Free Radical Research, August 2010; 44(8): 891–906

informa
healthcare

Oxidative damage induced by herbicides is mediated by thiol oxidation and hydroperoxides production

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(Received date: 21 January 2010; In revised form date: 20 April 2010)

Abstract
Toxicological and environmental issues are associated with the extensive use of agricultural pesticides, although the knowledge of their toxic effects as commercial formulations is still far from being complete. This work investigated the impact of three herbicides as commercial formulations on the oxidative status of a wild type *Saccharomyces cerevisiae* strain. With yeast being a well-established model of eukaryotic cells, especially as far as regards the stress response, these results may be indicative of potential damages on higher eukaryotes. It was found that herbicide-mediated toxicity towards yeast cells could be the result of an increased production of hydroperoxides (like in the case of the herbicides Pointer and Silglif) or advanced oxidation protein products and lipid peroxidation (especially in the case of the herbicide Proper Energy). Through a redox-proteomic approach it was found also that, besides a common signature, each herbicide showed a specific pattern for protein thiols oxidation.

Keywords: *Commercial formulations, pesticides, protein oxidation, redox-proteomics, Saccharomyces cerevisiae, 2D-PAGE*

Abbreviations: *AOPP, Advanced Oxidation Protein Products; BIAM, Biotinylated iodoacetamide; GPx, Glutathione Peroxidase; HRP, HorseRadish Peroxidase; IEF, IsoElectric Focusing; LPO, Lipid PerOxidation; MDA, MalonylDiAldehyde; P, Pointer; PE, Proper Energy; S, Silglif; SDS-PAGE, Sodium Dodecyl Sulphate– PolyAcrylamide Gel Electrophoresis; TH, Total Hydroperoxides; YPD, Yeast Peptone Dextrose; 2D-PAGE, Two-dimensional PolyAcrylamide Gel Electrophoresis.*

Introduction

Pesticides are unique chemicals designed to exert specific biological activities that can affect many non-target species. Nevertheless, they are intentionally released into the environment in large quantities, eventually leading to serious health and eco-toxicological damage that may be under-estimated because little information is provided on the effects of pesticides as complete formulations rather than pure active molecules.

In previous works of ours we compared the effects of three herbicides, namely Pointer (P), Silglif (S) and Proper Energy (PE), and their pure active ingredients (tribenuron methyl, glyphosate and fenoxaprop-P-ethyl, respectively) on a wild-type wine *Saccharomyces cerevisiae* strain isolated from the same geographical

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd.
DOI: 10.3109/10715762.2010.489111

area where these herbicides have been used during the last years [1,2]. We demonstrated that, when applied as complete formulations, herbicides have more serious effects on yeast cells mediated by oxidative perturbations [3]. Moreover, we found that herbicides could alter the yeast protein repertoires and induce oxidative modifications of proteins [3,4]. We identified the majority of yeast proteins undergoing herbicide-induced carbonylation [4], a well consolidated marker for oxidative stress, and showed that the effects of herbicides on yeast protein profiles shared homologies with those of known oxidants [4].

In order to obtain further insights on the mechanisms underlying the herbicide-mediated toxicity on yeast cells, in the present work we moved towards the analysis of other cell parameters related to oxidative injuries, such as glutathione (GSH) content, hydroperoxides production and protein oxidation. Particularly, we decided to investigate protein thiol oxidation, as thiols should be considered critical components in redox systems biology, regulating through their reversible/irreversible oxidation many biological functions [5]. We combined selective labelling of cysteine residues with two-dimensional electrophoresis (2D-PAGE) to evaluate the redox state of yeast proteins during herbicide stress.

We found that the tested herbicides could differently alter the examined yeast parameters, and the most deleterious effects were, in general, found to be caused by the herbicide PE. For all the three herbicides, however, minor effects were found on GSH and related compounds, whereas levels of lipid peroxidation (LPO), hydroperoxides production or generation of advanced oxidation protein products (AOPP) were more importantly affected. Considering thiol oxidation, each herbicide showed oxidative effects on yeast proteins, and we were able to find specific protein targets for oxidation besides a common oxidative signature. Our results indicate that herbicide-induced oxidative stress could impair metabolic fluxes of carbohydrates (thus eventually impairing the fermentative performance of wine yeast strains) as well as specific metabolic pathways.

Materials and methods

General materials

Unless otherwise indicated, all high quality reagents and antibodies were from Sigma-Aldrich (Milan, Italy). All water used was Milli-Q (Millipore, Bedford, MA).The herbicides P, S and PE were from commercial sources.

Yeast strain and culture conditions

S. cerevisiae K310 strain yeast cells were pre-cultured in yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C with rotary shaking (120 rpm) for 10 h. Then, an aliquot of the cell culture was inoculated in 150 mL of a modified YPD medium (YPDm) adjusted to a final pH of 4.5 by adding 0.2 M citrate/phosphate buffer and containing 100 g/L glucose, to obtain an initial concentration of 1×10^4 cells/mL. The cell suspension was then incubated at 28°C in the dark without shaking and allowing semi-anaerobic growth.

Commercial grade herbicides were singularly added to the culture medium at the beginning of the exponential growth phase (16^{th} hour of cell culture, ~ 3×10^6 cells/mL). P was added as a water dispersion to a final concentration of 100 mg/L; S was added as a water solution to a final concentration of 1 g/L; PE was added as a water emulsion to a final concentration of 500 mg/L. These concentrations were chosen in order to reproduce the conditions we adopted for our previous works [1–4].

Preparation of cell lysates

Yeast cells samples and culture supernatants were harvested at various moments according to K310 growth patterns observed in our previous investigations [1–4], as follows:

i) two samplings in the log phase, one 15 min after the addition of herbicides, indicated as [log (0h)] and one after 1 h of treatment, indicated as [log (1h)];
ii) one sampling in the mid-log phase (40^{th} h of growth for control, P and S; 116^{th} h for PE);
iii) one sampling at the end of the log phase (64^{th} h of growth for control and P; 90^{th} h for S; 142^{nd} h for PE); and
iv) one sampling after the entry into the stationary phase (90^{th} h of growth for control and P; 116^{th} h for S and 164^{th} h for PE).

Cell lysates were prepared as detailed elsewhere [6]; together with culture supernatants, they were immediately refrigerated for further analyses. Protein content was assessed according to Bradford's [7] method.

Transmission electron microscopy (TEM)

After 1 h of treatment with the herbicides P, S and PE or after growth in control conditions, samples of yeast cells were fixed in cold Karnovsky fixative for 2 h at 4°C, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 2 h at 4°C in 2% buffered OsO_4, dehydrated in a graded series of ethanol and embedded in Epon-Araldite. Ultra-thin sections cut with an LKB III ultramicrotome were collected on copper grids, stained with uranyl acetate and lead citrate and then photographed using a Philips

EM 208 electron microscope. At least 100 cells for each group were examined.

Detection of thiobarbituric acid reactive substances (TBARS)

The levels of TBARS in yeast cell lysates were determined following the method described by Do et al. [8]. Briefly, yeast cell lysates (0.1 mL containing 0.48 mg of proteins) were mixed with 0.4 ml of methanol (0.01% BHT) and 0.5 ml of 1% thiobarbituric acid (prepared in 1% sulphuric acid) and incubated for 15 min at 100°C. Samples were allowed to cool and after centrifugation (12 000 x *g*, 10 min) the absorbance at 532 nm was measured against a yeast lysate-free blank. Levels of TBARS, expressed as malonyldialdehyde (MDA) equivalent, were calculated using $\varepsilon 532 = 13\ 700\ M^{-1}\ cm^{-1}$ [8].

