RESEARCH ARTICLE

Mentha piperita essential oil induces apoptosis in yeast associated with both cytosolic and mitochondrial ROS-mediated damage

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Abstract

Mentha piperita (MP), also known as peppermint, is an aromatic and medicinal plant widely used in the food industry, perfumery and cosmetic, pharmacy and traditional medicine. Its essential oil (EO) displays antimicrobial activity against a range of bacteria and fungi. In this study, we found that MP EO lethal cytotoxicity is associated with increased levels of intracellular reactive oxygen species, mitochondrial fragmentation and chromatin condensation, without loss of the plasma membrane integrity, indicative of an apoptotic process. Overexpression of cytosolic catalase and superoxide dismutases reverted the lethal effects of the EO and of its major component menthol. Conversely, deficiency in Sod1p (cytosolic copper-zinc-superoxide dismutase) greatly increased sensitivity to both agents, but deficiency in Sod2p (mitochondrial manganese superoxide dismutase) only induced sensitivity under respiratory growth conditions. *Mentha piperita* EO increased the frequency of respiratory deficient mutants indicative of damage to the mitochondrial genome, although increase in mitochondrial thiol oxidation does not seem to be involved in the EO toxicity.

Introduction

Essential oils (EO) are complex mixtures of volatile compounds produced by plants. They exhibit a diverse array of pharmacological properties such as antispasmodic (de Sousa *et al.*, 2010), antioxidant (Yang *et al.*, 2010), antimicrobial (Reichling *et al.*, 2009), antiplasmid (Schelz *et al.*, 2006) anti-inflammatory and anticancer activities, and, being natural compounds, represent a class of growing interest (McKay & Blumberg, 2006; Bakkali *et al.*, 2008). Induction of apoptosis has also been demonstrated for several EO and EO components in different mammalian cell lines associated with their anticancer activity (Paik *et al.*, 2005). This is the case of eugenol, a major component of EO of *Eugenia caryophyllata,* which induces apoptosis in human promyelocytic leukaemia HL-60 cell line through reactive oxygen species (ROS) production (Yoo *et al.*, 2005). Menthol, one of the major components of *Mentha piperita* (MP) EO, has also been shown to display anticancer activity by inducing cell death, either by necrosis (in HL-60 cells) or apoptosis (in Caco-2 cell line) (Lu *et al.*, 2006; Faridi *et al.*, 2011). Cytotoxicity associated with EO has been attributed to different effects such as production of reactive species, changes in membrane fluidity and permeability (Ultee *et al.*, 1999; Hammer *et al.*, 2004; Tian *et al.*, 2012), tubulin polymeri-

zation (Faridi *et al.*, 2011), imbalance of ion transport and inhibition of protein function, among others (Bakkali *et al.*, 2008). *Mentha piperita* is a widely distributed aromatic and medicinal plant from the Mediterranean flora, and its EO has been shown to inhibit growth of several food spoilage yeast species (Araújo *et al.*, 2003), bacteria (Sartoratto *et al.*, 2004; Weseler *et al.*, 2005) and pathogenic fungi including *Candida albicans* (Işcan *et al.*, 2002; Pauli, 2006). However, the mechanisms responsible for the observed cytotoxic properties have not been investigated. The yeast *Saccharomyces cerevisiae* has been shown to induce an apoptotic cell death programme in response to several stress agents or physiological cues, in most cases associated with the production of ROS (Pereira *et al.*, 2008). Nonetheless, reports on the induction of apoptosis by EO in yeast are very scarce (Bakkali *et al.*, 2006).

In the present work, we investigated the presence of characteristic apoptotic cell death markers upon incubation with cytotoxic concentrations of MP-EO and studied the involvement of yeast antioxidant enzymes in the resistance to this oil. We demonstrated that death induced by MP-EO is accompanied by accumulation of mitochondrial ROS and nuclear DNA condensation, without significant loss of plasma membrane integrity, consistent with an apoptotic cell death process. We also showed that the overexpression of the antioxidant enzymes catalase, cytosolic copper–zinc superoxide dismutase (Sod1p) and mitochondrial manganese superoxide dismutase (Sod2p) was beneficial to the cell, while deletion of *SOD1* resulted in higher sensitivity to the EO or to its major component menthol.

