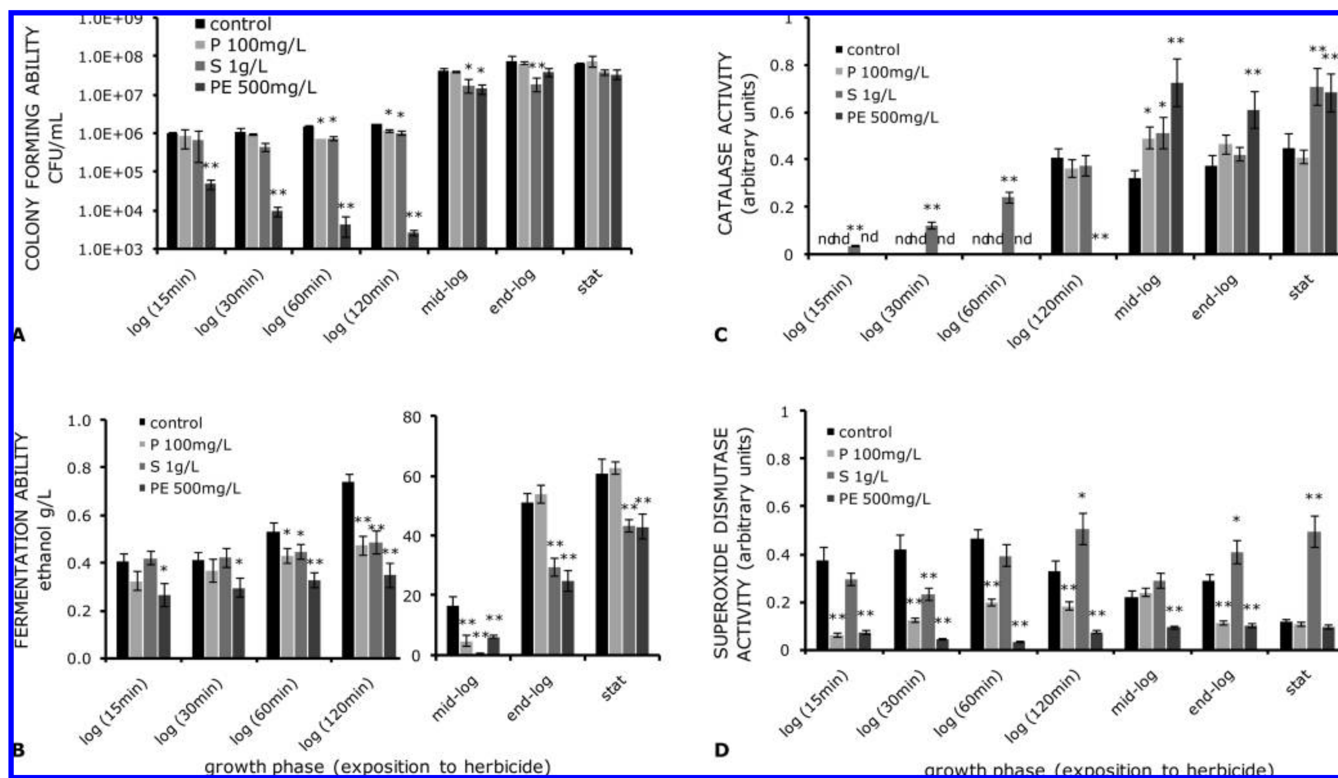

Figure 2. Colony-forming ability (**A**), fermentation ability (**B**), and enzymatic activity of catalase (**C**) and superoxide dismutase (**D**) of K310 in YPDm medium (glucose, 100 g/L; pH 4.5, incubation at 28 °C in semiaerobiosis) in the presence of herbicide formulations (P, 100 mg/L; S, 1 g/L; or PE, 500 mg/L) or in the absence of herbicides (control). Data reported are average values of three independent experiments carried out under identical conditions; standard deviations are indicated with vertical bars. ∗∗, $P < 0.01$; ∗, $P < 0.05$, compared with the control culture; nd, not detectable.

protein thiols in cells treated with herbicides during various phases of growth are reported. The results of the quantitative analysis, performed by detecting and summing the intensities of immunoreactive bands in different ranges of molecular weight, are shown in **Figure 6**.

