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Biochemical and Biophysical Research Communications 330 (2005) 271–278
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Yeast adaptation to 2,4-dichlorophenoxyacetic acid involves increased membrane fatty acid saturation degree and decreased *OLE1* transcription

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Received 21 February 2005

Abstract

Yeast cells adapted to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) exhibit a plasma membrane less susceptible to 2,4-D-induced disruption and are more tolerant than unadapted cells to lethal concentrations of the herbicide. These cells, adapted to grow in the presence of increasing concentrations of 2,4-D, were found to exhibit a dose-dependent increase of the saturation degree of membrane fatty acids, associated to the higher percentage of stearic ($C_{18:0}$) and palmitic ($C_{16:0}$) acids, and to the decreased percentage of palmitoleic (Δ9-*cis*$C_{16:1}$) and oleic (Δ9-*cis*$C_{18:1}$) acids. The decreased transcription of the *OLE1* gene (encoding the Δ9 fatty acid desaturase that catalyses the conversion of palmitic and stearic acids to palmitoleic and oleic acids, respectively) registered in 2,4-D adapted cells suggests that yeast adaptation to the herbicide involves the enhancement of the ratio of saturated ($C_{16:0}$ and $C_{18:0}$) to monounsaturated ($C_{16:1}$ and $C_{18:1}$) membrane fatty acids through a reduced *OLE1* expression.

Keywords: *Saccharomyces cerevisiae*; 2,4-D; Fatty acid unsaturation degree; Plasma membrane; *OLE1*; Stress response; Lipophilic acids; Herbicides

The auxin-like herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), widely used in agriculture and forestry during the last decades, may give rise to a number of toxicological problems in non-target organisms [1–4] (http://extoxnet.orst.edu/pips/24-D.htm) and has led to the emergence of resistant weeds [5]. The yeast *Saccharomyces cerevisiae* is a very useful eukaryotic experimental model organism for assessing 2,4-D toxicity under different environmental and physiological conditions [3,6]. The yeast responses to 2,4-D include the transient Pdr1p/Pdr3p-dependent transcriptional activation of *PDR5* and *TPO1* genes during the adaptation period preceding eventual cell division in the presence of 2,4-D [7]. These genes encode plasma membrane multidrug transporters, suggesting a role as active exporters of the anionic form of this lipophilic weak acid herbicide ($\log K_{ow} = 2.81$ [8]; $pK_a = 2.73$ [4]). Another crucial gene to yeast adaptation to 2,4-D is the *SPI1* gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein and highly transcriptionally activated during yeast adaptation to 2,4-D, in a Msn2p/Msn4p-dependent manner [9]. Other Msn2p/Msn4p-regulated genes, required for a more rapid adaptation to 2,4-D, include genes encoding molecular chaperones, heat shock proteins, and antioxidant enzymes, consistent with the pro-oxidant effect of this herbicide in yeast [9,10]. Adaptation to 2,4-D also involves the coordinated stimulation of vacuolar and plasma membrane H^+-ATPase activities, to counteract the dissipation of the physiological

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doi:10.1016/j.bbrc.2005.02.158

H^+-gradients across vacuolar and plasma membranes occurring under 2,4-D stress [11]. More insights into the global mechanisms of adaptation to 2,4-D were obtained by comparative expression proteomics, using two-dimensional gel electrophoresis [12]. Increasing amounts of several proteins were registered during the adaptation period preceding growth resumption under herbicide stress, namely the antioxidant enzyme Ahp1, heat shock proteins, enzymes involved in protein and mRNA degradation, carbohydrate metabolism and vacuolar function, and several enzymes involved in amino-acid biosynthesis. The increased abundance of amino-acid biosynthetic enzymes was hypothesized to contribute to counteract amino-acid depletion occurring in 2,4-D challenged cells as a consequence of the disturbance, by the highly lipophilic acid herbicide, of the vacuolar function and of the function of plasma membrane as a permeability barrier and a matrix for solute transporters [12]. Indeed, 2,4-D toxicity towards yeast cells is mainly due to the action of the undissociated form [6], suggesting that plasma membrane lipid bilayer is among the biological targets of 2,4-D, either through the direct interaction of this highly lipophilic form with membrane lipids, affecting membrane spatial organization [13], or through lipid peroxidation as a consequence of its action as a pro-oxidant agent [10]. Intracellular acidification and the accumulation of the counter-anion, as the result of 2,4-D dissociation in the neutral cytosol, are also among the deleterious effects of this herbicide [9,11].