Materials and methods

Strains

The following *S. cerevisiae* strains were used in this study: FY1679-28c (MATa *his3-200*, *ura3-52*, *leu2-1*, *trp1-63*), FY1679-28c (HIS3::[pRS403] URA3::[pRS406]), SOD1 (FY1679-28c HIS3::[pRS403], URA3::[pRS406(ADH2-SOD1)]), SOD2 (FY1679-28c HIS3::[pRS403], URA3::[pRS406 (ADH2-SOD2)), CCS1 (FY1679-28cHIS3::[pRS403(ADH2-CCS1)],URA3::[pRS406]), CTT1 (FY1679-28c HIS3::[pRS403 (ADH2-CTT11)],URA3::[pRS406]), CCS1+CTT1 (FY1679-28c HIS3::[pRS403(ADH2-CCS1)],URA3::[pRS406(ADH2-SOD1)]), FY*sod1*Δ (FY1679-28c sod1ΔKANMX4), FY*sod2*Δ (FY1679-28c *sod2ΔKANMXR*), FY*sod1*Δρ− (ρ⁻FY1679-28c *sod1ΔKanMX4*) and FY*sod2*Δρ− (ρ⁻ FY1679-28c *sod2ΔKANMXR*), FY*grx5*Δ (FY1679-28c grx5::kanMX4) (Piper, 1999; Harris *et al.*, 2003), W303-1A (MATa, *ade2, his3, leu2, trp1, ura3, can1*), W303-1A ρ^0 (MATa, *ade2, his3, leu2, trp1, ura3, can1,* ρ^0), W303 pYX232- mtGFP (Westermann & Neupert, 2000), BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15Δ0 ura3Δ0*) and the respective knockouts in *YAC1*, *NUC1* and *AIF1* genes (EUROSCARF, Frankfurt, Germany).

Growth conditions and treatments

Yeast cells were maintained in YPDA [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar]. For spot assays, cells were grown in YPD media (YPDA media without agar) or in synthetic complete medium [SC; 0.67% (w/v) Bacto-yeast nitrogen base w/o amino acids, 2% (w/v) glucose and 0.2% (w/v) Dropout mix] lacking the appropriate selective marker. After growth, cells were harvested at OD_{640} between 0.5 and 0.7 and washed with sterile water, and the cellular concentration adjusted to $OD_{640\ nm} = 0.3$. Three dilutions were prepared, and 5 μL of each dilution and of the nondiluted suspension were placed on plates with either YPDA or YPGA [similar to YPDA but replacing glucose for 3% (w/v) glycerol] and different MP-EO or menthol concentrations. The plates were left on the bench to dry for one night. After they were sealed with parafilm to prevent the escape of the oil and the plates were incubated at 30 °C. After 2 days, they were evaluated and photographs were taken. For resistance tests in the presence of copper, 0.5 mM of $CuSO_4.5H_2O$ was added into the growth media of the plates. To test for the presence of apoptotic markers, the cells were grown in YPD medium, harvested at OD_{640nm} between 0.5 and 0.7 and then incubated in YPD medium with 800 ppm (720 μg mL^{-1}) of MP-EO. At different incubations times, CFU counts were estimated and samples were taken for staining as described below.

MP-EO was obtained by steam distillation of the aerial part of MP plants grown in North of Portugal (Braga) and analysed by GC and GC/MS, as described elsewhere (Araújo *et al.*, 2003). Main components of the oil were (%): L-menthol – 50.67; L-menthone – 12.54; isomenthone – 10.10; 1.8-cineole – 9.32; menthyl acetate – 5.24; β-pinene – 1.35; (E)-caryophyllene – 0.62; germacrene D – 0.85 and viridiflorol – 0.38.

Detection of apoptotic markers

Mitochondria dysfunction monitored by ROS production was assessed using the MitoTracker Red CM-H2Xros staining. Cells of the wild-type strain were incubated with 0.4 μg mL^{-1} MitoTracker for 15 min at 30 °C (Pereira *et al.*, 2007). Nuclear staining was performed with 4,6-diamido-2-phenyl-indole (DAPI, 2 μg mL^{-1}) according to Madeo *et al.* (1997). For propidium iodide (PI) staining, cells were incubated with 5 μg mL^{-1} of PI for 10 min at room temperature (Pereira *et al.*, 2007). To determine the frequency of apoptotic phenotypes, at least 300 cells of three independent experiments were evaluated. The samples were observed in a Leitz Laborlux S Microscope with fluorescence apparatus accessories. The digital images were

acquired with the camera Leica DCF350FX and the LAS AF LEICA MICROSYSTEMS software.

Assessment of mitochondrial fragmentation

Mitochondria were visualized by fluorescence microscopy using a matrix-targeted GFP (Westermann & Neupert, 2000) in W303-1A strain. For these assays, cells were grown in SC medium without tryptophan.

Reproducibility of the results

All the experiments were repeated at least three times. The data reported for the CFU counts, apoptotic markers and formation of respiratory mutants are mean values with standard deviations.

