Induction of morphological changes in *Ustilago maydis* cells by octyl gallate

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The effects of octyl gallate on *Ustilago maydis* yeast cells were analysed in relation to its capacity to oxidize compounds (pro-oxidant actions). All phenolic compounds tested inhibited the alternative oxidase (AOX). However, only octyl gallate induced a morphological change in yeast cells and collapsed the mitochondrial membrane potential. In contrast to octyl gallate, propyl gallate and nordihydroguaiaretic acid caused only a negligible cell change and the membrane potential was not affected. Our findings show that structurally related phenolic compounds do not necessarily exert similar actions on target cells. Preincubation of *U. maydis* cells with trolox inhibited the change to pseudohyphal growth produced by octyl gallate. These results suggest that in addition to the inhibitory action of octyl gallate on the AOX, this compound induces a switch from yeast to a mycelium, probably through the formation of lipid peroxides.

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INTRODUCTION

Gallic acid, a natural plant triphenol that constitutes tannin, and some esters of gallate, especially propyl, octyl and lauryl gallate, are widely used as scavenging reactive oxygen species (ROS). The antioxidant effect of polyphenolic compounds is closely related to their hydrogen donor activity (Serrano *et al.*, 1998), and this is the case for gallate esters, which inhibit lipid peroxidation by donating hydrogen to peroxy and alkoxy radical intermediates. However, gallic acid compounds show various cytotoxic and antiproliferative effects on tissues and cells. Alkyl gallates, including octyl gallate, induce apoptosis with DNA fragmentation in hepatocytes (Inoue *et al.*, 1994; Nakagawa *et al.*, 1997) and some tumour cells (Serrano *et al.*, 1998); they also show trypanocidal effects (Koide *et al.*, 1998), and free radicals of propyl gallate inhibit the activity of some redox enzymes (Brzhevskaia *et al.*, 1966). These cytotoxic effects of gallate compounds are assumed to be due to the pro-oxidant action, not to their antioxidant capacity. Alkyl gallates also have antifungal activities against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Aspergillus niger* and *Candida albicans* (Kubo, 1999; Fujita & Kubo, 2002b; Hirasawa & Takada, 2004). In addition, propyl gallate inhibits the growth of microorganisms by inhibiting respiration and nucleic acid synthesis (Boyd & Beveridge, 1979). Similarly, nordihydro-

droguaiaretic acid (NDGA) strongly inhibits succinate cytochrome *c* reductase by depleting the thiol groups (Shi & Pardini, 1995). Therefore, these natural compounds could serve as useful alternatives or additives to conventional antifungals (Kim *et al.*, 2006).

Corn smut disease is caused by the phytopathogenic basidiomycete *Ustilago maydis*. The disease results in stunted maize plant growth and reduced yield, leading to severe economic losses (Martínez-Espinoza *et al.*, 2002). *U. maydis* is a dimorphic micro-organism, capable of producing different morphological forms (yeast or mycelium) in response to environmental stimulus. The change in morphology in *U. maydis* results from the activation of two conserved signalling pathways: a mitogen-activated protein kinase (MAPK) signalling cascade and a cyclic AMP-dependent pathway (D'Souza & Heitman, 2001). Both pathways are thought to transduce environmental signals, such as nutrient availability, presence of lipids, air exposure, acidic pH, and pheromones from cells of opposite mating type, during the transition from budding to filamentous growth (Bölker *et al.*, 1995; Klose *et al.*, 2004; Martínez-Espinoza *et al.*, 2004).

It has been shown that *U. maydis* is able to express a rotenone-insensitive NADH dehydrogenase and an alternative oxidase (AOX), which may play an important role in the yield of ATP synthesis (Juárez *et al.*, 2004) due to their non-proton motive character. It is also likely that the composition of the fungal electron transport chain depends on the metabolic state of the cell, and on developmental

Abbreviations: AOX, alternative oxidase; DCFDA, dichlorofluorescein diacetate; DHR123, dihydrorhodamine 123; NDGA, nordihydroguaiaretic acid; ROS, reactive oxygen species.

and environmental conditions (Juárez *et al.*, 2006). Thus the alternative respiratory enzymes might confer resistance against certain fungicides.

