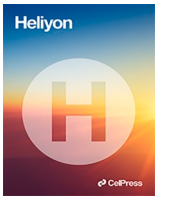
Heliyon 10 (2024) e34885

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Effect of *CUP1* copy number and pH on copper resistance of *Saccharomyces cerevisiae* enological strains

Jacopo Sica [a], Barbara Bovo [a], Chiara Nadai [a,b,*], Milena Carlot [a,b], Alessio Giacomini [a,b], Viviana Corich [a,b,c]

[a] *Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy*

[b] *Interdepartmental Centre for Research in Viticulture and Enology (CIRVE), University of Padova, Viale XXVIII Aprile 14, 31015, Conegliano, TV, Italy*

[c] *Department of Land, Environment, Agriculture and Forestry (TESAF), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy*

ARTICLE INFO

Keywords:
Cu detoxification system
Grape pomace
Wine
Metallothionein
Real-time PCR

ABSTRACT

The widespread use of copper-based pesticides in winemaking can affect wine fermentation. Therefore, it is crucial to assess the resistance levels of *Saccharomyces cerevisiae* wine strains in enological growth conditions. In the context of winemaking, grape juice is a complex environment capable of chelating copper and is characterized by a distinctly acidic pH. In this work, the effects of copper concentration on the growth of 10 *S. cerevisiae* strains, isolated from an enological environment, and one commercial starter were tested in YNB minimal medium and synthetic must, mimicking enological conditions.

In minimal medium, resistance to copper varied among yeasts (50–600 μM), revealing the presence of three resistance levels (high, intermediate, and low). Representative strains of the three groups were tested at a pH range from 5.2 to 3.0 at the copper concentration that showed a 20–25 % growth reduction. At pH range 5.2–4.5, a growth reduction was observed, while, conversely, a strain-specific recovery was observed at pH range 3.2–3.0.

In synthetic must, the strains showed higher copper resistance levels than in minimal medium (50–4000 μM). In both synthetic must and minimal medium, a significant logarithmic correlation was found between copper resistance and *CUP1* gene copy number. The copy number tended to better explain resistance in minimal medium compared to synthetic must. The results shed light on the role of *CUP1* copy number within an enological environment.

1. Introduction

Copper (Cu) is an essential micronutrient for prokaryotes and eukaryotes [1]. In trace amounts, it acts as a cofactor for many chemical reactions involved in the mitochondrial electron transport chain, iron homeostasis, oxidative stress protection, peptide hormone processes, pigmentation, and normal cell growth. Copper can also bind proteins, stabilizing their conformations [2]. However, Cu participates in redox reactions generating free radicals, which cause damage to lipids, proteins and DNA [3]. High levels

* Corresponding author. Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy.

E-mail address: chiara.nadai@unipd.it (C. Nadai).

https://doi.org/10.1016/j.heliyon.2024.e34885
Received 26 February 2024; Received in revised form 16 July 2024; Accepted 18 July 2024
Available online 20 July 2024

of Cu ions may also disrupt the normal conformations and functions of proteins.

In viticulture, the excessive use of copper –based herbicides, bactericides, and fungicides often exceeds the threshold limit of ecotoxicity (toxic effect against macro-, meso-, micro-fauna, and microorganisms) for this metal in the soils [4]. The control of downy mildew (*Plasmopara viticola*) and gray mold *(Botrytis cinerea*) is commonly achieved using copper-containing fungal pesticides (Cu oxychloride) [5,6]. Recently, the use of this heavy metal has increased due to the rise of organic and integrated grapevine cultivation practices, where Cu-based formulations are the main treatments [7]. The adverse impact of Cu has also been noted in winemaking, where excessive Cu residues in grape juices may cause lagging fermentation and detrimentally affect wine quality [8]. The maximum Cu contamination measured in European vineyard soils ranged from 435 to 1500 mg/kg, with the highest level reported in France [9, 10]. In Italy, total Cu accumulated in vineyard soils ranged from 9 to 945 mg/kg [11]. In grape, Cu concentrations ranged from 0.136 to 25.2 mg/kg, while in wine the range is 0.0005–1.010 mg/kg [12]. Cu concentration in wine generally decreased compared to that in the grape. Cu is reduced, forming insoluble sulphides, or adsorbed by yeast cells. In both cases, it is removed at the end of the process together with yeast lees [13]. The generally recommended "safe" total Cu concentration in wine, after fermentation, is between 0.3 and 0.5 mg/kg [14].

Copper homeostasis has been extensively studied in *Saccharomyces cerevisiae* [15–17]. This yeast species exhibits significant variability in Cu resistance, and the acquisition of this trait seems to be the result of environmental adaptation [18–20]. Several Cu uptake, efflux and chelation strategies have been developed by yeasts to control Cu ion homeostasis [21]. Regarding toxicity, Cu-sensitive strains do not change the metal concentration in wine, whereas resistant strains sensibly reduce this element by accumulating Cu inside the cell [22].

S. cerevisiae contains two metallothioneins, Cup1p and Crs5p. Their inactivation leads to copper sensitivity, while their overexpression confers resistance [23]. In particular, Cup1p seems to play a dominant role in neutralizing excess intracellular Cu. Copy number variation of the *CUP1* gene is commonly observed in *S. cerevisiae*, and several studies have suggested a positive correlation between high copper tolerance and an increased copy number of the *CUP1* gene as an adaptation strategy to deal with increasing environmental copper [24–26]. In fact, prolonged growth in the presence of high amounts of Cu leads to *CUP1* gene amplification, with a remarkable increase in resistance in multicopy strains [27].

Commonly, to assess the copper tolerance of yeast strains, tests have been conducted using standard growth media (YNB minimal medium). Few studies have evaluated copper resistance in enological conditions, where grape juice components can have an effect in modulating strain resistance, and the role of the *CUP1* gene copy number is still underexplored.

In this study, the copper tolerance of ten *S. cerevisiae* enological strains and a commercial reference strain was evaluated using microplate assays in minimal medium and synthetic must. To evaluate *S. cerevisiae* strains associated to a specific environment, eight enological strains isolated from fermenting grape juice and the related pomace, obtained from the same grape bunches, were chosen. Generally grape pomace contains high amounts of copper, and strains isolated from this matrix may have increased resistance to copper [12,28]. Moreover, two strains isolated from the vineyard, whose copper tolerance and *CUP1* gene copy number are known, were added to the analysis. The effect of pH variation on copper resistance was also considered. Real-time PCR allowed to determine the copy number of the *CUP1* gene and its association with copper resistance.

2. Materials and methods

2.1. Yeast strains

A total of 11 *S. cerevisiae* strains were used in this study (Table 1). Eight strains were obtained from fermenting juice and their grape pomace [29]. Among them, three were isolated from the Tocai friulano and five from the Glera variety of *Vitis vinifera* in the North-East of Italy. Strain P283 and R008, isolated from vineyards in the Conegliano–Valdobbiadene Prosecco superior and Piave Appellation of Origin winemaking regions, respectively [30,31], have been used as controls. The commercial wine yeast EC1118 was used as reference (Lallemand Inc., Montreal, Canada).

Table 1
S. cerevisiae strains used in this work.

Strain	Origin	Reference
GA	Glera juice	[29]
GB	Glera juice	[29]
GC	Glera pomace	[29]
GF	Glera juice	[29]
GH	Glera pomace	[29]
TA	Tocai friulano juice	[29]
TB	Tocai friulano pomace	[29]
TD	Tocai friulano pomace	[29]
P283	Vineyard strain	[30]
R008	Vineyard strain	[30]
EC1118	Industrial wine strain	Lallemand Inc.