In the present work, we examined the adaptive responses to 2,4-D occurring in *S. cerevisiae* at the level of membrane fatty acid composition and found that the ratio of saturated ($C_{16:0}$ and $C_{18:0}$) to monounsaturated ($C_{16:1}$ and $C_{18:1}$) fatty acids increases in herbicide adapted cells. Consistent with the increase of the ratio of saturated:unsaturated fatty acids in cells grown under 2,4-D stress, the level of transcripts from the *OLE1* gene was reduced in these stressed cells. The *OLE1* gene encodes the endoplasmic reticulum (ER) membrane-bound Δ9 fatty acid desaturase that catalyses the conversion of saturated fatty acids to monounsaturated species by introducing a double bound between carbons 9 and 10 of palmitoyl- and stearoyl-CoA to form palmitoleic and oleic acids, respectively [14]. The yeast cell population which exhibited the referred adaptive responses was less susceptible than the unadapted population to plasma membrane permeabilization and loss of viability induced by highly stressing concentrations of 2,4-D.

Materials and methods

Strain and growth conditions. The strain *S. cerevisiae* W303.1b (MATα, *ura*3-1, *leu*2-3, *his*3-11, *trp*1-1, *ade*l-2, and *can*1-100), one of the strains used in our laboratory for assessing herbicide toxicity and to investigate adaptive responses to 2,4-D [3,6,7], was used. Cells were batch-cultured at 30 °C, in liquid growth medium MM2, containing (per liter): 1.7 g yeast nitrogen base without amino-acids or NH_4^+ (Difco), 20 g glucose (Merck), 2.65 g $(NH_4)_2SO_4$ (Merck), 80 mg adenine (Sigma), 10 mg histidine (Merck), 10 mg leucine (Sigma), 20 mg tryptophan (Merck), and 20 mg uracil (Sigma). A stock solution of 2,4-D (2,4-D sodium monohydrate, Pestanal; Riedel-de Haën), prepared in dimethyl sulphoxide (DMSO) (0.8 M), was added to this medium to obtain the desired concentrations of 2,4-D. The media pH was adjusted to 3.0 with HCl 6 M. The final concentration of DMSO in the different growth media (including the unsupplemented medium, used as control) was kept equal to 0.05%(v/v), a concentration that has no detectable effect on yeast growth. Yeast cells used as inoculum were harvested by centrifugation in the middle of exponential growth (at the standardized culture optical density at 600 nm, $OD_{600nm} = 0.40 \pm 0.05$) carried out in the absence of herbicide. Cultures were centrifuged and the cells were resuspended in a suitable volume of MM2 liquid medium to give an initial culture $OD_{600nm} = 0.20 \pm 0.02$. Growth was monitored by measuring culture OD_{600nm} or the concentration of viable cells. This was assessed by counting colony forming units (CFU) obtained by plating 0.1 ml of the culture serial dilutions onto the surface of MM2 solid media (with 20 g agar/L; Iberagar, Barreiro, Portugal) followed by incubation of the inoculated agar plates during 72 h, at 30 °C. The specific growth rate (μ_g) was calculated by least-square fitting to the linear part of the semi-logarithmic plot of culture OD_{600nm} versus time.

Lipid extraction, transesterification, and fatty acid analysis. For the analysis of membrane fatty acids, yeast cells grown in MM2 medium (pH 3.0) or in this basal medium supplemented with increasing concentrations of 2,4-D (from 100 to 185 μM) were used. Cells were harvested by centrifugation in the mid-exponential phase of growth ($OD_{600nm} = 0.40 \pm 0.02$), washed twice with phosphate buffer (50 mM, pH 7.0), and stored at −20 °C until used. Total lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer [15]. Total fatty acid methyl esters (FAME) were prepared by incubation of the lipid extract in boron trifluoride/methanol, during 15 min at 95 °C, using the method of Morrison and Smith [16]. FAME were extracted with hexane. Analysis of FAME in hexane was performed using quadruple GC-MS System (HP6890, HP5973, Hewlett & Packard, Palo Alto, USA) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; ID: 0.32, 30 m; 0.25 μm film) was used for the separation of the FAME. GC conditions were as follows: injector temperature was held at 250 °C; the split flow was 1:10 and carrier gas was He; and the temperature program was: 80 °C, 1 min isotherm, 15 °C/min to 140 °C; and 4 °C/min to 280 °C. The MS conditions were: ionization mode EI; ionization energy 70 eV. The peak areas of the carboxylic acids in total ion chromatograms (TIC) were used to determine their relative amounts. The fatty acids were identified by GC-MS and co-injection with reference compounds obtained from Suppelco (Bellefonte, USA). The degree of saturation of the membrane fatty acids was defined as the ratio between the concentrations of the two saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) and the two unsaturated fatty acids (Δ9-*cis*$C_{16:1}$ and Δ9-*cis*$C_{18:1}$) determined in the extracts.

