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Short-term low temperature treatments of harvested wine grapes (cv. Vermentino) affect the volatile organic compound profile of the berries

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All relevant data are within the paper and its Supporting Information files.

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Abstract: In the recent years, due to the climate change and the effects of greenhouse gases average temperatures are increasing. Grapes cultivated in Mediterranean areas are exposed to high temperatures especially during the late growing season and at harvest. This may induce undesirable biochemical processes (e.g. aroma losses and oxidative reactions) with negative effects on the berry composition and specific quality traits of the resulting wine. In the present study the effects of short-term low temperature treatments on harvested grapes before vinification have been evaluated. Bunches of wine grapes cv. Vermentino have been hand-harvested and then refrigerated at 4°C and 10°C for 24 and 48 hours, while 22°C has been applied as control temperature. Grapes were analysed in terms of technological parameters (weight loss, total soluble solids, titratable acidity, pH and total polyphenols) and volatile organic compound profile by HS-SPME GC-MS. Low-temperature post-harvest treatments affect total polyphenols content of the berries and appear to reduce the heat-related aroma loss, increase the content of four volatile terpenoids and decrease the accumulation of ethyl acetate.

1. Introduction

Several challenges characterize the wine industry and have a marked impact on the production chain, final quality of the wines and consumer acceptance. One major problem, in particular in warm-temperate climates, is represented by the increase of the average temperatures, mainly due to the accumulation of greenhouse gases, that often leads to different pheno/physiological processes in grape berry and strongly affects berry development. High temperatures induce anticipated and unbalanced ripening and, at harvest, undesirable biochemical changes such as aroma losses and oxidative processes (Ribéreau-Gayon *et al*., 2006). This negatively affects grape composition and wine quality. Hence, it is crucial to find and develop effective strategies for mitigating these negative effects. One option is represented by the application of postharvest cool-

ing treatments of the bunches.

Postharvest protocols based on controlling (lowering) temperature are used for the management and storage of fresh horticultural crops with the main goal of prolonging commercial life and freshness (Tonutti, 2013). The effects of low temperature on harvested fruits are diverse and depend on a number of factors including the fruit type, pre-harvest factors, ripening stage, the applied temperature and the duration of the treatment (Kader, 1999). Both primary and secondary metabolisms are affected (Brizzolara *et al*., 2020), with changes in the composition and quality parameters, including those related to polyphenols and volatile organic compounds (VOCs) (Valenzuela *et al*., 2017, Brizzolara *et al.*, 2018). This is also the case of table grapes that, when stored at 0°C to prolong commercial life, show changes in the VOC profiles and related-metabolic pathways, resulting in altered overall flavours (Maoz *et al*., 2019).

With other goals, post-harvest treatments can also be applied on specific crops undergoing processing. This is the case of wine grapes on which techniques such as controlled dehydration, high carbon dioxide, ozone, ethylene and pre-cooling treatments have been applied or studied to modulate the composition of the harvested berries and the style of the resulting wines (Mencarelli and Tonutti, 2013; Becatti *et al*., 2014; Mencarelli and Bellincontro, 2018). Maintaining harvested wine grapes at low temperature is a practice that is already applied in certain production areas and for specific enological purposes. Low temperature treatments prior to vinification appear to have a positive effect on the aromatic profile of the wines, especially when white-skinned berries are processed. This empirical approach has, so far, very little scientific evidence and, differently from table grapes, just few studies report the effects of such treatments on technological parameters and secondary metabolism (including aroma compounds) of wine grapes. Marais (2003) showed that keeping grapes (cv. Pinotage) overnight at 10°C and then maintaining the same temperature during skin contact with the must prior to fermentation resulted in the production of the most typical and highest quality Pinotage wines, compared to the same treatments carried out at 15°C. This effect appears to be related to changes in ester metabolism occurring in the berries. Mencarelli and Bellincontro (2018) reported that following a 10°C treatment applied on wine grapes during post-harvest partial dehydration (a practice used to produce special wines, such as the "passiti") an up regulation of genes involved in the phenylpropanoid pathway occurs together with a slight increase of stilbenes and a decrease of polyphenol oxidase activity. The present study aimed at evaluating the effect of a short-term low temperature conditioning on harvested wine grapes cv. Vermentino in terms of technological parameters and VOCs profile.