When BIAM labeling was performed at pH 6.5 a small number of bands was detected both in standard conditions and in the presence of S and PE. In the case of P supplementation, instead, no immunoreactive bands were detected in the conditions we adopted. From a quantitative point of view, each sampling during S treatment showed a higher number of nonoxidized thiols (fold-change ranging from 2 to 4 vs the control), whereas in the presence of PE we observed, following a brief exposition to herbicide, a particularly significant decrease of free thiols at 15 min (no detectable bands) and 30 min (2-fold vs the control); nevertheless, for the following growth phases we observed an increase in BIAM-labeled thiols (fold-change ranging from 2 to 4 vs the control).

When BIAM labeling was performed at pH 8.5, a wider range of protein thiols were observable. The total number of immunoreactive bands increased consistently in the whole range of examined molecular weights. Grouping and summing the intensities of detected bands, we observed for P treatment quantitative and qualitative patterns very similar to those of the control. The only difference was the oxidation of thiols in proteins with MW higher than 116 kDa, observable only in control conditions and not in the presence of P. Quite a different pattern was found in the presence of S and PE: in these cases the total sum of BIAM-labeled thiols was reduced up to 4 times with respect to the control. Moreover, we found the absence of BIAM-labeled proteins with MW higher than 116 kDa, and a significant and consistent decrease in all of the investigated ranges of MW except that between 45 and 62 kDa in the presence of S and between 35 and 45 kDa in the presence of PE, indicating how both of these formulations can exert oxidative effects on protein thiols.

DISCUSSION

S. cerevisiae is probably the best studied eukaryote organism and a well-consolidated model in the study of responses to xenobiotics and oxidative stress; moreover, *S. cerevisiae* is a key microorganism for the vinification process. In a previous work (*9*) we compared the effects of the herbicides P, S, and PE with those sustained by their active ingredients (tribenuron methyl, glyphosate, and fenoxaprop-P-ethyl, respectively) on a wild-type wine yeast strain and proved that commercial formulations exert the most cytotoxic action in a dose-dependent manner. In the present work, we investigated if such cytotoxic effects could be mediated by oxidative mechanisms. For this reason we decided to evaluate various yeast parameters following the treatment of our yeast strain with P, 100 mg/L; S, 1g/L; or PE, 500 mg/L, the highest concentrations previously tested, to allow the observation of the most evident effects.

The involvement of oxidative mechanisms to mediate the damaging effects of herbicides has been first shown by the analysis of two classical enzymes involved in the oxidative stress response, catalase and SOD. The activation of catalase, observed following each herbicide treatment, is one of the most common cellular responses to redox alterations, because this enzyme is easily induced by a wide range of stimuli often related to the energy status of the cell. Particular attention should be paid to S, able to induce the catalase even when its activity was not detectable in control conditions during the early log phase of growth, when the high glucose concentration of culture medium can exert a negative pressure on antioxidant systems, catalase

