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Change in Kinetic parameters of commercial yeast in the presence of copper fungicides

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Abstract. The Serra Gaucha has a high rainfall occurrence during the ripening and harvest. It results in higher use of fungicides, being the copper-based ones, the most commonly used. High levels of this metal can cause stress to the yeast, thus, influencing on the fermentation kinetics. The aim of this study was to evaluate the fermentative behavior of oenological yeast in the presence of copper content in the must. The experiment used the following fungicides: Copper Sulfate, Copper Oxychloride and Copper Hydroxide. Samples were inoculated with *S. cerevisiae, S. cerevisiae* var. *bayanus* and *Torulaspora delbrueckii* and the fermentation was plotted for carbon dioxide production by time. The fermentative kinetics were assessed by the following kinetic parameters: the lag phase time, maximum rate of CO2 production and maximum production of CO2. The study showed that *T. delbrueckii* had higher sensitivity to copper regarding the lag phase. Copper oxychloride promoted greater latency period in yeast *T. delbrueckii* and *S. cerevisiae*. All copper fungicides decreased maximum rate of fermentation, and *T. delbrueckii* was the most affected. The maximum CO2 production during the fermentations significantly differed only in the presence of copper sulphate.

1. Introduction

The Hill Top Northeast Sierra (Sierra Gaucha) is the main wine-growing region of Rio Grande do Sul State and Brazil. This region, although considered climatically viable for the processing of grapes [1], has high rainfall occurrence during the ripening and harvest periods. The viability of the grape cultivation in this region requires preventive and frequent application of chemicals for the control of fungal diseases on the aerial part of the plant. Spraying fungicides and the use of resistant cultivars are some of the methods usually recommended for the management of some fungal diseases such as downy mildew [2]. The control method most frequently adopted by manufacturers is spraying contact and systemic fungicides [3], which require multiple applications during the growth and production cycle of the vine [4]. Moreover, in order to avoid damage caused by the rain, the harvest is, many times, anticipated and the fruit does not get to the ideal commercial ripening. The applications are even more frequent for *Vitis vinifera* grapes because those varieties are more susceptible to diseases and they are the most valued grapes in the market [5].

Since the late seventeenth century, copper has been used to fight fungal diseases of the vines, and its use was intensified at the end of the nineteenth century [6]. Since then, grape growers have continuously and repeatedly applied it to the vines in the form of spray. Copper-metal-containing substances are highly efficient against pathogens that can attack vines, mainly against downy mildew, commonly found in the Serra Gaucha region, powdery mildew and other varieties of pests and pathogens [7–9].

Copper (Cu) is an essential micronutrient for plants, animals and humans as a constituent of numerous enzymes and as a catalyst for redox reactions in various metabolic pathways [10]. However, its optimal concentration range is very narrow, and when higher than it, the metal can be inhibitory. For yeast cells, especially *Saccharomyces cerevisiae*, different levels of this metal in adverse conditions can cause stress. Therefore, it can influence cell growth and fermentative kinetics. The stress may compromise the plasma membrane and stop cell growth and reproduction, resulting in cell death [11]. Thus, there may be changes in the duration of the lag phase, the maximum rate of fermentation and complete degradation of sugars.

Studies show that the yeast has certain absorption capacity of heavy metals, especially yeast of the species *Saccharomyces cerevisiae*, which have significant results in the absorption of heavy ions. Different yeast species have different absorption rates of the Cu^{+2} metal and consequently different resistance to it [12–15].

The residual concentration in musts resulted from fruit, or use of fungal treatments, is related to copper rate applied throughout growing season, the quantity of the applications, and the amount of rainfall between application and harvest [16]. The current legislation in South Africa considers in the legal parameters any concentration up to 20 mg L^{-1} Cu^{2+} in the grape berries [17]. According to [18] metal concentrations found in wine are around 6 to 19 times lower than doses observed in the fruit itself, so it can be assumed that much waste is disposed over the winemaking process. According to [19], Cu concentrations can reach up to 3 mg L^{-1} in the must. However, the value found from this metal may be much higher, reaching values up to 15 mg L^{-1} and 26 mg L^{-1} [20–22] observed the presence of metal in musts of Concord and Isabel varieties, finding maximum values of 9.28 and 12.89 mg L^{-1}, respectively. [23] analyzed musts from eight different vineyards of Cabernet Franc in Serra Gaucha and found an average of 5.4 mg L^{-1}, and a range of 2.3 to 13.4 mg L^{-1} of Cu^{2+}, which was related to the amount and time of the application.