2.2. Determination of copper content

Copper concentration in juice (10 mL) and grape pomace samples (50 g) was determined by inductively coupled plasma optical emission spectrometer (ICP-OES) as described by Lante and colleagues [32]. A SPECTRO CIROS^{CCD}ICP (SPECTRO Analytical Instruments, Kleve, Germany) with axial plasma viewing was used. Grape pomace samples were previously dried and subjected to mineralization using sulfuric acid (Kjeldahl Method, AOAC). Calibration samples were prepared from a 1 g/L copper standard solution (Spectrascan-Teknolab A/S, Norway) diluted to concentrations ranging from 0.002 to 5 mg/L. Three independent measurements were performed for each sample.

2.3. Microtiter assays

A loopful of a 2-days-old culture from a YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 5 mL of YPD broth. A stationary phase culture with approximately 10^7-10^8 cells/mL, measured by spectrophotometry, was obtained after 24 h of incubation at 30 °C. Fifty μL of the yeast culture were resuspended in 5 mL of YPD broth for 4 h to obtain an exponential phase culture. Ten μL of yeast culture (in order to obtain a starting OD of 0.1) were inoculated into 96-wells microplates (Greiner Bio-One, Germany) filled with 300 μL of broth medium.

Growth at different Cu concentrations was performed in minimal medium, Yeast Nitrogen Base (YNB) (w/o aa 1.7 g/L, ammonium sulfate 5 g/L, dextrose 20 g/L, pH 5.2), supplemented with 50–1500 μM of $CuSO_4$ and in synthetic must [33] at pH 3.0 supplemented with 50–9000 μM of $CuSO_4$.

Growth at different pH was performed in YNB medium (pH 5.2) and in modified YNB at different pH values (4.5; 4.2; 3.8; 3.2 and 3.0). A 1 M HCl solution was used to modify the pH values.

The cell turbidity (OD_{600} nm) was monitored every 3 h using Spectra Fluor microtiter plate reader (Tecan, Mannedorf, Switzerland) incubated at 30 °C for up to 45–60 h. Before each measurement, a 60-s shake was performed for cell resuspension. Growth curves were performed in triplicate.

2.4. Real-time PCR quantification of CUP1 gene copy number

Genomic DNA extraction was performed as described by Bovo and colleagues [34]. Real-Time PCR for the quantification of *CUP1* copy numbers was performed on a CFX96 cycler – Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as described by Crosato and colleagues [24]. PCR primer pairs *CUP1* and *FBA1* were used. The amplification conditions were as follows: initial denaturation of DNA at 98 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 5 s, and annealing of primers at 58 °C for 40 s. All samples were analyzed in triplicate.

2.5. Data analysis

For each condition, from cell turbidity (OD_{600}) values the growth curve was obtained by averaging the data from three independent replicates. For each growth curve replica, the area under the curve (AUC) was estimated with trapezoidal rule. Subsequently, to permit comparisons between different strains, the AUC of the various curves was normalized on the AUC of the control condition curve, by division. In the copper trials, for the calculation of the "normalized AUC this condition corresponded to the curve at 0 μM of Cu. In the pH trials in presence of copper, for the calculation of the "normalized AUC this condition corresponded to the curve at the pH of standard YNB medium (5.2).

Furthermore, for each strain, the area under the curve determined by the values of normalized AUC across varying Cu or pH levels was computed. This was considered as an indicator of copper resistance (termed as 'normalized copper resistance') and pH resistance in presence of copper (referred to as 'normalized pH-copper resistance').

Data visualization, ANOVA, linear regressions and generalized linear models were performed using Python libraries matplotlib 3.8.0 [35] and statsmodels 0.14.1 [36].

3. Results and discussion

3.1. Copper resistance in minimal medium

In this study, a total of 10 *S. cerevisiae* strains collected from Italian winemaking regions [29,30], plus a commercial control strain, were considered (Table 1). To evaluate *S. cerevisiae* strains associated to a specific environment, eight strains were isolated from fermenting white grapes and their grape pomace after pressing. These strains, belonging to the same environment, shared the same origin, although, as Cu concentration is generally higher in grape pomace, this matrix should select the more resistant strains. The strains variability was analyzed in a previous work [29] starting from a very high number of isolates (198 colonies isolated from Tocai juice and grape pomace, and 188 collected from Glera variety). The analysis of mitochondrial DNA allowed for the identification of a total of 32 profiles (24 for Glera and 8 for Tocai), suggesting that genetic variability was not very high. The strains selected for this work were the most abundant during the isolation process, with frequencies always higher than 20 % in the yeast population [29].

The Cu concentrations in grape juice and pomace measured at the beginning of the fermentation period were not significantly different in Glera (3.29 ± 0.09 mg/kg and 3.60 ± 0.50 mg/kg, respectively), whereas in Tocai friulano the concentration was

significantly higher in grape pomace (4.48 ± 0.43 mg/kg) compared to juice (2.73 ± 0.05 mg/kg). These concentrations were in line with those found in literature [12]. It is common to find higher levels of Cu in grape pomace compared to juice due to the specific physical-chemical characteristics of the skin, which vary by grape variety, technological treatments or atmospheric conditions [28].

The strains P283 and R008, isolated from vineyards of Italian winemaking regions, were used as reference strains since their