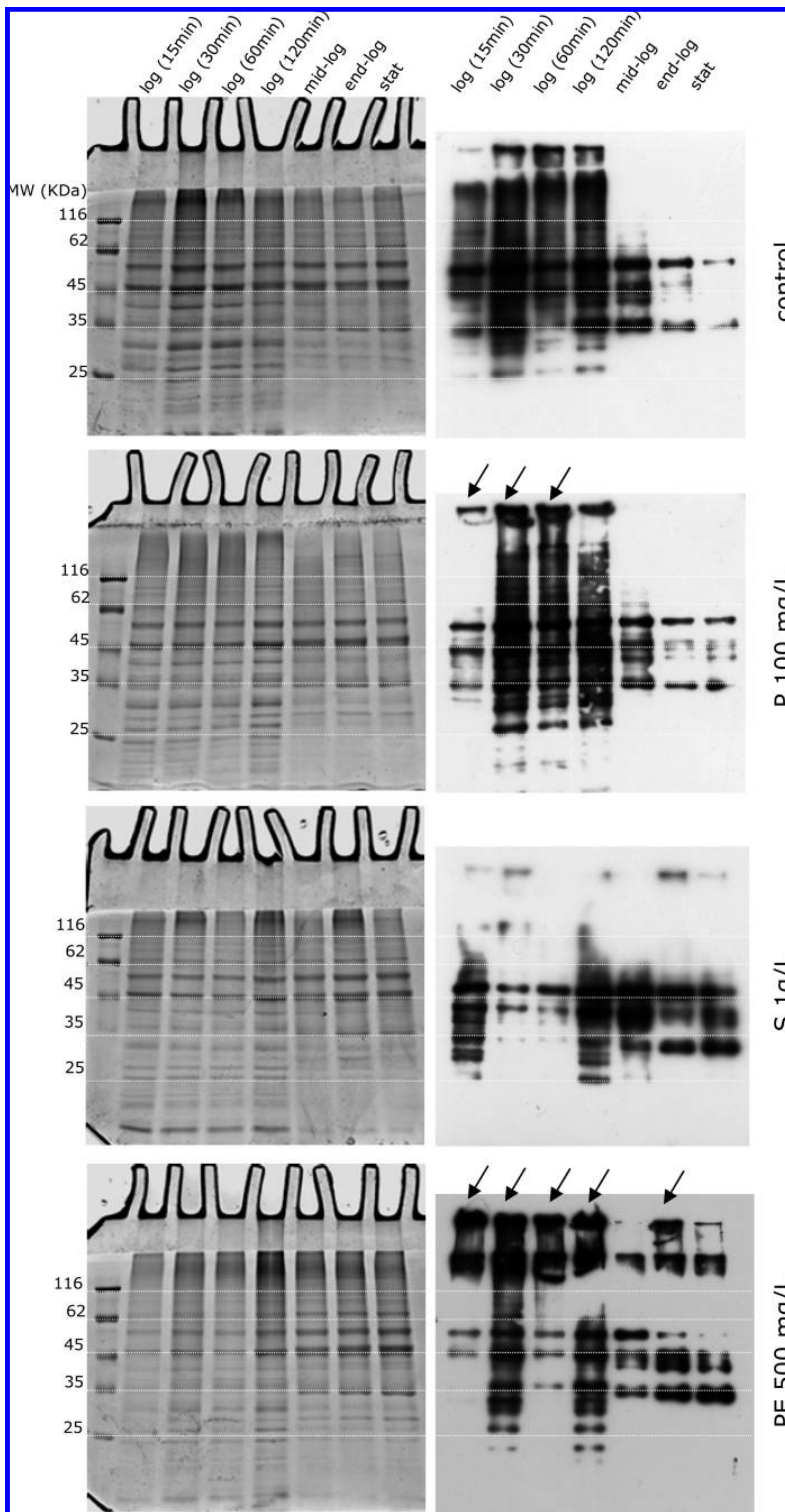

Figure 3. Coomassie-stained SDS-PAGE (left) and immunoblotting of carbonylated proteins (right) of K310 grown in standard conditions or subjected to herbicide supplementation. Arrows indicate carbonylated protein aggregates. Experiments were carried out in triplicate; only representative gels and films are reported.

included (*24*). On the other hand, the late activation we observed for PE-treated cells could be one of the mechanisms involved in growth resumption after the long period of latency induced on yeast cells by this herbicide. The general inactivation of SOD