2,4-D-induced-death experiments. Death experiments were carried out in MM2 medium supplemented with 300 μM of 2,4-D (pH 3.0), using a water bath at 30.0 ± 0.1 °C. Death medium was inoculated with mid-exponential yeast cells (6000 ± 500 CFU/ml) grown in the presence of increasing concentrations of 2,4-D, as described above. The loss of cell viability was followed by counting the colonies obtained after plating samples of 0.1 ml onto the surface of agarized MM2 growth medium, at suitable times. The specific death rates (μ_d) were calculated by least-square fitting to the linear part of the semi-logarithmic survival plots versus time (in min).

Assessment of the permeabilizing effects of 2,4-D. Plasma membrane permeabilization by 2,4-D was estimated by fluorescence microscopy, based on the influx of the fluorescent nuclear stain ethidium bromide

(EtBr), which is excluded by yeast cells with intact plasma membranes [17]. To assess cellular plasma membrane permeability to EtBr during cultivation of the yeast cell population in the presence of 165 μM of 2,4-D (at pH 3.0), cells were harvested by centrifugation, at suitable times during the lag- and exponential-phases of growth, and resuspended in fresh MM2 medium to obtain cell suspensions with $OD_{600nm} = 1.0 \pm 0.2$. EtBr (10 μg/ml) was added to these cell suspensions and incubation proceeded for 15 min, at 30 °C. The cell suspensions were then centrifuged, resuspended in 50 μl of the supernatant to obtain more dense cell suspensions (final $OD_{600nm} = 30 \pm 2$), and immediately examined under the microscope. A yeast cell population grown in the absence of the herbicide was used as the control, being negligible the number of control cells permeated by EtBr. To compare the permeabilizing effect of 2,4-D in the plasma membrane of yeast cells either or not previously adapted to the herbicide, exponential cells were harvested at $OD_{600nm} = 0.40 \pm 0.02$ after growth in the presence or absence, respectively, of 125 μM of 2,4-D, and were resuspended in fresh MM2 medium (pH 3.0) supplemented with increasing concentrations of 2,4-D (up to 1.6 mM) and 10 μg/ml EtBr. These cell suspensions were incubated for 15 min, at 30 °C, and then concentrated (to $OD_{600nm} = 1.0 \pm 0.1$) and immediately examined under the microscope. A Zeiss Axioplan microscope equipped with adequate epifluorescence interference filters (Zeiss BP 546 and Zeiss LP 590) was used. Fluorescence and phase-contrast images (with an amplification of 400×) were immediately acquired with a cooled CCD camera (Cool $SNAP_{FX}$, Roper Scientific Photometrics, Tucson, USA) and stored on a computer to be analysed later. Membrane permeability to EtBr was estimated based on a cell-by-cell analysis, using MetaMorph Imaging System 4.6.9 (Universal Imaging, Downingtown, USA), by measuring either the percentage of fluorescent cells in the yeast cell population and/or the total fluorescence intensity emitted by these fluorescent cells, determined pixel-by-pixel. The percentage of fluorescent cells was estimated as the ratio of the number of fluorescent cells present in each fluorescence image per the total number of cells present in the corresponding phase-contrast image (at least 300 cells were analysed for each test condition).

OLE1 mRNA quantification. Extraction of total RNA from yeast cells harvested during exponential phase of growth ($OD_{600nm} = 0.40 \pm 0.03$) carried out in the absence or presence (140 and 165 μM) of 2,4-D was performed according to the hot-phenol method and Northern blot hybridizations were carried out as described before [7]. Total RNA in each sample used for Northern blotting was approximately equal to 15 μg (assessed by measuring A_{260nm}). The specific DNA probe to detect *OLE1* transcripts was prepared by PCR amplification with the following primers: 5′-TACGCTATCCTTCGG TTGTGCT-3′ and 5′-AGACCTCTACGAGCGTCATAA-3′. This DNA probe, with 119 bp, expands from positions 534 to 653 in the *OLE1* coding region. The *ACT1* mRNA level was used as an internal control. Kodak Biomax MS films were exposed to the nitrocellulose membranes (Hybond-N, Amersham Biosciences, Buckinghamshire, UK) following the hybridization step and incubated with an intensifying screen at −70 °C for approximately 16 or 6 h to obtain *OLE1* or *ACT1* hybridization signals, respectively. The relative intensities in the autoradiograms were quantified by densitometry (ImageMaster TotalLab Software, version 3.0; Amersham Pharmacia Biotech, Uppsala, Sweden).