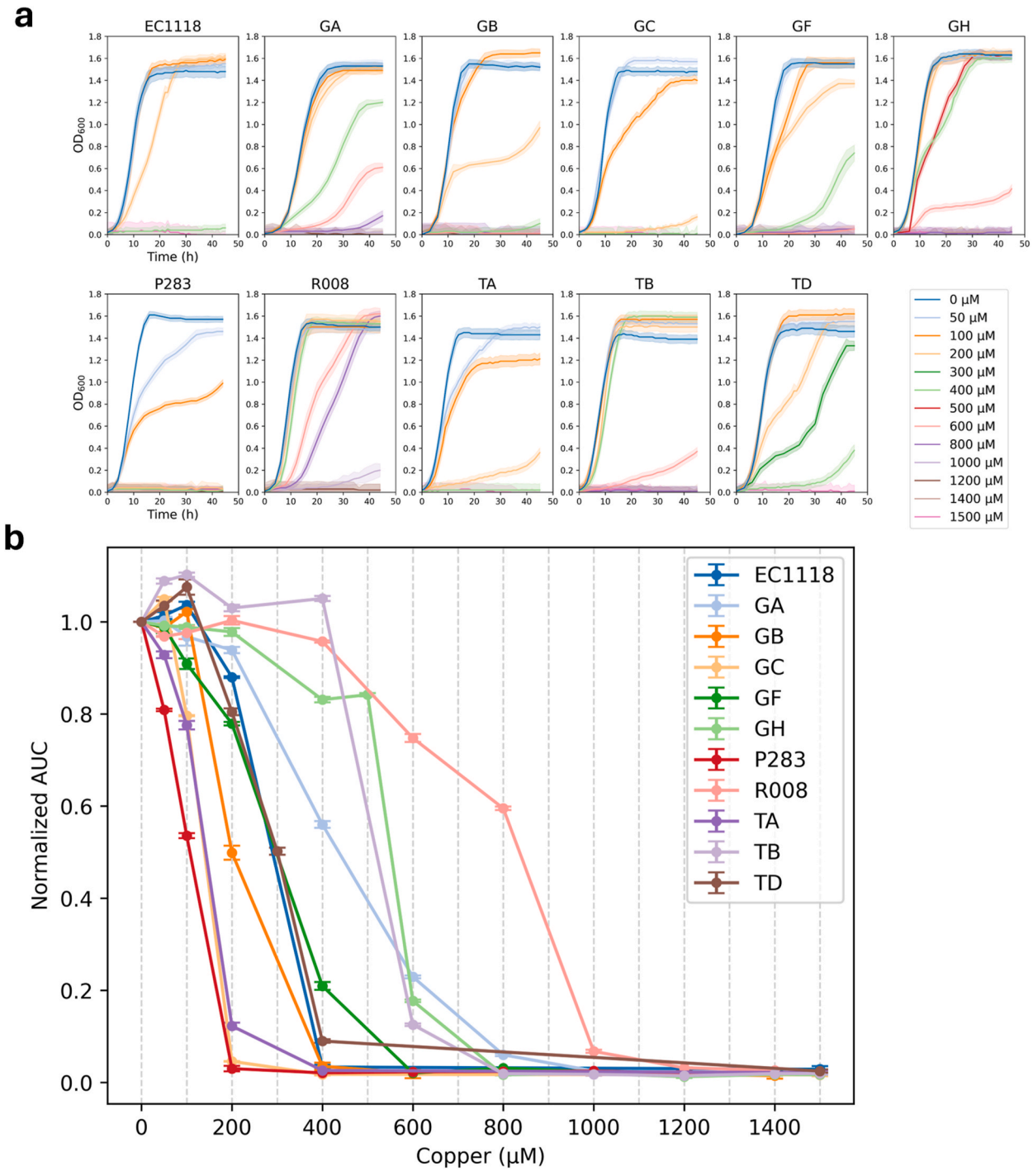


Fig. 1. a) Growth of *S. cerevisiae* strains under different copper doses in minimal medium. Data shows the average of triplicates growth curves ± standard error (shaded area around the curve). b) Normalized area under the growth curve (AUC) trend of *S. cerevisiae* strains over increasing copper concentration, in minimal medium. Each strain's trend is indicated with a different color, as shown by the legend in the figure. Average value points are plotted with standard error bars.

genomes have been fully sequenced [30,31]. The well-studied industrial strain EC1118 was used as a control strain.

To assess copper resistance, strain growth in minimal medium (YNB) was evaluated at different Cu concentrations ranging from 0 to 1500 μM. This range was determined based on trials reported by Crosato and colleagues [37], along with preliminary trials carried out with the laboratory strain S288c, that has been investigated to understand the mechanism of Cu toxicity [38], and strain EC1118 (data not shown).

The growth kinetics of the strains are reported in Fig. 1 a. When grown without Cu addition, the strains showed similar kinetics with OD_{600} maximum values between 1.50 and 1.68. None of the strains could grow at Cu concentrations higher than 1000 μM. At intermediate Cu concentrations, growth kinetics varied among strains. Previous work analyzing Cu resistance of strains isolated form the same winemaking region (North-East of Italy) evidenced that, in YNB minimal medium with 1000 μM $CuSO_4$ concentration, 88 out of

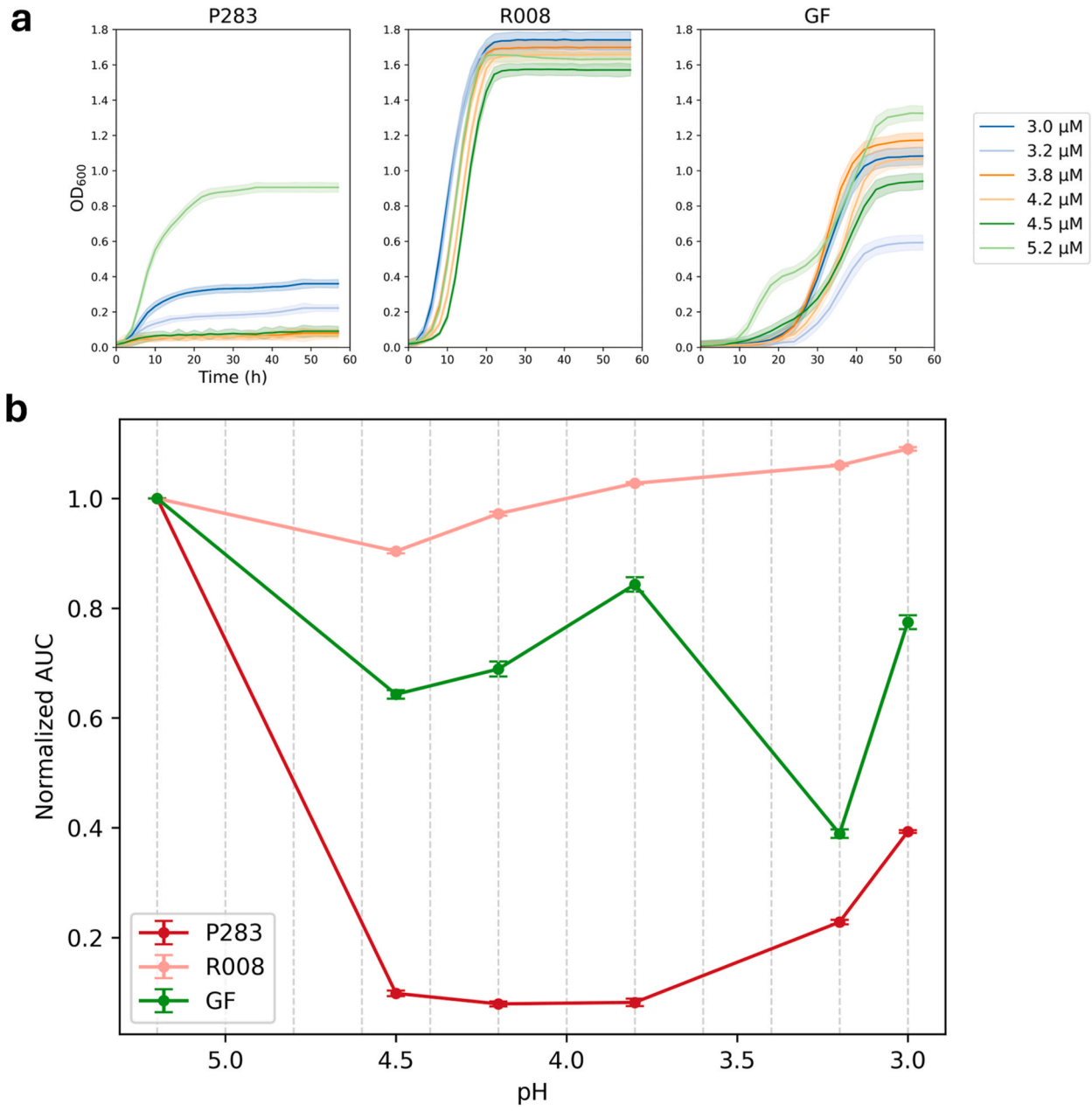


Fig. 2. a) Growth of *S. cerevisiae* strains in minimal medium at different pH values at the copper concentration that inhibits growth by 20–25 % (600 μM for R008, 200 μM for GF, 50 μM for P283). Data shows the average of triplicates growth curves ± standard error (shaded area around the curve). b) Normalized area under the growth curve (AUC) trend over decreasing pH, at the copper concentration that inhibits growth by 20–25 %, in minimal medium. Each strain's trend is indicated with a different color, as shown by the legend in the figure. Average value points are plotted with standard error bars.

190 (46 %) strains showed growth comparable to control condition (no Cu addition) [24]. At the same tested condition (YNB with 1000 μM $CuSO_4$), in a group of 63 isolates from a Brazilian winemaking region, 96 % of strains were able to grow [37].