2. Materials and Methods

Grapes samples and cooling treatments

Bunches of white-skinned wine grapes (*Vitis vinifer*a L.) cv. Vermentino were hand harvested in 2018 in correspondence of an average total soluble solid (TSS) value of 21°Brix. The grapes were collected from a commercial vineyard (Lodolina) located in the hills of Candia (Massa province, Tuscany, Italy. 44°02'197.6" N, 10°11'265.9" E). The vines are trained at simple Guyot, and all agronomic practices follow the disciplinary of production for the Appellation of Controlled Origin (DOC) Candia dei Colli Apuani. After harvest, grapes were immediately transported to the laboratory and selected based on absence of evident defects or diseases. Grapes were randomly distributed into six lots (of 5 kg each) and subjected to post-harvest low temperature treatments as follow: two lots were cooled at 4°C (±0.5) for 24 (4°C 24 h) and 48 (4°C 48 h) h; two other lots were cooled at 10°C (±0.5) for 24 (10°C 24 h) and 48 (48°C 48 h) h. The last two lots were used as a control and kept at 22°C (±0.5) for 24 (22°C 24 h) and 48 (22°C 48 h) h. Immediately after harvest (T0) and at the end of each treatment,30 berries per biological replicate (three biological replicates per lot) were collected and immediately analyzed for technological parameters. For VOCs analysis 30 berries per biological replicate (five biological replicates for each treatment) were homogenized and a NaCl buffer solution (1 M) has been added (1:1) by using an UltraTurrax (Mod. T25, IKA),immediately frozen in liquid nitrogen and stored at -80°C.

Technological parameters

The weight loss (WL) of 5 bunches from each lot was measured by using a technical balance. These 5 bunches were tagged and weighed at T0 and at the end of each treatment. For each of the three biological replicates a total of 30 berries were manually pressed and the obtained must was centrifugated

(8,000 rpm, 5 min, 22°C), filtered with syringe filters (0.22 µm pore size, 33 mm diameter, Sigma-Aldrich, Italy) and used for the following analyses: pH, using a pH meter (pH-metro GLP21; Crison Instruments); TSS employing an optical refractometer; titratable acidity (TA), titrating 7.5 mL of filtered must with 0.1 N sodium hydroxide (NaOH), expressed in g/L of tartaric acid equivalent. For each of the three biological replicates, 30 berries were powdered with liquid nitrogen and total polyphenols were then extracted from 250 mg of berries powder with 1.25 mL of 80 per cent methanol and then centrifugated at 4°C, 10,000 rpm for 15 min. The total polyphenols content (TPC) was then measured using the Folin-Ciocalteau method (Singleton and Rossi, 1965), expressed as mg of gallic acid equivalents (GAE) x 100 g^{-1} fresh weight.

HS-SPME GC-MS analysis

The pre-homogenized (as described above) samples were thawed and 10 g were weighed in a 20 mL glass crimpvial for headspace analysis (Cat. No. SU860049, Sigma-Aldrich, Italy) sealed with silicone septa for SPME (Cat. No. 27362, Sigma-Aldrich, Italy). The grape samples were incubated under agitation for 30 minutes at 40°C. VOCs were sampled at the same temperature for 30 min using an SPME fiber (50/30 µm, DVB/CAR/PDMS, 1 cm long; Supelco, Bellefonte, PA, USA). The fiber was desorbed into the injector of the GC set at 250°C for 5 min (splitless mode). A Clarus 680 Gas Chromatograph equipped with a split/splitless injector (PerkinElmer®, Waltham, Massachusetts) was used for the analysis. Volatiles were separated on a fused-silica capillary column (DB-Wax, 60 m, 0.32 mm ID, 0.25 µm film thickness; Restek, Bellefonte, PA). Helium was used as carrier gas with a flow rate of 1 mL min^{-1}. The GC-MS settings employed were the same adopted by Genova and Montanaro (2012). For the identification of the compounds, a mass spectrometer (Clarus 500 Mass spectrometer, PerkinElmer®, Waltham, Massachusetts) coupled to the GC was used. Each chromatogram was deconvoluted using AMDIS software (National Institute of Standards, Gaithersburg, MD, USA). Each peak was identified by comparing the experimental spectra with those of the National Institute for Standards and Technology (NIST98, Version 2.0, USA) data bank including only compounds with 75 per cent of identity or more. The peaks were quantified using TurboMass software (TurboMass®, Version 5.4.2 PerkinElmer Inc., USA, 2008), by integration of the peak's areas. The area of each peak was normalized on the sum of the areas of all peaks detected in the same chromatogram to eliminate variations in fiber adsorption. The efficiency of the fiber was monitored by running on daily bases a quality check (QC) sample, calculating the percent of variance in the total area of the QC chromatograms. For each sampling time and treatment five biological replicates were analyzed.