In this work we explore the underlying mechanism of the antifungal effects of octyl gallate in the phytopathogen *U. maydis*.

METHODS

Materials. Dihydrorhodamine 123 (DHR123) and DCFDA (dichlorofluorescein diacetate) were from Molecular Probes. Strain FB₂ was obtained from the American Type Cell Collection (Manassas, VA, USA). All other reagents were from Sigma.

Culture conditions. Growth of wild-type *U. maydis* strain FB₂ was as described previously (Juárez *et al.*, 2004). Briefly, cells were grown in YPD medium (1 % yeast extract, 0.25 % bactopectone, 1 % glucose, pH 4.7) at 28 °C with shaking at 220 r.p.m. After 24 h of culture, cells were harvested by centrifugation and washed twice with distilled water.

Morphological evaluation. Morphology was monitored by light microscopy. The cells were grown in YPD for 24 h in the presence of 30 µM each polyphenolic compound. Cells were photographed using a Nikon model TE300 digital camera.

Cell-wall staining. For cell-wall staining, 1 mg fluorescent brightener 28 ml⁻¹ (Sigma) was added to 1 ml cell culture (approx. 1×10^7 cells). The cell suspension was incubated for 5 min at 30 °C with agitation and washed twice in sterile distilled water. Cells were viewed under a fluorescence microscope with UV illumination by using different filter combinations (emission and excitation filters). Images were captured with a Nikon model TE300 digital camera.

Preparation of mitochondria. To prepare intact mitochondria, cells were suspended in isolation buffer (20 mM Tris/HCl, pH 7.0, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1 %, w/v, BSA), at a final ratio of 5 ml (g wet wt)⁻¹. The cells were disrupted with glass beads (450 µm) using 3 cycles of 25 s stroke at 35 s intervals in a bead beater at 0–4 °C. From this point, all steps were carried out at 4 °C. The broken cells were centrifuged for 10 min at 3000 g to remove the unbroken cells and cell debris. The supernatant was centrifuged at 12 000 g for 10 min, and the pellets containing mitochondria were washed once in isolation buffer and resuspended to a final protein concentration of 5 mg ml⁻¹. Mitochondria were maintained on ice and used within 3 h. Protein concentration in mitochondrial preparations was estimated by the Biuret method using BSA as standard. The integrity of the isolated mitochondria was determined by measuring the membrane potential and the respiratory control ratio.

Measurement of mitochondrial membrane potential. The mitochondrial membrane potential was monitored by measuring the changes in absorbance of safranin O (Akerman & Wikström, 1976) at the wavelength pair 533/511 nm. Since the system was not calibrated, the results show the relative changes in $\Delta\psi$, but not their absolute values. The incubation medium contained 125 mM sucrose, 65 mM KCl, 10 mM HEPES (pH 7.0), 2.5 mM KH₂PO₄, 5 mM MgSO₄, 0.5 mM EGTA, 8 µM safranin O and 1.0 mg mitochondrial protein ml⁻¹. The temperature was 30 °C, and the reaction was started by addition of 7 mM succinate or 0.5 mM NADH to 2.0 ml of the medium.

Mitochondrial respiration. Oxygen consumption was measured polarographically using a Clark-type electrode fitted to a 1.5 ml

water-jacket closed chamber. Isolated mitochondria (0.5 mg ml⁻¹) were suspended in a medium consisting of 125 mM sucrose, 65 mM KCl, 10 mM HEPES (pH 7.0), 2.5 mM KH₂PO₄, 5 mM MgSO₄, 0.5 mM EGTA, 0.1 % (w/v) BSA and 1 µM rotenone. All experiments were performed at 30 °C in air-saturated buffer.

Detection of ROS. The fluorescence of DHR123 and DCFDA were used to detect mitochondrial and cytosolic ROS levels, respectively. Briefly, 2×10^6 cells were incubated at 28 °C for 30 min in the dark in the presence of 2.5 µM DCFDA or for 10 min in the presence of 10 µM DHR123 (dissolved in ethanol). The cells were rinsed twice with 50 mM MOPS (pH 7.0) and the fluorescence intensity was measured.