Results

Death induced by MP EO is accompanied by apoptotic markers

Exposure of *S. cerevisiae* FY1679-28c (wild type) to 800 ppm (720 μg mL^{-1}) of MP-EO resulted in cell death that increased over time, the percentage of survival estimated by CFU counts reaching almost zero at 500 min. treatment time (Fig. 1). To address the type of cell death induced by this EO, a kinetic analysis of cellular markers associated with apoptotic cell death was carried out, which included assessment of ROS accumulation, chromatin condensation and plasma membrane integrity. ROS accumulation evaluated by MitoTracker Red CM-H2Xros staining, showed a drastic increase in the percentage of stained cells from the earliest time point, before any loss of cell viability could be detected. Consistent with the occurrence of mitochondrial damage, we also found that incubation with MP-EO resulted in a rapid fragmentation of the mitochondrial network that was observed using a mitochondrial-tagged GFP fusion (Fig. 2a). Chromatin condensation, assessed by 4,6-diamido-2-phenyl-indole (DAPI) staining, increased with time to near 40% of the cells at 500 min of exposure. Plasma membrane integrity, monitored by propidium iodide (PI) staining, was maintained at reduced levels even when cell viability was almost loss, and attained a maximum of 35% of damaged cells, for the longer treatment time. Overall the results suggest that the MP-EO induces cell death by an apoptotic process.

To further characterize the cell death process, we next tested the effect of the MP-EO on the cell viability of strains deficient in proteins known to be involved in apoptotic cell death induced by other stimuli, namely the

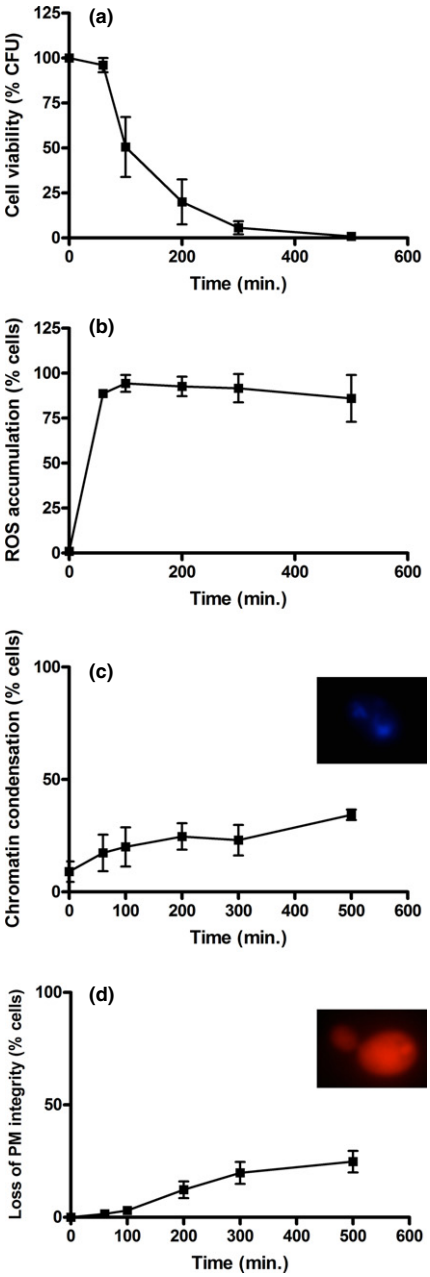

Fig. 1. Cell death induced by *Mentha piperita* essential oil is accompanied by the occurrence of apoptotic markers. Kinetic analysis of the cellular events associated with cell death of the FY1679-28c strain during 500 min. exposure to 800 ppm (720 μg mL^{-1}) *M. piperita* essential oil. (a) Cell viability (%) assessed by the number of colony forming units (CFU), (b) Percentage of cells exhibiting ROS accumulation monitored by MitoTracker Red CM-H2Xros staining. (c) Percentage of cells exhibiting chromatin condensation assessed by DAPI staining. The insert shows a photomicrography of a cell stained with DAPI, as an example of chromatin condensation. (d) Percentage of cells exhibiting plasma membrane integrity assessed by PI exclusion. The insert shows a photomicrography of a cell stained with PI, as an example of a PI positive cell. The data represent mean values with standard deviations of at least three independent experiments.