Results

Modification of membrane fatty acid composition in response to growth under 2,4-D stress

When a cell population of *S. cerevisiae* W303.1b, grown in the absence of 2,4-D stress, was used to inoculate the same growth medium (MM2; pH 3.0) supplemented with increasing inhibitory concentrations of this herbicide, a lag-phase, whose duration depended on the severity of herbicide stress, was observed (Fig. 1). After this period of adaptation to the deleterious effects of the herbicide, the cell population resumed inhibited exponential growth in the presence of 2,4-D (Fig. 1). Yeast cells resulting from growth with these moderately inhibitory concentrations of 2,4-D exhibited an increasingly higher content of the saturated fatty acids stearic acid ($C_{18:0}$) and, less significantly, palmitic acid ($C_{16:0}$), while the percentage of the unsaturated fatty acids palmitoleic acid ($\Delta 9\text{-}cis C_{16:1}$) and oleic acid ($\Delta 9\text{-}cis C_{18:1}$) suffered a decrease, compared to cells grown in the absence of the herbicide (Fig. 2). For the range of concentrations tested, the dose-dependent increase of the degree of saturation of membrane fatty acids in 2,4-D-adapted yeast cells correlated with the dose-dependent growth inhibitory effect of the herbicide (Fig. 3).

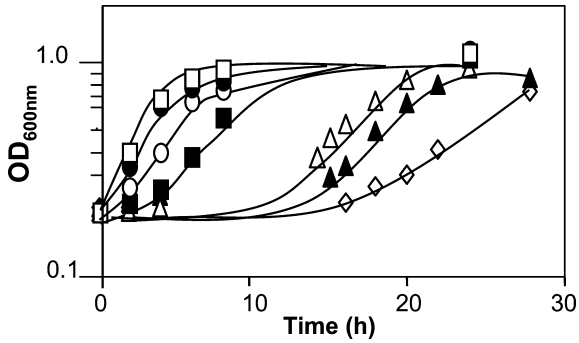

Fig. 1. Growth curves of *S. cerevisiae* W303.1b in MM2 medium (pH 3.0) supplemented with increasing concentrations of 2,4-D (μM): 0 (□), 100 (●), 125 (○), 140 (■), 155 (△), 165 (▲), and 185 (◊), at 30 °C. The yeast cell population used as inoculum was grown in the absence of 2,4-D. Results are averages from at least two independent growth experiments for each herbicide concentration.

Plasma membrane permeabilization induced by 2,4-D is less drastic in cells adapted to the herbicide

Cultivation of a yeast cell population, that has not been previously exposed to 2,4-D, in the presence of 165 μM of the herbicide, led to a drastic decrease of the percentage of the initial concentration of viable cells during the first 4 h of 2,4-D-induced latency (Figs. 4A and B). This loss of cell viability was accompanied by the increase of the percentage of cells that became fluorescent when ethidium bromide (EtBr) was externally added, due to the increased influx of this stain across plasma membrane (Fig. 4C). This observation is consistent with the disruption of plasma membrane permeability barrier in 2,4-D stressed cells. As soon as 2,4-D-adapted cells started duplication under herbicide stress, the fraction of the cell population, whose plasma