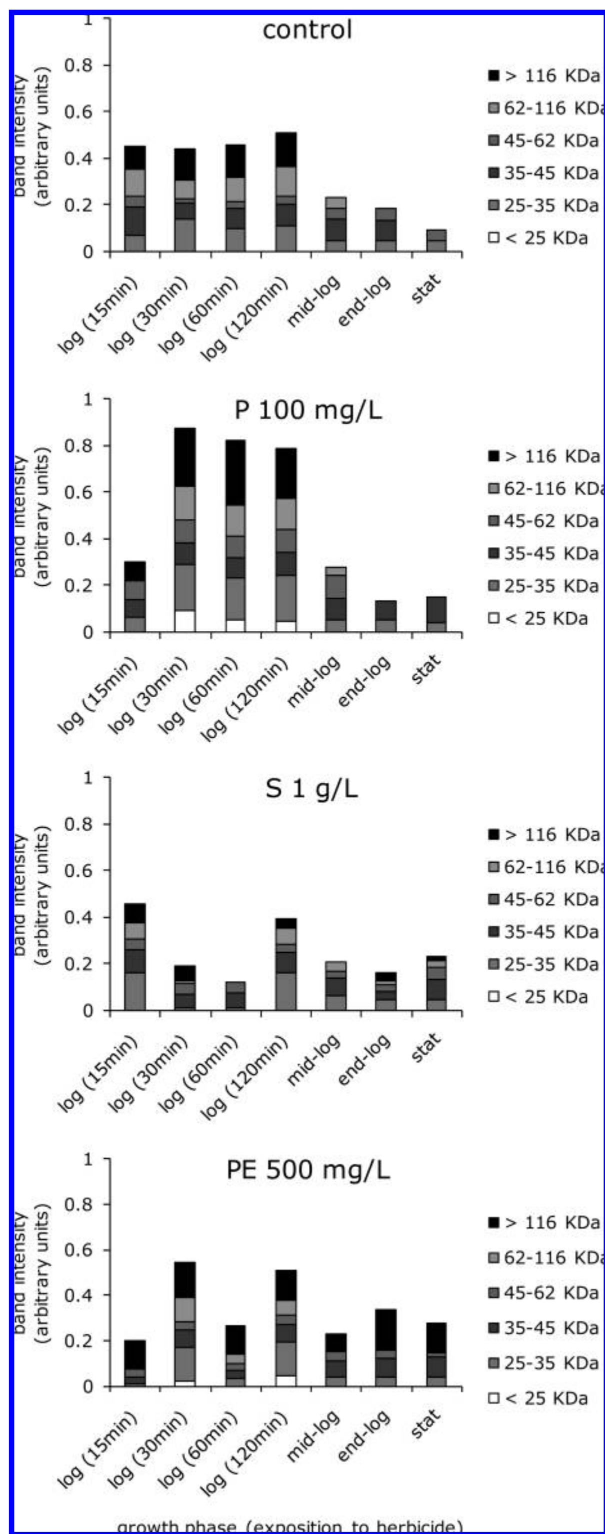

Figure 4. Quantitative analysis of carbonylated proteins of K310 grown in standard conditions or subjected to herbicides supplementation. Immunoreactive band intensities have been grouped in various ranges of molecular weight (as indicated in **Figure 3**), and their sum is reported in the graph. Values represent the average of three independent experiments.

observed after short herbicide treatments could be the result of oxidative damage of this enzyme, because it is known to be inactivated by various peroxides (*25*). This probably reflects the decreased ability of yeast cells to adapt efficiently to the oxidative stress encountered. A sudden decrease in SOD activity had been already associated with brief treatments of human erythrocytes with the herbicide 2,4,5-trichlorophenoxyacetic acid and to one of its metabolites (2,4,5-trichlorophenol) (*26*). Whereas SOD is considered to be an essential antioxidant enzyme, at the same time it can have pro-oxidant effects in vivo (*27*), and thus SOD inactivation can be read as a defense mechanism as well.

Literature reports about the activity of antioxidant enzymes after herbicide treatments are quite heterogeneous, probably reflecting different experimental conditions and/or tested concentrations. Other factors that must be borne in mind are the different ROS types that can be generated, their toxicity for the cells, and their detoxification rate as well as the involvement of enzymatic and nonenzymatic defense mechanisms. Only as a few examples, glyphosate alone did not alter catalase and SOD activity in the liver of rats (*28*), whereas SOD activity in roots and leaves of pea plants was not affected by the treatment with imazethapyr, an imidazolinone herbicide (*29*). Moreover, whereas catalase activation in yeast had been already associated with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (*17, 30*), in mice a commercial formulation containing 2,4-D and the single active ingredient did not alter catalase activity (*31*). Nevertheless, our data for S are in good agreement with a recent report for another glyphosate-based formulation (Roundup Ultra 360 SL) able to quickly induce catalase activity in human erythrocytes after very short treatments (*6*).

Redox−homeostasis alteration through PTMs of proteins is one of the main events related to oxidative stress. The introduction of carbonyl groups in proteins is widely accepted as a biomarker of oxidative stress, because this modification is nonenzymatic, stable, and easily detectable (*32–34*). Carbonyl groups can be introduced into proteins by a wide variety of mechanisms: directly, by interactions with reactive oxygen species (ROS), or indirectly, by reaction with reactive carbon species (RCS) generated in the cell during the ROS-mediated oxidation of molecules such as sugars and lipids.

