Vitis **52** (3), 149–155 (2013)

The influence of the copper content in grape must on alcoholic fermentation kinetics and wine quality. A survey on the performance of 50 commercial Active Dry Yeasts

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Summary

The effects of copper on the viability and fermentative activity of 50 active dry yeasts purchased on the northern Italian market were studied, and revealed that Copper excess may cause massive death of yeast cells, leading to a significant delay in the start and progress of alcoholic fermentation. A two-log units reduction in cell viability was observed when copper content of musts was around 20 mg·L^{-1}. Despite this, the difference noted in the kinetics after 20 days' fermentation was lower than that observed 48 hours after in the grape must. An excess of copper in must affected also the composition of the produced wines. The increase in acetic acid and in the sulphur dioxide concentration, observed in wines made using grape must with a high copper concentration, raises serious doubts both regarding the possibility of obtaining good wines from these raw materials and in relation to the progress of subsequent steps of winemaking, such as malolactic fermentation. While it is an important tool in preventing vine diseases, copper must be used very carefully to avoid serious troubles during wine fermentation, even if some yeasts seem more suited to ferment musts containing up to 20-30 mg·L^{-1} copper.

Key words: Alcoholic fermentation, copper, grape must, Active Dry Yeast.

Introduction

Copper is one of the oldest methods used in viticulture against vine diseases, and in the last few years, an increase in its use has been observed, as copper, together with sulphur, is one of the few instruments that organic vine growers can use against vine diseases. While copper is a useful micro-element, necessary to yeast growth (Ferreira *et al.* 2006), it has also been ascertained that excess copper in grape must leads to serious stress in *Saccharomyces cerevisiae* (Yasokawa *et al.* 2008, Liang and Zhou 2007). The mechanism of its toxicity and the resistance of yeast to its excess have already been described (Wang and Chen 2006).

The incidence of copper in the pattern of mineral elements of grapes in Trentino was described by Larcher and Nicolini (2008), who observed, on average, a copper concentration in grape musts around 11 mg·kg^{-1}. If this was an average value, it may happen that some lots of musts contain higher amounts of the metal. The practical implications of excess copper in grapes, and consequently in grape musts, are well known to winemakers: a reduction in the vitality of yeast biomass, slowing down of alcoholic fermentation, and a change in the features of wines. However at present, an exhaustive study of the sensitivity of the oenological yeasts available on the market to this element is still lacking.

In this work, attention was paid to the technological implications of excess copper in grape must, as sometimes observed in northern Italian wine-growing regions, where the climate favours the proliferation of fungal infections. Other authors have investigated the effect of the presence of copper residues on fermentation efficiency and volatile acidity, demonstrating that copper resistance is a strain dependent characteristic (Ferreira *et al.* 2006). We present a survey, conducted over a period of three years, on the effects of copper on the viability and fermentative activity of 50 active dry yeasts purchased on the northern Italian market.

Material and Methods

Chemical and microbiological analysis: The chemical composition of musts and wines (sugar, ethanol, titratable acidity, pH, and organic acid concentration) was determined using Near Infra-Red Spectrometry (FT-IR, WineScan 2000, FOSS Instruments, DK); total and free SO_2 concentrations were measured with a Crison Compact Titrator (Crison Instruments, E). Plate counts were performed according to the OIV methods (OIV 2011). Wallerstein Laboratory (WL) Agar Medium (Oxoid, UK) was used in the enumeration of total yeasts, and Lysine Agar (LA) (Oxoid, UK) in non-Saccharomyces yeast counts. All plates were incubated at 25 °C for four d. Lactic acid bacteria (LAB) were enumerated on Tomato Juice Agar (TJA) (Fluka, D), and acetic acid bacteria (AAB) on Kneifel Agar medium (CARR), both incubated at 25 °C for 10 d, LAB in anaerobic conditions (Anaerogen Kit, OXOID, UK), AAB in aerobic conditions. Yeast cell number during AF was counted by microscopic counts using a Bürker chamber; live/dead cells were counted after methylene blue staining (OIV 2011).

