Fungicides and sterol-deficient mutants of *Ustilago maydis*: plasma membrane physico-chemical characteristics do not explain growth inhibition

Agustín Hernández,† David T. Cooke, Mervyn Lewis and David T. Clarkson

Author for correspondence: Agustín Hernández. Fax: +32 16 321979.
e-mail: agustin.hernandez@bio.kuleuven.ac.be

Department of
Agricultural Sciences,
University of Bristol,
Institute of Arable Crops
Research, Long Ashton
Research Station, Bristol
BS18 9AF, UK

Plasma membrane vesicles from *erg11* and *erg2* sterol-deficient mutants and from wild-type *Ustilago maydis* sporidia treated with and without inhibitors of sterol 14 α -demethylase or sterol Δ^8 - Δ^7 isomerase (triadimenol and fenpropimorph fungicides, respectively) were purified by aqueous two-phase partitioning. Changes in plasma membrane lipid composition were mostly restricted to sterols and complex lipid-bound fatty acids (CLB fatty acids). There was a greater accumulation of abnormal sterols (14 α -methyl- or Δ^8 -unsaturated sterols) in plasma membranes from sterol-deficient mutants than from those treated with their fungicide counterparts. However, greater growth inhibition was observed on fungicide-treated wild-type than on mutants. Changes in CLB fatty acids were restricted to alterations in the relative proportion of linoleic acid (18:2) with respect to oleic acid (18:1). The 18:2 to 18:1 ratio found in CLB fatty acids in plasma membranes could be correlated to rates of sporidial growth but not to accumulation of a particular abnormal sterol or to the extent of sterol replacement. Plasma membrane permeability to protons was increased moderately in the mutants only. No changes were observed in plasma membrane fluidity. Plasma membrane H⁺-ATPase activity was increased up to twofold in those cases with lower growth rate. It was concluded that fungicide-induced growth inhibition in *U. maydis* was not due to accumulation of abnormal sterols in plasma membranes but probably due to intracellular ATP depletion by the H⁺-ATPase and that changes in 18:2 to 18:1 ratio in CLB fatty acids were not directly dependent on the plasma membrane physical state or lipid composition but were possibly part of a stress adaptation mechanism.

Keywords: corn smut, ergosterol, fatty acids, proton permeability, membrane fluidity

INTRODUCTION

In eukaryotic cells, sterols are present in great amounts in the plasma membrane, more than in any other organelle, reaching molar proportions of 1:1 with

phospholipids (Van der Rest *et al.*, 1995). Ergosterol biosynthesis inhibitors (EBIs) are widely used as fungicides. In the current view, their effectiveness in inhibiting fungal proliferation is based on limiting the supply of ergosterol for membrane construction while provoking the accumulation of biosynthetic precursors and abnormal sterols in membranes. The direct effects of this sterol replacement would be, on the one hand, an increase in the permeability of the plasma membrane, which would impair the regulation of cytosol composition, and, on the other hand, altered membrane fluidity that would, in turn, affect membrane-associated enzymic activity (Vanden Bossche *et al.*, 1983; Lees *et*

† **Present address:** Katholieke Universiteit Leuven, Laboratorium voor Moleculaire Celbiologie, Instituut voor Plantkunde, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium.

Abbreviations: CLB fatty acid, complex lipid-bound fatty acid; EBI, ergosterol biosynthesis inhibitor; Et-C, ethanol control treatment; Fen-T, fenpropimorph treatment; Tri-T, triadimenol treatment; UI, unsaturation index.

al., 1995). As a physiological counter-response, these changes in sterols would trigger changes in the fatty acid profile of cell membranes (Weete, 1987). However, several facts suggest that the mechanisms responsible for growth inhibition may be more complex. For example, EBIs are lethal to *Plasmodium falciparum*, a protozoan incapable of *de novo* sterol synthesis (Vanden Bossche, 1993). Also, to date, no key membrane-bound enzyme in fungal physiology has been found to be fluidity-sensitive. Moreover, different species show dramatic changes in growth inhibition at similar extents of replacement of normal sterols by abnormal ones (Loeffler & Hayes, 1992). Finally, a report pointed out that fatty acid changes may not be triggered by sterol replacement but merely accompany it (Weete *et al.*, 1991). This all suggests that a more critical look at the real effects that abnormal sterols have on the plasma membrane physico-chemical properties is needed both to understand the role of sterols in the membrane and the mode of action of fungicidal compounds.

This paper analyses the effects of the accumulation of abnormal sterols in the plasma membrane of *Ustilago maydis* and focuses on lipid composition, permeability to protons and fluidity of the lipid moiety. Comparison between mutant strains and fungicide treatments has been used to identify the specific effects of abnormal sterol accumulation.

METHODS

Strains and culture conditions. *U. maydis* (IMI 103761) was maintained in frozen aliquots with 9% DMSO at -70°C . Liquid cultures were inoculated with 75 mg (fresh wt) cells per 400 ml medium and cultured for 48 h in Minimal Medium (Hargreaves & Turner, 1989) on a rotatory shaker at 25°C . When appropriate, 2.5 μM triadimenol (a triazole) or 0.1 μM fenpropimorph (a morpholine) as ethanolic solutions were added to cultures of wild-type strain at the time of inoculation (treatments named Tri-T and Fen-T, respectively). Ethanol (0.025%, v/v), in the absence of fungicide, was also added to wild-type sporidia as a proper control (treatment Et-C). Mutant strains A14 and P51 were kind gifts of Dr J. A. Hargreaves (James *et al.*, 1992; Keon & Hargreaves, 1996) and were cultured without additions, as was the above-mentioned parental strain as a control (WT).

The growth of *U. maydis* sporidia in liquid media was monitored as the increase in light scattered at 500 nm (Pye Unicam SP1800). Growth rates were deduced from the slopes of observed optical density vs time during the linear phase of growth, as obtained by linear regression.

Plasma membrane purification. *U. maydis* sporidia were collected by centrifuging at 6000 *g* for 10 min (typical harvest, 12 g fresh weight). The biomass was mixed with 33 ml homogenization buffer (50 mM HEPES adjusted to pH 7.5 with KOH, 330 mM sucrose, 5 mM EDTA, 5 mM EGTA, 0.2% BSA, 0.2% casein hydrolysate, 1 mM PMSF, 2% choline, 5 mM DTT) and 25 g glass beads (0.125 mm diameter). Cells were homogenized in a Bead-Beater (Biospect Products), the homogenate filtered through nylon cloth (240 μm pore size) and centrifuged at 10000 *g* for 15 min. The pellet (unbroken cells, cell debris and intact mitochondria) was discarded and the supernatant centrifuged at 100000 *g* for

30 min to produce a microsomal pellet which was resuspended in 5 mM phosphate buffer, pH 7.8, 330 mM sucrose. Plasma membranes were isolated and purified using the aqueous two-phase polymer technique as previously described (Hernández *et al.*, 1994).