Glutathione assay. Total glutathione content, the sum of reduced and oxidized glutathione, was estimated by converting oxidized glutathione (GSSG) into glutathione with glutathione reductase, and then glutathione was quantified spectrophotometrically by monitoring the reduction of DTNB (Akerboom & Sies, 1981).

Measurement of lipid peroxidation. Lipid peroxidation was determined with the Calbiochem LPO assay kit. The coloured complex formed reached its maximum absorbance at 500 nm.

Protein determination. The protein content was determined by using the Biuret method using BSA as standard.

Data analysis. Experiments reported in this study were performed a minimum of three times. Results are expressed as means $\pm$ SEM or by a representative experiment. Data were considered significant at $P < 0.05$.

RESULTS

Important advances have been made in the understanding of energy metabolism and the function of AOX under physiological conditions in *U. maydis* yeast cells (Juárez *et al.*, 2006). Despite this, *U. maydis* is a dimorphic micro-organism, capable of producing different morphological forms in response to several environmental stimuli, and the ability to switch from a yeast-like form to a mycelium form correlates with pathogenesis (Martínez-Espinoza *et al.*, 1997). Stress conditions such as low pH (pH 3) along with nitrogen starvation induce the dimorphic transition (Ruiz-Herrera *et al.*, 1995).

Alkyl gallates are phenolic compounds that inhibit the AOX in systems ranging from isolated mitochondria (Shimoji & Yamasaki, 2005) to whole organisms (Juárez *et al.*, 2004). The AOX plays a role in protecting fungal cells against oxidative stress, and recently it has been implicated in promoting cryptococcal virulence (Akhter *et al.*, 2003). This aspect is important because many plants synthesize antioxidant polyphenolic compounds and inhibitors of fungal growth in response to fungal infections. In addition, mitochondria, major players in cell redox homeostasis and signalling, may also have a role in the morphological change.

To understand the importance of the AOX in *U. maydis*, yeast cells were cultivated in the presence of octyl gallate. Growing cells under this condition generally results in inhibition of AOX and an increase in the activity of the

cytochrome *c* oxidase pathway (Tanton *et al.*, 2003), but in *U. maydis* the respiratory activity through the cytochrome *c* oxidase decreased (data not shown). From a theoretical point of view, if the only target of octyl gallate is the AOX, it should not affect the growth of yeast cells, because this enzyme does not participate in the synthesis of ATP (Juárez *et al.*, 2006). In addition, octyl gallate elicited a concentration-dependent increase in the rate of state 4 oxygen consumption (approx. 30 %), probably due to an uncoupling effect (data not shown). Therefore, we determined how different polyphenolic compounds (propyl gallate, octyl gallate and NDGA; Fig. 1) affected cellular respiration and growth.

Octyl gallate inhibits growth and stimulates the morphological change to pseudohyphal growth on rich medium

The inhibition of growth by polyphenolic compounds in *U. maydis* yeast cells is shown in Fig. 2. When exponentially growing cells in YPD medium were exposed to different concentrations of propyl gallate, octyl gallate and NDGA, the growth, measured in terms of biomass yield, was slightly reduced with propyl gallate and NDGA. However, when yeast cells were cultivated in the presence of octyl gallate, there was a dramatic decrease in biomass. Surprisingly, exposure of yeast cells to 40 µM octyl gallate induced a morphological change to pseudomycelium, and at higher concentrations of octyl gallate (60 µM), cells started to produce the melanin-like pigment (data not shown). In all cases, the pH of the medium increased, after 24 h growth, from 4.7 to 5.5 (octyl gallate) or 6.0 (propyl gallate and NDGA).

Mitochondrial dysfunction may mediate the switch to pseudohyphal growth by phenolic compounds

It is widely assumed that alkyl gallates are specific inhibitors of the AOX (Siedow & Grivin, 1980). Similar to the alkyl gallates, NDGA also has antifungal properties (Jensen *et al.*, 1992). So, it is likely that NDGA will display an inhibitory action on *U. maydis* AOX due to its structural similarity to alkyl gallates. In the presence of 3 µM

Fig. 1. Structures of the polyphenolic compounds used in this study.