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Table 1

Chemical composition of the musts used in the fermentation trials before the addition of copper

Must set	Reducing sugars ($g \cdot L^{-1}$)	pH	Total acidity ($g \cdot L^{-1}$ as tartaric acid)	Yeast assimilable nitrogen ($mg \cdot L^{-1}$)	Total SO_2 ($mg \cdot L^{-1}$)	Cu^{2+} ($mg \cdot L^{-1}$)
1	240	3.70	5.83	189	42	1.5
2	249	3.03	6.40	127	21	5.0
3	252	3.27	7.40	122	26	5.0

Active Dry Yeast sampling: The ADY samples delivered to the Microbiological Laboratory of the Edmund Mach Foundation (I) were purchased by the cooperative wineries associated to CAVIT Group (Ravina – Trento, Italy) from: AEB spa (I), Anchor Yeast (SA), Danstar Ferment (D), Enartis srl (I), Enologica Vason srl (I), Esseco srl (I), Essedielle (I), Ever srl (I), Ferrari srl (I), IOC (F), Laffort oenologie (F), Lallemand. (CA), Maurivin Yeast Australia (AU), Oliver Ogar Italia (I), Pascal Biotech (F), and Perdomini spa (now Pall Filtration & Separation spa). More information on each ADY batch analyzed is available at http://www.iasma.it/servizi_context.jsp?ID_LINK=111&area=6

Active Dry Yeast rehydration: ADY were rehydrated following OIV methods (OIV 2011). 10 g of each ADY sample was dispersed in 90 g peptone water (Mycological Peptone, OXOID, UK), then homogenised using a Stomacher 400 Blender (UK) for 60 s. Samples were stored at 25 °C in a water bath for 15 min. Both operations were repeated twice.

Fermentation tests: Yeast alcoholic fermentation (AF) was performed in 1 L 'Chardonnay' grape must samples (see Tab. 1 for its composition) at 20 °C. Musts were stirred before each weighing with the use of a magnetic stirrer placed at the bottom of bottles. 0.2 mL of rehydrated ADY were inoculated to each must to start fermentation. The progress of alcoholic fermentation was evaluated through the mass loss related to CO_2 production (BELY *et al.* 1990). The addition of copper, in the form of copper sulphate salt (Sigma, D) was performed 24 h before yeast inoculums.

Statistical analysis: Statistical analysis of the data was carried out using Statistica 7.1 software (StatSoft Inc., USA).

Results and Discussion

Microbial composition of ADY preparations: The main microbiological features of the ADYs tested are given in Tab. 2. All the ADY samples complied with OIV limits regarding viable yeast (> 10^{10} $cfu \cdot g^{-1}$) and lactic acid bacteria cell counts (<10^5 $cfu \cdot g^{-1}$) (OIV 2011). Mould and non-Saccharomyces yeasts were never detected. However, the large differences in total yeast counts and in the live/dead cell ratio in all ADY samples suggests that significant differences can be found be-