In this work, we observed that the herbicides P and PE affected protein carbonylation from both from qualitative and quantitative points of view. Levels of carbonylated proteins were increased by short herbicide treatments during the log phase of growth, and proteins in MW ranges higher than 116 kDa and lower than 25 kDa were selectively found to be oxidized in treated cells. Thus, protein oxidation should be seen not as a random event, but the carbonylation of specific proteins could regulate, depending on their redox state, fundamental cellular events (*35, 36*). Cells with large amounts of carbonylated proteins could be subjected to alterations in proteasome and chaperone activities; therefore, they could have to cope with impaired cellular protein turnover and functionality. Moreover, carbonylation can alter the protein folding and, as a consequence, the proper function of molecules (*32–34*). Yeast cells subjected to P and PE contained carbonylated protein aggregates. This is a well-known consequence of protein oxidation, because highly carbonylated proteins tend to aggregate and form structures that are resistant to degradation and accumulate as damaged or unfolded proteins. Such aggregates tend to inhibit proteasome activity as well (*33*). On the contrary, the lower levels of protein carbonyls in the case of S supplementation could be explained by the high activity in these cells of catalase, an enzyme that has been recently found to protect in vivo yeast proteins from H_2O_2-induced carbonylation (*37–39*).

Sulfur-containing amino acids are among the best susceptible targets for a wide range of ROS (*40*). Proteins sulfhydryl groups play a fundamental role in numerous cell processes, and their redox state is involved in both structure and proper function of