Lipid analysis. Plasma membrane lipids were extracted as described (Cooke *et al.*, 1991). Briefly, in an Eppendorf tube, chloroform/methanol (0.75 ml, 1:2, v/v) was added to resuspended membranes (0.5 ml) along with 60 μl 2 M KCl; also, β -cholestanol (20 μl , 0.1 mg ml^{-1}) and methyl heptadecanoate (30 μl , 0.1 mg ml^{-1}) were added as internal standards for sterol and fatty acid analysis, respectively. Chloroform (0.25 ml) was added, the mixture shaken and centrifuged at 10000 *g* for 6 min. The chloroform layer was retained, evaporated to dryness under N_2 and made up to 100 μl with chloroform.

Sterols were analysed by GC using an SE52-bonded capillary column with H_2 as carrier gas (1 ml min^{-1}) and a temperature programme of 120 – 265°C at $10^{\circ}\text{C min}^{-1}$. The injector and detector temperatures were 250 and 320°C , respectively. Identification of individual sterols was done by comparing their respective mass spectra with those already published (James *et al.*, 1992). Mass spectra were obtained with a Kratos MS80 system (Kratos Analytical). The quantification of the peaks was done by flame ionization detection and comparison with the internal standard.

Complex lipid-bound fatty acids (CLB fatty acids) were quantified by GC analysis. An aliquot of the chloroform extract was evaporated to dryness under nitrogen and transmethylated with 0.5% (w/v) freshly prepared sodium methoxide dissolved in dry methanol and heated at 70°C for 10 min. The resultant fatty acid methyl esters were extracted with hexane, evaporated to dryness under nitrogen, dissolved in ethyl acetate and analysed by GC with a flame ionization detector attached using a RSL 500-bonded capillary column and helium as the carrier gas (1 ml min^{-1}). The temperature programme was 170 – 200°C at $2^{\circ}\text{C min}^{-1}$. Injector and detector temperatures were 250 and 300°C , respectively. In the case of free fatty acids, a similar aliquot was dried under nitrogen, dissolved in 20% (v/v) methanol in ether and methylated with an excess of diazomethane in ether for 10 min. After this period, the diazomethane and the solvents were evaporated, the lipids redissolved in ethyl acetate and analysed as described above. The peaks were quantified with a flame ionization detector and identified by comparison with authentic standards.

Unsaturation index (UI) values were calculated using the formula:

$$\text{UI} = (\Sigma\text{M} + 2\Sigma\text{D} + 3\Sigma\text{T})/100$$

where ΣM , ΣD and ΣT are the sum of the percentages of mono-, di- and tri-unsaturated fatty acids, respectively.

Phospholipids were analysed by HPLC, using a three-solvent system as described by Christie (1986). The chloroform extract was injected into an Econosphere silica 3 μm column (150 $\times$ 4.6 mm) and its components detected with an evaporative light-scattering detector. Phospholipids were quantified by comparing peak areas with those of known standards.

Passive diffusion constants for protons. The generation of a Mg-ATP-dependent ΔpH was assayed by monitoring the change in fluorescence emission of the fluorescent probe, 9-amino-6-chloro-2-methoxyacridine (Molecular Probes), as described by Coupland *et al.* (1991). The change in fluorescence emission was measured at 485 nm with excitation at 415 nm and recorded using a chart recorder.

The accumulation of protons into a vesicle can be described as the resultant of two fluxes:

$$J_{H^+net} = J_{H^+in} - J_{H^+out}$$

The inward flux will be driven by the H^+ -ATPase, while the outward flux will be due to passive diffusion, obeying Fick's law. If T_{H^+} is the initial velocity of proton transport by the H^+ -ATPase, $\Delta[H^+]$ is the concentration of protons inside minus those outside the vesicle and $K_{D_{H^+}}$ is the diffusion constant for protons through the lipid bilayer, we can rewrite:

$$J_{H^+net} = T_{H^+} - \Delta[H^+] \times K_{D_{H^+}}$$

In time, an equilibrium is reached in which the rates of proton transport inside the vesicle and the outward diffusion are equal, then:

$$J_{H^+net} = 0 \quad T_{H^+} / \Delta[H^+] = K_{D_{H^+}}$$

Membrane fluidity measurements. Fluidity determinations were done by steady-state fluorescence polarization as described by Cooke *et al.* (1991) using DPH (1,6-diphenyl-1,3,5-hexatriene) as the fluorescent probe. Membranes were diluted with 40 mM HEPES, 100 mM KCl, 5 mM $MgSO_4$ and 0.1 mM EGTA, to give 100 μ g protein in a total volume of 1 ml. The probe was added as 1 μ l of a 1 mM solution in tetrahydrofuran (THF), 1 μ l THF was added to controls. After eliminating excess THF under nitrogen, samples and controls were incubated at 25 °C for 1 h before measuring. The steady-state fluorescence polarization (P) was calculated according to the relationship:

$$P = (I_{VV} - I_{VH}G) / (I_{VV} + I_{VH}G)$$

where, I refers to the fluorescence intensity through polarizers orientated vertically (V) or horizontally (H) with respect to the plane of polarization of the excitation beam. G (the grating correction factor) is described by the ratio I_{HV}/I_{HH} . The excitation wavelength was 360 nm with 5 nm slit width, and emission was 430 nm with 10 nm slit width.

ATPase assays. The medium consisted of 100 mM MES adjusted to pH 6.5 with Tris, 0.0125% (w/v) Triton X-100, 1 mM sodium azide, 0.1 mM sodium molybdate, 50 mM potassium nitrate, 3 mM magnesium sulphate, 3.5 mM ATP (sodium salt) and 2–5 μ g membrane protein in a total volume of 240 μ l. Assays were run for 10 min at 37 °C. Under these conditions, the concentrations of Mg-ATP and free Mg^{2+} were 2.5 and 0.5 mM, respectively. The reaction was terminated by adding the stopping reagent for phosphate determination.

Other methods. Protein concentration was determined by the method of Bradford (1976) using thyroglobulin as the standard.

Statistics. Except where indicated, all experiments were done at least in triplicate. The actual number (n) of experiments is indicated in each case. Determinations within a single experiment were typically repeated three times. Data shown in the present work are means of the values obtained in different experiments ($\pm$ SE). Statistical differences were analysed by LSD (least significant difference) at 95% level of confidence.