Therefore, it can be speculated that the resistance percentage of strains seems to be related to the winemaking isolation area.

To better evaluate differences between strains, the normalized AUC (NAUC) was computed for each growth curve at increasing

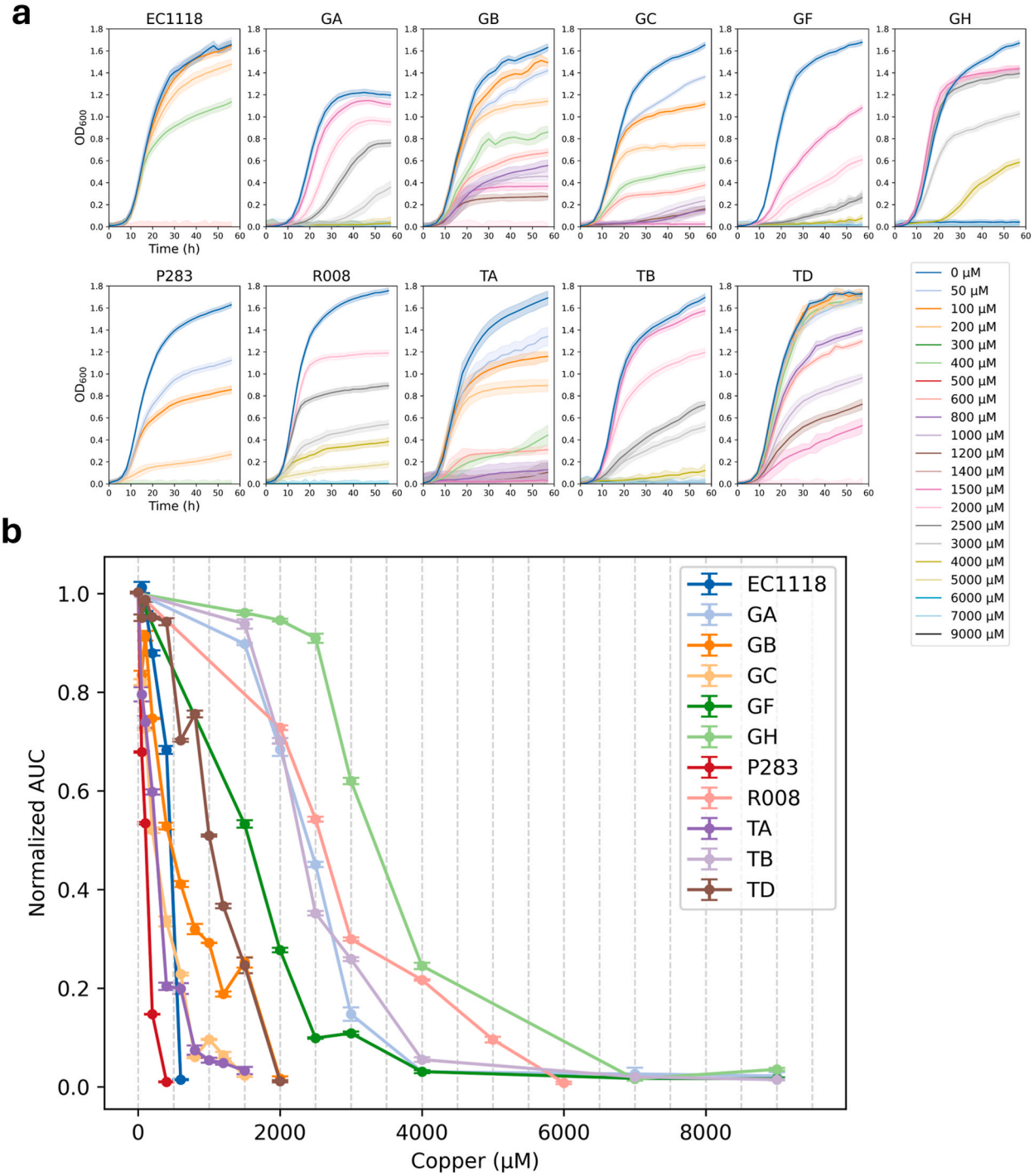


Fig. 3. a) Growth of *S. cerevisiae* strains under different copper doses in synthetic must. Data shows the average of triplicates growth curves ± standard error (shaded area around the curve). b) Normalized area under the growth curve (AUC) trend over increasing copper concentration in synthetic must. Each strain's trend is indicated with a different color, as shown by the legend in the figure. Average value points are plotted with standard error bars.

copper concentrations. This was done by calculating the ratio between the AUC of a given curve and the AUC of the growth curve in absence of copper (Fig. 1 b).

Strains GA, GH, TB and R008 showed good growth at Cu concentrations in the range 500–800 μM, making them the most resistant. In this group, excluding the reference strain R008, two out of three were isolated from grape pomace (GH, TB). Strains GB, GF, TD and EC1118 were well adapted at intermediate concentrations (200–400 μM), while the growth of GC, TA and P283 were strongly affected at 100–200 μM.

3.2. Growth at different pH values in presence of copper

In winemaking, grape juice typically has an acidic pH ranging between 2.75 and 4.25 [39]. Therefore, evaluating the effect of pH on yeast copper toxicity is crucial. Acidity influences Cu oxidation state, as well. Copper exists in two oxidation states: Cu(I)/Cu^{+} (cuprous ion) and Cu(II)/Cu^{++} (cupric ion). At low pH levels (typically below pH 6), the cupric form (Cu^{++}) is more prevalent and is the most toxic. At neutral pH, both Cu^{+} and Cu^{++} can exist, with the dominant form depending on other factors such as the presence of ligands. In alkaline conditions, Cu often exists more as Cu^{+} (cuprous) and, in the form of Cu hydroxide, tends to precipitate (typically at pH 6.5–12) or complex with common anions including SO_4^{2-}, OH^{-}, PO_4^{3-}, HCO_3^{-}, NO_3^{-}, and CO_3^{2-} [40]. Therefore, at the same Cu concentration, alkaline conditions are associated to low toxicity levels.

The effect of pH variation was tested in the presence of Cu in an acidic environment. Given the variability in copper resistance among the strains, three strains were chosen to evaluate the effect of pH, each representing one of the three levels of copper resistance (high, intermediate, and low). Each strain was grown at the copper concentration that resulted in a comparable reduction of NAUC with respect to the other two (Fig. 1 b). The chosen strains were R008 (resistant), GF (intermediate), and P283 (sensitive). At copper concentrations of 600 μM (R008), 200 μM (GF), and 50 μM (P283) 20–25 % reduction in NAUC values was obtained. The chosen limited percentage, compared to larger reductions, allows for the observation of further growth decrease due to pH levels.

Each strain was grown in minimal medium at different pH levels. The pH range (from 5.2 to 3.0) was chosen to include the value typical of grape juice. To evaluate if pH variations themselves influenced yeast growth, the same pH range was tested without Cu addition (Supplementary Fig. 1). No notable variations in growth kinetics were observed.

In the presence of Cu, the tested pH range had different effects on growth (Fig. 2 a). Strain P283 showed the highest growth kinetics variation, whereas R008 showed the lowest. Intermediate variations were found in GF. Considering the NAUC calculated at different pH values, with respect to the original minimal medium pH 5.2 (Fig. 2 b), all strains showed a decrease when transitioning from pH 5.2 to pH 4.5.

Beyond pH 4.5, NAUC values of strain R008 progressively increased, reaching a maximum at pH 3.0. Similarly, strain GF NAUC values increased up to pH 3.8, and this value was confirmed at pH 3.0. For strain P283, the increase in NAUC values occurred only when the pH was below 3.8.