Fig. 2. Biomass yield of *U. maydis* yeast cells grown in the presence of phenolic compounds: propyl gallate (nPg), octyl gallate (nOg) and NDGA.

antimycin A, both propyl and octyl gallates completely inhibited mitochondrial respiration at 5 µM, whereas full inhibition of respiration was attained at 10 µM NDGA (Fig. 3). The effects of phenolic compounds on glutamate+malate-supported respiration were similar to those observed for succinate oxidation (data not shown). These results are in agreement with some studies showing that polyphenolic compounds and gallate esters have similar characteristics, exhibiting the same inhibitory activity against the AOX and comparable antioxidant properties (Shimoji & Yamasaki, 2005).

Unfortunately, very little information exists on the mechanism by which phenolic compounds inhibit the

Fig. 3. Oxygen uptake by isolated *U. maydis* mitochondria. The additions to yeast mitochondria were 7 mM succinate (Suc), 3 µM antimycin A (Ant), 1 mM AMP, 5 µM propyl gallate (nPg), 5 µM octyl gallate (nOg) and 15 µM NDGA. nAO, nano-atom-grams of oxygen.

AOX, although sequestering of iron ions from the enzyme active site or competition between the inhibitor and ubiquinone for the active site have been proposed. It is worth mentioning that other mechanisms and protein targets should be taken into consideration, since it has been shown that propyl gallate inhibits oxygen consumption in isolated rat hepatocytes (Nakagawa & Tayama, 1995).

Since the mitochondrial membrane potential plays an important role in mitochondrial functions, we examined the effect of phenolic compounds on this potential. In the absence of succinate there was no membrane potential. At 5 μ M, propyl gallate and NDGA had no effects on the membrane potential generated by succinate (data not shown), but octyl gallate collapsed the potential (Fig. 4). Further addition of CCCP induced a small dissipation, whereas antimycin A produced a full collapse of the membrane potential due to inhibition of the respiratory chain (Fig. 4). From a bioenergetic point of view, it is important to realize that electron flow through the AOX is not coupled to proton pumping and hence the effect of octyl gallate is probably associated with an increase in membrane permeability or inhibition of the cytochrome pathway. We suggest that at low concentrations, the effect of octyl gallate is due to its uncoupling properties. These multiple actions are similar to certain dinitrophenols which exhibit both inhibition of respiration and uncoupling activity (Miyoshi *et al.*, 1990).

Generation of oxidative stress is believed to be involved in the antifungal effects of octyl gallate (Fujita & Kubo, 2002b). When cells in YPD medium were exposed to different concentrations of octyl gallate, the growth yield

was significantly reduced (Fig. 2). In addition, concentrations of octyl gallate around 40 μ M also induced a morphological change (Fig. 5b). Next, we looked at *U. maydis* by fluorescence microscopy using calcofluor, a dye that has been used in this organism to visualize cell-wall regions (Klose *et al.*, 2004). A common phenotype was observed when cells were grown in YPD as budding yeasts (Fig. 6a). In contrast, cells grown in YPD medium supplemented with 30 μ M octyl gallate underwent the switch to pseudomycelium, with visible septa and branched hyphae (Fig. 6b).

To determine whether octyl gallate confers oxidative stress on *U. maydis* cells, we tested its effect on cellular ROS production in yeast cells. It has been reported in several systems that propyl gallate, octyl gallate and NDGA increase the production of ROS (Yoshino *et al.*, 2002; Shi

Fig. 4. Effect of octyl gallate on mitochondrial membrane potential. Membrane potential was measured with safranin O. At the times indicated by an arrow, 7 mM succinate (Suc), 5 μ M octyl gallate (nOg), 1 μ M CCCP and 3 μ M antimycin A (Ant) were added.