RESULTS

Growth characteristics of mutants and fungicide-treated sporidia

Culture of wild-type sporidia in the presence of 2.5 μ M triadimenol slowed growth by a factor of two, and 0.1 μ M fenpropimorph inhibited growth by a factor of

about 1.3 compared with the ethanol control (samples Tri-T, Fen-T and Et-C, respectively; Table 1). Addition of ethanol, the solvent of both fungicides, did not produce significant changes in growth rate compared with cultures with no additions (WT; Table 1). On the other hand, mutants affected on the sterol 14 α -demethylase or the sterol Δ^8 - Δ^7 isomerase did not show a significant decrease in growth rate, compared with WT (Table 1). The morphology of the sporidia was altered in similar ways when fungicide-treated cells were compared with mutants, i.e. sporidia were smaller than wild-type and swollen (A14, Tri-T) or appeared as long unseparated chains (P51, Fen-T) (data not shown) and was in accordance with previous reports (Girling, 1991; James *et al.*, 1992; Keon & Hargreaves, 1996). After 52 h of culture, stationary phase had not been reached with any of the treatments or by any of the mutants (data not shown).

Sterol composition of plasma membranes

The total amount of free sterols in plasma membranes of *U. maydis* was significantly greater in the case of P51, but not in any other strain or treatment (Table 5).

The different sterols present in plasma membrane preparations were identified by mass spectrometry. In control sporidia (Et-C and WT), ergosterol, ergosta-5,7-dien-3 β -ol and ergosta-5-en-3 β -ol appeared in the proportion 15:5:1 and represented more than 90% of total free sterols in plasma membranes. The rest corresponded mostly to traces of ergosta-5,8,22-trien-3 β -ol and eburicol and to other unknown sterols (Table 2). The presence of 0.025% ethanol in the culture medium only produced minor changes in the proportion of ergosta-5,7-dien-3 β -ol, compared with WT (Table 2).

Compared with Et-C, triadimenol-treated sporidia (Tri-T) showed a 2.6-fold decrease in ergosta-5,7-dien-3 β -ol and a sevenfold decrease in ergosta-7-en-3 β -ol (Table 2). However, no significant differences were found in the proportion of ergosterol, compared with Et-C plasma membranes. Eburicol was the major 14 α -methylated sterol (12%). Curiously, the proportion of ergosta-5,8,22-trien-3 β -ol also increased significantly, which was also observed in A14 (twofold increase) and P51 (18-fold increase) sporidia plasma membranes. In the mutant strain A14, a small but significant decrease in ergosterol was found and larger decreases were observed for ergosta-5,7-dien-3 β -ol (fivefold smaller) and ergosta-7-en-3 β -ol (3.5-fold smaller), compared with WT. The presence of 14 α -methylated sterols accounted for about 34% of the total free sterols in these plasma membrane preparations, eburicol being the most abundant. The proportion of abnormal sterols in Tri-T plasma membranes was somewhat less than that in A14 plasma membranes (26%), but no significant differences were observed in any particular 14 α -methylated sterol, when compared with A14 (Table 2).

In the other mutant strain, P51, a minute amount of ergosterol was detected but no traces of the other two

Table 1. Relevant biological characteristics of *U. maydis* strains and treatments

Genetic lesions, sterol biosynthetic steps inhibited by the fungicides used in this work, and concomitant effects on growth. Triadimenol and fenpropimorph were ethanolic solutions; final concentration of ethanol, 0.025 % (v/v). Significant differences at 95 % level of confidence, as evaluated by LSD, compared with: *, Et-C; †, WT; ‡, A14; §, P51.

Strain/ treatment	Relevant genotype	Addition to culture medium	Step affected	Growth rate ($\Delta OD_{500} h^{-1} \pm SE$) ($n = 2$)
Et-C	Wild-type	Ethanol (0.025 %, v/v)	None	0.35 ± 0.01
Tri-T	Wild-type	Triadimenol (2.5 μM)	Sterol C-14 α -demethylase	$0.18 \pm 0.06^{* \dagger \ddagger}$
Fen-T	Wild-type	Fenpropimorph (0.1 μM)	Sterol Δ^8 - Δ^7 isomerase	$0.28 \pm 0.01^{* \S}$
WT	Wild-type	None	None	0.38 ± 0.02
A14	<i>erg11</i>	None	Sterol C-14 α -demethylase	0.33 ± 0.03
P51	<i>erg2</i>	None	Sterol Δ^8 - Δ^7 isomerase	$0.34 \pm 0.00^{\ddagger}$

Table 2. Type and proportion of free sterols in *U. maydis* plasma membrane fractions

Accumulation of abnormal sterols in plasma membranes of sterol-deficient mutants and fungicide-treated sporidia. Data, expressed as percentage (w/w) of total sterols, are means $\pm SE$ of four independent experiments. See Table 1 legend for symbols.

Sterol	Et-C	Tri-T	Fen-T	WT	A14	P51
Ergosterol	65.9 ± 0.6	61.5 ± 3.5	$27.4 \pm 0.9^{\S}$	69.1 ± 2.2	$58.1 \pm 4.7^{\dagger}$	$2.1 \pm 0.5^{\ddagger}$
Ergosta-5,7-dien-3 β -ol	$25.3 \pm 1.3^{\dagger}$	$9.9 \pm 0.7^{* \ddagger}$	$3.5 \pm 1.3^{* \S}$	18.4 ± 1.8	$3.8 \pm 1.4^{\dagger}$	—
Ergosta-7-en-3 β -ol	4.3 ± 0.4	$0.6 \pm 0.2^{* \ddagger}$	$1.5 \pm 0.3^{* \S}$	4.5 ± 0.4	$1.3 \pm 0.2^{\dagger}$	—
14-Methylfecosterol	—	6.8 ± 0.6	—	0.6 ± 0.4	$7.4 \pm 0.9^{\dagger}$	—
Obtusifolol	—	7.5 ± 1.1	—	—	8.6 ± 1.4	—
Eburicol	1.6 ± 0.3	$11.7 \pm 2.1^{*}$	$0.4 \pm 0.1^{*}$	1.3 ± 0.4	$17.9 \pm 4.3^{\dagger}$	$0.5 \pm 0.0^{\ddagger}$
Ergosta-5,8,22-trien-3 β -ol	0.9 ± 0.1	$1.5 \pm 0.3^{*}$	$13.2 \pm 0.8^{* \S}$	1.1 ± 0.0	$2.1 \pm 0.3^{\dagger}$	$20.0 \pm 1.5^{\ddagger}$
Ergosta-8,22-dien-3 β -ol	—	—	14.7 ± 0.6	—	—	16.2 ± 1.2
Ergosta-8-en-3 β -ol	—	—	$38.3 \pm 0.9^{\S}$	—	—	52.7 ± 2.2
Unknown sterols	1.9 ± 1.2	0.5 ± 0.2	1.0 ± 0.3	5.0 ± 0.6	1.0 ± 0.3	8.6 ± 2.9

normal sterols (Table 2). Δ^8 -Unsaturated sterols represented 89 % of free sterols in these plasma membranes. In particular, ergosta-8-en-3 β -ol accounted for more than 50 % of the sterols. The plasma membrane of Fen-T sporidia showed a 2.5-fold reduction in the amount of ergosterol, as compared with Et-C, and further reductions in the other two major normal sterols: sevenfold for ergosta-5,7-dien-3 β -ol and threefold for ergosta-7-en-3 β -ol (Table 2). However, Fen-T plasma membranes accumulated significantly lesser amounts of abnormal sterols, compared with P51. Thus, in Fen-T plasma membranes, only 66 % of the sterols were Δ^8 -unsaturated, ergosta-8-en-3 β -ol (38 %) being the major sterol in Fen-T plasma membranes.

