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The effect of the fungicide captan on *Saccharomyces cerevisiae* and wine fermentation

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Abstract. Fungicides, particularly those used during grape maturation, as captan, can affect the natural yeast population of grapes, and can reach grape must affecting wine fermentation. The objective of the present work was to study the effect of captan on the viability and fermentative behavior of *S. cerevisiae*. *S. cerevisiae* (BY4741) on exponential phase was treated with captan (0 to 40 μM) for different periods, and their cell viability analyzed. Cell membrane integrity, thiols concentration, and reactive oxygen species (ROS) accumulation was determined. The fermentation experiments were conducted in synthetic must using wine yeast strain Y904. The results showed that under aerobic conditions, 20 μM of captan reduce 90% of yeast viability in 6 hours. Captan treated cells exhibited alteration of membrane integrity, reduction of thiol compounds and increase in intracellular ROS concentration, suggesting a necrotic and pro-oxidant activity of the fungicide. Fermentative experiments showed that concentrations above 2.5 μM captan completely inhibited fermentation, while a dose dependent fermentation delay associated with the reduction of yeast viability was detected in sub-inhibitory concentrations. Petit mutants increase was also observed. In conclusion, the captan induces yeast necrotic cell death on both aerobic and anaerobic conditions causing fermentation delay and/or sucking fermentations.

1. Introduction

Captan (N-cyclohex (trichloromethylthio)-4-ene-1, 2-di carboximide) is a broad spectrum fungicide of the phtalimide class, widely used to control several grapevine diseases, as downy mildew, phomopsis, black rot, ripe rot, and bitter rot. With a pre-harvest interval of 24 h to 30 days depending on specific national legislations, experimental data showed that Captan residue in grapes varies between 0.74 to 22 mg/kg [1], and can reach grape must, and decline but persist during fermentation [2].

Captan reaction with thiol groups has been pointed as the main mode of action on phytopathogenic fungi, been responsible for the reduction of enzymatic activities, respiration, physiological changes, and fungal death [3]. In the presence of exposed thiol groups, captan oxidize thiols and is hydrolyze to its reactive thiophosgene ($SCCl_3$) moiety, and the 1,2,3,6-tetrahydrophthalimide ring [4]. In vitro genotoxicity studies indicated that phtalimide fungicides were associated to point mutations, and gene conversion [5,6], been classified as potential human carcinogen. However, based on *in vivo* and molecular analysis in mammals they were re-classified as non-genotoxic [7].

As other broad-spectrum fungicides, captan can affect non-target microorganisms, among which epiphytic and wine yeasts [8–11]. A comparison of the toxicity of 25 vineyard pesticides on the growth of wine yeast (*Saccharomyces cerevisiae*) showed that captan one of the more toxics [8]. Moreover, the presence of captan in grape must can drastically reduce yeast viability causing fermentation delay, wine cloudiness, and stuck fermentations [12]. Although used for more than 60 years, the mechanism of action and the effect of captan on both target and non-target organisms is poor and most studies just describe its toxicity on mammals.

Considering that captan is used to control grape diseases with a pre-harvest interval as low as 24 h, depending on specific national legislations, and consequently reach wine must, the aim of the present study was to determine captan minimal inhibitory concentration, and evaluate the effect of captan on *S. cerevisiae* under actively aerobic growth and wine fermentation.

2. Material and methods

2.1. Yeast strains and media

Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) reference strain (Euroscarf,, Frankfurt, Germany), and Y904 wine strain (ABB, Brazil) were used. Other wine yeast strains were used to determine minimum inhibitory concentration under fermentative conditions: EC1118, QD145, CY3079 (Lalvin, Denmark), Rouge (Fermol, France), and BP725 (Maurivin, Australia). Yeasts were cultured at 28°C with orbital shaking (150 rpm) in YEPD broth (1% yeast extract, 2% peptone, 2% glucose, pH 6.5). Captan (Pestanal®) was purchased from Sigma-Aldrich, and stock solutions (10 mM in dimethylsulfoxide, DMSO) were prepared just before each experiment.