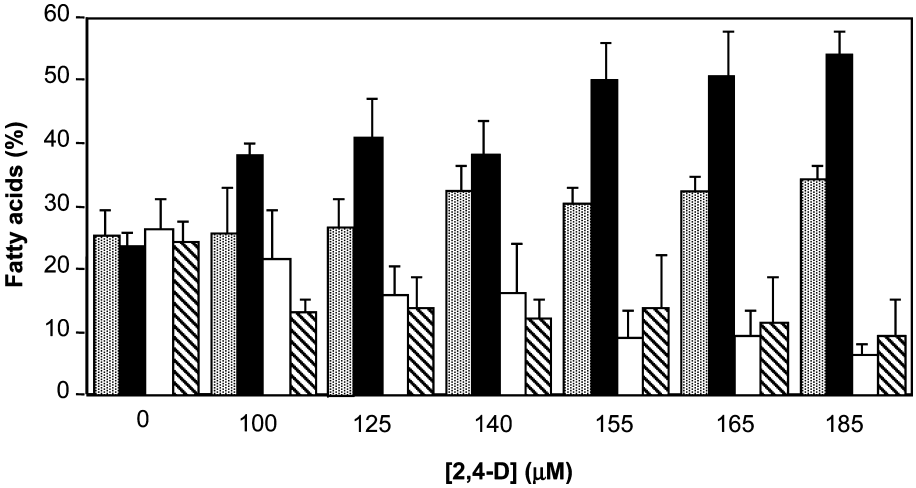

Fig. 2. Fatty acid profiles, described as the percentage of palmitic ($C_{16:0}$, ▨), stearic ($C_{18:0}$, ■), palmitoleic ($C_{16:1}$, □), and oleic ($C_{18:1}$, ▧) acids, of *S. cerevisiae* W303.1b cells harvested in middle exponential growth (culture $OD_{600nm} = 0.40 \pm 0.02$) carried out in MM2 medium (pH 3.0, 30 °C) or in this medium supplemented with increasing concentrations of 2,4-D. The corresponding growth curves are shown in Fig. 1. Bars represent the standard deviation from the average of at least two fatty acid analysis using cells from at least two independent growth experiments for each herbicide concentration.

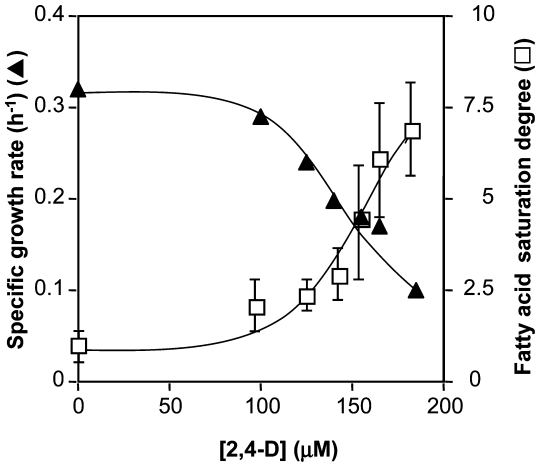

Fig. 3. Effects of the addition of increasing concentrations of 2,4-D to the growth medium, on the specific growth rate (▲) and on the degree of saturation of membrane fatty acids (□) of exponentially growing cells of *S. cerevisiae* W303.1b. These herbicide concentrations led to reduction of the specific growth rate (▲) which was calculated based on the growth curves shown in Fig. 1. Fatty acid degree of saturation was estimated using the fatty acid profiles shown in Fig. 2.

membrane was permeable to EtBr, decreased (Fig. 4C). This observation suggests a recovery of membrane organization in the dividing population, following adaptation to 2,4-D. To compare the permeabilizing effect of lethal concentrations of 2,4-D towards yeast cell populations either or not adapted to the herbicide, the corresponding cell populations were exposed for 15 min to increasing lethal concentrations of 2,4-D in the presence of EtBr. The dose-dependent increase of plasma membrane permeability to EtBr was more evident when cells that had not been previously exposed to the herbicide were tested, compared with adapted cells (Fig. 5). These 2,4-D-adapted cells, with a plasma membrane less prone to 2,4-D-induced permeabilization (Fig. 5), were found to exhibit a modified fatty acid composition (Figs. 2 and 3) and were remarkably more resistant to 2,4-D-induced death than cells that had not been previously exposed to the herbicide (Fig. 6).

The levels of OLE1 transcripts decrease in 2,4-D-grown cells

The levels of mRNA produced from the *OLE1* gene decreased significantly in cells of *S. cerevisiae* W303.1b grown in the presence of 140 or 165 μM of 2,4-D (1.9- or 2.3-fold, respectively), compared to unstressed cells (Fig. 7). Since the *OLE1* gene encodes the yeast Δ9 fatty acid desaturase that converts the CoA esters of $C_{16:0}$ and $C_{18:0}$ into the corresponding monounsaturated fatty acids [14], these results are consistent with the increase registered in the saturation degree of membrane fatty acids of the same yeast cell populations cultivated under 2,4-D stress (2.2- and 3.4-fold, respectively) (Fig. 3).

Discussion

Many biological processes that are vital for the yeast cell take place at the plasma membrane. Many studies have shown that cell performance to cope with environmental stresses that affect plasma membrane organization and function depends upon maintenance of its physical characteristics which depends on the packing of fatty acyl chains [18]. In the present work, we show experimental evidences supporting the conclusion that cells of *S. cerevisiae* adapted to the lipophilic acid