A semi-quantitative pH measurement, using litmus paper, was conducted after 60 h of growth (end of the trial). For all tested strains, under all conditions, the pH value ranged between 2.5 and 3.0, indicating that yeast strains acidify the medium during the growth.

Wang and colleagues [41] reported that for Cu, Cd and Zn, the biosorption capacity of *S. cerevisiae* at pH 4.5 is higher than that at pH 2.5 and pH 3.5. They suggested that electrostatic attraction to negatively charged functional groups present on the cell surface is the first step for heavy metal to interact with yeast cells. At pH 4.5, the most important group capable of binding Cu is phosphate, that is largely present in the plasma membrane as a component of yeast phospholipids. Therefore, the toxicity level is higher at pH 4.5 than at lower tested pH values. This could explain the obtained results, namely a common reduction in growth observed in all tested yeasts when the pH was lowered from 5.2 to 4.5, and the variable growth trends at pH below 4.5. Additionally, Wang asserted that another key active molecular group is the carboxyl, which is found in organic acids and functions to chelate Cu ions. At low pH, *S. cerevisiae* cells display an adaptive stress response, which involves both the activation of the plasma membrane H1-ATPase, regulating intracellular pH and homeostasis, and the induction of Pdr12, a plasma membrane carboxylate efflux pump [42]. Therefore, the different strain trends reported at pH values above 4.5 down to 3.0 could be attributed to differences in acidification abilities among the strains.

3.3. Copper resistance in synthetic must

The effect of the Cu concentration on the growth of *S. cerevisiae* strains was evaluated in synthetic must. Generally, the strains showed higher copper resistance levels compared to minimal medium (Fig. 3 a).

In fact, the range of Cu concentrations tested was between 0 and 9000 μM. This range is definitely wider than that reported in the literature for natural must, ranging from 2 to 370 μM [12].

When strains were grown without Cu addition, similar kinetics were observed for all strains except GA. The OD_{600} maximum value was above 1.6 for most strains, whereas strain GA only reached 1.2. No strains were able to grow at Cu concentrations higher than 7000 μM. The higher levels of strain resistance indicated the presence of a greater quantity of chelating agents in the synthetic must than in minimal medium. Synthetic must is composed of free amino acids and peptides, that are known to chelate Cu [43], and 3 g/L of tartaric acid, another copper chelating molecule [12,44]. Although the most resistant strain in synthetic must was GH (while in minimal medium it was R008), the differences in resistance levels between strains remained generally consistent (Fig. 3 b).

The most resistant strains were GH, R008, GA and TB as they were able to grow in Cu concentrations ranging from 2000 to 5000 uM. Strains GB, GF and TD were well adapted at intermediate concentrations (500–1500 μM), while the growth of GC, EC1118, TA and P283 was strongly influenced above 500 μM. Considering Cu concentrations generally present in natural must (2–370 μM), all the

strains would not be influenced by the lowest level, while the highest concentration would affect the low resistant group, totally inhibiting the growth of strain P283 (NAUC 0.01) and strongly influencing the growth of TA and GC (NAUC 0.20 and 0.34, respectively).

To compare resistance levels between minimal medium and synthetic must, the normalized copper resistance (NCR) value was calculated for each strain under each condition (Fig. 4), as the area under the NAUC curves reported in Figs. 1 b and Fig. 3 b.

All tested strains deviated from the identity line (represented by the dashed line) except for P283, the least resistant among the strains. This indicates that only P283 exhibited the same levels of copper resistance in both minimal medium and synthetic must.

A significant relationship ($R^2 = 0.753$, $p < 0.001$) was observed between the values of NCR in minimal medium and synthetic must. This confirms that strains more resistant in minimal medium also exhibited high resistance in synthetic must. The distribution of strains revealed the presence of the same three groups (high, intermediate, and low resistance) as highlighted in previous analyses. In the high resistance group (GH, R008, TB, and GA), R008 revealed to be more resistant than GH in minimal medium compared to synthetic must. Among the intermediate strains (GF, TD, EC1118, and GB), resistance levels were very similar in minimal medium, but showed notable differences in synthetic must. Specifically, EC1118 was more resistant than GB in minimal medium compared to synthetic must.

3.4. Determination of *CUP1* gene copy number

To identify a relationship between the resistance levels of the strains and the *CUP1* gene copy number, a specific Real-time PCR protocol has been applied [24]. For relative quantification, the reference gene *FBA1*, encoding an isoform of the enzyme fructose 1, 6-bisphosphate aldolase involved in glycolysis, conserved in all *S. cerevisiae* strains and present in single copy in the sequenced strains, was chosen [45].

P283 was considered the reference strain, since genomic sequencing data showed that this strain carried a single copy of the *CUP1* gene in its haploid genome [30]. Except for P283, all strains had more than 2 copies of the *CUP1* gene (Table 2).

Strain R008 had the highest copy number as confirmed by genome sequencing [30]. Strains GH and TA had 13.8 and 3.5 copy number, respectively, whereas the other strains had between 4 and 6 copies, with no significant differences. It was not possible to determine the *CUP1* gene copy number for strain GA. This could be due either to the absence of the *CUP1* gene or, more likely, to a polymorphism in the primer binding region of the *CUP1* sequence. Consequently, GA was excluded from subsequent analyses correlating *CUP1* with growth values in the presence of Cu.

To evaluate the effect of the *CUP1* gene on strain growth in minimal medium in the presence of Cu, the NCR value was compared with the respective *CUP1* copy number for each strain (Fig. 5 a): a positive logarithmic relationship (pseudo-$R^2 = 0.995$, $p < 0.001$) was found.

Considering strains positioned at the initial part of the curve, a slight increase in copy number significantly enhanced the NCR value. For strains with high copy numbers, further increases in the number of copies had a limited influence on NCR value. Within the group with the same copy number (GC, GB, EC1118, GF, TD, and TB), variable NCR values were observed, particularly for TB, which

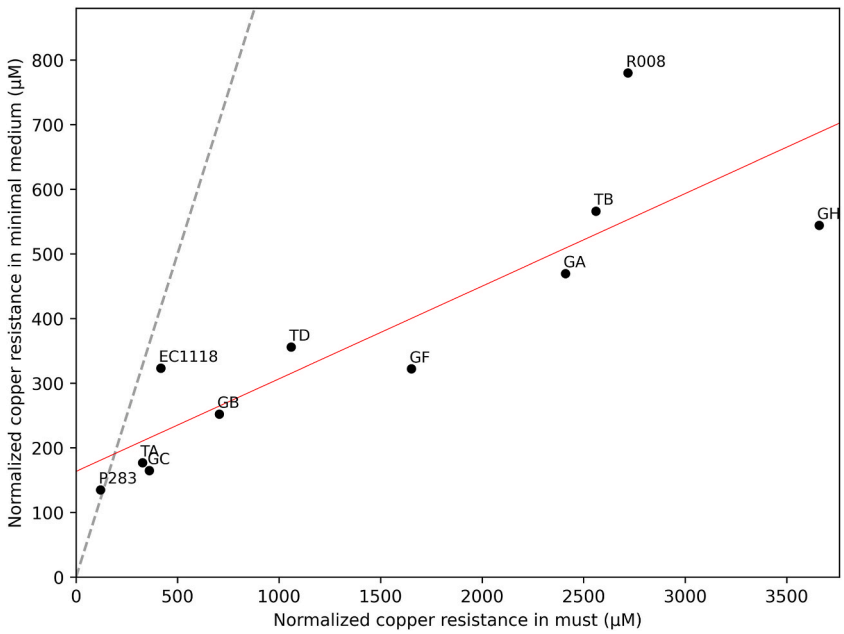


Fig. 4. Relationship between normalized copper resistance measured in minimal medium and in synthetic must. Data were fitted with a linear regression model (red line). Dashed line represents the identity line. Each dot represents the average value calculated for the strain.