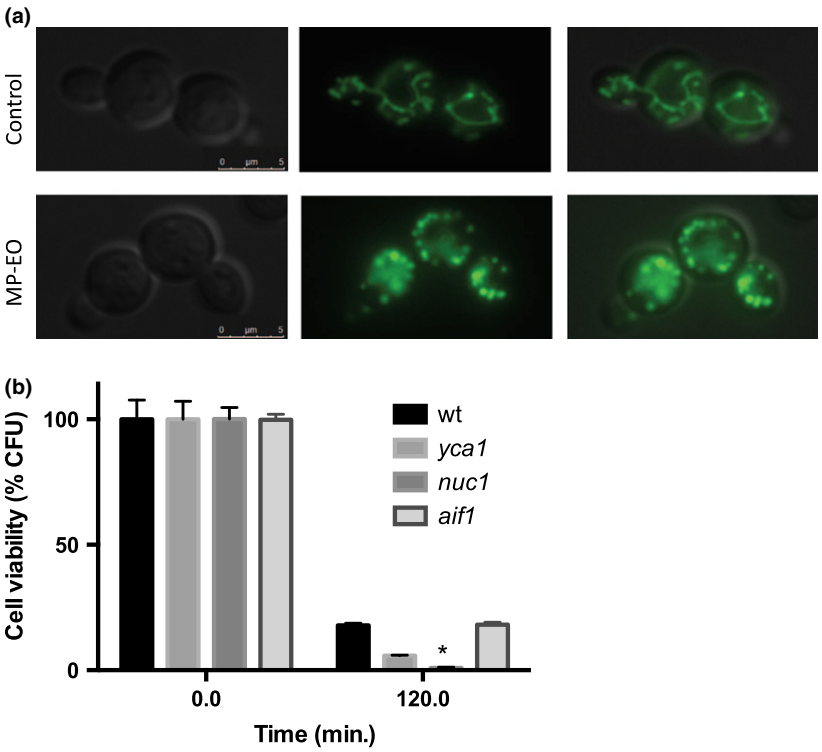

Fig. 2. Cell death induced by *Mentha piperita* essential oil is accompanied by mitochondrial fragmentation and does not depend on Yca1p, Nuc1p or Aif2p. (a) Photomicrographs obtained by differential interference contrast (DIC) and fluorescence microscopy of cells expressing mitochondrial matrix-targeted GFP (mtGFP) before (upper panels) and after exposure to 800 ppm (720 μg mL^{-1}) *M. piperita* essential oil for 30 min. (lower panels). Left panels – DIC images; middle panels – green fluorescence images; right panels – merged images. Representative images are shown. (b) Cell survival of *Saccharomyces cerevisiae* BY4741 and *yca1*Δ, *nuc1*Δ and *aif1*Δ after exposure to MP-EO (percentage of cfu on YEPDA plates; 100% corresponds to the number of cfu at time 0). Cells were incubated for 120 min with 800 ppm (720 μg mL^{-1}) *M. piperita* essential oil. Values are means ± SD of three independent experiments; $P > 0.05$ (*nuc1*Δ vs. BY4741 cells); two-way ANOVA.

yeast metacaspase Yca1p, the apoptosis inducing factor, Aif1p, and the yeast Endo G ortholog, Nuc1p (Madeo *et al.*, 2002; Wissing *et al.*, 2004; Büttner *et al.*, 2007). Absence of Yca1p and of Aif1p (*yca1*Δ and *aif1*Δ mutants) had no effect, whereas deficiency of Nuc1p (*nuc1*Δ mutant) exacerbated MP-EO-induced cell death (Fig. 2b). *NUC1* gene encodes a mitochondrial protein with DNase/RNase activity involved in apoptotic DNA degradation, but under respiration repression conditions its deletion has also been described to sensitize cells to death (Büttner *et al.*, 2007). Regarding the yeast metacaspase and Aif1p, although these are commonly recognized apoptotic regulators, many cell death stimuli have been described to induce apoptotic cell death independently of their activity, namely ethanol (Kitagaki *et al.*, 2007). Our results seem to indicate that none of these proteins are involved in the cell death process induced by MP-EO.

Mentha piperita EO induces 'petite' formation

Mitochondrial DNA is particularly susceptible to ROS (Piper, 1999) that can induce genome damage with ensuing loss of respiratory chain function and formation of 'petite' mutants (rho- mutants). As we observed accumulation of ROS at mitochondria from the earliest times of exposure to MP-EO (Fig. 1b), we aimed to elucidate whether mitochondrial genome damage occurs upon exposition to this EO. For such, colonies obtained in the YPD plates from viability assays performed with 400 ppm (360 μg mL^{-1}) MP-EO were tested for ability to grow on YPG for 48 h at 30 °C and the percentage of 'petites' was determined. We found that for cell cultures exposed to this concentration of EO, for which no significant decrease in CFU was observed, the percentage of respiratory deficient cells increases considerably over time (Table 1), evidencing the occurrence of mitochondrial damage at the genome level.