CLB fatty acids

Plasma membranes from *U. maydis* showed a wide range of fatty acids in their complex lipids (Table 3). These included from palmitic (16:0) to erucic acids (22:1). CLB fatty acid content showed a tendency to increase in A14 plasma membranes, while in plasma membranes from Tri-T, Fen-T and P51 only about 75 %

of the value corresponding to the appropriate controls, Et-C and WT, respectively, was found (Table 5).

The 18-carbon fatty acids were the most abundant (Table 3). In combination, they comprised 65 % of the total; this proportion was constant irrespective of the strain or treatment. However, there were important changes in the proportions of oleic and linoleic acids among the treatments and in strain P51, but not in A14, compared with Et-C and WT, respectively. Furthermore, the proportions of oleic acid decreased and the proportions of linoleic acid increased in corresponding amounts in all these cases. In particular, the 18:2/18:1 ratio remained in the range 0.8–1.4 with Et-C, WT and A14 but increased up to 6.6 in Tri-T. Stearic acid did not follow this pattern and significant variations were found only in Δ^8 - Δ^7 isomerase defective cells. Linolenic acid represented a mere 1.3 % of the total CLB fatty acids and showed no significant variations.

The shortest fatty acids analysed (palmitic and palmitoleic acids) represented a constant proportion in any given strain or treatment (Table 3). The sum of these fatty acids remained around 32 % of the total.

Table 3. Proportion of CLB fatty acids in *U. maydis* plasma membranes

Changes in plasma membrane CLB fatty acids in sterol-deficient mutants and in fungicide-treated sporidia. Data, expressed as percentage (w/w) of the total, are means $\pm$ SE of four independent experiments. See Table 1 legend for symbols.

Fatty acid	Et-C	Tri-T	Fen-T	WT	A14	P51
16:0	31.1 $\pm$ 0.7	32.4 $\pm$ 1.6	29.5 $\pm$ 1.7	29.3 $\pm$ 1.1	30.1 $\pm$ 2.9	26.0 $\pm$ 1.2
16:1	1.1 $\pm$ 0.4	2.3 $\pm$ 0.3*	1.9 $\pm$ 0.9	2.0 $\pm$ 0.4	2.4 $\pm$ 1.2	1.6 $\pm$ 0.1
18:0	12.9 $\pm$ 0.7†	10.4 $\pm$ 3.0‡	8.2 $\pm$ 0.9§	20.0 $\pm$ 1.3	17.3 $\pm$ 1.2	11.9 $\pm$ 1.3†
18:1	22.7 $\pm$ 1.9	6.9 $\pm$ 0.9*‡	11.7 $\pm$ 1.8*	24.4 $\pm$ 0.9	21.0 $\pm$ 4.7	18.8 $\pm$ 6.2
18:2	27.9 $\pm$ 1.6†	42.4 $\pm$ 3.6*‡	43.3 $\pm$ 2.6*	20.5 $\pm$ 1.7	24.4 $\pm$ 3.0	38.1 $\pm$ 7.8†
18:3	1.1 $\pm$ 0.2	1.4 $\pm$ 0.3	1.4 $\pm$ 0.6	1.3 $\pm$ 0.4	1.8 $\pm$ 0.8	0.6 $\pm$ 0.1
20:0	1.4 $\pm$ 0.1	1.0 $\pm$ 0.2	1.2 $\pm$ 0.1	1.0 $\pm$ 0.0	1.3 $\pm$ 0.0†	0.8 $\pm$ 0.1
20:1	0.9 $\pm$ 0.1	2.1 $\pm$ 0.5‡	1.8 $\pm$ 0.2*	0.7 $\pm$ 0.1	0.7 $\pm$ 0.2	1.2 $\pm$ 0.3
22:0	0.7 $\pm$ 0.0	0.5 $\pm$ 0.3	0.8 $\pm$ 0.1§	0.7 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1†
22:1	0.2 $\pm$ 0.1	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1	0.2 $\pm$ 0.2	0.6 $\pm$ 0.3	0.5 $\pm$ 0.1
18:2/18:1	1.3 $\pm$ 0.1†	6.6 $\pm$ 1.3*‡	3.9 $\pm$ 0.6*	0.8 $\pm$ 0.1	1.4 $\pm$ 0.4	3.6 $\pm$ 1.6
UI	0.84 $\pm$ 0.02†	1.01 $\pm$ 0.06*‡	1.06 $\pm$ 0.04*	0.72 $\pm$ 0.03	0.79 $\pm$ 0.04	1.00 $\pm$ 0.10†

Table 4. Proportion of free fatty acids in *U. maydis* plasma membranes

Changes in plasma membrane free fatty acids in sterol-deficient mutants and in fungicide-treated sporidia. Data, expressed as percentages (w/w) of the total, are means $\pm$ SE of four independent experiments. See Table 1 legend for symbols.

Fatty acid	Et-C	Tri-T	Fen-T	WT	A14	P51
16:0	31.8 $\pm$ 2.1	22.7 $\pm$ 3.2*	26.7 $\pm$ 2.3	25.1 $\pm$ 2.5	20.6 $\pm$ 2.1	23.1 $\pm$ 1.3
18:0	22.8 $\pm$ 2.8	13.6 $\pm$ 0.9*	15.1 $\pm$ 1.4*§	28.0 $\pm$ 0.8	24.1 $\pm$ 4.9	24.4 $\pm$ 3.2
18:1	12.1 $\pm$ 1.0†	8.2 $\pm$ 2.5	7.2 $\pm$ 1.1*	7.6 $\pm$ 1.3	4.9 $\pm$ 0.8	5.6 $\pm$ 1.2
18:2	20.1 $\pm$ 3.2†	34.1 $\pm$ 6.2	27.7 $\pm$ 4.9	30.8 $\pm$ 3.9	41.0 $\pm$ 6.5	36.2 $\pm$ 2.1
18:3	0.8 $\pm$ 0.1†	1.8 $\pm$ 0.4*‡	1.4 $\pm$ 0.4	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2	1.0 $\pm$ 0.2†
20:0	5.7 $\pm$ 0.5†	7.2 $\pm$ 1.8	10.1 $\pm$ 1.4*§	3.7 $\pm$ 0.4	3.9 $\pm$ 0.5	4.8 $\pm$ 0.7
20:1	2.1 $\pm$ 0.1†	3.9 $\pm$ 0.9‡	3.6 $\pm$ 0.7*§	0.7 $\pm$ 0.1	1.6 $\pm$ 0.3†	1.2 $\pm$ 0.3
22:0	2.7 $\pm$ 0.2†	4.8 $\pm$ 1.2‡	5.8 $\pm$ 0.8*§	1.8 $\pm$ 0.3	1.1 $\pm$ 0.1	2.2 $\pm$ 0.3
22:1	1.9 $\pm$ 0.4	3.8 $\pm$ 1.6	2.4 $\pm$ 1.0	1.9 $\pm$ 0.4	2.3 $\pm$ 0.9	1.6 $\pm$ 0.3
18:2/18:1	1.7 $\pm$ 0.4	8.5 $\pm$ 5.4	4.6 $\pm$ 1.7	4.7 $\pm$ 1.4	9.7 $\pm$ 2.8	7.7 $\pm$ 1.9
UI	0.653 $\pm$ 0.057	0.911 $\pm$ 0.085*	0.772 $\pm$ 0.085	0.755 $\pm$ 0.070	0.927 $\pm$ 0.121	0.864 $\pm$ 0.037