Table 2
Relative *CUP1* gene copy number of *S. cerevisiae* strains. Different letters indicate statistically significant differences between strains (ANOVA, $p < 0.05$).

Strain	*CUP1* copy number
P283	2.0^{f}
R008	29.4^{a}
GB	4.1^{d}
GC	4.2^{d}
GF	5.4^{c}
GH	13.8^{b}
TA	3.5^{e}
TB	4.2^{d}
TD	5.0^{cd}
EC1118	5.6^{c}

was isolated from grape pomace with high copper content. This demonstrated that there must be other mechanisms involved in resistance, as reported by Crosato and colleagues [24]. The tolerance of yeast to copper can be modulated by reducing Cu uptake through cell surface adsorption [46] and activating the oxidative stress response. The latter leads to the induction of superoxide dismutase *SOD1* [47] and the inactivation of genes such as *CTR1*, *FRE1*, and *FRE7*, responsible for Cu reduction (from Cu^{++} to Cu^{+}) and import (in the Cu^{+} form) [48]. Moreover, the Cu homeostasis pathway, leading to copper sulfide generation and CuS biomineralization on the cell surface, can participate preventing copper-induced toxicity [49].

The three strains tested at different pH values in YNB minimal medium showed different *CUP1* copy number (R008 29.4, GF 5.4 and P283 2.0). Considering NAUC values at different pH, results indicated that the higher the *CUP1* copy number the higher the NAUC values. Although the number of strains tested in this condition is limited, the growth trend at different pH indicate that the *CUP1* copy number influenced the strain's ability to grow at acidic pH. In fact, at pH lower than 5.2 (control condition) the same amount of copper determined a higher toxicity. Therefore, a higher *CUP1* copy number ensure an increased copper resistance.

To evaluate the effect of the *CUP1* gene on strain growth in synthetic must in the presence of Cu, the NCR value was compared with the respective *CUP1* copy number for each strain (Fig. 5 b): a positive logarithmic relationship (pseudo-$R^2 = 0.713$, $p < 0.001$) was found, as well.

Indeed, once again, strains with high copy numbers were the most resistant. These results were expected since a linear and significant correlation between NCR in minimal medium and synthetic must was found. However, evaluating the most resistant strains (R008 and GH), the *CUP1* copy number affected copper resistance differently in synthetic must compared to minimal medium. In fact, GH, with a significantly lower copy number than R008, was more resistant in synthetic must. Within the group with the same copy number (GC, GB, EC1118, GF, TD, and TB), strains arrangement differed between synthetic must and minimal medium. This means that the composition of the must in the presence of Cu had a different impact on the growth of these strains with respect to the minimal medium. TB exhibited higher resistance even in synthetic must despite its low *CUP1* copy number. Overall, the copy number tends to better explain resistance in minimal medium compared to synthetic must, where strain resistance was generally higher.

4. Conclusions

In this work, strains isolated from fermenting grape juice and the related grape pomace, obtained from two different varieties, were investigated to evaluate Cu resistance relative to *CUP1* gene copy number. Although only Tocai variety grape pomace had a significantly higher Cu concentration than juice, two out of the three most resistant strains were isolated form grape pomace, one from Glera and one from Tocai. These findings did not support the use of grape pomace as an isolation source of copper resistant strains interesting for winemaking.

In minimal medium, strains showed different levels of resistance that positively correlated, following a logarithmic trend, with the number of *CUP1* gene copies. Hence, a higher number of copies resulted in a less pronounced increase in resistance levels. In synthetic must, except for strain P283, yeasts showed significantly higher resistance levels. Experiments in minimal medium at different pH values demonstrated that in the pH range 3.2 to 3.0 copper resistance increased. These findings contribute to explaining the high resistance levels observed in synthetic must and suggests that in a more complex environment, such as natural grape must, resistance levels will be even higher.

In synthetic must, a similar correlation between *CUP1* copy number and resistance levels was observed with respect to minimal medium, although the correlation of *CUP1* copy number with strains resistance is stronger in minimal medium than in a complex environment like synthetic must. Therefore, screening for *CUP1* copy number could be introduced as a method for evaluating copper resistance in strain selection for winemaking. Further studies are necessary to understand the other mechanisms influencing copper resistance in the grape juice environment and their relationship with the copper detoxification system.

Funding

This work was supported by University of Padova [grant number CPDR115444].

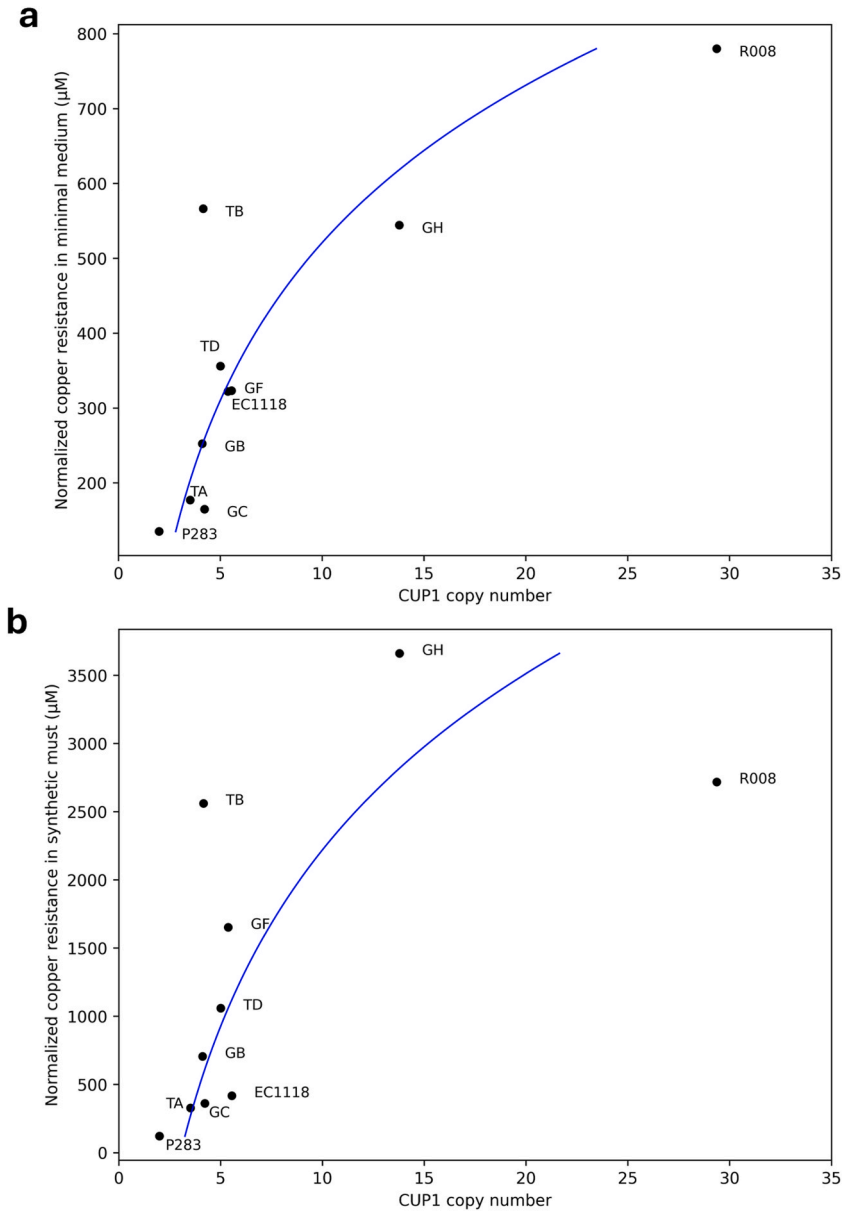


Fig. 5. Relationship between normalized copper resistance and *CUP1* copy number, in minimal medium (a) and in synthetic must (b). Data were fitted with a generalized linear regression model for gamma distribution and with 'log' link function (blue line). Each dot represents the average value calculated for the strain.