The antioxidant defence enzymes Sod1p, Sod2p and Ctt1p increase resistance to *M. piperita* EO

Cells counteract oxidative stress by producing antioxidant enzymes and free radical scavengers. Superoxide dismutase (SOD), catalase and peroxidases, which are able to neutralize ROS before they can induce serious damage to cellular components, are the first line of defence against oxidative damage in all aerobic cells. Antioxidant defences in *S. cerevisiae* are similar to those of higher organisms; this yeast posses both Cu.ZnSOD (Sod1p), present in the cytosol, and the MnSOD (Sod2p), present in the mitochondrial lumen (Moradas-Ferreira *et al.*, 1996). It also has two catalases, A and T (encoded by *CTA1* and *CTT1* genes, respectively), the cytosolic form (Cat T) being the most important for oxidative defence. These enzymes can

Table 1. Percentage of respiratory deficient mutants present in YPD cultures exposed to 400 ppm (360 μg mL^{-1}) *Mentha piperita* essential oil for different time periods

Time (min)	CFUs (%)	Respiratory deficient cells (%)
0	100.0	1.0 ± 0.9
30	91.8 ± 2.9	6.1 ± 0.6
60	96.0 ± 4.3	10.4 ± 0.6
90	91.6 ± 9.2	13.9 ± 4.1
120	100.0 ± 9.2	14.6 ± 4.3

be induced by oxidative stress leading to increase in resistance due to the increase of the repair activities and reducing the damage caused by ROS (Harris *et al.*, 2003). It has been shown that catalase is not essential in normal conditions, because a double mutant (acatalasaemic mutant) grew with a similar rate as the wild-type strain; however, it becomes important in acquiring tolerance to oxidative stress in the cell adaptative response (Izawa *et al.*, 1996).

To determine the relevance of the ROS accumulation induced by MP-EO (Fig. 1) in the mediation of the toxic effects of the EO, we used strains either deleted in *SOD1* and *SOD2,* or overexpressing *SOD1*, *SOD2* and *CTT1* and grew them on media with and without the EO. *Δsod1* strain displayed an increased sensitivity to the presence of the MP-EO when compared to the wild-type strain, while oppositely *Δsod2* strain showed higher resistance to the EO (Fig. 3a). In addition, the *Δsod1*ρ^- and *Δsod2*ρ^- strains displayed a similar phenotype as their respiratory-competent counterparts, and likewise, a wild-type *S. cerevisiae* strain, and its ρ^0 mutant displayed similar sensitivity to the MP-EO (Fig. 3a). These results indicate that although MP-EO induces ROS accumulation at the mitochondria, the primary cause of loss of viability is not ROS production by the mitochondrial electron transport chain, the ρ^- and ρ^0 strains lacking an assembled respiratory chain. A similar behaviour was found for *Δsod1, Δsod1*ρ^-, *Δsod2* and *Δsod2*ρ^- mutants when they were incubated with the major component of MP-EO, menthol, suggesting that the effects of the EO can be attributed mainly to this component (Fig. 3c), in agreement with the results from Işcan *et al.* (2002). The differences in the phenotypes of *Δsod1* and *Δsod2* strains could be due to the reported higher levels of Sod1p in glucose-grown cells when compared to Sod2p, the latter being subject to glucose repression (Piper, 1999), and as a consequence to a more determinant role of the former enzyme under the assayed conditions. Thus, we tested *Δsod1* and *Δsod2* strains on glycerol medium with MP-EO or menthol (Fig 3b and d). In fact, in this medium both *Δsod1* and *Δsod2* cells displayed decreased resistance indicating that MP-EO and menthol, apart from being able to induce oxidative stress in the absence of an active electron transport chain, will also enhance oxidative damage at the mitochondria, associated with the high activity of the electron transport chain under respiratory conditions. In YPD medium, *SOD2* deletion not only did not induce a higher sensitivity to MP-EO and menthol, but it actually seemed to have a protective effect. These results may be explained by the fact that Δ*sod2* strain displays an increased expression of *SOD1* (Peter Piper, unpublished results) and emphasis once more the role of Sod1p.

To further evaluate the role of ROS accumulation in the mediation of the toxic effects of the MP-EO and the relevance of these effects at cytosol level, we then tested strains that overexpress *SOD1*, *SOD2* and *CTT1* when grown on glycerol. When these strains were grown in the presence of 800 ppm (720 μg mL^{-1}) MP-EO, an increased resistance was observed for all the strains overexpressing the enzymes when compared with the wild-type strain (Fig. 4a). Regarding *SOD1,* this gene was also overexpressed simultaneously with its dedicated chaperone Ccs1p, which loads the Cu^{2+} ions to Sod1p apoprotein to avoid potential toxicity associated with the over expression of *SOD1* alone due to the accumulation of an inactive protein form (Harris *et al.*, 2003). However, under our experimental conditions this co-expression proved to be beneficial only when copper was added to the culture medium (Fig 4b). In fact, addition of copper ions to the medium could increase resistance of both *SOD1* overexpressing strains. Likewise, increasing the copper concentration in the medium could increase resistance even for the wild type, which could be due to direct effect of iron in the dismutation of superoxide or could suggest that the protein levels of Sod1p may not be the limiting factor. A similar conclusion could also be obtained from the assay where we only overexpressed *CCS1,* which could efficiently increase resistance to MP-EO (Fig. 4a and b). The overexpressing mutants were also tested with menthol, and a sensitivity and resistance pattern identical to that obtained for MP-EO was observed (Fig. 4c). Once more the results indicated that the effects of MP-EO, at least to a great part, could be attributed to its major compound.