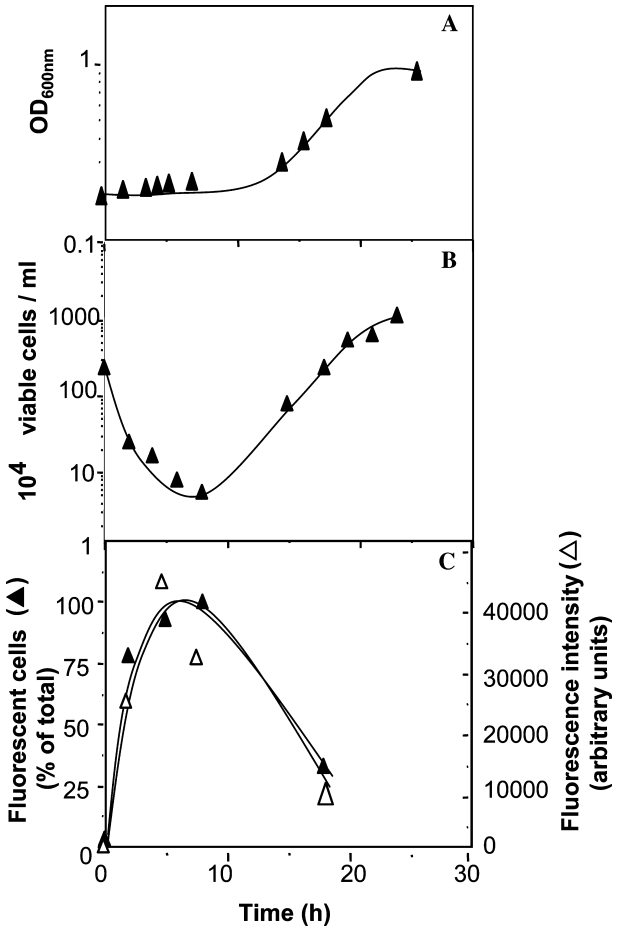

Fig. 4. (A) Culture optical density at 600 nm, (B) concentration of viable cells, assessed by the number of colony forming units, and (C) permeability of the plasma membrane to the fluorescent stain ethidium bromide (EtBr), during cultivation of *S. cerevisiae* W303.1b, at 30 °C, in medium MM2 (pH 3.0) supplemented with 165 μM of 2,4-D. Plasma membrane permeability (C) was assessed by fluorescence microscopy, as (▲) the percentage of fluorescent cells in the yeast population or (△) the total fluorescence intensity (in arbitrary units) emitted by these cells, due to influx of EtBr. The yeast cell population used as inoculum was grown in the absence of 2,4-D. Results are representative of at least two independent growth experiments.

herbicide 2,4-D exhibit a higher saturation degree of the principal fatty acyl chains in this organism [18]. To examine the full extent of the dependence of the saturation degree of membrane fatty acids on the presence of 2,4-D, fatty acid composition was determined over a wide range of herbicide concentrations. Data indicated that the presence of 2,4-D leads to the modification of yeast fatty acid profile by increasing, in a dose-dependent manner, the ratio of saturated ($C_{16:0}$ and $C_{18:0}$) to monounsaturated ($C_{16:1}$ and $C_{18:1}$) fatty acids. The modifications registered can essentially be attributed to alterations occurring at the level of plasma membrane since the bulk majority of these fatty acids are located at this cell membrane [19]. The plasma membrane of 2,4-D adapted cells, with a lower fatty acid unsaturation

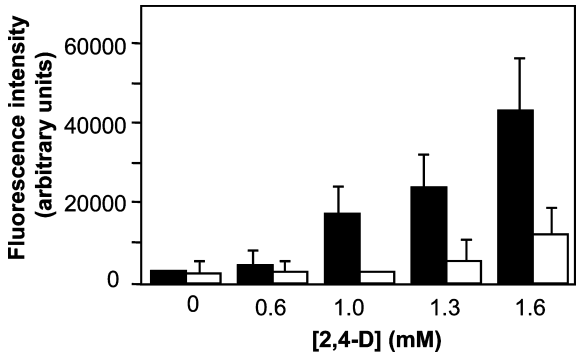

Fig. 5. Effect on plasma membrane permeability to ethidium bromide (EtBr) of a 15-min exposure to increasing concentrations of 2,4-D of cells of *S. cerevisiae* W303.1b either or not adapted to 2,4-D. The two different yeast populations tested resulted from growth, at 30 °C, in medium MM2 (pH 3.0) in the absence (■) or presence (□) of 125 μM of 2,4-D and harvested in the middle of exponential growth at culture $OD_{600nm} = 0.40 \pm 0.02$. The corresponding growth curves are shown in Fig. 1. Plasma membrane permeability was assessed by fluorescence microscopy as the total fluorescence intensity (in arbitrary units) emitted by the fluorescent cells in the stressed yeast cell population, due to influx of EtBr. Bars represent the standard deviation from the average of the fluorescence intensity of the many cells analysed from at least two independent experiments for each herbicide concentration.