Similarly, and independent of the strain or treatment chosen, the percentages of long-chain fatty acids, namely arachidic, eicosenoic, behenic and erucic acids, were small and constant, their sum representing just about 3% of the total (Table 3).

Free fatty acids

In one of the mutant strains, A14, free fatty acids were more abundant than in WT, while in the others they were less abundant (Table 5). Similar contrasts were seen in Fen-T plasma membranes, where free fatty acid abundance declined, and in Tri-T plasma membrane, where it was unchanged relative to the control Et-C. In the samples analysed there was an unexplained difference between the WT and the Et-C control, a point that emphasizes the minor significance of this category of lipids.

Free fatty acids included all those already found as CLB, with the exception of palmitoleic acid (Table 4). Palmitic acid represented 20–30% of the total free fatty acids (Table 4). No significant differences were found in any strain or treatment with the exception of Tri-T, where a slight decrease was encountered, compared with Et-C.

Fatty acids with 18 carbons, again, represented the majority in any strain or treatment studied (Table 4). The order of abundance was slightly different and stearic acid (18:0) was found in greater amounts than 18:1, while the relative amounts of linoleic acids (18:2) were about 30%. Linolenic acid (18:3) was a minor component, ranging from 0.4 to 1.8%. The sum of the proportions presented a wider range than among the bound fatty acids (51–70%). However, no pattern for the changes was found and only a few significant differences were noticeable. In particular, stearic acid decreased with both fungicide treatments to values

Table 5. General lipid composition of *U. maydis* plasma membranes

Data are means $\pm$ SE [μg (mg protein) $^{-1}$] of four independent experiments. See Table 1 legend for symbols.

	Et-C	Tri-T	Fen-T	WT	A14	P51
PE	141.8 $\pm$ 8.2	141.3 $\pm$ 28.2	115.0 $\pm$ 6.4	125.3 $\pm$ 10.6	154.4 $\pm$ 9.2	116.9 $\pm$ 12.6
PC	81.4 $\pm$ 10.1	64.1 $\pm$ 7.8	57.5 $\pm$ 4.7§	79.1 $\pm$ 8.4	89.1 $\pm$ 18.5	94.0 $\pm$ 3.2
Free sterols	153.4 $\pm$ 18.1	187.7 $\pm$ 40.9	247.7 $\pm$ 81.2	146.6 $\pm$ 21.3	145.6 $\pm$ 20.2	265.5 $\pm$ 36.6†
CLB fatty acids	295.8 $\pm$ 19.1	224.6 $\pm$ 26.0*‡	211.2 $\pm$ 30.5*	334.2 $\pm$ 18.6	413.8 $\pm$ 46.0	243.2 $\pm$ 36.5†
Free fatty acids	47.4 $\pm$ 3.7†	41.9 $\pm$ 9.8‡	32.1 $\pm$ 3.7*	66.0 $\pm$ 6.1	94.0 $\pm$ 6.5†	45.5 $\pm$ 5.5†

Table 6. Biophysical parameters of *U. maydis* plasma membranes

Changes in plasma membrane fluidity and passive diffusion constant for protons observed in sterol-deficient mutants and in fungicide-treated sporidia. Data are means $\pm$ SE of three independent experiments. See Table 1 legend for symbols.

	Et-C	Tri-T	Fen-T	WT	A14	P51
Fluidity parameter 'P'	0.266 $\pm$ 0.010	0.283 $\pm$ 0.010	0.293 $\pm$ 0.007	0.280 $\pm$ 0.004	0.266 $\pm$ 0.002†	0.288 $\pm$ 0.003
K _{DH} ⁺ (min ⁻¹)	0.675 $\pm$ 0.061	0.851 $\pm$ 0.053‡	0.798 $\pm$ 0.073§	0.705 $\pm$ 0.085	2.232 $\pm$ 0.152†	1.366 $\pm$ 0.057†

Table 7. H⁺-ATPase hydrolytic activity and percentages of vanadate inhibition found in *U. maydis* plasma membranes

Differences between strains and fungicide treatments of *U. maydis* sporidia. ATPase specific activity in $\mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$. Assays performed in the presence and absence of 10 μM vanadate ($\pm$ SE, $n = 3$). See Table 1 legend for symbols.

	Et-C	Tri-T	Fen-T	WT	A14	P51
– Vanadate	1.828 $\pm$ 0.071	3.687 $\pm$ 0.289*‡	2.966 $\pm$ 0.334§	1.854 $\pm$ 0.016	1.504 $\pm$ 0.204	2.147 $\pm$ 0.058†
+ Vanadate	0.789 $\pm$ 0.025	1.460 $\pm$ 0.159*‡	1.236 $\pm$ 0.091*	0.780 $\pm$ 0.035	0.657 $\pm$ 0.117	1.068 $\pm$ 0.068‡
Inhibition (%)	56.8 $\pm$ 0.9	60.6 $\pm$ 2.0	57.9 $\pm$ 2.0	57.9 $\pm$ 1.5	56.9 $\pm$ 2.3	50.3 $\pm$ 2.5†

about 1.5-fold smaller and linolenic acid increased about twofold in 14 α -demethylase-defective cells. No changes were detected in the relative amounts of oleic and linoleic acids, except for a small decrease in oleic acid for Fen-T sporidia.

The presence of long-chain fatty acids (20–22 carbons long) among the free fatty acids was much greater than among bound ones. The sum of the percentages ranged from 8 to 21 %, in contrast to 3 % found in the CLB fatty acid fraction (Tables 4 and 3, respectively). Nevertheless, with minor exceptions, no clear differences were found between strains or treatments (Table 4).

Major phospholipids

In all membranes analysed the ratio of PE to PC was constant at about 1.8 (Table 5).