Table 2

Main microbiological parameters of the ADYs investigated over the 3 years

ADY	Supplier	Must set	Alive yeast (10^{10} $cfu \cdot g^{-1}$)	Live/dead yeast ratio	LAB ($cfu \cdot g^{-1}$)
1	A	1	3.3	2.38	5.0 x 10^3
2	A	1	3.5	2.46	4.0 x 10^3
3	B	3	3.3	3.38	6.7 x 10^2
4	B	1	4.1	2.43	2.8 x 10^4
5	C	1	2.8	4.27	< 5
6	D	1	4.2	6.76	4.5 x 10^3
7	E	2	4.5	4.85	1.6 x 10^4
8	E	2	4.1	3.74	< 5
9	B	1	3.0	1.52	5.0 x 10^2
10	F	3	4.2	3.98	2.3 x 10^4
11	G	3	2.4	1.56	< 5
12	H	1	2.6	5.44	< 5
13	H	1	3.9	5.92	< 5
14	E	2	4.1	3.01	2.5 x 10^2
15	E	3	5.1	2.57	1.3 x 10^3
16	E	3	3.5	3.48	4.5 x 10^4
17	E	1	2.8	2.43	5.0 x 10^2
18	E	1	1.3	1.89	4.3 x 10^4
19	I	3	3.8	2.95	3.8 x 10^3
20	I	1	2.1	2.27	1.1 x 10^5
21	I	1	4.1	2.83	8.8 x 10^3
22	I	2	2.9	4.40	3.2 x 10^5
23	I	2	3.6	2.20	3.8 x 10^3
24	I	2	3.4	1.76	2.2 x 10^3
25	I	1	3.6	2.62	4.5 x 10^3
26	I	1	2.6	1.70	7.8 x 10^4
27	I	2	3.1	3.83	1.8 x 10^3
28	L	1	3.8	2.22	< 5
29	L	1	3.2	2.95	< 5
30	L	1	3.2	3.35	< 5
31	L	1	5.3	2.72	5.0 x 10^2
32	G	1	3.8	3.19	< 5
33	B	1	4.2	3.29	< 5
34	L	1	3.9	3.71	< 5
35	L	2	2.3	1.73	1.2 x 10^4
36	L	2	3.8	3.60	6.8 x 10^3
37	L	2	2.2	1.70	5.6 x 10^4
38	L	3	3.3	3.08	2.4 x 10^4
39	G	2	3.4	3.30	< 5
40	G	1	4.6	2.29	< 5
41	M	2	3.3	5.46	< 5
42	M	2	4.2	4.76	< 5
43	M	2	3.7	5.21	< 5
44	L	1	3.5	1.89	< 5
45	A	1	2.6	4.29	< 5
46	A	1	1.7	1.50	1.3 x 10^5
47	A	1	3.4	2.58	1.0 x 10^1
48	A	1	5.4	2.67	7.4 x 10^5
49	A	2	3.3	2.23	3.9 x 10^4
50	A	1	2.1	0.80	5.1 x 10^4

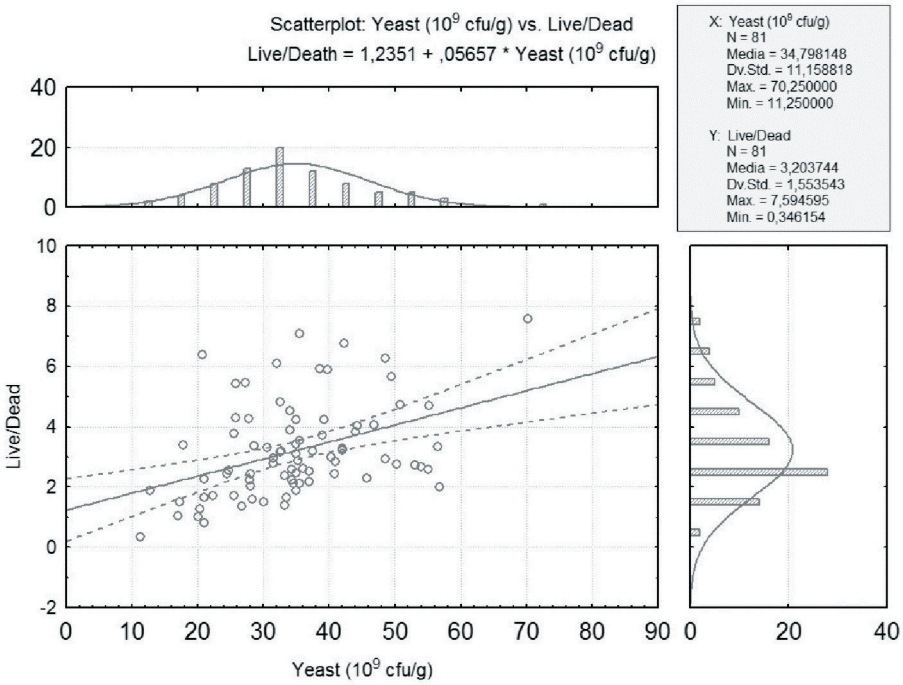

Fig. 1: Correlation between yeast cell concentration in the ADY samples and the percentage of live cells determined by microscopic count and methylene blue staining. Pearson's test. $p < 0.5$.