AOPP

AOPP were measured using the microplate assay by Witko-Sarsat et al. [9]. Calibration was performed using chloramine-T, which absorbs at 340 nm in the presence of potassium iodide. In test wells, 200 µL of yeast cell lysates diluted 1:20 in PBS and in standard wells 200 µL of chloramine-T solutions (ranging from 0–100 mM) were distributed on a 96-well microtiter plate; 10 µL of 1.16 M potassium iodide and hence 20 µL of acetic acid were added to each well. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a 200 µL PBS blank. Being the absorbance of chloramine-T at 340 nm linear up to 100 mM, AOPP concentrations are expressed as mmol/L chloramine-T equivalents.

Total hydroperoxides (TH)

TH were measured in yeast culture supernatants with a d-ROMs Kit (Diacron srl, Italy) using the method described by Buonocore et al. [10]. The assay estimates the total amount of hydroperoxides in a 10 µL sample by using a spectrophotometric procedure. Briefly, hydroperoxide groups are attacked by iron and decompartmentalized from transport protein in 1 mL of a pH 4.8 acetate buffer to catalyse reactive oxygen metabolites formation by Fenton's reaction. The peroxy- and alkoxy-radicals produced, whose quantities are directly proportional to peroxides in the yeast culture supernatants, are hence chemically trapped by 10 µL of chromogen (N,N-dyethyl-p-phenyldiamine) in an electron-transfer process leading to the formation of the radical cation of the chromogen. The purple colour resulting from the reaction is then monitored over time in a UV-VIS spectrophotometer (Perkin Elmer l 16, Norwalk, CN) at 505 nm. Results obtained in conventional Carr units (1 Carr unit is equal to a concentration of 0.08 mg/dL of H_2O_2) are expressed as mg H_2O_2 per litre of culture supernatant.

Non-protein thiol assay (GSH)

Cellular non-protein thiol content was determined as described in Beutler [11]. Since GSH represents more than 90% of the non protein –SH, the latter will be referred to as GSH. In brief, aliquots of yeast cell lysates (0.24 mg of proteins) were re-suspended in 150 µl of a metaphosphoric acid solution [1.67% (v/v) metaphosphoric acid, 0.2% (w/v) EDTA, 30% (w/v) NaCl], kept on ice for 5 min and centrifuged (12 000 × *g*, 5 min). The non-protein thiol content was measured spectrophotometrically in the supernatant, at 412 nm, using 5,5-dithiobis(2-nitrobenzoic acid) $\varepsilon 412 = 13\ 600\ M^{-1}\ cm^{-1}$ [11].

GSSG assay

Aliquots of yeast cell lysates (0.24 mg of proteins) were re-suspended in 150 µl of a metaphosphoric acid solution [1.67% (v/v) metaphosphoric acid, 0.2% (w/v) EDTA, 30% (w/v) NaCl], kept on ice for 5 min and centrifuged (12 000 × *g*, 5 min). 2-vinylpyridine was added to derivatize GSH [12] and samples were kept in ice for 1 h. GSSG content was then assayed as reported by Tietze [13].

Glutathione peroxidase (GPx) activity

Yeast cell lysates (0.12 mg of proteins) were centrifuged (12 000 × *g*, 5 min at 4°C). GPx (E.C. 1.11.1.9) activity was determined spectrophotometrically in the supernatants as described by Beutler [11].

Glutathione reductase (GR) activity

GR (E.C. 1.8.1.7) activity was followed spectrophotometrically at 340 nm by monitoring the oxidation of NADPH at 30°C as described previously [14] using aliquots of yeast cell lysates containing 10 µg proteins.

Thiol derivatization with biotinylated iodoacetamide (BIAM) and two-dimensional gel electrophoresis (2D-PAGE)

Aliquots of yeast cell lysates containing 75 µg of proteins were derivatized in MES-Tris buffer (pH 6.5) containing 200 µM BIAM for 15 min in the dark. The labelling reaction was quenched by the addition of 2 mM β-mercaptoethanol (final concentration) [15].

After BIAM labelling, yeast cell lysates were denatured in 40 µL of the IEF denaturing buffer and

loaded onto 11 cm Immobiline strips (3.0–10.0 non-linear pH, Bio-Rad, Milan, Italy) together with 180 μL of the immobiline rehydration buffer. IEF was then run according to manufacturer's instructions. SDS-PAGE separation step was performed on 4–12% polyacrylamide linear gradient Criterion XT™ Pre-cast Gels (Bio-Rad, Milan, Italy). The obtained 2D-gels were washed and equilibrated in a transfer buffer (50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20% (v/v) methanol) and protein transfer from 2D gels onto nitrocellulose membranes was carried out using a semi-dry Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm^2 for 75 min. Protein transfer was checked by staining of membranes with 0.2% (w/v) Ponceau S in 3% (v/v) trichloroacetic acid.

BIAM-labelled proteins were detected with horseradish peroxidase (HRP)-linked streptavidin (dilution 1:10 000), followed by a chemiluminescence reaction using the Immun-starTM HRP kit (Bio-Rad).

In parallel, yeast samples containing 50 μg of proteins were resolved according to the same procedures on 4–12% polyacrylamide Criterion XT™ Pre-cast Gels to produce silver stained maps (data not shown).

Image analysis

Digitalized images of stained gels and immunoblotting films were acquired (Image Scanner, Amersham Biosciences; Milan, Italy) and analysed with Image Master™ Platinum (Amersham Biosciences; Milan, Italy).

Protein identification

BIAM-labelled immunoreactive proteins in films were first matched to the corresponding silver stained maps, then identified by gel matching with reference proteomic maps of the K310 strain already produced and characterized in our laboratory [4,6].

Analysis of proteolytic activity (SELDI proteomic profiling)

Total yeast protein extracts were analysed with the SELDI ProteinChip to obtain a quantitative profile of peptides ranging from 2–16 kDa in molecular mass. Hydrophobic (H50) ProteinChip Arrays were used and treated with 10% ACN, 0.1% TFA as binding buffer according to the manufacturer's bioprocessor protocol (Bio-Rad). One microlitre of CHCA (Bio-Rad) as the energy adsorbing molecule was added to each spot. ProteinChip Arrays were read on a SELDI-ToF/MS (ProteinChip SELDI System Personal Edition, Bio-Rad) acquiring peptide spectra and measuring masses and intensities of peptide peaks. The spectra were smoothed, baseline subtracted, externally calibrated, while peak intensities were normalized with the total ion current of m/z starting from 1.5 kDa.

Statistics

All of the experiments were carried out in triplicate. Data are presented as average values ± standard deviation; at least a p-value ≤ 0.05 was considered to be significant for analysis of variance. For 2D-PAGE, Western Blot and SELDI proteomic analyses, only representative gels, films and spectra are shown.

Results

Yeast cell morphology

TEM microscopy highlighted structural effects of herbicides on yeast cell morphology. If compared to the control (Figure 1A), yeast cells challenged with P (Figure 1B) or S (Figure 1C) showed alterations at the nuclear level, particularly in chromatin (Figure 1B) which appears marginated (Figure 1C) in about half of the cells. Cytoplasmic components (vacuoles, mitochondria) and cell wall organization did not appear altered. On the contrary, ~ 90% of PE-treated cells showed vacuolization at the nuclear and cytoplasmic level (Figure 1D) causing loss of cell integrity and cell death. It is thus tempting to speculate that, although more specific assays are necessary, PE-treated cells could be apoptotic, since apoptosis is characterized by DNA damage, chromatin condensation and fragmentation and also linked to oxidative stress [16].

MDA levels

MDA levels in yeast cells were significantly affected by the presence of herbicides during the whole period under investigation (Figure 2A). P and PE induced a sudden and statistically significant increase in MDA levels immediately after the addition to yeast culture [log (0h)] and after 1 h treatment, when MDA levels in PE-treated cells were 4-times higher than in the control. At the following samplings, collected when yeast cells were in the mid-log, end-log and stationary phases of growth, MDA levels were always higher in herbicide-treated cells than in the control culture, with S and PE showing the most important effects.