Oxidative damage to mitochondria may be caused at least partially by a depletion of glutathione in the mitochondrial matrix. Grx3p, Grx4p and Grx5p are mitochondrial monothiol glutaredoxins (CGFS active site) and may also have specialized functions related to iron homoeostasis and iron–sulphur cluster synthesis in mitochondria (Rodríguez-Manzaneque *et al.*, 1999, 2002; Herrero & de la Torre-Ruiz, 2007). Among the three, deficiency in Grx5p originates the most sensitive phenotype to oxidative damage (Rodríguez-Manzaneque *et al.*, 1999). To further assess the relevance of oxidative damage at mitochondrial level, we tested *Δgrx5* mutant. Our results show

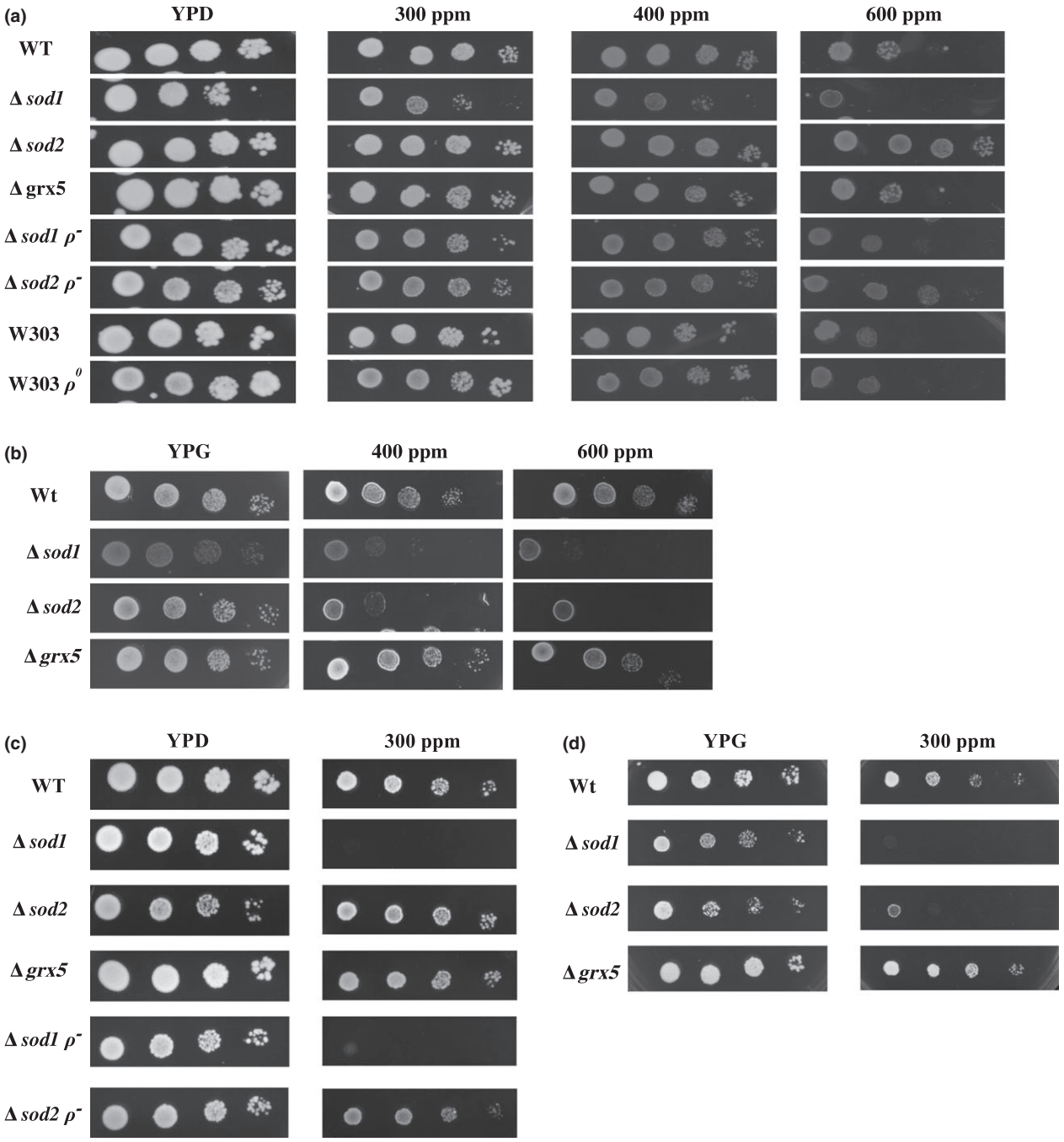

Fig. 3. Deficiency in Sod1p but not Sod2p or Grx5p, increases sensitivity to the essential oil of *Mentha piperita* and to its major constituent menthol. (a) Effect of *M. piperita* essential oil on growth of FY1679-28c (WT), *Δsod1, Δsod1*ρ$^-$, *Δsod2, Δsod2*ρ$^-$ and *Δgrx5* isogenic strains and of W303-1A and W303-1A ρ^0 strains in glucose medium. First column: control plates on YPD media; second, third and fourth columns: YPD plates with 300, 400 and 600 ppm (270, 360 and 540 μg mL^{-1}) *M. piperita* essential oil, respectively. (b) Effect of *M. piperita* essential oil on growth of FY1679-28c (WT), *Δsod1*, *Δsod2* and *Δgrx5* isogenic strains in glycerol medium. First column: control plates on YPG media; second and third columns: YPG plates with 400 and 600 ppm (360 and 540 μg mL^{-1}) *M. piperita* essential oil, respectively. (c) Effect of menthol on growth of FY1679-28c (wild type), *Δsod1, Δsod1*ρ$^-$, *Δsod2, Δsod2*ρ$^-$ and *Δgrx5* strains in glucose medium. First column: control plates on YPD media; second column: YPD plates with 300 ppm (270 μg mL^{-1}) menthol. (d) Effect of menthol on growth of FY1679-28c (wild type), *Δsod1, Δsod2* and *Δgrx5* strains in glycerol medium. First column: control plates on YPG media; second column: YPG plates with 300 ppm (270 μg mL-1) menthol. Representative results of at least three independent experiments are shown.