tween commercial ADYs, as they can be purchased on the market. On average, the live/dead cell ratio, determined by haemocytometry, was quite low. Some information is also provided by the correlation found between the 3 parameters considered in Tab. 2. When comparing the live cell counts and the live/dead ratio (Fig. 1), performing a Pearson's test on the whole dataset, a broad dispersion can be observed, even though the linear correlation between the parameters is significant. We could not determine if the presence of high number of dead cells could suggest that even the living cells are stressed of in bad physiological conditions, but in the trials without copper addition (see later) the fermentation did not show any delay. The presence of contaminants was distributed randomly, and no statistical correlation was found between the yeast cell counts and the presence of lactic acid bacteria in the ADY, that was also variable in different ADYs from the same producer. LAB detected in ADY preparations were not subjected to identification, and they were not able to grow in wines used in the trials (MLF was never detected in wines, even if no SO_2 was added to musts). Anyway, considering the relevance of wine spoilage due to lactic bacteria (Lonvaud-Funel 1999), their content should be taken into account in the choice of an ADY, especially when malolactic fermentation must be avoided.

The effects of copper on the evolution of alcoholic fermentation: Fig. 2 shows a box plot about the progress of sugar degradation after 48 h by the whole set of ADYs in the three sets of musts. The correlation between the amount of copper in the grape must and fermentative activity after 48 h is clearly evident. The non-enriched musts contained between 1.3 (set 1) and 5 (set 2 and set 3) $mg \cdot L^{-1}$ copper. These concentrations are within the common range of copper residues observed in

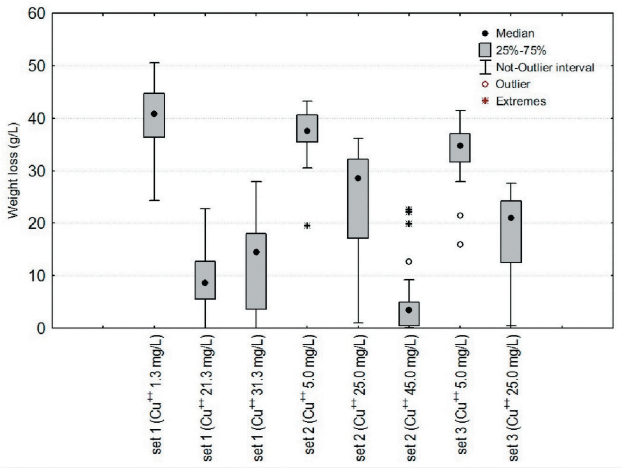

Fig. 2: Box plot of the progress of alcoholic fermentation after 48 h. Data are expressed as weight loss due to CO_2 production.

the Trentino area (Pellegrini *et al.* 2010). The fermentation kinetics of all yeast strains showed few differences in the three musts in which copper was not added, that were due to the different composition of the grape musts in the three sets. The must 1 showed the highest pH and nitrogen concentration, combined with lowest sugar concentration; these characteristics favoured the yeast growth, and allowed the highest fermentation rate. When 20 $mg \cdot L^{-1}$ copper was added to this must, yeast activity decreased dramatically, being virtually absent when the copper concentration was 45 $mg \cdot L^{-1}$. Interestingly, the data dispersion shows that copper resistance was significantly different in the commercial yeast strains. Intervals including 50 % of observations (grey bars in Fig. 2) and the non-outlier range when the copper concentration was between 20 and 30 $mg \cdot L^{-1}$ were very large. When copper concentration was