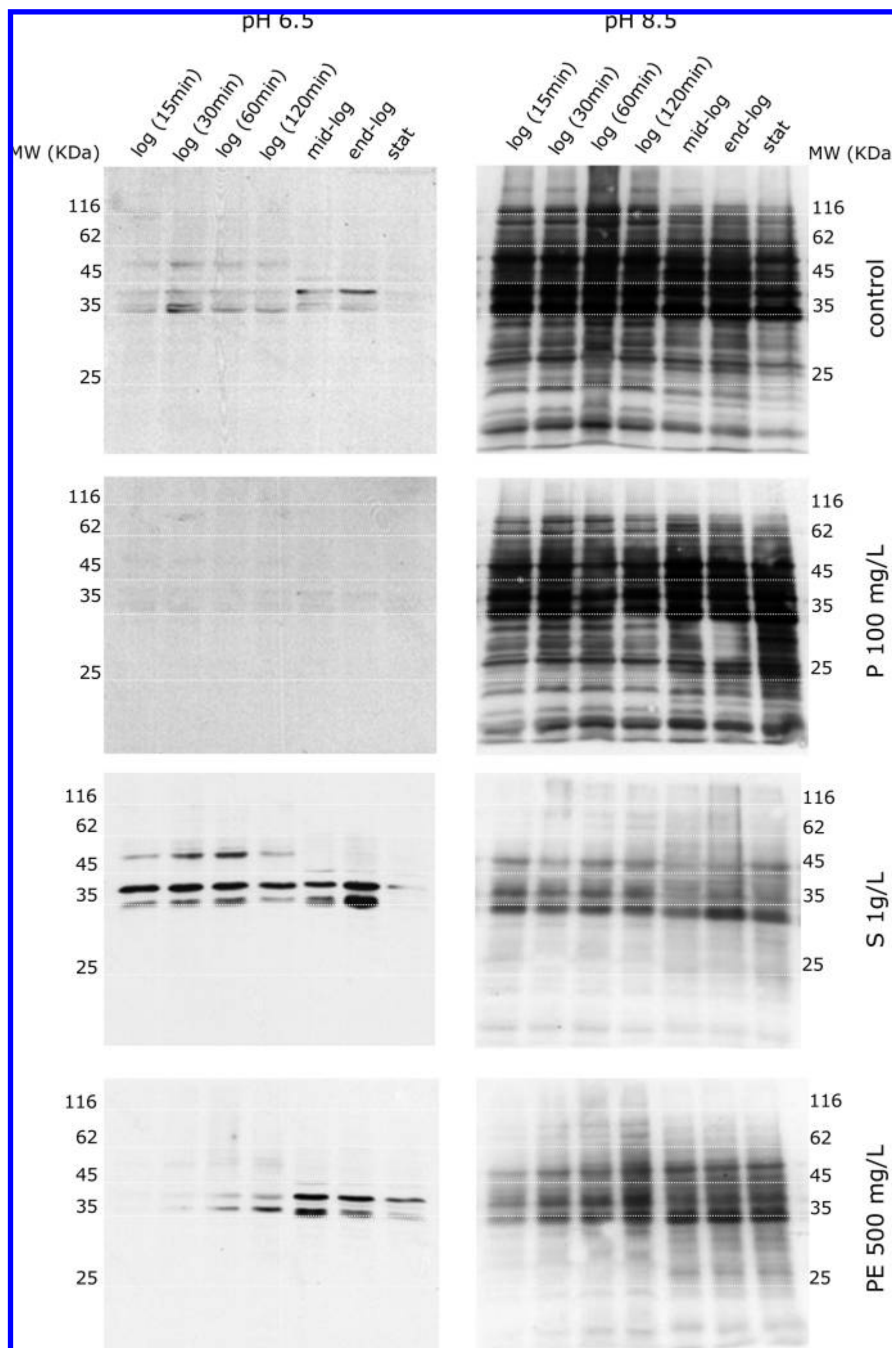

Figure 5. Immunoblots of protein free thiol groups at pH 6.5 (left) or at pH 8.5 (right) of K310 grown in standard conditions or subjected to herbicides supplementation. Molecular weights were obtained by using a standard in the corresponding Coomassie-stained replica gels (not reported). Experiments were carried out in triplicate; only representative films are reported.

many receptors, enzymes, and transcription factors. Their oxidation often leads to alterations in conformation and catalytic activity of proteins (*41, 42*); notwithstanding, these events do not necessarily have to be seen as damaging. On the one hand, thiol oxidation could be a "random" event; on the other hand, it can be part of finely tuned processes that protect proteins from irreversible oxidation or activate specific functions related to stress response. Often, cysteines, as in the case of glyceraldehyde-6-phosphate dehydrogenase, are in the active site of molecules and their oxidation could act as a protection; on the contrary, their irreversible oxidation could furnish the cell a signal for protein degradation (*43*), and critical cysteine residues have been shown to play a role in the regulation of cell death in yeast (*44*).

We observed that the three herbicides tested were able to differently oxidize *S. cerevisiae* thiol protein groups. P exerted only slight effects, and an indirect confirmation of its oxidizing power was obtained only for thiols with lower pK_a. In the case of cells treated with S and PE we clearly demonstrated a wide oxidative damage of thiols, with significant differences in almost the entire protein repertories.

However, it was also possible to hypothesize a beneficial involvement of proteins containing thiols with lower pK_a in the oxidative stress response. Such proteins were protected from oxidation during the resumption of growth in the PE-treated cells and during the entire period of observation in the S-treated cells. This allowed us to suppose that catalase may play also a