AOPP levels

AOPP levels in the presence of the herbicides P and S were found to be almost unaffected in respect to the control (Figure 2B). The most striking finding was when we treated yeast cells for 1 h with PE (Figure 2B). At this time point, we found AOPP levels 11-times higher

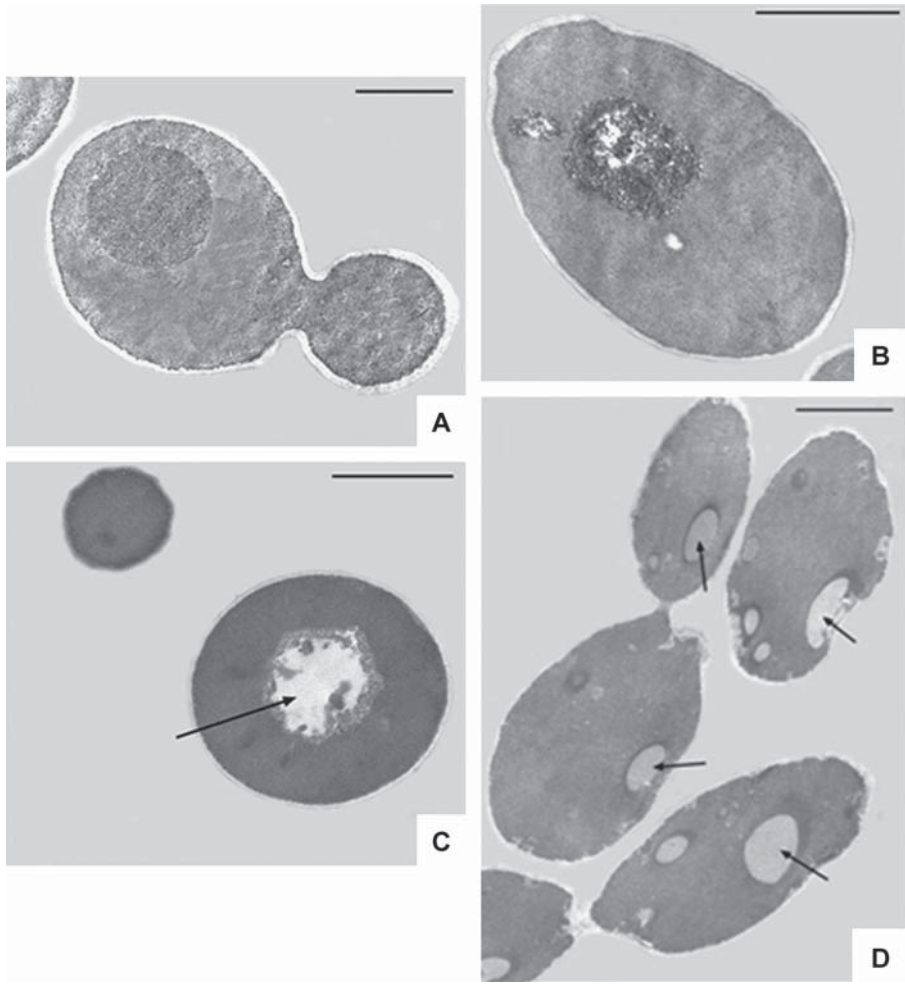

Figure 1. TEM micrographs of *S. cerevisiae* K310. (A) K310 cultured under control conditions (YPDm), bar 0.6 μm. The cell shows a normal morphology at nuclear and cytoplasmic level; cell wall organization appears maintained. (B) K310 cultured in YPDm + 100 mg/L P, bar 0.9 μm. The cell shows an evident change in chromatin status. (C) K310 cultured in YPDm + 1 g/L S, bar 0.75 μm. Chromatin appears marginated (arrow). (D) K310 cultured in YPDm + 500 mg/L PE, bar 0.75 μm. The cells highlighted ultrastructural alterations at nuclear and cytoplasmic level (arrows) causing cell death.

in PE-treated cells than in the control. At the following samplings, differences were no more significant.

TH levels

TH levels, measured in yeast culture supernatants, were significantly higher in herbicide-treated cultures than in the control (Figure 2C). In the case of P and S a similar pattern was found during the entire period of observation. In the case of PE, on the contrary, TH levels were similar to those of control during the log and mid-log phases of growth, whereas they were increased at the end-log and stationary phase.

GSH, GSSG, GSH/GSSG, GPx and GR activities

Yeast levels of the anti-oxidant GSH were almost unaffected by herbicide treatments (Figure 3A). The only exception was the sudden increase in GSH we registered after 1 h treatment with P. On the contrary, levels of oxidized glutathione (GSSG) (Figure 3B) were higher in S- and PE-treated cells with respect to the control in almost all the samplings at different phases of growth. For P- treated cells, we found a significant increase in the levels of GSSG only at the stationary phase of growth. As a consequence, the ratio GSH/GSSG (reported in Figure 3C) showed a significant decrease in both S- and PE-treated cells for all the samplings, and also for the herbicide P at the stationary phase of growth.

The activity of GPx, reported in Figure 3D, was unaffected by short treatments of K310 with all the tested herbicides in respect to the control. However, at the mid-log, end-log and stationary phases of growth, the herbicides S and PE caused a statistically significant decrease in the activity of GPx. The analysis of GR activity, reported in Figure 3E, indicated only minor changes induced by the tested herbicides. In particular, an increased GR activity was found in P-treated cells during the log-phase of growth, whereas GR activity was decreased by the presence of S and PE at the end-log samplings.

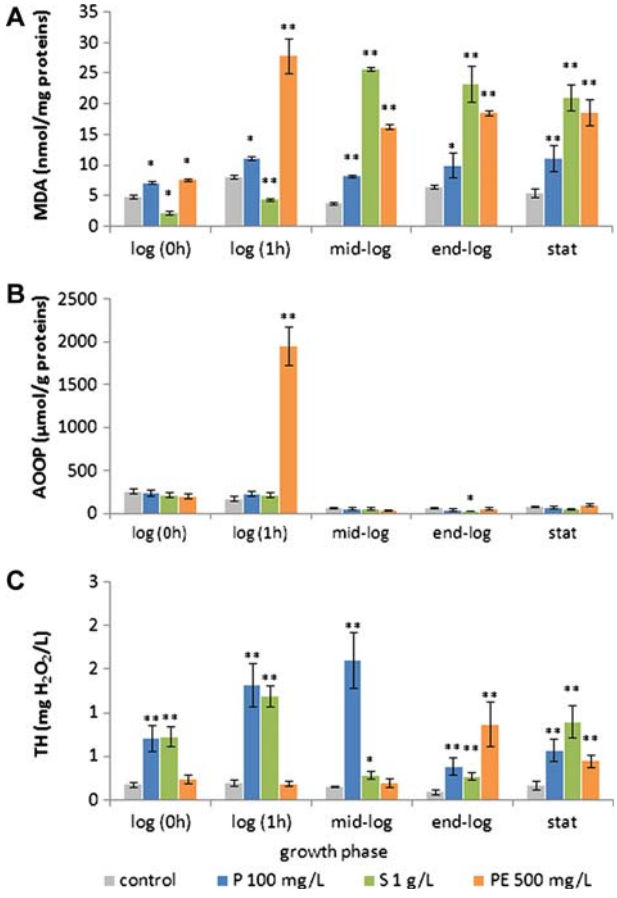

Figure 2. MDA, AOPP and TH levels under control conditions or after treatment with 100 mg/L P, 1 g/L S or 500 mg/L PE. Data reported are average values of three independent experiments carried out under identical conditions; standard deviation is indicated with vertical bars. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ compared with the control culture.

BIAM-labelled yeast proteins

We undertook a proteomic analysis to identify yeast protein thiols undergoing oxidation following herbicide treatment. For this reason, yeast cells were challenged for 1 h with 100 mg/L P, 1 g/L S or alternatively 500 mg/L PE. Hence protein thiol oxidation patterns found in the presence of herbicides were compared with the one obtained in control conditions. The choice of herbicide concentrations and time of treatment were based on our previous observations on herbicide-induced toxic effects and protein carbonylation in K310 cells [1–4].