Data availability statement

All the relevant data are included in the manuscript and supplementary material.

CRediT authorship contribution statement

Jacopo Sica: Writing – review & editing, Investigation, Formal analysis. **Barbara Bovo:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Chiara Nadai:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Milena Carlot:** Investigation. **Alessio Giacomini:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Viviana Corich:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Luciano Magro and Massimo Cagnin (La-Chi laboratory, DAFNAE, Legnaro) are gratefully acknowledged for ICP-OES analyses. The authors wish to thank Roberto Calzavara for growth test trials. The topics covered in this work fall within the VITAE Project [CUP C23C23000240001].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34885.

References

[1] J.T. Rubino, K.J. Franz, Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function, J. Inorg. Biochem. 107 (1) (2012) 129–143.
[2] R.A. Festa, D.J. Thiele, Copper: an essential metal in biology, Curr. Biol. 21 (21) (2011) R877–R883.
[3] S. Puig, D.J. Thiele, Molecular mechanisms of copper uptake and distribution, Curr. Opin. Chem. Biol. 6 (2) (2002) 171–180.
[4] B. Karimi, V. Masson, C. Guilland, E. Leroy, S. Pellegrinelli, E. Giboulot, P. Maron, L. Ranjard, Ecotoxicity of copper input and accumulation for soil biodiversity in vineyards, Environ. Chem. Lett. 19 (2021) 2013–2030, https://doi.org/10.1007/s10311-020-01155-x, 2021.
[5] A. Aziz, P. Trotel-Aziz, L. Dhuicq, P. Jeandet, M. Couderchet, G. Vernet, Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew, Phytopathology 96 (11) (2006) 1188–1194, https://doi.org/10.1094/PHYTO-96-1188.
[6] D. Judet-Correia, C. Charpentier, M. Bensoussan, P. Dantigny, Modelling the inhibitory effect of copper sulfate on the growth of *Penicillium expansum* and *Botrytis cinerea*, Lett. Appl. Microbiol. 53 (5) (2011) 558–564.
[7] M.R. Provenzano, H. El Bilali, V. Simeone, N. Baser, D. Mondelli, G. Cesari, Copper contents in grapes and wines from a Mediterranean organic vineyard, Food Chem. 122 (4) (2010) 1338–1343.
[8] G. Kovačič, M. Lešnik, S. Vršič, An overview of the copper situation and usage in viticulture, Bulg. J. Agric. Sci. 19 (1) (2013) 50–59.
[9] S. Ruyters, P. Salaets, K. Oorts, E. Smolders, Copper toxicity in soils under established vineyards in Europe: a survey, Sci. Total Environ. 443 (2013) 470–477.
[10] N. Mirlean, A. Roisenberg, J.O. Chies, Metal contamination of vineyard soils in wet subtropics (southern Brazil), Environ. Pollut. 149 (1) (2007) 10–17.
[11] K.A. Mackie, T. Müller, E. Kandeler, Remediation of copper in vineyards–a mini review, Environ. Pollut. 167 (2012) 16–26.
[12] J. Wang, T. Ma, M. Wei, T. Lan, S. Bao, Q. Zhao, Y. Fang, X. Sun, Copper in grape and wine industry: source, presence, impacts on production and human health, and removal methods, Compr. Rev. Food Sci. Food Saf. 22 (3) (2023) 1794–1816, https://doi.org/10.1111/1541-4337.13130.
[13] M.A. García-Esparza, E. Capri, P. Pirzadeh, M. Trevisan, Copper content of grape and wine from Italian farms, Food Addit. Contam. 23 (3) (2006) 274–280, https://doi.org/10.1080/02652030500429117.
[14] A.C. Clark, G.R. Scollary, Determination of total copper in white wine by stripping potentiometry utilising medium exchange, Anal. Chim. Acta 413 (1) (2000) 25–32.
[15] J. De Freitas, H. Wintz, J.H. Kim, H. Poynton, T. Fox, C. Vulpe, Yeast, a model organism for iron and copper metabolism studies, Biometals 16 (1) (2003) 185–197.
[16] A.R. Fernandes, I. Sá-Correia, Comparative effects of *Saccharomyces cerevisiae* cultivation under copper stress on the activity and kinetic parameters of plasma-membrane-bound H -ATPases *PMA1* and *PMA2*, Arch. Microbiol. 171 (4) (1999) 273–278.
[17] H. van Bakel, E. Strengman, C. Wijmenga, F.C. Holstege, Gene expression profiling and phenotype analyses of *S. cerevisiae* in response to changing copper reveals six genes with new roles in copper and iron metabolism, Physiol. Genom. 22 (3) (2005) 356–367.
[18] M. Azenha, M.T. Vasconcelos, P. Moradas-Ferreira, The influence of Cu concentration on ethanolic fermentation by *Saccharomyces cerevisiae*, J. Biosci. Bioeng. 90 (2) (2000) 163–167, https://doi.org/10.1016/S1389-1723(00)80104-8.
[19] C. Fiore, J. Arrizon, A. Gschaedler, J. Flores, P. Romano, Comparison between yeasts from grape and agave musts for traits of technological interest, World J. Microbiol. Biotechnol. 21 (2005) 1141–1147.
[20] T. Shinohara, H. Furuya, F. Yanagida, T. Miki, Ecological distribution and phenotypic diversity of *Saccharomyces cerevisiae* strains from the Wine–Producing area in Yamanashi, Japan, Microbiol. Cult. Collect. 19 (2003) 69–80.
[21] T. Nevitt, H. Öhrvik, D.J. Thiele, Charting the travels of copper in eukaryotes from yeast to mammals, Biochim. Biophys. Acta Mol. Cell Res. 1823 (9) (2012) 1580–1593.
[22] V. Brandolini, P. Tedeschi, A. Capece, A. Maietti, D. Mazzotta, G. Salzano, A. Paparella, P. Romano, *Saccharomyces cerevisiae* wine strains differing in copper resistance exhibit different capability to reduce copper content in wine, World J. Microbiol. Biotechnol. 18 (6) (2002) 499–503, https://doi.org/10.1023/A:1016306813502.
[23] L.T. Jensen, W.R. Howard, J.J. Strain, D.R. Winge, V.C. Culotta, Enhanced effectiveness of copper ion buffering by *CUP1* metallothionein compared with *CRS5* metallothionein in *Saccharomyces cerevisiae*, J. Biol. Chem. 271 (31) (1996) 18514–18519.
[24] G. Crosato, C. Nadai, M. Carlot, J. Garavaglia, D.R. Ziegler, R.C. Rossi, J. De Castilhos, S. Campanaro, L. Treu, A. Giacomini, V. Corich, The impact of *CUP1* gene copy-number and XVI-VIII/XV-XVI translocations on copper and sulfite tolerance in vineyard *Saccharomyces cerevisiae* strain populations, FEMS Yeast Res. 20 (4) (2020) foaa028, https://doi.org/10.1093/femsyr/foaa028.
[25] G.M. Adamo, M. Lotti, M.J. Tamas, S. Brocca, Amplification of the *CUP1* gene is associated with evolution of copper tolerance in *Saccharomyces cerevisiae*, Microbiology 158 (9) (2012) 2325–2335.
[26] P.K. Strope, D.A. Skelly, S.G. Kozmin, G. Mahadevan, E.A. Stone, P.M. Magwene, F.S. Dietrich, J.H. McCusker, The 100-genomes strains, an *S. cerevisiae* resource that illuminates its natural phenotypic and genotypic variation and emergence as an opportunistic pathogen, Genome Res. 25 (5) (2015) 762–774.
[27] M. Karin, R. Najariant, A. Haslinger, P. Valenzuelat, J. Welcht, S. Fogelt, Primary structure and transcription of an amplified genetic locus: the *CUP1* locus of yeast, Proc. Natl. Acad. Sci. USA 81 (1984) 337–341.
[28] M. Fregoni, G. Corallo, Il rame nei vigneti italiani, Vignevini 5 (2001) 35–40.
[29] B. Bovo, C. Nadai, W.J.F. Lemos Junior, M. Carlot, A. Giacomini, V. Corich, The different physical and chemical composition of grape juice and marc influence *Saccharomyces cerevisiae* strains distribution during fermentation, J. Food Sci. 83 (8) (2018) 2191–2196, https://doi.org/10.1111/1750-3841.14274.