2.2. Yeast growth and fermentations

For aerobic experiments, yeasts from overnight cultures were inoculated in YPD broth, and grown to mid-exponential phase (OD_{600} ~0.7) at 28 °C with orbital shaking (150 rpm). Cells were harvest by centrifugation,

washed twice with 0.9% NaCl, and cell density adjusted to 10^7 cells/ml in minimal medium (0.67% Yeast Nitrogen Base without aminoacids, 2% glucose, with 20 mg/L histidine, methionine, and uracil, and 60 mg/L leucine, pH 6.5). Control and captan-treated cultures were incubated for 6 hours at 28 °C with shaking (150 rpm).

For fermentation experiments, yeasts were grown to stationary phase on YEPD broth for 48 hours at 28 °C with shaking (150 rpm), collected by centrifugation, washed with 0.9% NaCl. Yeast inoculation was standardized at 5×10^6 cells/ml in 100 ml of MS300 medium [13] in 250 ml Duran flasks with Müller valves. Fermentation were conducted at 24 °C with periodical agitation, and monitored by CO_2 release.

2.3. Analytical techniques

The viability of Captan-treated and untreated yeasts was determined by spot assay. Cultures were diluted at 10-fold series, and aliquots (10 μl) of each dilution were spotted onto YEPD plates. Colony were enumerated after 48 h incubation at 28°C, and expressed as percentage of colony forming units (CFU) compared with the control (untreated cells).

Respiration deficient yeasts (petit) were determined by plating on YEPD and YEPG (1% yeast extract, 2% peptone, 3% glycerol, 2% agar, pH 6.5) media, and confirmed by sticking YEPD colonies on YEPG medium.

Total, protein and non-protein thiols were measured by the DTNB (5,5'-dithiobis-(2-nitribenzoic acid)) according with [14]. Total proteins were quantified by the Bradford method.

Cell membrane integrity and ROS accumulation were determined with LIFE/DEAD FungalLight Yeast Viability kit (Invitorgen), and the dihydroethidium and dihydrorhodamine 123 oxidant sensitive dyes, respectively, using a flow cytometry FACSCalibur (Becton-Dickinson) instrument equipped with an argon-ion laser emitting at 488 nm.

Wine total acidity (g tartaric acid/L), volatile acidity (g acetic acid/L), residual sugar (g/L), alcohol (% v/v), and density (20/20) were determined according to the International Organization of Vine and Wine methods [15]. Glycerol concentration was quantified by an enzymatic method (Megazyme).

2.4. Statistical analyses

All analyses were carried out in triplicate, and the results expressed as mean values ± standard deviation. The SPSS 20.0 software for Windows (Chicago, IL, USA) was used for the analysis of variance (ANOVA), and means comparison.

3. Results and discussion

Initial experiments showed that captan minimal inhibitory concentration (MIC) for aerobic exponential growing cells of *S. cerevisiae* on SD medium was 40 μM, but a 6 hours treatment with 20 μM captan resulted in 97.7% reduction in yeast viability (Table 1). This concentration and time exposure was adopted to analyze captan effect on the reference strain *S. cerevisiae* BY4741.

A comparison between control (untreated) and captan-treated cells (Table 1) showed a significant reduction of both cellular protein and non-protein thiol groups. This result confirms captan non-specific thiol reactivity as one of the most important direct effects of the phtalimide fungicides [3]. Moreover, captan-treated cells exhibited a drastic loss of membrane integrity, a typical necrotic behavior [16], which can be the result of the modification of membrane protein structures by protein sulfhydryl-disulfide transitions, and consequent increase in membrane permeability [17].

Table 1. Viability, thiol content, membrane integrity, and ROS accumulation on control and 20 μM captan treated (6 hours) exponential growing yeast cells.

	Control	Captan	Difference
Viability (%)	100.00	5.30**	−97.7%
NP-SH[1]	54.17	7.37**	−86.4%
P-SH[1]	7.14	2.91**	−59.2%
Membrane integrity[2]	97.2	18.3**	−78.90%
DHE fluorescence[3]	14.7	70.4**	4.8 ×
DHR fluorescence[3]	13.2	39.4**	3.0 ×

[1] μM cysteine/μg protein; [2] percentage of cells (10000 cells evaluated); [3] median fluorescence; ** significantly different (> 0.01) from control.