Membrane fluidity and proton permeability

The mobility of the probe DPH in the plasma membrane was not changed by any of the fungicide treatments and a slightly lesser fluidity was observed only in A14 plasma membranes (Table 6). No changes were observed in

proton permeability in *U. maydis* plasma membranes from fungicide-treated sporidia (Table 6). In contrast, proton passive diffusion constants for plasma membrane vesicles from A14 and P51 mutant strains were three- and twofold greater, compared with WT, respectively (Table 6). However, these increases in proton permeability did not prevent membrane vesicles from forming proton gradients *in vitro* (data not shown). Addition of fungicides to isolated plasma membrane vesicles had no effect on proton permeability (data not shown).

Plasma membrane H⁺-ATPase

ATPase activity by the plasma membrane proton pump was twofold greater in vesicles derived from triadimenol treated sporidia and 1.6-fold in Fen-T samples (Table 7). In contrast, A14 mutant showed no differences in ATPase activity and P51 exhibited only a slight increase. The vanadate-sensitivity was not affected. Thus, all samples presented a 55–60 % inhibition by 10 μM vanadate, with the exception of P51, which showed a slightly greater sensitivity.

DISCUSSION

In vivo characteristics

The observed growth rates confirmed that *U. maydis* can cope with the presence of abnormal sterols in its membranes. The fungicide concentrations used in this study were carefully chosen to be non-lethal while still producing abnormal sterol accumulation comparable with that observed in the mutants (Loeffler & Hayes, 1992; James *et al.*, 1992; Keon & Hargreaves, 1996). However, the two fungicide treatments had more profound effects as growth inhibitors than the mutations, although, as was later verified (Table 2), the effects of the fungicide treatments on the sterol composition of the plasma membranes were somewhat less than those produced by the mutations. This suggests that these fungicides have other important effects, additional to those on sterol biosynthesis.

Sterols

The sterol profiles were greatly altered in all the strains and treatments and were consistent with a partial blockage of 14 α -demethylase enzyme activity in the case of A14 and Tri-T sporidia, and lack of Δ^8 - Δ^7 isomerase activity for P51 and Fen-T sporidia (Table 2). The sterol composition of the plasma membranes from corresponding mutants and fungicide treatments were similar enough to provide good ground for further comparisons. Treatment of the wild-type with 0.1 μ M fenpropimorph resulted in the substitution of the normal Δ^7 sterols by their Δ^8 counterparts, but no $\Delta^{8,14}$ -diene sterols were found. Lack of such sterols is in agreement with previous work that described fenpropimorph as a specific inhibitor of sterol Δ^8 - Δ^7 isomerase at low concentrations (Loeffler & Hayes, 1992; Girling, 1991). Some authors postulate that inhibition of sterol Δ^{14} reductase is the basis of the lethal effects of fenpropimorph to *Saccharomyces cerevisiae* (Lai *et al.*, 1994; Kelly *et al.*, 1994). However, in the present conditions, where no inhibition of Δ^{14} reductase activity was apparent, this would not explain why the growth of Fen-T sporidia was slower.

It is generally agreed that sterols play a significant role in the maintenance of the membrane properties. However, the existence of sterol-deficient mutants that can grow in the absence of exogenously added sterols at nearly the same rate as the wild-type suggests that bulk sterol replacement is not lethal. Moreover, a recent report on EBI-sensitive and -resistant strains of *Uncinula necator* showed that the extent of sterol replacement on both strains upon addition of triadimenol was very similar (Debieu *et al.*, 1995), and basically identical results were reported for *Erysiphe graminis* (Senior *et al.*, 1995). On the other hand, A14 is hypersensitive to lanosterol demethylase inhibitors, such as azole fungicides, although this step is partially blocked in this mutant (Keon & Hargreaves, 1996). Therefore, the prospect of an alternative mode of action for certain fungicides remains open.

The low content of ergosterol in the plasma membrane of the P51 mutant (33-fold less ergosterol than wild-type, Table 2) is surprising since the genetic lesion is a deletion of the Δ^8 - Δ^7 isomerase gene (J. A. Hargreaves, personal communication). Previous analysis of this mutant failed to identify any ergosterol, and, in this work, no ergosterol peak was found in the GC traces corresponding to the P51 mutant microsomes (data not shown). Since plasma membranes were purified sevenfold from microsomes, according to enzyme markers, and 17-fold if we take into consideration the enrichment in sterols (Hernández *et al.*, 1994), it is easy to understand how the small amounts of ergosterol detected in these particular plasma membrane preparations can be overlooked if whole-cell sterols are analysed. Ergosterol plays several roles in the growth and physiology of fungi as defined by Rodriguez *et al.* (1985). The growth of sterol auxotrophs is precluded unless hormonal amounts of ergosterol (1–10 ng ml⁻¹) are present. In this context, it seems plausible that P51 owes its ability to grow at a rate similar to the wild-type to the presence of low amounts of ergosterol in its plasma membrane. In research on *Gibberella fujikuroi* (Nes & Heupel, 1986), it was proposed that there may be alternative genetic compartments for the synthesis of ergosterol, which would explain the ability of the P51 mutant to synthesize ergosterol in the absence of the Δ^8 - Δ^7 isomerase enzyme. Moreover, this hypothesis has been reinforced by the ability of a yeast mutant to synthesize ergosterol, despite two different mutations affecting the pathway (Nes & Dhanuka, 1988).

Phospholipids

As expected, phosphatidyl ethanolamine was more abundant in *U. maydis* plasma membranes than phosphatidyl choline (Hernández *et al.*, 1994). No treatment or mutation affected the amounts or the proportions of major phospholipids in plasma membranes of *U. maydis* (Table 5). This is in accordance with previous studies where, although triarimol or propiconazole had severe effects on the sterol composition of *U. maydis* or *Taphrina deformans*, respectively, no effect was observed in the corresponding phospholipid fractions (Ragsdale, 1975; Weete *et al.*, 1985).