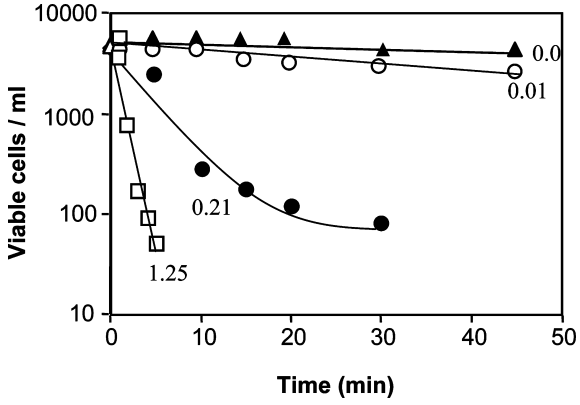

Fig. 6. Comparison of the loss of cell viability during incubation, at 30 °C, in MM2 medium (pH 3.0) supplemented with a lethal concentration (300 μM) of 2,4-D of different cell populations of *S. cerevisiae* W303.1b. The cell populations tested were harvested in the mid-exponential phase of growth ($OD_{600nm} = 0.40 \pm 0.02$), at 30 °C, in MM2 medium (pH 3.0) (□) or in this same medium supplemented with increasing 2,4-D concentrations of moderate toxicity: 100 (●), 125 (○) or 165 (▲) μM. The corresponding growth curves are shown in Fig. 1. The specific death rates (in min^{-1}), calculated for each cell population tested, are indicated in the figure, close to the corresponding death curve.

degree, was found to be more resistant to herbicide-induced permeabilization than the plasma membrane of unadapted cells which was apparently impermeable to the fluorescent stain ethidium bromide, used to estimate the level of 2,4-D-dependent plasma membrane disorganization. Consistently, the marked decline in the viability of a population of untreated cells exposed to lethal concentrations of the herbicide was less drastic when a

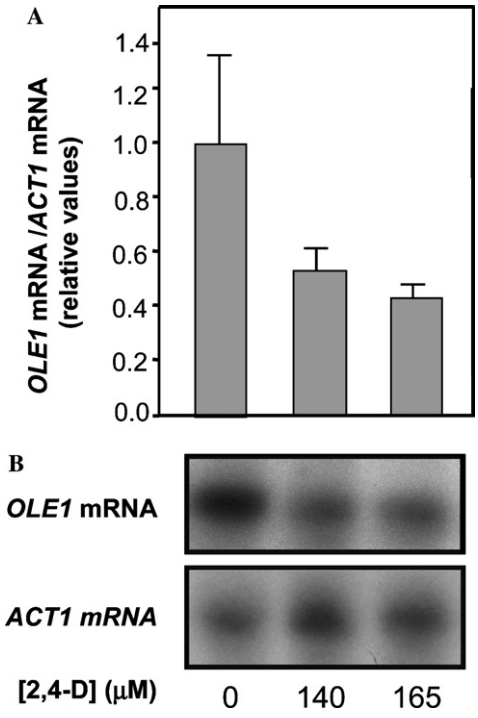

Fig. 7. Levels of *OLE1* and *ACT1* mRNA produced in *S. cerevisiae* W303.1b cells harvested in the middle of exponential growth ($OD_{600nm} = 0.40 \pm 0.03$) carried out, at 30 °C, in MM2 medium (pH 3.0) supplemented with the indicated increasing concentrations of 2,4-D. The corresponding growth curves are shown in Fig. 1. (A) Relative values of *OLE1* mRNA/*ACT1* mRNA were quantified by densitometry of the (B) autoradiograms. The relative mRNA ratio of exponential cells that had not been exposed to the herbicide was set as 1. Bars represent the standard deviation from the average of at least two independent growth experiments for each herbicide concentration with two Northern blot experiments each.

population previously exposed to concentrations of 2,4-D of moderate toxicity was tested. Although the mechanism of adaptation to 2,4-D involves many cellular functions and components, as detailed in the Introduction, the maintenance within physiological limits of membrane organization and integrity, through changes at the level of fatty acid composition, is likely to contribute to the global adaptive process. Indeed, it is known that the packing of fatty acyl chains determines to a large extent the membrane fluidity, the decreasing extent of the unsaturation degree leading to a more ordered structure and a decrease in fluidity [18]. Besides the direct effect of 2,4-D as a highly lipophilic agent, that is expected to disturb membrane organization thus affecting its biological function as a permeability barrier and a matrix for membrane enzymes, this herbicide has a dose-dependent pro-oxidant action in the yeast [10]. It is known that the loss of membrane integrity can also arise through oxy-radical mediated lipid peroxidation, unsaturated fatty acids being more susceptible [20]. The extent of cellular damage induced by heat shock or by oxidative stress induced by H_2O_2 [21], copper [22,23] or cadmium [23] was related to membrane lipid composition and correlated positively with increasing unsaturation of the fatty acid components. Indeed, both cadmium- and copper-induced disruption of plasma membrane was markedly accelerated in *S. cerevisiae* cells with plasma membranes enriched with polyunsaturated fatty acids (PUFA) [22,23]. It was thus proposed in the above-referred studies that the sensitivity to the different stresses of yeast cells with a PUFA enriched plasma membrane is attributable to membrane damage associated with increased membrane fluidity and oxygen-derived free radical attack of membrane lipids. The adaptive response described in the present study, occurring at the level of fatty acid unsaturation degree in yeast cells coping with 2,4-D, is therefore consistent with the hypothesis that yeast cells' resistance to 2,4-D toxicity involves the reduction in the level of increase of plasma membrane fluidity and oxygen-derived free radical attack of membrane lipids resulting from 2,4-D exposure.