The technical approach adopted was based on the derivatization of non-oxidized protein thiol groups with BIAM followed by streptavidin immunostaining. As a consequence, oxidation could be indirectly evaluated as a lack of signal in the immunoblots. Labelling was performed at pH 6.5 in order to highlight those redox-sensitive proteins that have a pK_a lowered by charge interactions with surrounding amino acid residues and can exist as thiolate anions at pH values near the neutrality [15]. Among these proteins, many have a protective role in the oxidative stress response [15].

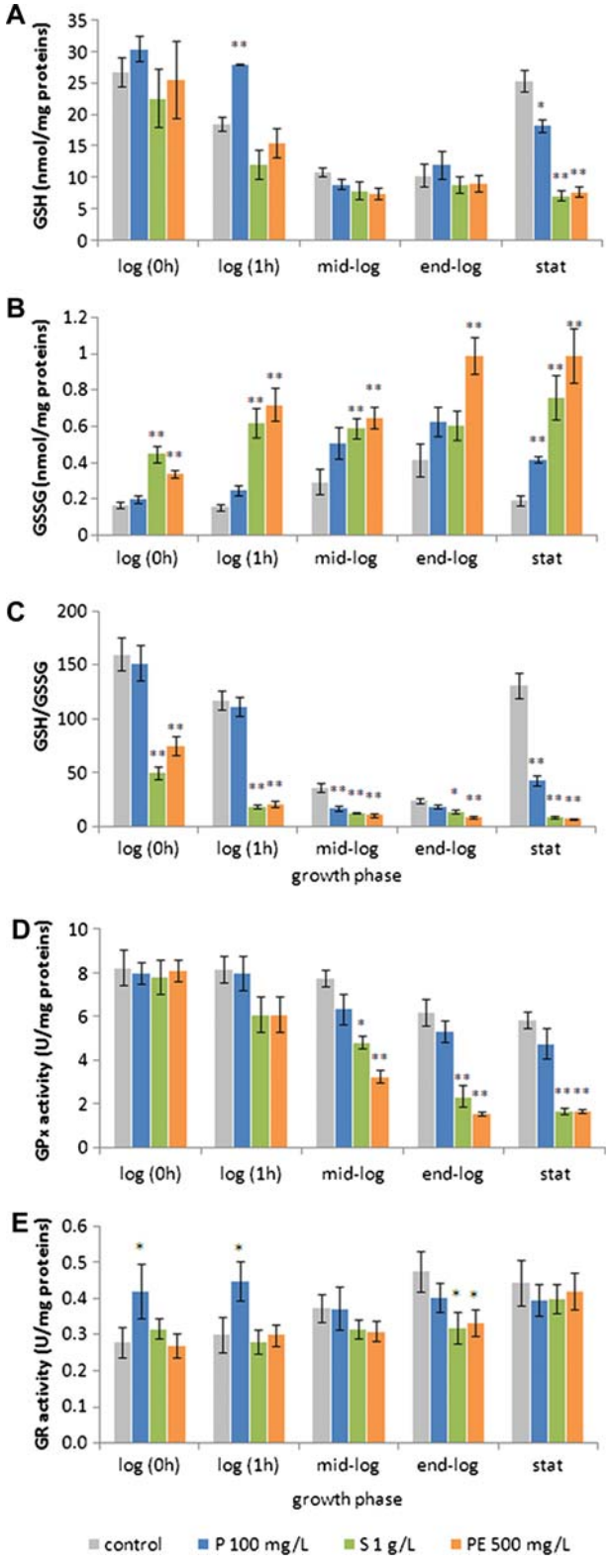

Figure 3. Levels of GSH (A), GSSG (B), GSH/GSSG (C) and enzyme activities of GPx (D) and GR (E) under control conditions or after treatment with 100 mg/L P, 1 g/L S or 500 mg/L PE. Data reported are average values of three independent experiments carried out under identical conditions; standard deviation is indicated with vertical bars. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ compared with the control culture.

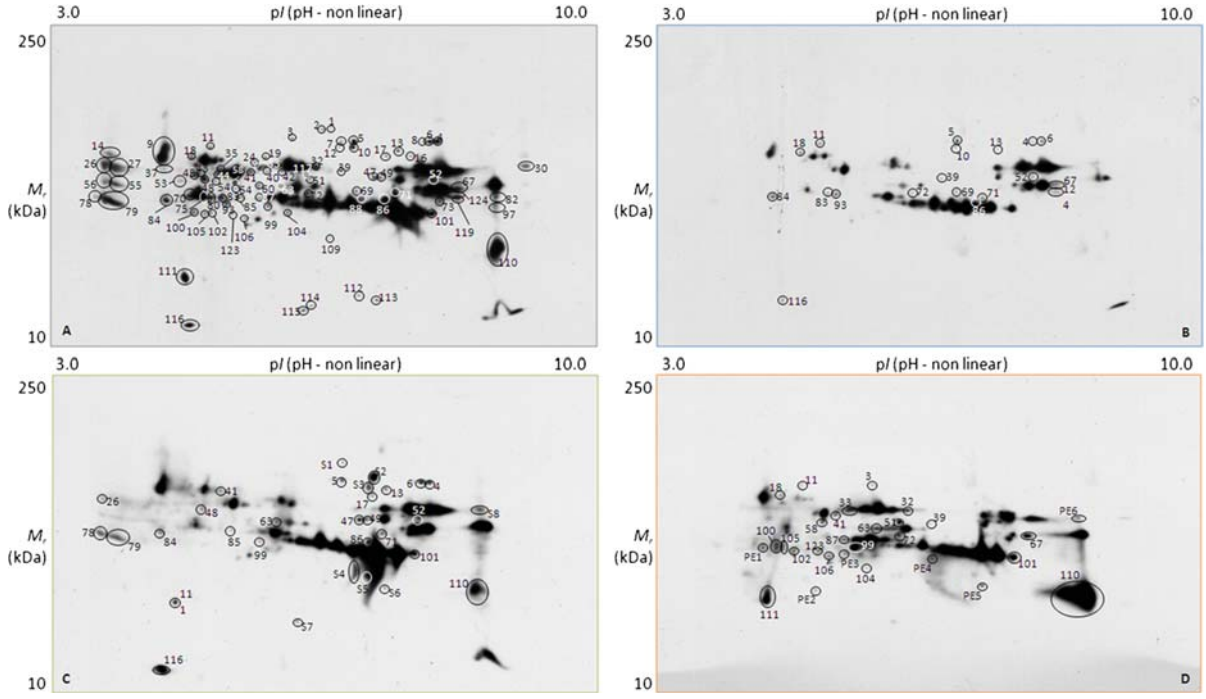

Figure 4. BIAM-labelled protein thiols in total *S. cerevisiae* K310 lysates under control conditions (A) or after 1 h treatment with 100 mg/L P (B), 1 g/L S (C) or 500 mg/L PE (D) immuno-revealed with streptavidin-HRP. Eighty-three protein spots, indicated with numbers, were found to be oxidized by the tested herbicides, i.e. they were found under control conditions (A) but were missing in the Western Blot films in the presence of P (B), S (C) or PE (D). Proteins identified by gel matching with a silver stained reference map (reported in Figure 6) are described in Table I.

In Figure 4A, the Western Blot films of BIAM-labelled thiols in control conditions is reported. In Figures 4B–D, the films of BIAM-labelled thiols in cells treated for 1 h with the herbicides P, S and PE are reported, respectively.

One hundred and twenty-four protein spots were found to be BIAM-immunostained in control conditions, 59 in the presence of P, 72 in the presence of S and 70 in the presence of PE (Figure 5). Forty-one protein spots were found as BIAM-immunostained in all the conditions tested, meaning that these proteins did not undergo thiol oxidation in the presence of herbicides. Hence, these spots were not further considered in our analysis.