Statistical analysis

Each set of replicates was tested to detect outliers performing principal component analysis (PCA) employing Metaboanalyst online tool (Chong *et al*., 2019).

One-way ANOVA was performed on technological parameter and GC-MS data following a post hoc Tukey's honestly significant difference (HSD) test (with $p = \leq 0.05$) for multiple comparation using GraphPad Prism version 7 (GraphPad Software, La Jolla California USA).VOCs revealing statistically significant differences between treatments were then analyzed by means of partial least square discriminant analysis (PLS-DA) using Metaboanalyst online too (Chong *et al*., 2019).

3. Results

Considering technological parameters, as expected all samples lost weight after 24 and 48 h, following both cooling treatments (4 and 10°C) and control conditions (22°C) (Table 1). The WL percentage was higher in the control, which showed the highest value after 48 h. The lowest WL value was recorded for grapes cooled at 4°C for 24 h. The WL of grapes cooled at 10°C for 24 h was not significantly different from samples kept at 4 and 22°C for the same time. Both cooled samples at 48h showed significantly lower WL values than the respective control. Compared to T0 samples the pH values were slightly lower in all samples except for grapes kept at 22°C for 48 h, while TA values significantly decreased only in berries kept for 24h at 4°C and in the 48 h control sample (Table 1). With the exception of this latter sample, compared to T0 a general reduction of TSS values was observed in comparison with T0 sample. TPC was significantly lower in comparison to T0 in control berries kept at 22°C for 24 and 48 h (Table 1). Low temperature treatments induced variable effects on this parameter with increases in 10°C 24 h and decreases in 10°C 48 h samples.

The grapes VOCs profile was acquired by HS-SPME GC-MS. A total of 35 VOCs has been detected. Among

Table 1 - Technological parameters in Vermentino wine grapes at harvest (T0) and after post-harvest treatments at 4°C for 24 h (4°C 24 h), 10°C for 24 h (10°C 24 h), 4°C for 48 h (4°C 48 h) and 10°C for 48 h (10°C 48 h). 22°C is the temperature of the control samples

Technological parameters	T0	4°C 24 h	10°C 24 h	22°C 24 h	4°C 48 h	10°C 48 h	22°C 48 h
Weight loss (%)	-	1.4±1.5 c	2.3±0.6 bc	4.5±1.9 b	2.8±1.5 bc	4.5±1.5 b	9.3±2.1 a
pH	3.46±0.02 b	3.40±0.0 c	3.33±0.0 d	3.34±0.0 d	3.39±0.0 c	3.36±0.01 d	3.50±0.0 a
Titratable acidity (g/L^{-1})	4.6±0.2 ab	4.2±0.0 c	5±0.1 a	4.5±0.0 b	5±0.1 a	4.7±0.0 a	3.7±0.0 c
Total soluble solid (° Brix)	21±0.0 a	19±0.0 b	16.5±0.4 d	19±0.0 b	17.6±0.3 c	18±0.0 c	20.4±0.2 a
Total polyphenols content (GAE/100 gr FW)	613.6±60.4 b	550±47.3 bc	722.7±84.5 a	560.4±9 c	555.6±83 bc	492.1±77.2 c	363.3±46.7 d

Different letters indicate statistically significant differences at p≤0.05 according to the results of the Tukey's HSD test. Values are the mean of three biological replicates +/- SD.