As previously observed in another thiol-reactant fungicide Mancozeb [18], captan-treated cells exhibited an important increase in ROS concentration. ROS accumulation can be related to the reduction of glutathione, the most important reducing compound in yeasts [19]. Moreover, ROS accumulation can induce apoptotic cell death, which in turns can be responsible for the difference between the percentage of necrotic cells (78.9%) and loos of culture-ability (97.7%).

Captan MIC determined in synthetic must (MD300) on seven *Saccharomyces* strains was 5 μM, except for BP725 (2.5 μM) and F15 (10 μM). This values are similar to those reported by Cabras et al [2] that observed complete inhibition of *S. cerevisiae* and *Kloeckera apiculata* by 6.7 μM Folpet, a sister molecule of captan.

Captan MIC values under fermentation conditions are lower than those defined for aerobic active growing cells. Evaluation of pH and osmotic effect on captan MIC values, showed that where glucose concentration (2–20%) has not effect, the reduction of pH values directly affect captan toxicity varying from 20 μM at pH 6.5 to 5 μM at pH< 5.0. Acidity and pH affects the dissociation of captan on their thiophosgene and tetrahydrophtalic acid moieties [20].

Fermentation experiments with different concentrations of captan were conducted in MD300 medium using the wine strain Y904. The results showed that 10 μM captan completely inhibited fermentation, but in lower dosages the fungicide determined a dose dependent delay effect with an increase of 1, 3 and 9 day in the presence of 1.25, 2.5 and 5 μM of captan, respectively (Fig. 1). Conversely, the maximum fermentation rate increased from 1.04, in the control, to 1.39 gCO_2/100ml/day in the 2.5 μM captan treatment. Fermentation delay, inhibition of cell development and reproduction, and modification of wild yeasts population dynamics during wine fermentations, by low concentrations of phtalimide fungicides was previously reported [12].

The evaluation of viable yeast population (UFC/ml) and fermentation behavior in control, 0.625 and 2.5 μM captan confirmed a fermentation delay, particularly evident

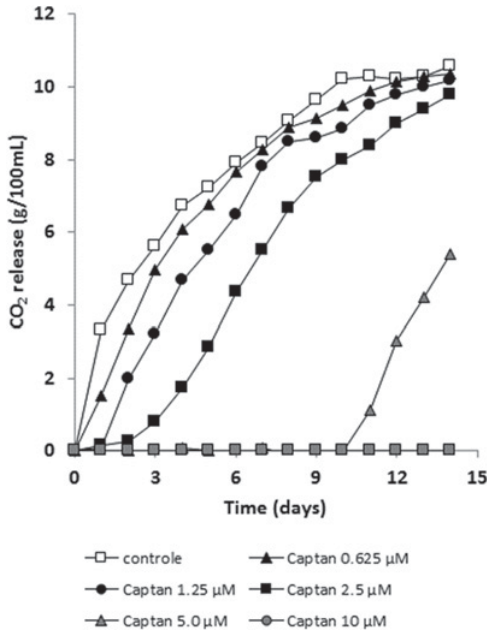

Figure 1. Fermentation behavior in increased concentrations of captan. Values are mean of three replications.

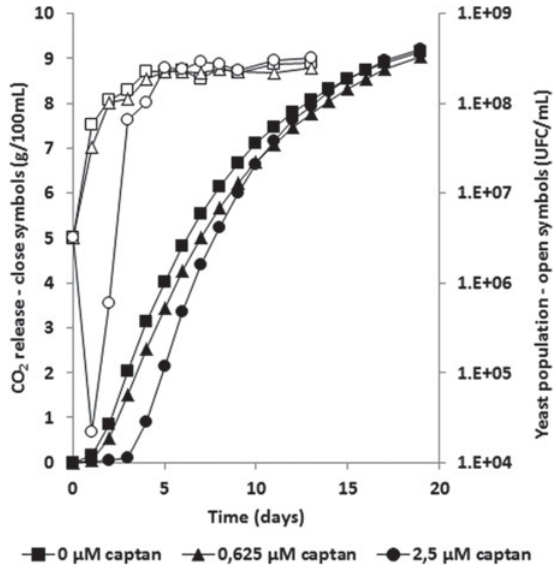

Figure 2. Yeast population and fermentation kinetics in sub-inhibitory concentrations of captan. Values are mean of three replications.