Although labelling with BIAM does not allow the identification of the exact oxidative modification occurring in proteins, this method offers indeed an easy and global evaluation of thiol oxidation. In the present work, 83 protein spots were found to be specifically oxidized by herbicides, because of their lack in BIAM immunostaining films in herbicide-treated samples vs the control. Each herbicide showed a different selectivity and particularly six spots were oxidized in the presence of P; five spots in the presence of S; and nine spots in the presence of PE. Twelve spots were found to be oxidized either in the presence of P or S; eight spots either in the presence of P or PE; and five spots either in the presence of P or PE. More importantly, 38 protein spots were found to be commonly oxidized after the treatment with each herbicide tested (Figure 5). Also, eight protein spots appeared as BIAM-immunostained only in the presence of S (protein spots from S1 to S8 in Figure 4C) and six only in the presence of PE (protein spots from PE1 to PE6 in Figure 4D).

BIAM immunostained films were matched with the corresponding replica silver stained proteomic maps (data not shown) and 28 proteins were identified by gel matching with a proteomic reference map of K310 proteins (reported in Figure 6) already produced and characterized in our laboratory. Once identified, proteins were grouped according to their biological functions, and listed in Table I. We found that the yeast proteins undergoing thiol oxidation in the presence of the herbicides P, S or PE were mostly enzymes involved in carbohydrate metabolism. In this group, proteins coded by genes *ald2*, *eno2*, *fba1*, *hxk2*, *pdb1*, *pdc1*, *pfk1*, *pgk1*, *sec53* and *tdh3* were identified. Other proteins were found in the cell rescue and defence (*kar2*, *mnn4* and *ssb1*); protein synthesis (*sup1* and *tef1*); cellular transport (*pho84* and *vma2*); amino acid metabolism (*met6*, *sam2* and *trp5*); cellular components (*tub2*); proteins with binding functions (*lia1*); nucleoside/nucleotide metabolism (*ado1*); and protein fate (*pre9*).

Among the few protein spots found as BIAM-immunostained in the presence of herbicides but not in the control, two protein spots were identified in the presence of S exclusively. These were the transketolase 1 (*tkl1*) (spot S3) and the cytosolic superoxide dismutase (*sod1*) (spot S7).

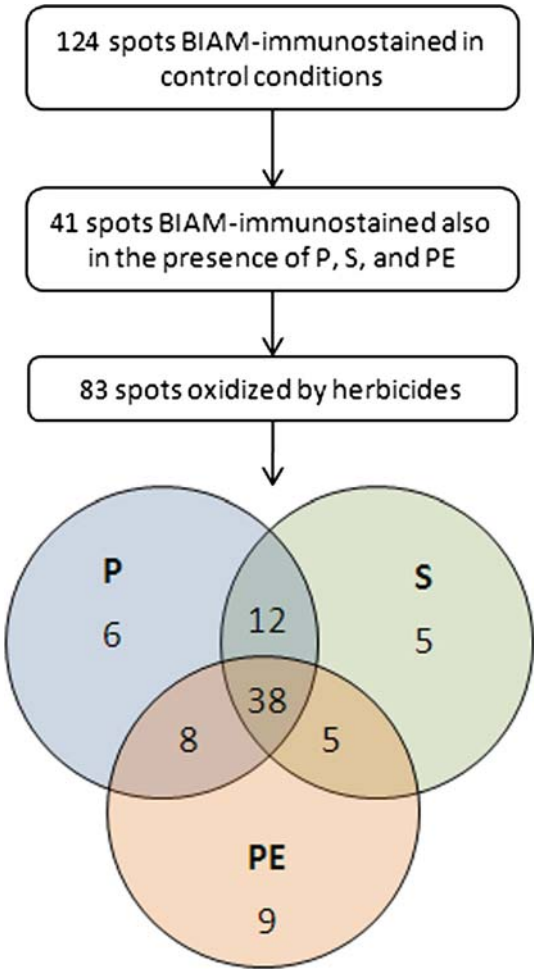

Figure 5. Selectivity of herbicide-induced thiol oxidation of yeast proteins in the presence of 100 mg/L P, 1 g/L S or 500 mg/L PE.

Analysis of proteolytic activity

In order to evaluate the proteolytic activity in yeast under the tested conditions, K310 cells were collected after 1 h treatment with the herbicides P, S, PE or under control conditions and cell lysates were prepared in the presence of vacuolar protease inhibitors [6]. Samples were analysed with reverse phase H50 ProteinChip arrays, allowing the revelation of the hydrophobic peptidic component of samples and spectra obtained were compared to evaluate if the treatment with the tested herbicides could differently affect the yeast peptide repertoire. The analysis was carried out in triplicate sets, showing optimal reproducibility of spectra and the most interesting peaks were detected in the 2-16 kDa *m/z* range (Figure 7). Overall, the treatment of K310 yeast cells with the herbicides P and PE caused a decrease in the intensities and number of peaks with respect to the control, whereas an opposite phenomenon was found in the presence of the herbicide S. This suggested a decreased (in the presence of P and PE) and increased (in the case of S) proteolysis of yeast proteins, respectively.

Discussion

Oxidative stress occurs when the rate at which ROS are generated exceeds the capacity of the cell to remove them by anti-oxidants. When an increased ROS production is concomitant with a reduction of anti-oxidant systems, cells have to cope with oxidative damage of proteins, DNA and lipids. A large body of evidence suggests that herbicides may promote toxic events via the intermediate release of ROS which, because of their extreme reactivity, lead to the formation of lesions in target molecules. Although this notion was established for an array of herbicides, including some of those employed in the present study [17,18], very little information is at present available on the identity of the radical species involved for the specific herbicides. In particular, formation of superoxide and H_2O_2 was documented in neuronal cells and shown to be implicated in the lethal response resulting from pesticide exposure [19]. Light-dependent formation of singlet oxygen was rather shown to mediate the toxic effects induced by herbicides in plant cells [20–22].

Herbicides, together with many other environmental contaminants, can cause oxidative damage by producing ROS that can react with various biological macromolecules, eventually damaging them and leading to cell death. For this reason, the analysis of cell oxidative status is crucial to the understanding of mechanisms underlying herbicide-mediated damage. Nevertheless, the importance of evaluating the potential toxicity of complete pesticide formulations, rather than just testing their active ingredients, began to be appreciated only recently. As an example, some authors investigated the toxicity of the herbicide glyphosate and some of its formulations, sold worldwide under a variety of commercial names. Importantly, Géhin et al. [23] and Hultberg [24] reported that glyphosate, alone or as its formulation Roundup, can alter the cell antioxidant status. Also, it has been found that surfactants may interfere with phospholipid bilayers of biological membranes, impairing all the cell processes requiring intact membrane structure [25].

In this work, we implemented with new experiments our previous results on oxidative damage [1–4] in order to get a more complete view of yeast cells anti-oxidant status after treatments with the commercial herbicides P, S and PE. We focused our attention on classical parameters related to oxidative injuries, such as GSH and GSH-related compounds and LPO, as well as on oxidative modifications of proteins.

TH content represents a measure of the overall oxidative stress, given that it takes into account intermediate oxidative products of lipids, peptides and amino acids. Our analysis strongly indicated that, globally, P- and S-mediated toxicity towards yeast cells could be the result of an increased production of hydroperoxides.

