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Necrotic cell death induced by dithianon on *Saccharomyces cerevisiae*

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ABSTRACT

Dithianon is a broad-spectrum anthraquinone fungicide used to control several diseases of grapes, apples, and other fruits and vegetables. Its mode of action is described as multi-site and associated to thiol-reactivity. As other fungicides can affect non-phytopathogenic organisms as yeasts and bacteria, with impact on microbial population, diversity, and fermentation processes. In this context, we study the effect of dithianon on the model organism and fermentative yeast *Saccharomyces cerevisiae* in order to elucidate the mechanisms involved in yeast cell death., and explain its interference on wine fermentation kinetics. Thus for, we analyzed cellular protein and non-protein thiols, membrane and cell wall integrity, reactive oxygen species accumulation, mitochondrial membrane potential, and phosphatidylserine externalization. The results showed that when exponentially aerobic growing cells of *S. cerevisiae* are submitted to acute dithianon treatment they loss cell wall and membrane integrity, dying by necrosis, and this behavior is associated to a depletion of reduced proteic and non-proteic thiol groups. We also detected an important increase of cellular reactive oxygen species (ROS) associated to mitochondrial membrane potential modifications on dithianon treated cells. ROS accumulation was not associated to apoptotic cell death, but can be responsible for intracellular damages. Moreover, necrotic cell death induced by dithianon explains its effect on the kinetics of wine fermentations.

1. Introduction

Dithianon (5,10-dioxobenzo[g][1,4]benzodithiine-2,3-dicarbonitrile) is a synthetic fungicide of the quinone family used in agriculture since 1962 (Berker et al., 1963). It has a broad spectrum contact acting fungicide with good adherence to the surface of leaves and fruits. It is recommended for the control of several fungal diseases of apples, pears, grapes, and many other fruits, as anthracnose (*Colletotrichum sp., Elsinoe ampelina*), mildew (*Plasmopara viticola*), phomopsis (*Phomopsis viticola*), among others.

The mode of action of dithianon is not completely understand, being described as multi-site fungicide that reacts in a non-specific way with sulfhydryl groups leading to structural modification of proteins and enzymatic inhibition. In filamentous fungus, dithianon inhibits mycelial growth and conidial germination (Amponsah et al., 2012; Mirković et al., 2015). Studies on Ehrlich ascites carcinoma and yeast cells showed that dithianon inhibits respiration and fermentation affecting several thiol enzymes of the glycolytic pathway as hexokinase, phosphofructokinase, and glyceraldeyde-3-phosphate dehydrogenase (Sturdik and Drobnica, 1980). Similar effect was observed on flight muscle mitochondria of bumblebee (*Bombus terrestris* L.) which showed a dose dependent inhibition of respiration by dithianon associated with the inhibition of succinate dehydrogenase and glycerol-3-phosphate dehydrogenase (Syromyatnikov et al., 2017).

Although considered as a slightly hazardous fungicide with a LD_{50} of 640 mg/kg (WHO, 2010), toxicological studies showed that dithianon has in vitro cytotoxic effect and affect cell transforming activity of BLAB/c 3 T3 cells (Perocco et al., 1993), and has a complex pattern of CYP induction or suppression in various tissues, suggesting its possible toxic/cotoxic and cocarcinogenic potential (Paolini et al., 1997). Moreover, Pozzetti et al. (1999)) showed that the activity of testosterone hydroxylase of liver microsomes derived from male mice was increased when they were treated with acute doses of dithianon, while in females an inactivating effect was observed.

Dithianon, like other broad-spectrum fungicides, has important impact in non-target microorganisms like soil bacteria (Liebich et al., 2003), and non-phytopathogenic fungi like epiphytic yeasts (Vadkertiová and Sláviková, 2011; Gildemacher et al., 2006). The high incidence of late fungal diseases of grapes in Brazilian subtropical conditions has led grape growers to use fungicides, including dithianon, very close to harvesting, and consequently fungicide residues are frequently present in grapes and juices (Alves et al., 2014). Informal reports from winemakers and experimental wine fermentation showed that the presence of dithianon in grape musts, even at low

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concentrations, reduces yeast viability and interfere in the fermentations process causing weak and/or stuck fermentations, and generating off-flavors and aromas (Jahn et al., 2015). Wine fermentation problems were also reported for other multisite fungicides used for the control of late fungal diseases of grapes (Cus and Raspor, 2008; Noguerol-Pato et al., 2014; Scariot et al., 2016).

In this context, the present study attempts to better understand the cell death mechanism of dithianon on fungi by studying its effect on aerobically grown model and industrial fungus *Saccharomyces cerevisiae*, focusing on its thiol reactivity, membrane and cell wall integrity, ROS accumulation, mitochondrial membrane potential, and apoptotic markers. Moreover, the study aims to explain the previously observed effect of the fungicide on wine fermentations.

2. Material and methods

2.1. Yeast strains and media

Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) strain and its isogenic mutants Y0233 (*aif1*::kanMX4), Y01217 (*nuc1*::kanMX4) and Y06233 (*yca1*::kanMX4) were obtained from Euroscarf (Frankfurt, Germany). Yeast were growth in YEPD broth (2% yeast extract, 1% peptone, 2% glucose, pH 6.5) or SD medium (0.67% Yeast Nitrogen Base without amino acids, 2% glucose, with 20 mg/l histidine, methionine, and uracil, and 60 mg/l leucine, pH 6.5) at 28 °C with constant shaking (150 rpm).