45 mg·L^{-1}, its detrimental effect prevailed over the fermentative activity of the ADY, which was however retained by some of them even in this harsh environment. The extreme response of the ADYs to copper stress is shown in Fig. 3. On comparing the fermentation curve of two yeasts (ADY 2 and ADY 29) , it is evident that ADY 29 showed a 1, 2 or 3 d lag-phase when copper was present in 5.0, 25.0 and 35.0 mg·L^{-1} respectively, and after this lag phase its fermentation rate was the same until the total consumption of all the sugar, while ADY n°2 showed a reduction in fermentation rate when copper content was 25.0 mg·L^{-1}, and required more than 10 d to start alcoholic fermentation, and 45 d to finish it, when copper concentration was 35.0 mg·L^{-1}. Considering that the initial cell concentration and the composition of must were the same, the different behaviours can be attributed to the ability of some *Saccharomyces cerevisiae* strains to survive and ferment in the presence of copper, confirming the observations of Ferreira *et al.* (2006). This observations shows that the choice of the right ADY is crucial when there is a risk of high copper content in the grape must.

The fermentation progress observed after 20 d, when alcoholic fermentation should be far over, is shown in Fig. 4. In the majority of musts where copper was added, most strains recovered the initial delay in alcoholic fermentation, with the exception of the musts in which copper concentration was 45.0 mg·L^{-1}. When no copper was added, most of the alcoholic fermentation had been accomplished, and the differences were due to the chemical composition of the grape musts. In copper-enriched musts, the progress data were more dispersed, and the presence of some outlier data indicates that certain ADYs showed a higher sensitiv-