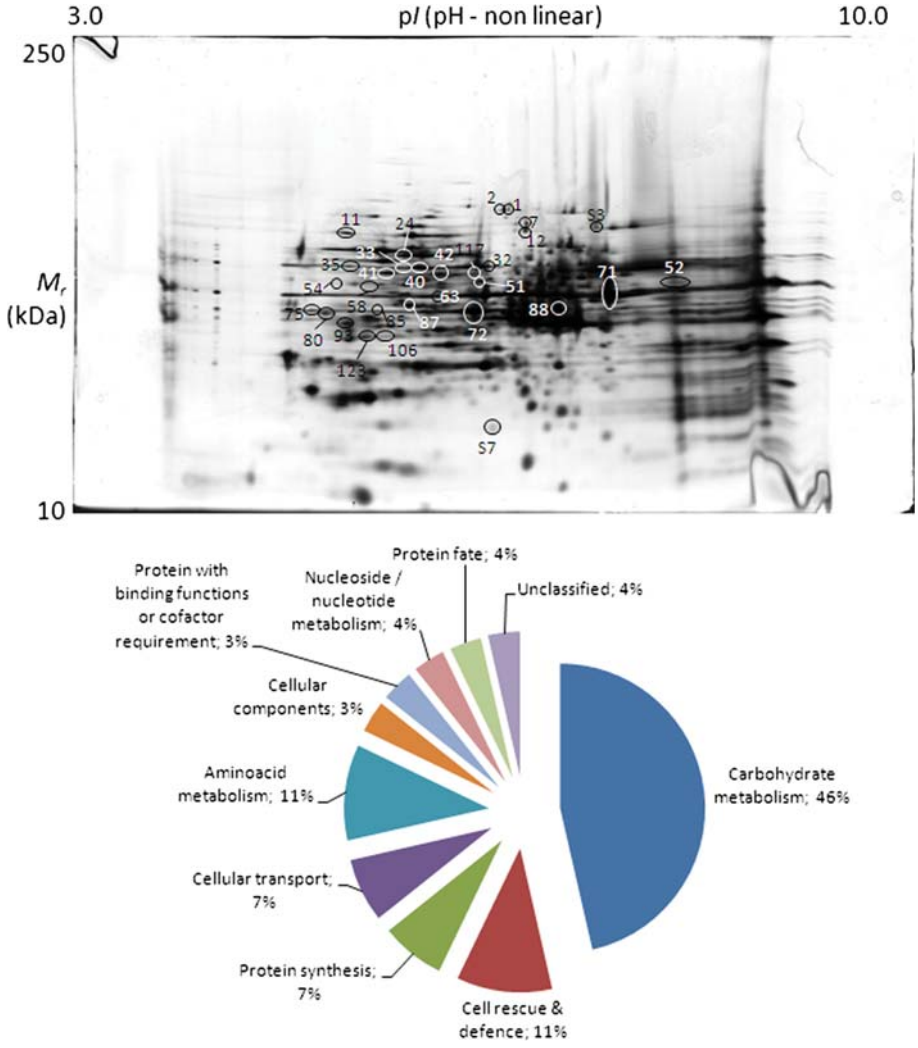

Figure 6. Silver stained yeast protein reference map of K310 cultured under control conditions and functional classification of identified proteins, indicated with numbers and described in Table I.

LPO is among the main outcomes of ROS injury to membranes and accumulation of MDA is regarded as a reliable biomarker of the degree of LPO during oxidative stress [26,27]. It has been shown that LPO may be involved in pesticide toxicity [28,29], like in the case of the pesticides endosulfan and zineb [19] and glyphosate [30]. Enhanced LPO could be detected after treatment with the glyphosate-based herbicide Roundup Ultra in liver cells of pregnant rats [31] and in human erythrocytes [32] and Costa et al. [33] found oxidative damage to lipids both in the liver and muscle tissues of tadpoles treated with glyphosate and Roundup. These findings were confirmed also in our work for P, S and PE, supporting the hypothesis of LPO phenomena in the presence of these herbicides. P and PE had faster actions than S on yeast cells, but PE and S had more important effects under the quantitative point of view. In particular, PE induced a very rapid increase in MDA levels just after 1 h treatment (MDA levels 4-times higher than in the control). This confirmed what we have already observed for PE, which is the most cytotoxic herbicide among the three tested on K310 cells [1–4] and the one able to randomly oxidize yeast proteins. Because LPO products can affect the activity of well-known anti-oxidant enzymes, such as catalase and superoxide dismutase (SOD) [34], the alterations on the activity of these enzymes we found in a previous work treating K310 with the herbicides P, S and PE [3] can now be explained.

The simultaneous determination of AOPP provided information about another aspect of protein involvement in free radical reactions, namely oxidized proteins that have lost their oxidant properties. AOPP have been designated as oxidatively modified protein cross-linking products [8] and are defined as dityrosine containing cross-linked protein products (thus excluding protein aggregates formed as a result of disulphide links following subtle oxidative stress) [35]. In particular, AOPP can result, even if not exclusively, from the use of chlorinating agents, and have spectral characteristics corresponding to several chromophores, especially dityrosine and carbonyl protein derivatives [36]. We found that the only herbicide able to increase

Table I. Yeast proteins containing oxidized thiols after 1 h treatment with 100 mg/L P, 1 g/L S or 500 mg/L PE

Spot#	Gene	AN[a]	Protein (ENTRY)[a]	loc.[b]	Biological processes and comments	Oxidation
Cell rescue and defence						
11	*kar2*	P16474	78 kDa glucose-regulated protein homologue (GRP78)	ER	Post-translational protein targeting; response to unfolded protein stress	S
117	*mnn4*	P36044	Protein MNN4 (MNN4)	PM	Putative positive regulator of mannosylphosphate transferase, involved in mannosylphosphorylation of N-linked oligosaccharides	P, S, PE
24	*ssb1*	P11484	Heat shock protein SSB1 (HSP75)	C	Protein biosynthesis; Stress response	P, S, PE
S7	*sod1*	P00445	Superoxide dismutase [Cu-Zn] (SODC)	C, Mt	Removal of superoxide radicals $2\ O_2^{\cdot -} + 2\ H^+ = O_2 + H_2O_2$	CTR, P, PE
Carbohydrate metabolism						
42	*ald2*	P47771	Aldehyde dehydrogenase [NAD(P)+] 1; (ALDH2)	C	Involved in ethanol oxidation and beta-alanine biosynthesis; uses NAD^+ as the preferred coenzyme; expression is stress-induced and glucose-repressed	P, S, PE
63	*eno2*	P00925	Enolase 2 (ENO2)	C	Glycolysis and Gluconeogenesis; Phosphopyruvate hydratase that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; expression is induced in response to glucose	P
72	*fba1*	P14540	Fructose-bisphosphate aldolase (ALF)	C, Mt	Glycolysis and Gluconeogenesis; Catalyses conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone- phosphate; locates to mitochondrial outer surface upon oxidative stress	S
40	*hxk2*	P04807	Hexokinase-2 (HXKB)	C, Mt, N	Glycolysis; It catalyses the phosphorylation of glucose in the cytosol; predominant hexokinase during growth on glucose; functions in the nucleus to repress expression of HXK1 and GLK1 and to induce expression of its own gene	P, S, PE
93	*pdb1*	P32473	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (ODPB)	Mt	Pyruvate metabolism; E1 beta sub-unit of the pyruvate dehydrogenase (PDH) complex, which is an evolutionarily-conserved multi-protein complex found in mitochondria	
32,33,35	*pdc1*	P06169	Pyruvate decarboxylase isozyme 1 (PDC1)	C, N	Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subject to glucose-, ethanol- and auto- regulation; Involved in amino acid catabolism	P, SP, SP, S, PE
1,2	*pfk1*	P16861	6-phosphofructokinase sub-unit alpha (K6PF1)	C	Glycolysis; Alpha sub-unit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes	P, S, PEP, S, PE
71	*pgk1*	P00560	Phosphoglycerate kinase (PGK)	C, Mt, PM	Glycolysis and Gluconeogenesis; It catalyses transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP	PE
106	*sec53*	P07283	Phosphomannomutase (PMM)	C	Involved in synthesis of GDP-mannose and dolichol-phosphate-mannose; required for folding and glycosylation of secretory proteins in the ER lumen	P, S
	tdh3	P00359	Glyceraldehyde-3-phosphate dehydrogenase 3 (G3P3)	C, CW, Mt	Glycolysis and Gluconeogenesis; Tetramer that catalyses the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; Also involved in apoptosis and oxygen and reactive oxygen species metabolic process	P, S, PE
S3	*tkl1*	P23254	Transketolase 1 (TKT1)	C	It catalyses conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids.It is also required for efficient use of fermentable carbon sources and indirectly involved in the response to reactive oxygen species (ROS) through its involvement in determining intracellular NADPH levels	CTR, P, PE