Thus, this study aimed to assess the sensitivity of different oenological commercial yeast *Saccharomyces* and *non-Saccharomyces* copper content present in wine.

2. Materials and methods

2.1. Must

It was used 6L of must, homogenized with 20% of fermentable sugars at pH 3.15. The juice was obtained from grapes of Isabel and Bordô varieties (hybrids and *Vitis labrusca)*. They were harvested from the IFRS experimental station – Campus Bento Gonçalves, and the plants had known and controlled phytosanitary treatments during the growing season. The juice was obtained from the processes of destemming, crushing, pasteurization (85 °C/2 min), enzyme addition (commercial pectinase 20 mg L^{-1}, two hours at about 45° C), filtration, pasteurization (85°C/1 min) and packaging in glass containers. We chose to use these varieties to be widely disseminated and adapted to the climate and soil of the Serra Gaucha, representing a significant portion of the grapes processed in the region [24]. Moreover, they are hardy varieties whose needs of phytosanitary treatments are smaller than the varieties Vitis vinifera [25], reducing risks of interference must in the experiment.

2.2. Microorganisms

The samples were inoculated with three different commercial yeast: *Saccharomyces cerevisiae var. cerevisiae* (Zymaflore X5, Laffort, France), *Saccharomyces cerevisiae var. bayanus* (La Claire SP 665/P, Perdomini-IOC, Italy), e *Torulaspora delbrueckii* (Zymaflore Alpha, Laffort, France). The yeast was acclimatized and hydrated with distilled water at 35 °C for 15 minutes, except *Torulaspora d.* 30 °C, as recommended by the suppliers. After acclimation, the yeast was inoculated to the juice using the same dose for all the different yeasts (30 g hL^{-1}).

2.3. Cupric fungicides

The added copper levels were established based on a previous biography, so the experiment aimed concentrations of 6 and 12 mg L^{-1} of the metal. The following were used fungicides: Copper Sulfate Pentahydrate [Molecular Formula: $CuSO_4.5H_2O$] (Commercial name: Microsal); Copper Oxychloride [Molecular Formula: $Cu_2Cl(OH)_3$] (Commercial name: Recop) and Copper hydroxide [Molecular Formula:$Cu(OH)_2$] (Commercial name: Ellect).

2.4. Fermentation

The fermentations were conducted in quadruplicate in containers of 45 mL at 25 °C, and they were plotted by the production of carbon dioxide (CO_2) as a function of time, due to loss of mass daily. The fermentation was monitored by measuring the mass balancing static on containers every 12 hours, over approximately 6 days. The fermentation was considered complete when evolution of CO_2 (mass loss) stopped.

Table 1. - Interference from copper fungicides on the lag phase (hours) of yeast.

	Copper hydroxide	Copper Sulfate	Copper oxychloride
S. cerevisiae	22.44 cB	23.03 cAB	23.40 cA
T. delbrueckii	54.74 aB	54.27 aB	58.87 aA
S. cerevisiae var. bayanus	27.32 bA	27.21 bA	27.39 bA

Values with different capital letters in the single columns (fungicides) and different small letters in the single rows (yeast) are statistically different. Tukey's test ($p < 0.05$).

2.5. Kinetic parameters

The fermentative kinetics were assessed by the following kinetic parameters according to [26]: Time of lag phase (hours) by production of CO_2, the maximum rate of CO_2 production (g L^{-1} h^{-1}) and maximum CO_2 production (g L^{-1}). Those parameters were obtained through the nonlinear sigmoidal fit of the modified Gompertz equation (equation 1) according [27], which importance for the activity in oenological research has been reported by [28,29].

$$y = A exp \left\{ -\exp \left[\frac{\mu_m \cdot \epsilon}{A} (\lambda - t) + 1 \right] \right\} \cdot \qquad (1)$$

It was used Software "Predictive Modelling Program Integrated Tools" USDA [30] for evaluation of the model and obtaining parameters.

2.6. Experimental design and statistical analysis

The experiment was designed in a full factorial arrangement (3 × 3 × 3) with three factors: 3 pesticides, 3 contents copper present in the must (0, 6 and 12 mg L^{-1}) and 3 commercial yeast. The results were evaluated by analysis of variance (ANOVA) at 1% probability followed by Tukey test ($p <$ 0.05) through software Assistant [31].