on the highest captan concentration (Fig. 2). The delay to begin fermentation in 2.5 μM captan treatment was associated to a drastic initial decrease in the overall viable yeast population, which fall from 5×10^6 to 2.2×10^4 CFU/ml (99.6%) in the first 24 hours. However, the cells that remain viable rapidly adapted and grew, reaching 5×10^8 CFU/ml, the same population of the control, at the fifth day. Yeast adaptation to stress depends on the mode of action of the stressing agent. Studies on *S. cerevisiae* adaptation to the thiol-reactive and pro-oxidant fungicide Mancozeb led to the identification of 286 genes that provide protection, most of which involved in oxidative stress response, protein degradation, and carbohydrate/energy metabolism, and

Table 2. Wine fermentation parameters, cell mass and cellular thiols in the absence and presence of captan.

Parameter*		Captan	
	Control	0.625μM	2.5μM
Density	1.0050^{A}	1.0055^{A}	1.0023^{A}
pH	3.59^{A}	3.62^{A}	3.60^{A}
Ethanol (% v/v)	12.27^{B}	12.10^{B}	13.20^{A}
Glycerol (mg/L)	4.74^{B}	4.79^{B}	5.01^{A}
Residual Sugar (g/L)	17.71^{A}	16.62^{A}	9.42^{B}
Cellular T-SH[1]	4.69^{B}	5.32^{B}	11.57^{A}
Cell mass (gdw/L)[2]	2,47^{B}	2,69^{AB}	3,25^{A}
Total acidity[3]	7.55^{B}	7.75^{AB}	7.92^{A}
Volatile acidity[4]	0.82^{A}	0.72^{B}	0.58^{C}

* Values are the means of three replicates, and different letters in each line are significantly different at the 0.05 level according to ANOVA by Tukey's test. [1]Total thiol groups (mg cysteine/mg protein); [2] g dry weight/L; [3] g tartaric acid/L; [4] g acetic acid/L.

controlled by the major yeast oxidative stress regulator, Yap1p [21].

A large number (24.5%) of small colonies were observed at the end of fermentations with 2.5 μM of captan. These colonies were unable to grow on glycerol as only carbon source and confirmed as petite (respiratory deficient) mutants. The highest frequency of petite mutants can be attributed to the direct mutagenic action of the tiophosgene moiety of the captan molecule [4], to high ROS accumulation, and the adaptive advantage of mitochondrial defective petite yeast under the stress condition imposed by the fungicide [22].

A comparison of fermentation parameters in the absence and presence of captan showed non-significant differences in wine density and pH (Table 2). However, wines obtained from 2.5 μM captan treatments exhibited significantly higher concentrations of ethanol, glycerol, and total acidity, lower concentration of residual sugar, and a dose dependent decrease of volatile acidity. Yeast cells from 2.5 μM captan treatments recovered at the end of fermentation showed higher concentration of total thiol groups, mainly glutathione. This increase may be part of the adaptation of yeast to glutathione (G-SH) depletion by the captan, as well as a response to ROS accumulation in the presence of the fungicide. In this sense, it is important to note that although progressively degraded in must and wines, phtalimide fungicides and their degradation products can remain at the end fermentation[2], and affect yeast behavior.

In summary, the data reported in this study showed that 20 μM of captan drastically reduce the viability of aerobic active growing cells of *S. cerevisiae* in just 6 hours treatments. Captan treated cells exhibited loss of membrane integrity, reduction of protein and non-protein thiol compounds and increase in intracellular ROS concentration, indicating necrotic and pro-oxidant activity. Under wine fermentation condition, pH stimulated fungicide dissociation resulting in a 4× reduction of MIC values. Fermentation delay caused by 2.5 μM captan was associated to the reduction of yeast viability. However, those cells that survived the initial shock adapted to the fungicide, reassumed growth, and finished fermentation. Wines obtained from 2.5 μM captan treatments showed higher concentrations of ethanol, glycerol, and total acidity, and lower volatile acidity. Moreover, yeast cells

from 2.5 μM captan treatments recovered at the end of fermentation showed higher concentration of total thiols, and almost 25% of them were respiratory deficient petite mutants.

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