(Continued)

Table I. (Continued)

Spot#	Gene	AN[a]	Protein (ENTRY)[a]	loc.[b]	Biological processes and comments	Oxidation
Protein synthesis						
58	*sup1*	P12385	Eukaryotic peptide chain release factor sub-unit 1 (ERF1)	C	Polypeptide release factor (eRF1) in translation termination	P, S
52	*tef1*	P02994	Elongation factor 1-alpha (EF1A)	C, Mt	Binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes	PE
Cellular transport						
51	*pho84*	P25297	Inorganic phosphate transporter PHO84 (PHO84)	PM	Phosphate metabolism; High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter	P, S
41	*vma2*	P16140	V-type proton ATPase sub-unit B (VATB)	V, C	Sub-unit B of the eight-sub-unit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites.Involved in protection from oxidative stress	P
Amino acid metabolism						
7	*met6*	P05694	5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase (METE)	C	Methionine biosynthesis	P, S, PE
87	*sam2*	P19358	S-adenosylmethionine synthetase 2 (METK2)	Unknown	Methionine biosynthetic process	P, S
12	*trp5*	P00931	Tryptophan synthase (TRP)	C, N	Tryptophan biosynthetic process	P, S, PE
Cellular components						
54	*tub2*	P02557	Tubulin beta chain (TBB)	C	It associates with alpha-tubulin (Tub1p and Tub3p) to form tubulin dimer, which polymerizes to form microtubules	P, S, PE
Protein with binding functions or cofactor requirement (structural or catalytic)						
80	*lia1*	P47120	Deoxyhypusine hydroxylase (DOHH)	C, N	Deoxyhypusine hydroxylase, a HEAT-repeat containing metalloenzyme that catalyses hypusine formation; binds to and is required for the modification of eIF5A	P, S, PE
Nucleoside/nucleotide metabolism						
85	*ado1*	P47143	Adenosine kinase (ADK)	C, N	Purine metabolism (AMP from adenosine); Required for the utilization of S-adenosylmethionine (AdoMet); may be involved in recycling adenosine produced through the methyl cycle	P, PE
Protein fate						
123	*pre9*	P23638	Proteasome component Y13 (PSA4)	C, N	Alpha 3 sub-unit of the 20S proteasome, the only non-essential 20S sub-unit; may be replaced by the alpha 4 sub-unit (Pre6p) under stress conditions to create a more active proteasomal isoform	P, S
Unclassified						
75	*sgt2*	Q12118	Small glutamine-rich tetratricopeptide repeat-containing protein 2 (SGT2)	C	Protein of unknown function; contains tetratricopeptide (TPR) repeats, which often mediate protein–protein interactions; has similarity to human SGT, which is a cochaperone that negatively regulates Hsp70	P, S, PE

[a]Protein ENTRY and AN (Accession Number) according to the protein knowledgebase UniProtKB (http://www.uniprot.org/)
[b]Cellular localization, as follows: C (cytosol); CW (cell wall); ER (endoplasmic reticulum); Mt (mitochondrion); N (nucleus); PM (plasma membrane); V (vacuole).

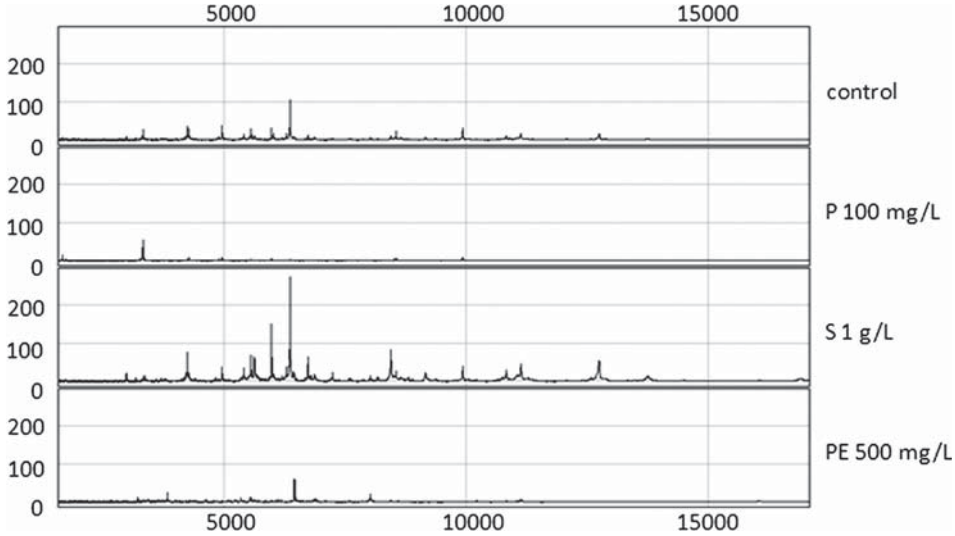

Figure 7. Proteolytic activity in K310 cells: analysis of low molecular weight protein profiles in K310 cells through SELDI ProteinChip technology, as described in the Materials and methods section. The *y*-axis represents the normalized peak intensity, while the *x*-axis represents the *m*/*z* ratio. Only representative spectra from triplicate sets is reported.

significantly AOPP levels in yeast was PE after 1 h treatment. This result strongly supported the hypothesis that the damage on yeast cells mediated by PE could be exerted by producing extensive protein aggregation, which can in turn explain the dramatic effects observed for classical biological parameters of yeast growth, colony forming ability, metabolism and protein carbonylation we observed in our previous investigations [1–4]. In these previous works of ours, we showed that herbicide-induced carbonylation of yeast proteins is a dynamic phenomenon that might lead to the formation of carbonylated protein aggregates [3] or, in the case of short treatments with PE, to the random oxidation of yeast proteins [4]. Such an important oxidative damage may negatively affect, in turn, proteolysis of damaged proteins. For this reason, in this work we implemented our results and, specifically, we monitored proteolysis after 1 h treatment with the tested herbicides, because this was the time point when the highest carbonylation was found [4]. The methodological approach used for the evaluation of proteolytic activity is based on the use of the new SELDI ProteinChip array technology, which allowed us to obtain quali-quantitative profiles (spectra) of yeast low molecular weight peptides. Superimposition of spectra clearly indicated that, both under the qualitative and the quantitative point of view, proteolysis in P- and PE-treated cells was lower than in control conditions, suggesting that the extensive carbonylation of proteins may inhibit the yeast proteolysis of damaged proteins.

GSH plays a main role in the protection from oxidative stress [37,38], heat shock [39] and detoxification of xenobiotics [39], providing cells with a reducing milieu and conferring unique redox and nucleophilic properties [40]. Moreover, GSH can be a storage compound to be used during starvation of yeast cells, since it can be a source of sulphur [40] or nitrogen [41].

In our analyses, we were not able to find important effects of P, S or PE on GSH levels in K310 strain. It is in fact known that, in common with higher eukaryotes, treatment of exponentially growing yeast cells with oxidants for short periods may not lead to marked alterations of GSH levels [42,43]. The only exception was found after 1 h treatment with P, when GSH levels were statistically higher in herbicide-treated cells than in the control. Probably, only in the case of P, GSH can exert a protective role in yeast cells and this supports our previous observations showing that the cell recovery after the supplementation of P is faster than in the presence of S and PE [1,2]. The increased activity of GR we found during the log-phase after treatment with the herbicide P correlated well with the higher levels of GSH found in P-treated cells than under control conditions. Also, this finding is corroborated by the increased levels of transketolase 1 (TKT1), an enzyme involved in the pentose phosphate pathway, that we found during the proteomic analysis of P-treated K310 cells [4], whose over-expression ensure cells more NADPH to counteract oxidative stress.