In yeast, unsaturated fatty acids are formed by the Ole1 protein that converts long-chain saturated fatty acyl-CoA substrates into monounsaturated species [14]. The Ole1 fatty acid desaturase plays a central role in the formation of membrane lipids and in the control of the physical properties of membrane bilayers [14,24]. The encoding gene *OLE1* is known to be regulated by the presence of unsaturated fatty acids, through a complex system of controls at both the levels of transcription and mRNA stability [14,24,25]. In the present work, we show the first experimental evidence indicating that *OLE1* expression may be regulated in response to 2,4-D induced stress. This is the first time that the presence of an environmental stress agent is demonstrated to lead to a coordinate reduced level of *OLE1* transcripts and decrease of the unsaturation degree of membrane fatty acids, even though the importance of the increase of the unsaturation degree of membrane fatty acids in decreasing yeast tolerance to heavy metals, supraoptimal temperatures or H_2O_2, has been recognized before [21–23]. However, based on a microarray analysis, the decrease of *OLE1* transcript levels was already reported to occur as part of the yeast global response to amphotericin B, an antifungal polyene which acts by increasing plasma membrane permeability [26]. The decline of *OLE1* expression was also observed in yeast mutants affected in ergosterol biosynthetic pathway, being considered a compensatory response to increased membrane fluidity due to reduced ergosterol levels [27]. However, in the present work, the gene *OLE1* could not be definitively identified as a determinant of yeast resistance to 2,4-D because the respective null mutant is not viable in growth medium lacking unsaturated fatty acid supplementation [28,29].

Given the central role of Ole1p in membrane lipid composition and in the control of the physical properties of membrane lipid bilayers, it is not surprising that

several regulatory systems are in operation for the control of this enzyme activity in yeast [14,24,30]. One of the regulatory mechanisms involves changes in the half-life of the *OLE1* mRNA [25]. The degradation of the *OLE1* mRNA, involving the removal of its 5′ cap by Dcp1p followed by 5′–3′mRNA digestion by Xrn1p, is considered to be an important component of the expression level of this gene [24,25]. Essential mRNA stability elements were identified within the transmembrane loop coding regions of *OLE1* and the association of the nascent Ole1p polypeptides with the endoplasmic reticulum membrane lipid bilayer was suggested to be a mechanism for controlling mRNA stability through some type of membrane fluidity regulation mechanism [24]. Interestingly, our study involving the quantitative analysis of the yeast proteome showed that the concentration of Dcp1 increased more than 7-fold during the period of adaptation to 2,4-D, maintaining higher levels in cells exponentially growing in the presence of the herbicide compared to unstressed cells [12]. This suggests that the observed drop in the concentration of *OLE1* transcripts in response to moderate stress induced by 2,4-D may, at least partially, be a consequence of differences occurring in transcript decay mediated by Dcp1p. It is thus possible that, as recently proposed for the role of initiation-mediated decay of transcripts encoding chaperone proteins in yeast cells exposed to heat-shock [31], the tuning of *OLE1* expression through the regulation of the decay of the encoding mRNAs may have an important physiological role in the adaptive response occurring at the level of plasma membrane fatty acid unsaturation degree of yeast cells challenged with 2,4-D.

Acknowledgments

This research was supported by contract QLK3-CT-1999-00041 of the European Comission within its Fifth Framework Programme, and by FEDER and Fundação para a Ciência e Tecnologia (FCT)/Portugal (contracts POCTI/AGG/38110/2001, POCTI/AGR/45347/2002, and POCTI/BIO/38115/2001, and grants to M.G. Cabral (PRAXIS XXI/BD/21707/99) and to M.C. Teixeira (FSRH/BPD/14484/03).

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