3. Results and discussion

The study showed effect of different cupric products on the duration the lag phase, and *T. delbrueckii* presented the greatest sensitivity. There was also differentiation between products. Copper oxychloride promoted longer lag phase in *T. delbrueckii* and *Saccharomyces cerevisiae*, but it did not affect *S. bayanus* (Table 1). For all yeasts there was no significant difference between the control and the dose of 12 mg.L^{-1} Cu present in the must. [32] stated that there was no inhibition for activity at a dose of 16 mg L^{-1} copper in the first 100 hours of fermentation.

All copper fungicides caused decrease on the values of maximum rate of fermentation for all species. *T. delbrueckii* was the most affected species by the presence of the products. [33] stated that musts containing 20 mg L^{-1} added copper reduced their fermentative activity drastically. There were significant differences in

Table 2. Interference of cupric fungicides on Fermentation Maximum Rate (g L^{-1} h^{-1}) of the yeasts.

	Copper hydroxide	Copper Sulfate	Copper oxychloride
S. cerevisiae	2,01 bA	2,00 bA	2,00 bA
T. delbrueckii	1,91 bA	1,47 cB	1,33 cC
S. cerevisiae var. bayanus	2,27 aA	2,18 aA	2,20 aA

Values with different capital letters in the single columns (fungicides) and different small letters in the single rows (yeast) are statistically different. Tukey's test ($p < 0.05$).

Table 3. Effects of fungicides and their doses applied on the maximum rate of fermentation.

	0 mg L^{-1}	6 mg L^{-1}	12 mg L^{-1}
Copper hydroxide	2,19 aA	2,08 aA	1,92 aB
Copper Sulfate	2,18 aA	1,87 bB	1,59 bC
Copper oxychloride	2,16 aA	1,83 bB	1,51 bC

Values with different capital letters in the single columns (concentration) and different small letters in the single rows (fungicides) are statistically different. Tukey's test ($p < 0.05$).

Table 4. Interference of Fungicides cupric dose on the studied kinetic parameters.

	Lag (h)	μ_{max}(g $L^{-1}h^{-1}$)	CO_{2Max} (g L^{-1})
0 mg L^{-1}	32,4 b	2,19 a	99,98 a
6 mg L^{-1}	36,96 a	1,93 b	99,5 a
12 mg L^{-1}	36,72 a	1,67 c	96 b

Values with different letters in the single columns are statistically different. Tukey's test ($p < 0.05$).

sensitivity to cupric products in the following decreasing order: copper oxychloride > Copper sulfate > copper hydroxide (Table 2).

Among the products, the oxychloride and sulfate copper showed a higher effect on fermentation than copper hydroxide. The hydroxide had effect only at a dose of 12 mg L^{-1}, while the other products showed effects also at the dose of 6 mg L^{-1} (Table 3) on all the yeasts except for *S. cerevisiae*.

It was observed that the dose of 12mg L^{-1} of copper caused interference at the maximum carbon dioxide production (CO_2Max) compared to control (Table 4). [34] found similar results in their work. The fermentation rate (μmax) was decreased whenever the dose of 12mg L^{-1} was used in comparative to the dose 6 mg L^{-1}.

The maximum CO_2 production during the fermentations significantly differed only in the presence of copper sulphate to *T. delbrueckii* (Table 5). Whereas treatment with other products in different concentrations did not differ from the control treatment. [35] reported a decrease of approximately 80% of CO_2 production in the first 10 days when using copper sulphate at a dose of 32 ppm of copper.

4. Conclusions

The effect of copper on yeasts is dependent on the fungicide that gives rise to the metal in the must. Different yeasts behave differently in the presence of copper in the must, and the yeast *T. delbrueckii* demonstrated greater sensitivity to copper compared the yeast *Saccharomyces.*

Table 5. Interference of cupric fungicides for producing carbon dioxide Maximum (g L^{-1}).

	Copper hydroxide	Copper Sulfate	Copper oxychloride
S. cerevisiae	101,17 aA	100,54 aA	100,66 aA
T. delbrueckii	99,28 aA	91,88 bB	96,18 bA
S. cerevisiae var. bayanus	99,16 aA	99,90 aA	98,54 abA

Values with different capital letters in the single columns (fungicides) and different small letters in the single rows (yeast) are statistically different. Tukey's test ($p < 0.05$).

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