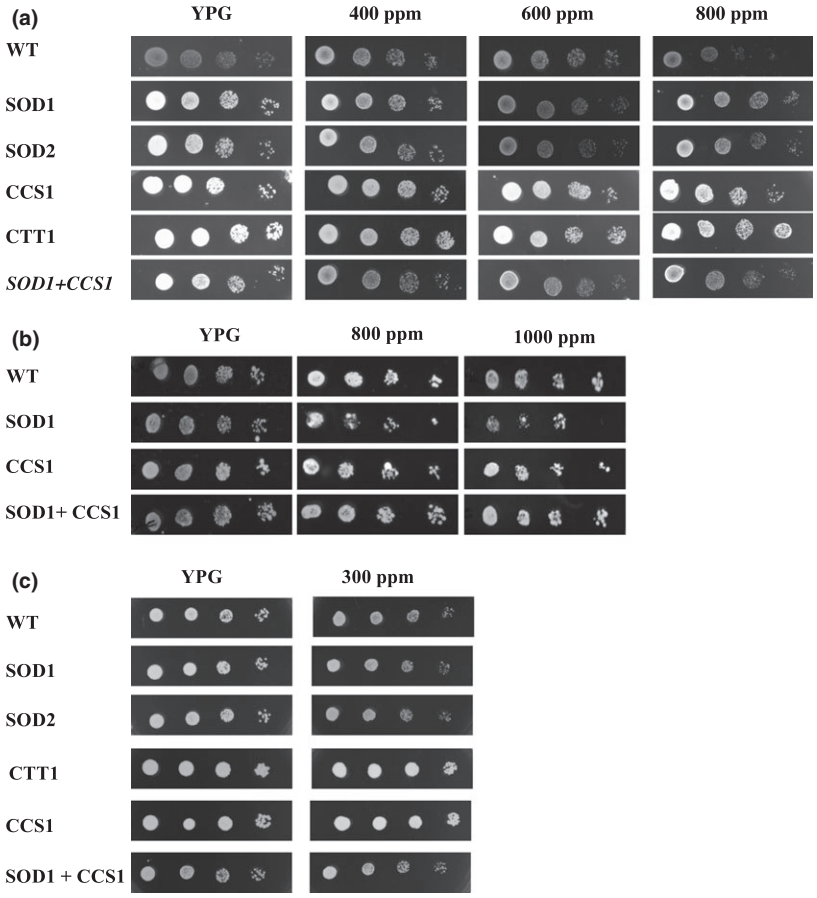

Fig. 4. Increased activity of Ctt1p and Sod1p, but not of Sod2p, leads to higher resistance to *Mentha piperita* essential oil and to menthol. (a) Effect of *M. piperita* essential oil on growth of FY1679-28c (HIS3::[pRS403] URA3::[pRS406]) (WT), SOD2, CTT1, SOD1, CCS1 and SOD1+CCS1 strains. First column, control plates on YPG media; second, third and fourth columns, YPG plates with 400, 600 and 800 ppm (360, 540 and 720 μg mL^{-1}) *M. piperita* essential oil, respectively. (b) Effect of *M. piperita* essential oil on growth of WT, SOD1, CCS1 and SOD1+CCS1 strains on YPG medium supplemented with Cu^{2+}. First column, control plates on YPG media; second column, YPG plates with 800 ppm (720 μg mL^{-1}) *M. piperita* essential oil, third column, YPG plates with 1000 ppm(900 μg mL^{-1}) *M. piperita* essential oil. (c) Effect of menthol on growth of WT, SOD2, CTT1, SOD1, CCS1 and SOD1+CCS1 strains. First column, control plates on YPG media; second column, YPG plates with 300 ppm (270 μg mL^{-1})menthol.

that deletion of *GRX5* does not induce sensitivity to MP-EO nor to menthol alone, suggesting that significant protein oxidative damage at mitochondria level is not occurring neither in cultures growing under fermentative conditions nor in actively respiring cells (Fig. 3).

Discussion

Commercially available EO extracted from aromatic and medicinal plants are widely used in the food industry (as aroma extracts and preservatives), in perfumery and cosmetic (as fragrances) and in pharmacy and natural products in traditional medicine (Pauli, 2006). Many of these applications rely on empiric knowledge. However, it is important to develop a better understanding of their mode of biological action and underlying mechanisms. In the present study, we have shown that MP-EO induces cell death in *S. cerevisiae* accompanied by ROS accumulation, mitochondrial fragmentation and chromatin condensation without extensive loss of plasma membrane integrity, suggesting the involvement of an apoptotic pathway. It has previously been shown that the *Mentha spicata* EO (Güney *et al.*, 2006) induces apoptosis in mammals' cells, so our results extends the capacity to induce apoptosis to another species of the *Mentha* genus and to yeasts.