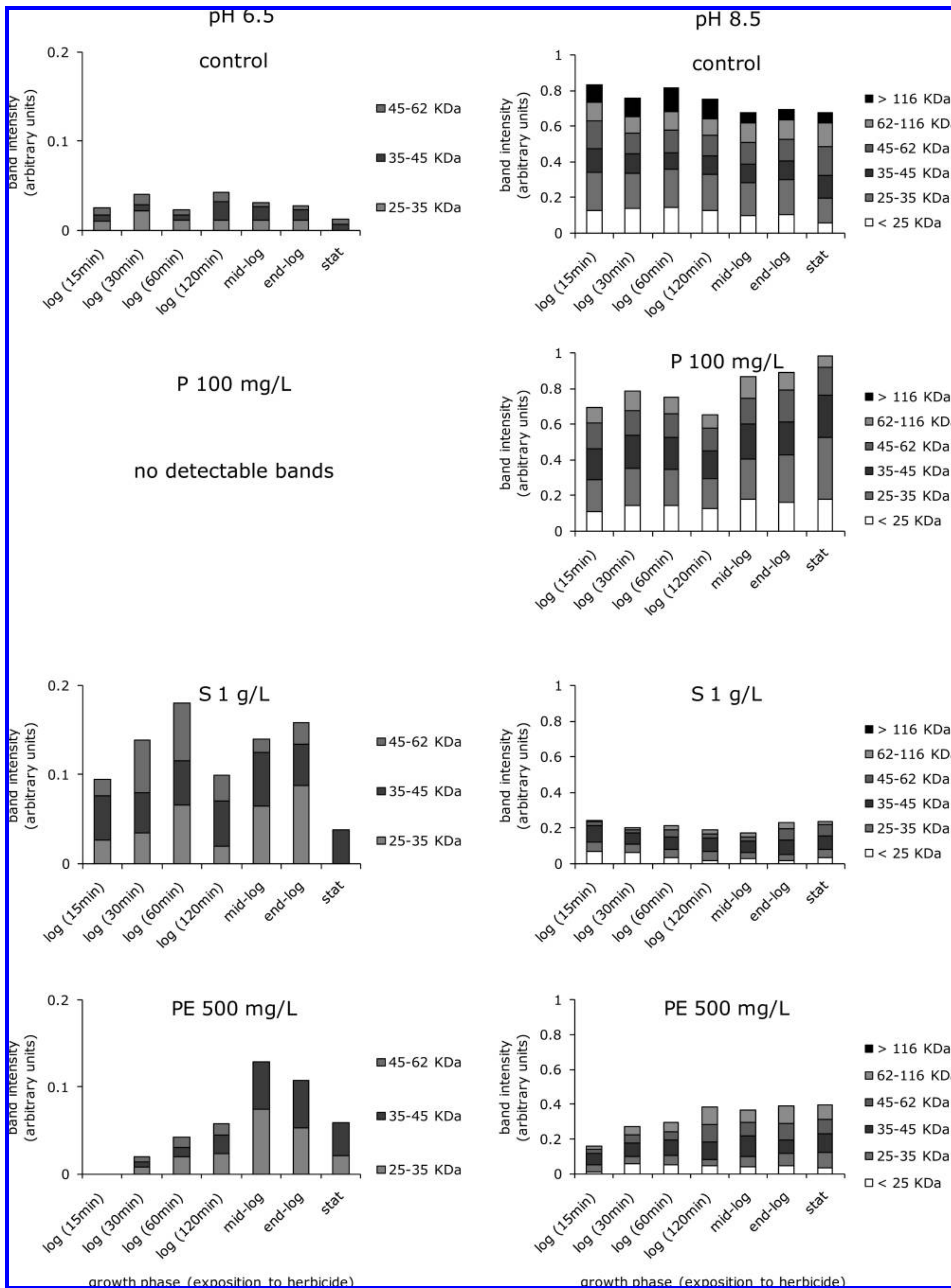

Figure 6. Quantitative analysis of protein free thiol groups at pH 6.5 (left) or at pH 8.5 (right) of K310 grown in standard conditions or subjected to herbicides supplementation. Immunoreactive band intensities have been grouped in various ranges of molecular weight (as indicated in **Figure 5**), and their sum is reported in the graph. Values represent the average of three independent experiments.

positive role in the response to herbicide stress, protecting thiols from oxidation.

Considering *S. cerevisiae* K310 strain not only as a model cell but also its potential application as fermentation starter in the production of wine, one of the most important parameters to be evaluated is the ethanol production. Our investigations allowed us to highlight how significant amounts of herbicides can inhibit initial vinification steps and have negative impacts on yeast fermentative ability. This phenomenon has been already reported during both wine and beer production (*45, 46*) and, on the basis of our findings, it can be now read in the light of oxidative PTMs of proteins. Both carbonylation and thiol oxidation of key enzymes involved in the fermentative processes could in turn have negative consequences for the biological processes that enological *S. cerevisiae* strains are supposed to properly carry out.

ABBREVIATIONS USED

BIAM, biotinylated iodoacetamide; P, Pointer; PAGE, polyacrylamide gel electrophoresis; PE, Proper Energy; PTM, post-translational modification; RCS, reactive carbon species; ROS, reactive oxygen speices; S, Silglif; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; YMB, yeast medium buffer; YPDm, yeast peptone dextrose modified; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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