Fatty acids

In *U. maydis*, although long-chain fatty acids were well represented, the bulk of the CLB fatty acids was comprised of medium-chain acids (16–18 carbons long) (Tables 4 and 5). The composition of the fatty acids is one of the most variable parameters in fungal membranes; their profile changes in many adaptation mechanisms, such as cold, ethanol or the presence of toxins (Weete, 1974). In the case of *U. maydis*, the profile does not change for most of the CLB fatty acids after fungicide treatment or in mutants. However, important changes were found among samples in CLB oleic and linoleic acids. The sum of the proportions of these two fatty

Fig. 1. Correlation between growth inhibition and changes in the linoleic/oleic fatty acid ratio of *U. maydis* plasma membrane complex lipids. Data of growth rates from Table 1 and data of 18:2/18:1 ratio from Table 3. Correlation coefficient, $r = 0.93$.

acids was maintained approximately constant with respect to the rest of the bound fatty acids, but the ratio between them changed dramatically from one strain (or treatment) to another (Table 3). Similar changes in plasma membrane CLB fatty acids have been reported in *U. maydis* in response to triarimol (an azole antifungal; Ragsdale, 1975), in *Candida albicans* treated with 0.1 μ M ketoconazole (Vanden Bossche, 1993) and in *T. deformans* treated with propiconazole (Weete *et al.*, 1985, 1991). Surprisingly, in the present study, normal sterol depletion was not the primary cause for these changes. Despite the fact that the plasma membranes of the mutants were equally, or more affected, in sterol composition than those from fungicide treatments, the latter displayed the greatest changes in 18:2/18:1 ratio. Nevertheless, these changes followed inversely the order of growth reduction (Fig. 1) showing a high correlation coefficient ($r = 0.93$). Similar changes in the abundance ratio of unsaturated fatty acids have been found as part of the adaptation processes of yeasts to stress conditions. For example, *S. cerevisiae* increases the amount of oleic acid at the expense of palmitoleic acid as a response to the presence of decanoic acid (a toxin produced during fermentation) in the growth medium (Alexandre *et al.*, 1996) and a similar response is observed in the presence of ethanol (Sajbidor & Grego, 1992). Also, work on *T. deformans* suggested that fungicide-induced fatty acid unsaturation was not dependent on abnormal sterol accumulation, although it could not be correlated with growth rate (Weete *et al.*, 1991). Therefore, in the context of the present work, the changes in linoleic to oleic acid ratio can be better understood as a stress response rather than a direct consequence of the presence of non-functional sterols. The origin of this stress condition may relate, in part, to the presence of abnormal sterols, but other detrimental effects, brought about by fungicide treatments, appear to enhance the stress effect and this, in turn, triggers further changes in fatty acid unsaturation. The mode of action cannot be elucidated from the current data, but this hypothesis would be in agreement with lethal effects for azoles and morpholines observed in bacteria (Baldwin, 1983; Kalam & Banerjee, 1995) and in *P. falciparum*, the

protozoan responsible for malaria, even though *Plasmodium* spp. are not able to synthesize sterols *de novo* (Vanden Bossche, 1993).

Free fatty acids did not show any significant differences in the proportion of individual species of acids (Table 4); the only changes being a slight shift towards the longer-chain fatty acids at the expense of medium-length ones in the fungicide treatments. It is worth noting that similar results to these were obtained in plasma-membrane-enriched fractions of *T. deformans* cells treated with propiconazole (Weete *et al.*, 1985).

Biophysical characteristics of the vesicles

Fluidity measurements revealed no significant differences for most of the strains and treatments (Table 6). The only exception was the A14 strain, which had a slightly more rigid membrane. This lack of effect on the microviscosity of the membrane is inconsistent with the hypothesis that fungicide-induced changes in fluidity are responsible for detrimental changes in activity of membrane-bound enzymes.

Despite this lack of difference in fluidity, some changes were observed in terms of passive permeability to protons in the mutant strains A14 and P51. These modest increases in the passive diffusion constant of protons did not prevent formation of proton gradients *in vitro* (data not shown). Assuming membrane thickness to be 7.5 nm (Van der Rest *et al.*, 1995), the diffusion coefficients for A14 and P51 strains would be about 1×10^{-4} and 6×10^{-5} cm s⁻¹, respectively, while for both controls and fungicide-treated sporidia it would correspond to about 3×10^{-5} cm s⁻¹. These values are all similar to those found in a wide range of preparations (Deamer & Nichols, 1983). Moreover, a recent report relating maximum bacterial growth temperature to membrane ion permeability has determined that proton permeabilities at 30 °C were at least fivefold greater in psychrophiles than in mesophiles (Van de Vossenberg *et al.*, 1995). Therefore, changes in permeability to protons are unlikely to explain growth inhibition.

ATPase activity and toxic stress

The activity of the plasma membrane proton pump was found to increase substantially in those cases with a lower growth rate, i.e. in plasma membrane vesicles from fungicide-treated sporidia. The maintenance of a proton gradient across the plasma membrane is an expensive process that can consume 40–60% of the cellular ATP (Serrano, 1991). In weak-acid-stressed yeast cells, it has been shown that increases in H⁺-ATPase activity were associated with lesser biomass yields (Viegas & Sá-Correia, 1991). Moreover, a recent report has demonstrated that stressed yeast cells activate their plasma membrane activity but do not increase their ATP synthesis, producing a concomitant depletion of cell ATP which restricts growth (Holyoak *et al.*, 1996). Therefore, in this context, the effects on growth produced by the addition of fungicides to the culture

medium of *U. maydis* can be explained by a toxic-stress-induced increase in plasma membrane H⁺-ATPase activity that would deplete ATP stocks. The characteristics of this H⁺-ATPase activation will be published in a separate communication (manuscript in preparation).

In the opinion of the authors, this is the first report comparing lipid composition and biophysical characteristics of pure plasma membranes from fungicide-treated cells and their analogous mutants. The present data indicate that, in *U. maydis*, accumulation of abnormal sterols in the plasma membrane do not compromise the overall functionality of the membrane and that the fungicidal effect of two compounds with different points of action in the sterol biosynthetic pathway cannot be explained on the basis of disruption of plasma membrane properties. On the other hand, growth impairment may be more related to ATP depletion provoked by toxic-stress-activation of the plasma membrane H⁺-ATPase. Also, the frequently observed increase in CLB fatty acids upon addition of fungicides is not provoked by the presence of abnormal sterols in plasma membranes, but maybe associated with impairment of growth.

ACKNOWLEDGEMENTS

We thank J. A. Hargreaves and J. P. R. Keon for the gift of the strains used and helpful discussions, and L. E. Hernández for critical reading of the manuscript. A.H. was the recipient of a Beca de Formación de Investigadores from Basque Government (Spain).