[30] L. Treu, C. Toniolo, C. Nadai, A. Sardu, A. Giacomini, V. Corich, S. Campanaro, The impact of genomic variability on gene expression in environmental *Saccharomyces cerevisiae* strains, Environ. Microbiol. 16 (5) (2014) 1378–1397, https://doi.org/10.1111/1462-2920.12327.
[31] A. Viel, J.L. Legras, C. Nadai, M. Carlot, A. Lombardi, M. Crespan, D. Migliaro, A. Giacomini, V. Corich, The geographic distribution of *Saccharomyces cerevisiae* isolates within three Italian neighboring winemaking regions reveals strong differences in yeast abundance, genetic diversity and industrial strain dissemination, Front. Microbiol. 8 (2017) 1595, https://doi.org/10.3389/fmicb.2017.01595.
[32] A. Lante, G. Lomolino, M. Cagnin, P. Spettoli, Content and characterisation of minerals in milk and in Crescenza and Squacquerone Italian fresh cheeses by ICP-OES, Food Control 17 (3) (2006) 229–233.
[33] C. Delfini, J.V. Formica, Isolation selection and purification of wine yeasts, in: Wine Microbiology: Science and Technology, Dekker, New York, US, 2001, pp. 193–218.
[34] B. Bovo, C. Andrighetto, M. Carlot, V. Corich, A. Lombardi, A. Giacomini, Yeast population dynamics during pilot-scale storage of grape marcs for the production of Grappa, a traditional Italian alcoholic beverage, Int. J. Food Microbiol. 129 (3) (2009) 221–228.
[35] J.D. Hunter, Matplotlib: a 2D graphics environment, Comput. Sci. Eng. 9 (3) (2007) 90–95.
[36] S. Seabold, J. Perktold, Statsmodels: econometric and statistical modeling with python, in: Proceedings of the 9th Python in Science Conference, vol. 57, 2010, pp. 10–25080, 61.
[37] G. Crosato, M. Carlot, A. De Iseppi, J. Garavaglia, L.M.N. Pinto, D.R. Ziegler, R.C. de Souza Ramos, R.C. Rossi, C. Nadai, A. Giacomini, V. Corich, Genetic variability and physiological traits of *Saccharomyces cerevisiae* strains isolated from "Vale dos Vinhedos" vineyards reflect agricultural practices and history of this Brazilian wet subtropical area, World J. Microbiol. Biotechnol. 34 (2018) 1–14, https://doi.org/10.1007/s11274-018-2490-z.
[38] D. Yasokawa, S. Murata, E. Kitagawa, Y. Iwahashi, R. Nakagawa, T. Hashido, et al., Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis, Environ. Toxicol. 23 (5) (2008) 599–606.
[39] G.H. Fleet, G.M. Heard, Yeasts-growth during fermentation, in: G.H. Fleet (Ed.), Wine Microbiology and Biotechnology, Harwood Academic Publishers, Chur, Switzerland, 1993, pp. 42–43.
[40] J.D. Cuppett, S.E. Duncan, A.M. Dietrich, Evaluation of copper speciation and water quality factors that affect aqueous copper tasting response, Chem. Senses 31 (7) (2006) 689–697.
[41] J. Wang, C. Chen, Biosorption of heavy metals by *Saccharomyces cerevisiae*: a review, Biotechnol. Adv. 24 (5) (2006) 427–451, https://doi.org/10.1016/j.biotechadv.2006.03.001.
[42] M. Casal, S. Paiva, O. Queirós, I. Soares-Silva, Transport of carboxylic acids in yeasts, FEMS Microbiol. Rev. 32 (6) (2008) 974–994, https://doi.org/10.1111/j.1574-6976.2008.00128.x.
[43] H. Shi, Y. Jiang, Y. Yang, Y. Peng, C. Li, Copper metabolism in *Saccharomyces cerevisiae*: an update, Biometals 34 (2021) 3–14, https://doi.org/10.1007/s10534-020-00264-y.
[44] A.C. Clark, E.N. Wilkes, G.R. Scollary, Chemistry of copper in white wine: a review, Aust. J. Grape Wine Res. 21 (3) (2015) 339–350.
[45] C. Nadai, S. Campanaro, A. Giacomini, V. Corich, Selection and validation of reference genes for quantitative real-time PCR studies during *Saccharomyces cerevisiae* alcoholic fermentation in the presence of sulfite, Int. J. Food Microbiol. 215 (2015) 49–56, https://doi.org/10.1016/j.ijfoodmicro.2015.08.012.
[46] X. Sun, L. Liu, Y. Zhao, T. Ma, F. Zhao, W. Huang, J. Zhan, Effect of copper stress on growth characteristics and fermentation properties of *Saccharomyces cerevisiae* and the pathway of copper adsorption during wine fermentation, Food Chem. 192 (2016) 43–52, https://doi.org/10.1016/j.foodchem.2015.06.107.
[47] F. Abe, T. Miura, T. Nagahama, A. Inoue, R. Usami, K. Horikoshi, Isolation of a highly copper-tolerant yeast, *Cryptococcus* sp., from the Japan Trench and the induction of superoxide dismutase activity by Cu^{2+}, Biotechnol. Lett. 23 (2001) 2027–2034, https://doi.org/10.1023/A:1013739232093.
[48] D. Dialynaki, A. Stavropoulou, M. Laskou, D. Alexandraki, The essential liaison of two copper proteins: the Cu-sensing transcription factor Mac1 and the Cu/Zn superoxide dismutase Sod1 in *Saccharomyces cerevisiae*, Curr. Genet. 69 (2023) 41–53, https://doi.org/10.1007/s00294-022-01258-8.
[49] W. Yu, R.A. Farrell, D.J. Stillman, D.R. Winge, Identification of *SLF1* as a new Cu homeostasis gene involved in copper sulfide mineralization in *Saccharomyces cerevisiae*, Mol. Cell Biol. 16 (1996) 2464–2472, https://doi.org/10.1128/MCB.16.5.2464.