ROS accumulation seems to play an important role in mediating the toxic effects of the MP-EO and of its principal component menthol, with damage induced both at the cytosolic and mitochondrial levels. In fact, overexpression of *SOD1*, *SOD2* and *CTT1* could increase resistance to both the EO and menthol, indicating that survival is limited by oxidative stress. According to these results, the addition of copper, a cofactor of Sod1p, to the culture medium resulted in a decrease of the lethal effect of this EO. The higher sensitivity of *Δsod1* opposite to *Δsod2* mutants, conserved also in their respective *Δsod1*ρ^- and *Δsod2*ρ^- strains, further suggest that both agents can act as redox cycling compounds and their effects do not depend on an active respiratory transport chain, although they are enhanced under respiratory conditions.

Compounds like MP-EO and methol can partition into the mitochondrial inner membrane where – by interfering with electron transfer to and from ubiquinone – they enhance free electron 'leakage' from the respiratory chain,

electrons that combine with molecular oxygen to produce superoxide ($O_2^{\cdot-}$). A number of other moderately lipophilic compounds, notably ethanol, sorbate and benzoate, are thought to cause oxidative stress this way and also increases the frequency of petites (Ibeas & Jiminez, 1997; Piper, 1999). Further sustaining similarities between the ethanol and MP-EO effects, we observed that MP-EO-induced cell death shares many common features with death induced by ethanol, namely the induction ROS production, mitochondrial fragmentation and chromatin condensation in a process independent of Yca1p, Aif1p and Nuc1p (Kitagaki *et al.*, 2007).

The results also suggest that significant protein oxidative damage at mitochondria level does not occur, as Grx5p glutaredoxin did not display a protective role. On the contrary, the MP-EO seems to induce damage to the mitochondrial genome as it increased the frequency of respiratory deficient strains.

In summary, the results indicate that MP-EO induces apoptotic cell death in *S. cerevisiae*, having the ability to exert pro-oxidant effects both at the cytosol and at the mitochondria, with loss of cell viability relating with both effects. As this EO and menthol are widely used [worldwide use of menthol is estimated to be 30–32 000 metric tonnes per annum (Kamatou *et al.*, 2013)] our results support that care should be taken to avoid detrimental effects, which might arise from its abusive use.

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