REFERENCES

- Alexandre, H., Mathieu, B. & Charpentier, C. (1996). Alteration in membrane fluidity and lipid composition, and modulation of H⁺-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. *Microbiology* **142**, 469–475.
- Baldwin, B. C. (1983). Fungicidal inhibitors of ergosterol biosynthesis. *Biochem Soc Trans* **11**, 659–663.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Christie, W. W. (1986). Separation of lipid classes by high-performance liquid chromatography with the ‘mass detector’. *J Chromatogr* **361**, 396–399.
- Cooke, D. T., Munkonge, F. M., Burden, R. S. & James, C. S. (1991). Fluidity and lipid composition of oat and rye shoot plasma membrane: effect of sterol perturbation by xenobiotics. *Biochim Biophys Acta* **1061**, 156–162.
- Coupland, D., Cooke, D. T. & James, C. S. (1991). Effects of 4-chloro-2-methylphenoxypropionate (an auxin analogue) on plasma membrane ATPase activity in herbicide-resistant and herbicide-susceptible biotypes of *Stellaria media* L. *J Exp Bot* **42**, 1065–1071.
- Deamer, D. W. & Nichols, J. W. (1983). Proton-hydroxide permeability of liposomes. *Proc Natl Acad Sci USA* **80**, 165–168.
- Debieu, D., Coriococtet, M. F., Steva, H., Malosse, C. & Leroux, P. (1995). Sterol composition of the vine powdery mildew fungus, *Uncinula necator* – comparison of triadimenol-sensitive and resistant strains. *Phytochemistry* **39**, 293–300.
- Girling, I. J. (1991). *The mode of action of morpholine fungicides and their mechanisms of selectivity between fungal species*. PhD thesis, University of Bristol.
- Hargreaves, J. A. & Turner, G. (1989). Isolation of the acetyl-CoA synthase gene from the corn smut pathogen, *Ustilago maydis*. *J Gen Microbiol* **135**, 2675–2678.
- Hernández, A., Cooke, D. T. & Clarkson, D. T. (1994). Lipid composition and proton transport in *Penicillium cyclopium* and *Ustilago maydis* plasma membrane vesicles isolated by two-phase partitioning. *Biochim Biophys Acta* **1195**, 103–109.
- Holyoak, C. D., Stratford, M., McMullin, Z., Cole, M. B., Crimmins, K., Brown, A. J. P. & Coote, P. J. (1996). Activity of the plasma membrane H⁺-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. *Appl Environ Microbiol* **62**, 3158–3164.
- James, C. S., Burden, R. S., Loeffler, R. S. T. & Hargreaves, J. A. (1992). Isolation and characterization of polyene-resistant mutants from the maize smut pathogen, *Ustilago maydis*, defective in ergosterol biosynthesis. *J Gen Microbiol* **138**, 1437–1443.
- Kalam, A. & Banerjee, A. K. (1995). Action of the fungicide tridemorph on the glucose, lactate and succinate dehydrogenase activities of some tridemorph-sensitive and tridemorph-resistant bacteria. *Pestic Sci* **43**, 41–45.
- Kelly, D. E., Rose, M. E. & Kelly, S. L. (1994). Investigation of the role of sterol Δ^{8→7}-isomerase in the sensitivity of *Saccharomyces cerevisiae* to fenpropimorph. *FEMS Microbiol Lett* **122**, 223–226.
- Keon, J. P. R. & Hargreaves, J. A. (1996). An *Ustilago maydis* mutant partially blocked in P450_{14DM} activity is hypersensitive to azole fungicides. *Fungal Genet Biol* **20**, 84–88.
- Lai, M. H., Bard, M., Pierson, C. A., Alexander, J. F., Goebel, M., Carter, G. T. & Kirsch, D. R. (1994). The identification of a gene family in the *Saccharomyces cerevisiae* ergosterol biosynthesis pathway. *Gene* **140**, 41–49.
- Lees, N. D., Skaggs, B., Kirsch, D. R. & Bard, M. (1995). Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* – a review. *Lipids* **30**, 221–226.
- Loeffler, R. S. T. & Hayes, A. L. (1992). Effects of sterol biosynthesis inhibitor fungicides on growth and sterol composition of *Ustilago maydis*, *Botrytis cinerea* and *Pyrenophora teres*. *Pestic Sci* **36**, 7–17.
- Nes, W. R. & Dhanuka, I. C. (1988). Inhibition of sterol synthesis by Δ⁸-sterols in a sterol auxotroph of yeast defective in oxidosqualene cyclase and cytochrome P-450. *J Biol Chem* **263**, 11844–11850.
- Nes, W. R. & Heupel, R. C. (1986). Physiological requirements for biosynthesis of multiple 24β-methyl sterols in *Giberella fujikuroi*. *Arch Biochem Biophys* **244**, 211–217.
- Ragsdale, N. N. (1975). Specific effects of triarimol on sterol biosynthesis in *Ustilago maydis*. *Biochim Biophys Acta* **380**, 81–96.
- Rodriguez, R. J., Taylor, F. R. & Parks, L. W. (1985). Multiple functions for sterols in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **837**, 336–343.
- Sajbidor, J. & Grego, J. (1992). Fatty acid alterations in *Saccharomyces cerevisiae* exposed to ethanol stress. *FEMS Microbiol Lett* **93**, 13–16.
- Senior, I. J., Hollomon, D. W., Loeffler, R. S. T. & Baldwin, B. C. (1995). Sterol composition and resistance to DMI fungicides in *Erysiphe graminis*. *Pestic Sci* **45**, 57–67.
- Serrano, R. (1991). Transport across yeast vacuolar and plasma

membranes. In *The Molecular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics*, pp. 523–585. Edited by J. N. Strathern, E. W. Jones & J. R. Broach. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Vanden Bossche, H. (1993). Antiprotozoal activity of ergosterol biosynthesis inhibitors: focus on Trypanosomatidae and Plasmodiidae. *Microbiology-Europe* 1, 20–28.

Vanden Bossche, H., Willemsens, G., Cools, W., Marichal, P. & Lauwers, W. (1983). Hypothesis on the molecular basis of the antifungal activity of N-substituted imidazoles and triazoles. *Biochem Soc Trans* 11, 665–667.

Van der Rest, M. E., Kamminga, A. H., Nakano, A., Anraku, Y., Poolman, B. & Konings, W. N. (1995). The plasma membrane of *Saccharomyces cerevisiae*: structure, function and biogenesis. *Microbiol Rev* 59, 304–322.

Van de Vossenberg, J. L. C. M., Ubbink-Kok, T., Elferink, M. G. L., Driessen, A. J. M. & Konings, W. N. (1995). Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea. *Mol Microbiol* 18, 925–932.

Viegas, C. A. & Sá-Correia, I. (1991). Activation of plasma

membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid. *J Gen Microbiol* 137, 645–651.

Weete, J. D. (1974). *Fungal Lipid Biochemistry. Monographs in Lipid Research*, vol. 1. New York: Plenum.

Weete, J. D. (1987). Mechanisms of growth suppression by inhibitors of ergosterol biosynthesis. In *Ecology and Metabolism of Plant Lipids*, pp. 268–285. Edited by G. Fuller & W. D. Nes. ACS Symposium Series 325. Washington, DC: American Chemical Society.

Weete, J. D., Sancholle, M., Touze-Soulet, J. M., Bradley, J. & Dargent, R. (1985). Effects of triazoles on fungi. III. Composition of a plasma membrane enriched fraction of *Taphrina deformans*. *Biochim Biophys Acta* 812, 633–642.

Weete, J. D., Sancholle, M., Patterson, K. A., Miller, K. S., Huang, M. Q., Campbell, F. & Van den Reek, M. (1991). Fatty acid metabolism in *Taphrina deformans* treated with sterol biosynthesis inhibitors. *Lipids* 26, 669–674.

Received 26 March 1997; revised 11 June 1997; accepted 8 July 1997.