Dithianon was purchased from Sigma-Aldrich (St. Louise, Missouri) and was diluted in DMSO at a concentration of 10 mM.

2.2. Viability assay

Yeast cells were growth until mid-exponential phase, cells were harvested by centrifugation, washed with 0.9% NaCl solution and cellular concentration was adjusted to 1×10^7 cells/ml in SD medium. Cells treated with dithianon in different concentrations (0 to 4 μM) and control (untreated cells) were incubated for 180 min (3 h) in orbital shaker (150 rpm; 28 °C), and cell viability was determined by spot assay. Cultures were diluted at tenfold series and aliquots of 10 μl were spotted in YEPD plates. Plates were incubated at 28 °C for 48 h and the number of colonies formation unities (c.f.u) was determined, and the results were expresses as percentage compared with the control.

To evaluate the effect of cysteine, glutathione and acid ascorbic addition on yeast survival, mid-exponential yeasts cells were incubated in SD medium with 0 to 5 mM of each antioxidants for 30 min, cells were harvested by centrifugation, washed, and treated with 2 μM of dithianon or without dithianon. Cell viability was determined as mention above.

2.3. Thiol content assay

Sulfhydryl groups content on yeast cells was determinate by the DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) assay described by Sedlak and Lindsay (1968)). Protein-thiol (P-SH) levels were measured by subtraction of non-protein-thiol (NP-SH) contents from the total sulfhydryl content, following the procedure described by Demasi et al. (2006)).

2.4. Cell wall sensitivity assay

The influence of dithianon on cell wall sensitivity to enzymatic degradation was evaluated by the spheroplast method (Brennan et al., 2013). Briefly, dithianon-treated and control yeasts were harvested, washed and diluted to an OD_{600nm} of 1,0 in TE buffer (50 mM Tris-HCl; 5 mM EDTA, pH 7.5), and 5 μg/ml of Lyticase (β-1,3 glucanase from *Arthrobacter luteus*; Sigma-Aldrich) were added to cell suspension. The samples were maintained at 28 °C, and the OD_{600nm} was measured every 30 min. The results for biological triplicate were express as percentage compared with the start value.

2.5. Cell membrane integrity, ROS accumulation, mitochondrial membrane potential, and phosphatidylserine exposition

Cell membrane integrity, ROS accumulation and apoptotic cells were evaluated by flow cytometry in a FACSCalibur flow cytometer (Becton-Dickinson) equipped with an argon-ion laser emitting at 488 nm. Flow cytometer data of 10,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc).

Cell membrane integrity was determined using the LIFE/DEAD FungaLight Yeast Viability kit (Invitrogen) that includes SYTO9 and PI dyes. Staining and flow cytometer assay followed manufacturing instructions.

Intracellular ROS accumulation was performed utilizing dihydroethidium (DHE, Sigma-Aldrich). Stock solution of DHE was prepared in DMSO (5 mg/ml). Dithianon-treated or control samples were staining with a final concentration of 5 μg/ml DHE, and incubated for 30 min at room temperature. Samples were evaluated by flow cytometry using FL3 (488/670).

To evaluate mitochondrial membrane potential (Δψm), cells obtained as described above were suspended in SD medium, and stained with 175 nM of 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) for 30 min at 30 °C in the dark. After staining, cells were analyzed by flow cytometry using FL1 filter.

Apoptotic cells were measured by quantification of phosphatidylserine externalization using Annexin V-PE/7AAD apoptosis detection kit (BD Pharmingen). Yeast cells preparation, staining and flow cytometer analysis followed the methodology described by Scariot et al. (2016)).

3. Results and discussion

In a first experiment we determined that yeast exposure to 10 μM dithianon for 3 h resulted in 100% lethality (data no shown). Thus in a subsequent experiment, we evaluate the dosage effect of dithianon on the viability of exponentially aerobic growing cells of *S. cerevisiae* BY4741 in lower concentrations. As can be observed in Fig. 1A, dithianon resulted in a dose dependent reduction on cell viability causing 84 and 98% decrease of yeast colony forming units in 3 h treatments with 2 and 4 μM dithianon, respectively, and were selected for further experiments. Sub-lethal (80–90% mortality) concentrations are used to stud the mode of action of fungicides in order to avoid the non-specific effects caused by the excess of product (Scariot et al., 2016; Arce et al., 2010a; Yang et al., 2011). Dose-time experiments showed a drastic decline of the percentage of yeast colony forming units after 1 h of treatment, but no further reduction was observed in longer exposure times (Fig. 2B), suggesting a contact effect of the fungicide on yeasts. Allinson et al. (1999)) showed that dithianon can persist on the skins of treated grapes (1 mg/L) under grape canopy for at least two weeks, and the fungicide is transferred to grape juice where it has a half-life of 2.8 to 4.6 h depending on grape variety.

It is important to emphasize that the highest concentration of the fungicide used in the experiments (10 μM or 0.003 mg/l) is approximately 300-fold lower than the field spray application dosage recommended for fruits, and within the limits of maximum residual levels (MRL) for apples, grapes, peaches, pears, and other fruits, stablished by the Codex Alimentarius (FAO, 2018). The high mortality caused by low concentrations of dithianon and it persistence on grape skins and musts (Yang et al., 2011) explains the drastic effect of this fungicide on epiphytic yeast populations (Vadkertiová and Sláviková, 2011; Gildemacher et al., 2006), and wine fermentation (Alves et al., 2014).

As the primary mode of action of dithianon on fungi and mammalian cells has been attribute to its reactivity with thiol groups (Sturdik