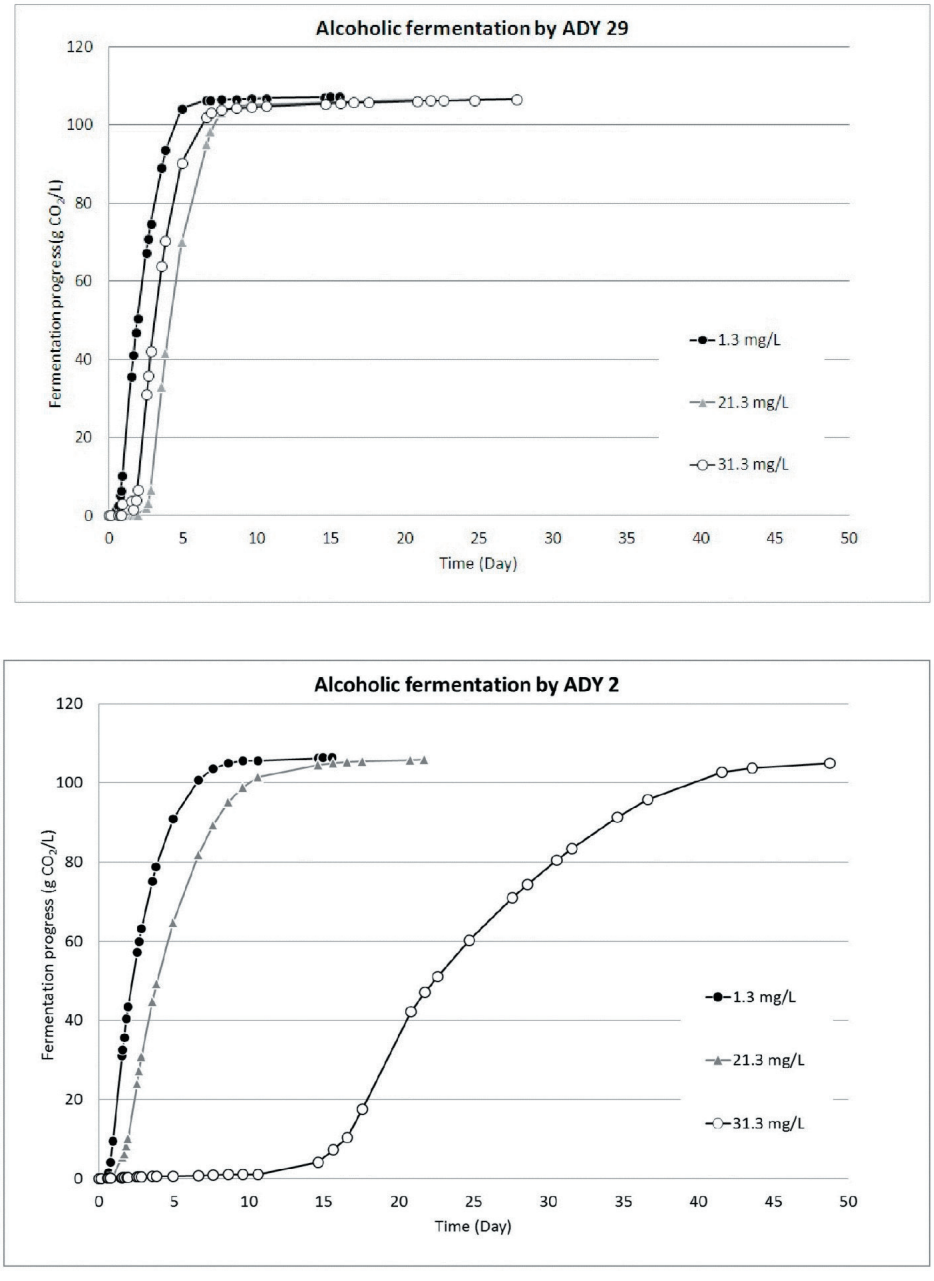

Fig. 3: Fermentation kinetics of two ADYs with different resistance to the copper concentration in grape must. A: ADY 29 – Copper resistant strain; B: ADY 2 – Copper sensitive strain.

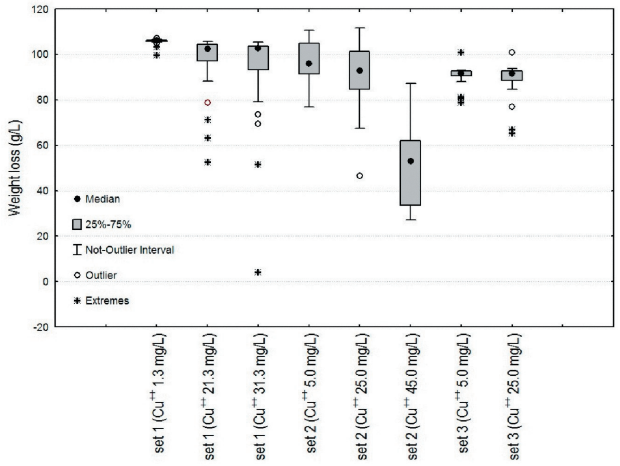

Fig. 4: Box plot of the progress of alcoholic fermentation after 20 d. Data are expressed as weight loss due to CO_2 production.

ity to copper, preventing them from effectively achieving a full degradation of sugar. A stuck fermentation gives rise to the possibility of subsequent microbial spoilage of wine, to its instability as the result of the presence of sugar residues (Bartowski 2009, Fleet 1992), and to a delay in the production process.

The microscopic counts of live/dead yeast cells ratio provide additional information about the impact of copper on yeast. The number of alive yeast cells after 24 h (Fig. 5A) in low-copper must was between 4 and 10 × 10^6 cells·mL^{-1}, as expected. According to the observations in Fig. 2, an increased copper concentration lowered cell viability to values below 3 × 10^6, and the delay in sugar consumption. Figs 5B and 5C explain the nature of this phenomenon. When copper was added (Fig. 5C), the dramatic decreases in the live/dead cell ratio (Fig. 5B), and the negative balance between the number of inoculated cells and the cells found in must show that the copper ion led to significant cell death, the intensity and rate being truly remarkable and rarely observed with other antimicrobial agents (Guzzon *et al.* 2011). Data for the other must sets agreed with these observations, indicating a cell death percentage after 24 h ranging between 80 % and 95 %, when copper was added to the grape must (data not shown). Despite the detrimental effects of copper on yeast viability in the first stages of alcoholic fermentation, the results observed suggest that yeast inhibition is not permanent and decreases in time and with the progress of alcoholic fermentation. A plausible reason for this behaviour can be found in the ability of yeast cells to chelate ions (Wang and Chen 2006) even in wine in wines (Nicolini and Larcher 2003), and the fact that copper precipitate in wine (Garcia-Esparza *et al.* 2006). The accomplishment of alcoholic fermentation observed in most of the high copper tests (Fig. 2), and the renewal of yeast cell density (Fig. 5) suggests that the abundant yeast biomass added to the must while inoculating ADYs supplies a sufficient number of cells to detoxify the copper, allowing the growth and alcoholic fermentation of the surviving cells.