Fig. 5. Morphological response of *U. maydis* to octyl gallate and trolox. (a) Control cells (no treatment); (b) cells treated with octyl gallate (40 μ M); (c) cells treated with octyl gallate (40 μ M) and trolox (200 μ M). The images were taken 20 h after the addition of the compounds. Scale bar, 10 μ m.

Fig. 6. *U. maydis* yeast cells stained with calcofluor. Cells were observed for morphological changes after 24 h of growth in liquid medium (YPD). Fluorescent brightener 28 (1 mg ml^{-1}) was mixed with 1 ml cell culture (approx. 1×10^7 cells), incubated for 5 min at 30°C with agitation, and washed twice in sterile distilled water. Cells were viewed under a fluorescence microscope with UV illumination and the photographs were taken with a Nikon model TE300 digital camera. (a) Control cells; (b) cells treated with $30 \text{ }\mu\text{M}$ octyl gallate.

& Pardini, 1995). However, under our experimental conditions there was no evidence of such an increase when yeast cells were loaded with either DHR123 or DCFHDA (data not shown).

Oxidative stress as a factor in the morphological change in *U. maydis*

In an attempt to understand the mechanisms underlying such diverse actions, we examined the chemistry of the interactions between octyl gallate and cellular components. Previously, it was reported that octyl and nonyl gallates induced membrane injury and ROS generation in *S. cerevisiae* (Fujita & Kubo, 2002a). In this case, α -tocopherol did not eliminate the production of ROS or the lethality of these compounds. Since this organism does not have an AOX, these effects should occur by different mechanisms.

Several reports have suggested that glutathione is involved in the yeast to hyphae transition in *C. albicans* (Thomas *et al.*, 1991) and *Aureobasidium pullulans* (Jürgensen *et al.*, 2001). In *S. cerevisiae*, glutathione and its oxidized disulfide form, GSSG, participate in essential physiological functions, such as synthesis of DNA and proteins, transport and detoxification of xenobiotics and endogenous toxic metabolites, as well as in cell defence against ROS (Penninckx & Elskens, 1993). Thomas *et al.* (1991) supported the idea that alterations in glutathione metabolism play a key role in the differentiation process.

There were no significant changes in total glutathione following treatment of *U. maydis* cells with octyl gallate (Fig. 7), but the addition of $10 \text{ }\mu\text{M}$ propyl gallate or NDGA to yeast cells caused a slight depletion of cellular glutathione. In addition, when the glutathione content of the cells was reduced to 60% of the control values by treatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, there was no induction of the morphological change. Thus, dimorphic changes are not due to a decrease in glutathione content.

Evidence of oxidative processes was obtained using the lipid peroxides assay, which revealed a 10-fold increase in the peroxide content after treatment with $40 \text{ }\mu\text{M}$ octyl gallate (Fig. 8). Pretreatment of cells with lipophilic antioxidant (trolox), a scavenger of lipid peroxides, protected cells from the octyl gallate-mediated morphological change. In addition, at $30 \text{ }\mu\text{M}$ octyl gallate, the presence of $200 \text{ }\mu\text{M}$ trolox inhibited the switch to pseudomycelium, but growth was still slowed down (Fig. 5c). These results indicate that octyl gallate has various biological activities. In this case, it was assumed that the cytotoxicity of octyl gallate was due to oxidative membrane damage, probably because of its pro-oxidant action. This is in agreement with previous observations

Fig. 7. Effect of polyphenolic compounds propyl gallate (nPg), octyl gallate (nOg) and NDGA on total glutathione levels. *U. maydis* yeast cells were pretreated with $10 \text{ }\mu\text{M}$ each compound or $200 \text{ }\mu\text{M}$ buthionine sulfoximine (BSO, positive control) for 3 h. Controls were prepared from cells treated only with ethanol (vehicle) for the same length of time.

Fig. 8. Lipid peroxidation. The production of lipid peroxides was quantified using the Calbiochem LPO Assay kit. Cells were incubated in the presence of 30 μ M octyl gallate (nOg) with or without 200 μ M trolox.

reporting the fungicidal activity of alkyl gallates on *S. cerevisiae* (Fujita & Kubo, 2002a).