The lower GSH levels found in herbicide-treated cells vs control after the entry into the stationary phase could reflect, on the contrary, differences in metabolic switching from fermentative to respiratory conditions, as recently demonstrated in a brewing yeast strain during industrial fermentation [44].

The presence of herbicides often resulted in oxidation of GSH. We found statistically significant increases in GSSG levels for all the herbicides tested, but especially for S and PE even after very short treatments (15 min and 1 h). This let us hypothesize the use of GSH as a free-radical scavenger, resulting

in its oxidation to GSSG [38]. It could also be a consequence of increased LPO as well, since lipid peroxides may react with GSH and lead to an increase in GSSG [45].

Another important parameter to be taken into account is the GSH/GSSG ratio, which gives a measure of the cell redox power [46–48]. The GSH/GSSG ratio can be substantially decreased by oxidants [38], pointing to a shift in the cell to a more oxidized state. We found consistent decreases in GSH/GSSG ratio as a common response of yeast cells to S and PE during the whole period investigated, while the effects of P were limited to the mid-log and stationary phases of growth. Such an alteration can result, in turn, in a decreased cell viability, and can thus explain our previous observations on herbicide-mediated toxicity towards K310 yeast cells [1–4].

Although GSH is an important antioxidant reacting directly with radicals and electrophiles, it can also act as a source of electrons for GPx, which catalyses the reduction of hydrogen peroxide, as well as organic peroxides generated during LPO, resulting in the formation of GSSG [38,49–51]. Yeast GPx activity towards both H_2O_2 and organic hydroperoxides has been demonstrated; interestingly, both these activities could be induced by a shift from anaerobic to aerobic growth conditions [52]. In the present work, we found that yeast GPx was almost unaffected by short treatments with the herbicides tested, since the lower activity observed in the presence of S and PE after 15 min or 1 h was not statistically significant. Nevertheless, this decrease became consistent at the following samplings for both herbicides, with very similar patterns. Analogous results were obtained by Takizawa et al. [53], who found a decreased GPx activity following treatment of endothelial cells with paraquat, a herbicide used as a model for the induction of oxidative stress both *in vitro* and *in vivo* [54], suggesting an oxidative injury of this enzyme by ROS. As a consequence, the acceleration of hydrogen peroxide production by the inactivation of GPx, which in turn increases oxidative stress, would result in cell damage. Considering the beneficial effects exerted by this enzyme on the protection from LPO, we could hypothesize that the lower GPx activity found in yeast cells treated with S and PE at the mid-log, end-log and stationary phases of growth could be one of the causes for the increased levels of MDA.

The rapid advancement of proteomic technologies allowed us to monitor global changes in redox state of thiol proteins in stressed cells [55]. If compared to DNA and lipids, proteins are the most abundant cellular targets for oxidation [56] and post-translational modification of proteins is one of the main events due to oxidative imbalances, making cysteine thiols very prone to be oxidized [15,57].

Thiol oxidation has a pivotal role during oxidative stress, since it has an increasingly recognized role in protein structure/function and redox signalling [58–66]; however, changes in protein structure must not be seen as solely detrimental, as they can activate specific protein functions or protect critical residues from irreversible oxidation. Nevertheless, a wide range of chemically reactive sulphur species can be generated, propagating the oxidative imbalance in the form of pro–anti-oxidant redox cascades [65].

In the present work, we combined the use of BIAM with 2D-PAGE to evaluate if herbicide treatments could oxidize yeast protein thiols. Either in the case of treatment of K310 cells with P, S or PE, we were able to find a lower number of BIAM-immunostained spots than in the control, thus pointing out herbicide-mediated oxidative damage towards yeast protein thiols. Particularly, 83 protein spots were found to be oxidized in the presence of herbicides and a common signature consisting of 38 protein spots oxidized in each of the conditions tested was also highlighted (Figure 5).

Once classified according to their functions, we found that many of the proteins containing oxidized thiols were involved in carbohydrate metabolism (Figure 6), confirming what was recently observed by other authors [67–69]. Our results globally indicated that thiol oxidation could coordinate the metabolic response to herbicide-induced oxidative stress and regulate fluxes of carbohydrates [69]; this could eventually have, in turn, negative relapses on the fermentative performance of yeasts. The susceptibility of key glycolytic enzymes to oxidative stress is well documented by numerous studies [70,71]. We also provided information in this direction with the demonstration that ROS generated by the cocktail ascorbic acid/iron as well as H_2O_2 potently inhibit hexokinase or glyceraldheyde 3 P-dehydrogenase [72]. Loss of enzyme activity was associated with oxidation of critical –SH groups [73,74]. It was therefore interesting to note that each of the herbicides tested in this study effectively oxidized thiols in both of the above enzymes. In addition, glyceraldheyde 3-P dehydrogenase may have a regulatory role as a sensor of oxidative stress conditions [75]. Recent studies suggest that oxidative inhibition of specific glycolytic enzymes, including glyceraldheyde 3-P dehydrogenase, enables cells to redirect their carbohydrate flux from glycolysis to the pentose phosphate pathway thereby leading to formation of NADPH [76]. Under these conditions, NADPH is particularly important as it provides the reducing potential for most antioxidants and redox regulatory enzymes.

Thiol specific oxidation was observed, also, for some proteins involved in the stress response or covering specific important functions in yeast cells, in good agreement with results from Le Moan et al. [67]. This is the case with the molecular chaperone GRP78, oxidized in the presence of S, and MNN4 and HSP75, oxidized in the presence of P, S and PE.

Among the few proteins found to be oxidized in control conditions we were able to identify, in the case of S, two proteins with a well-recognized importance in the response to oxidative stress: the cytosolic superoxide dismutase (SODC) and the transketolase 1 (TKT1). For SODC, the same response was observed recently in yeast cells exposed to hydrogen peroxide [69], which confirmed also our previous observations pointing out a beneficial effects of SOD in the response of K310 to S-induced stress [3].

We also found that proteins with specific cell functions could undergo thiol oxidation following herbicide treatments. The protein coded by *ado1* was found to be selectively oxidized in the presence of P and PE, also like that found by Le Moan et al. [67] in yeast exposed to hydrogen peroxide. Recently, this protein was found to be induced during the early log phase of growth in a lager brewing yeast [44], as it is involved in the activated methyl cycle thus providing methyl groups in various biosynthesis reactions [77]. This is one of the events observed when yeasts are grown in a fermentable medium, which causes the cells to grow bigger and increase their capacity for protein synthesis at a higher rate until a new cell cycle begins. They initiate or shift their carbon metabolism towards glycolysis and in the presence of oxygen, sterols and other membrane constituents are being synthesized [78].

VATG, coded by *vma2*, was found to be thiol oxidized in the presence of P. This protein is an ATP-synthase sub-unit involved in the vacuole acidification and in the metabolism of glycogen The over-expression of different sub-units of this enzyme has been previously associated in yeast to 2,4-dichlorophenoxyacetic acid stress [79] and proposed as a mechanism to counterbalance the dissipation of transmembrane proton gradient. It has been already associated to the H_2O_2 stress response in *S. pombe* [80] as well as to the response of herbicide-induced stress [4].

Overall, we can hypothesize that thiol oxidation of specific proteins could impair fundamental cellular functions that are necessary to yeasts to properly cope with herbicide-induced stress.

Declaration of interest: This work was partly supported by Siena Biotech, Fondazione Monte dei Paschi 2008, and TLS Orf-0108 grants. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 2 June 2010.