Impact of the copper content in grape must on the characteristics of the wines produced: Yeast metabolism has an important impact on wine characteristics and, as seen before, a high copper concentration in must significantly affects yeast activity. Variations in wine composition due to must copper content were evaluated. The wine compounds more affected by copper addition to grape must are listed in Tab. 3. Data in Tab. 3 refer only to fermentation accomplished in 20 d, while stuck fermentation data were discarded. Acetic acid

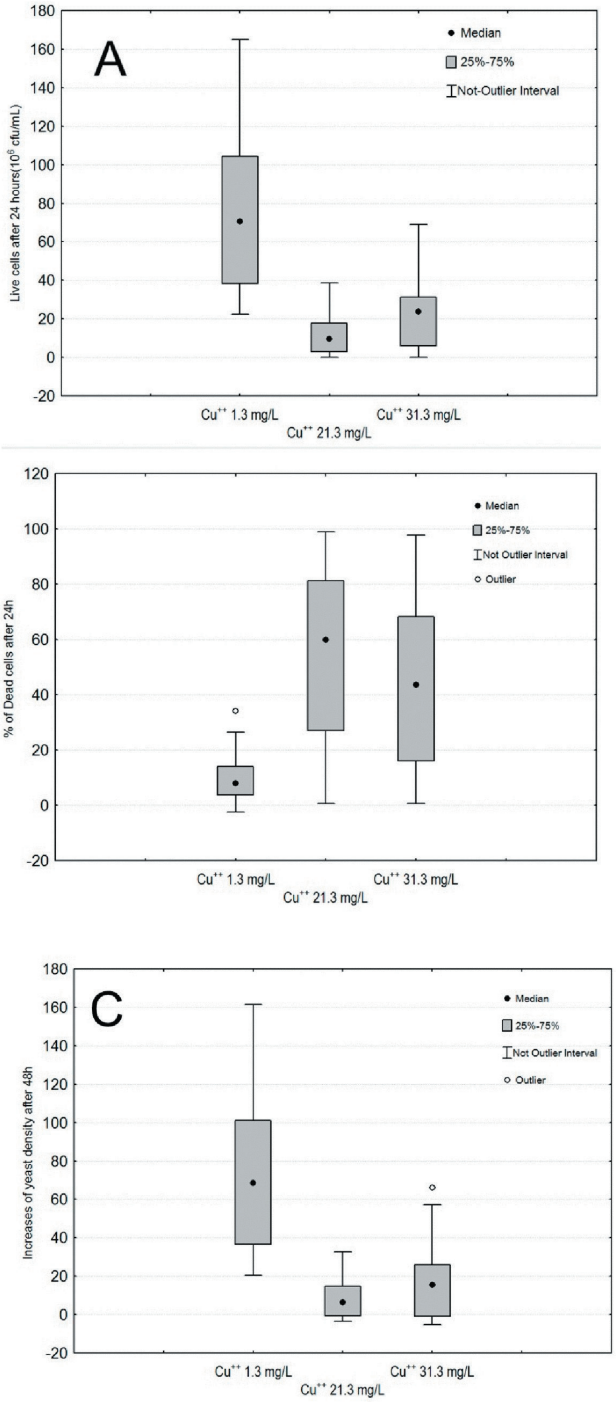

Fig. 5: Box plot of microbiological observations performed on yeast biomass after addition to the grape must. A. Yeast cell concentration after 24 h grape in must. B. Percentage of dead cells out of the total number of yeast cells counted using microscopic observation after 24 h in grape must. 3. Variation in cell number as compared to the initial inoculum after 48 h in grape must.