DISCUSSION

Phenolic compounds have multiple biological activities, including antibacterial, antiviral and antifungal actions, which may result, at least in part, from their pro-oxidation capacity and their ability to interact with lipid bilayers. In this sense, phenolics can act as antioxidants under oxidative stress conditions, but if they are applied to cells under normal growth conditions, the active phenolic compounds can interfere with the cellular redox homeostasis, resulting in oxidative stress and growth inhibition. This is the case for *S. cerevisiae*, in which the antifungal activities of phenolics (e.g. catechin and epigallocatechin gallate) are associated with the production of cellular oxidative stress (Maeta *et al.*, 2007). Furthermore, it is possible that many of the proposed biological effects of phenolic compounds depend on the cellular environment. For example, octyl gallate has been suggested to be both an antioxidant (Nakayama *et al.*, 1993) and a pro-oxidant (Roy *et al.*, 2000), depending on its concentration and cellular conditions. So, it was interesting that among the phenolic compounds tested only octyl gallate induced a morphological change in *U. maydis*. Since the mechanism behind this effect is not known, we focused on the ability of octyl gallate to generate oxidative stress.

While all compounds inhibited AOX activity in cells (data not shown) and isolated mitochondria, only octyl gallate disrupted the membrane potential in isolated mitochondria, probably because of its ability to act as surfactant. Inhibition of cell growth might be the result, in part, of

mitochondrial dysfunction in these cells. Interestingly, there was no evidence of an increase in the production of ROS when *U. maydis* yeast cells were exposed to octyl gallate, as measured by the fluorescence of DHR123 or DCFHDA; nor did octyl gallate affect the total glutathione level, whereas propyl gallate and NDGA decreased it.

To get a better understanding of the mechanism underlying this process, we looked for the effects of octyl gallate on cellular signalling and/or toxicity due to its interaction with cellular membranes and the formation of lipid peroxides. We assumed that when yeast cells are exposed to high concentrations of octyl gallate, this compound will partition into the various cell membranes, producing an increase in lipid peroxidation. In this regard, we investigated the effect of trolox on the morphological change induced by octyl gallate. The majority of yeast cells exposed to octyl gallate exhibited the switch from budding to pseudohyphal growth, whereas in yeast cells exposed to octyl gallate plus trolox this change was partially inhibited, suggesting that the production of lipid peroxides might be involved in this process. This result is not surprising because trolox, a water-soluble α -tocopherol analogue with a carboxylic group replacing the lipophilic tail, is frequently used as an antioxidant to inhibit lipid peroxidation in cellular membranes. In accordance with this, it has been established that the oxidative stress produced by octyl gallate kills *S. cerevisiae* via direct or indirect inhibition of plasma membrane H^+ ATPase (Fujita & Kubo, 2002b). Interestingly, free fatty acids have similar characteristics to gallate esters and exhibit the same tendency to induce the morphological transition (Klose *et al.*, 2004). Alkyl gallates and free fatty acids are thought to behave as detergents, disturbing the stable structure of lipid membrane bilayers, including the plasma membrane.

Little is known about the defence response of *U. maydis*. However, from a general perspective, different types of defence responses should be important for *U. maydis*, because its ability to deal with different ambient conditions appears to play a critical role in growth and virulence. It has been suggested that polyphenolic compounds may exert their effects through interactions with specific proteins of the cell signalling pathways (Williams *et al.*, 2004). In *U. maydis*, cAMP signalling is critical for cell morphology and filamentous growth (Kahmann *et al.*, 2000). In addition, several studies have shown that lipids induce the dimorphic transition on *U. maydis*. Klose *et al.* (2004) suggested that growth in the presence of lipids promotes a filamentous phenotype that resembles the infection cell type found in plants. In addition, the ability of the fungus to respond to lipids depends on both the cAMP and the Ras/MAPK signalling pathways which are known to regulate mating, filamentous growth and pathogenesis in *U. maydis*. Therefore, the identification of the signal transduction pathway modulated by octyl gallate will contribute to the characterization of how this polyphenolic compound exerts its morphological effects.

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