Table 3

Chemical composition (mean ± sd) of wines with and without the addition of copper to grape must

Year	Copper concentration in must ($mg \cdot L^{-1}$)	Acetic acid ($g \cdot L^{-1}$)	Sulphur dioxide ($mg \cdot L^{-1}$)	Ethanol (%)	Ethanol yield (%)
2005	1.3	0.20±0.07	27.1±11.3	14.0±0.2	0.59
	21.3	0.48±0.15	34.7±13.9	13.8±0.3	0.58
	31.3	0.50±0.18	55.5±19.5	13.7±0.2	0.55
2006	5.0	0.27±0.10	30.7±6.5	13.8±0.1	0.60
	25.0	0.52±0.14	54.8±12.3	13.6±0.5	0.59
	45.0	0.68±0.27	57.5±16.3	13.0±1.2	0.57
2007	5.0	0.30±0.06	19.5±6.7	11.8±0.6	0.64
	25.0	0.77±0.13	29.6±1.9	12.1±0.1	0.64

concentration increased when musts had high copper concentration. In wines obtained using non-enriched musts, acetic acid content ranged between 0.2 and 0.3 $g \cdot L^{-1}$, *i.e.* the usual concentration in wine. If copper content was around 20 $mg \cdot L^{-1}$, the acetic acid content of wine was around 0.5 $g \cdot L^{-1}$ and up to 0.77 $g \cdot L^{-1}$ (Tab. 3). An increase in acetic acid after alcoholic fermentation is generally recognised as a yeast stress signal (Erasmus *et al.* 2003, Bely *et al.* 2005). The data therefore agree with the microbiological data, also confirming that over and beyond the detoxification effect exerted by yeast cells in the first step of fermentation, the musts containing a high copper concentration remained an environment unsuitable for *Saccharomyces cerevisiae* activity.

Similar behaviour was observed in relation to sulphur dioxide excretion by yeasts during alcoholic fermentation. The high concentration of SO_2 produced after fermentation with added copper is entirely produced by yeasts, as sulphur dioxide was not added to the must at the beginning of fermentation. The production of a large amount of sulphur dioxide by ADYs, and the wide variability in their metabolism has already been observed (Guzzon *et al.* 2010). However, the link between sulphur dioxide concentration and the copper content of must is evident, and must be taken into consideration carefully, especially due to its implications in subsequent malolactic fermentation.

Conclusions

Excessive copper residue in grape musts may exert harmful consequences on the evolution of alcoholic fermentation and of the wines produced. Copper excess may cause massive death of yeast cells, leading to a significant delay in the start and in the progress of alcoholic fermentation. The reduction in cell viability is around two log units if the copper content of musts increases to 20-25 $mg \cdot L^{-1}$. Despite this, observations carried out after 20 d fermentation suggested that many yeast strains can straighten up the copper toxicity, as the extent of the differences noted in the fermentative kinetics were lower than that observed 48 h after yeast inoculation in grape must. However, excess copper in must affected the composition of the wines. The increase in acetic acid and sulphur dioxide content in wines made from must having high copper content made rise to serious doubts, both as regards the possibility of obtaining good wines from them, and in relation to the progress of subsequent steps in the production process, such as malolactic fermentation. While it is an important tool in preventing vine disease, copper must be used very carefully to prevent serious problems during the evolution of wine fermentation, even if conducted with the assistance of selected yeast cultures.

Acknowledgements

The authors wish to acknowledge CAVIT Scarl Ravina - Trento (Italy) for providing ADY samples and for the financial support.

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Received July 24, 2012