them, 14 terpenes, 7 esters, 4 alcohols, 3 aldehydes, 2 alkanes, 2 benzene derivates, 1 ether, 1 alkane and 1 phenol were identified. One-way ANOVA test was run on the whole VOCs dataset: a total of 11 compounds resulted significantly different (p≤0.05) between treatments (data not shown). Among the 11 statistically significant VOCs, 5 compounds of interest, known for their impact on grapes and wine aroma, were present and so used for a PLS-DA analysis. These compounds were three sesquiterpenes (cadinene, cubebene and isoledene) and the monoterpene dihydro-citronellol, which are generally associated with floral and spicy notes, and ethyl acetate, which is considered an off-flavor and associated with the anaerobic metabolism. The PLS-DA was carried out separately for the two sampling times. Cadinene, cubebene and isoledene, dihydro-citronellol and ethyl acetate levels were used as predictor variables, while the different treatments and T0 were used as response variable. The effect of the 24 h treatment is reported in figure 1. After 24 h of treatment, the model explained 51.6 per cent of the variability present in the dataset and in this projection the different treatments and T0 samples partially overlaps (Fig. 1A). Figure 1B reports the VIP scores for the employed features. The highest score is attributed to cubebene, which seems to be strongly accumulated in berries held at 10°C. Noticeably, the level of all the terpenoids considered is higher in the cooled grapes, regardless the temperature, with the only exception of the monoterpene dihydro-citronellol which showed the lowest level in 4°C sample. Interestingly, the most marked variation for the three sesquiterpenes (cubebene, cadinene and isoledene) is observed when comparing cooled with T0 samples. On the other hand, control grapes kept at 22°C are characterized by an accumulation of ethyl acetate. A slight accumulation of this compound is found also in grapes held at 10°C (Fig. 1B).The effect of 48 h treatment is reported in figure 2. The model explains 55.3 per cent of the variability with still an overlapping of the treatments (Fig. 2A). Fig. 2B reports the VIP scores for the employed features. The highest score is attributed to isoledene, which is markedly accumulating in berries kept at 10°C. Furthermore, cooled grapes showed again a higher content of terpenoids comparing with the T0. As far as ethyl acetate is concerned, this compound shows the highest level in the control grapes and the lowest in T0 samples.

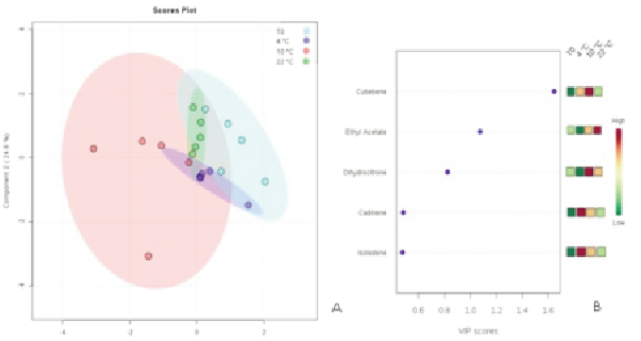

Fig. 1 - A) Partial least squares discriminant analysis (PLS-DA) performed on VOCs detected in Vermentino grapes following cooling treatment at 4 and 10°C for 24 h and control treatment at 22°C. Cadinene, cubebene, dihydrocitronellol, isoledene and ethyl acetate levels were used as predictor variables while the different treatments and T0 were used as response variables. Each color represents different treatment with five replicates. 95% confident intervals are presented in ellipses. B) The variable importance in projection scores of PLS-DA (VIP scores). The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

4. Discussion and Conclusions

In detached fruits, WL progresses with time and is dependent on the vapour pressure deficit, the evapo-

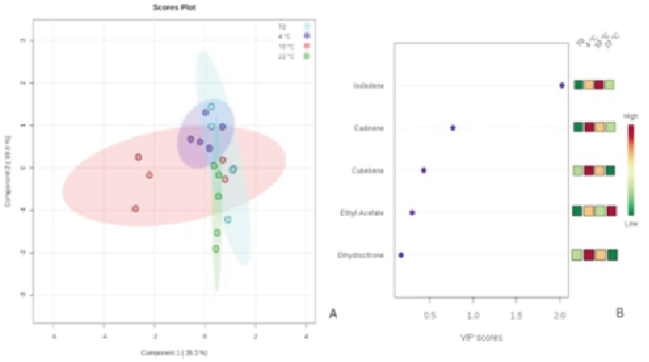

Fig. 2 - A) Partial least squares discriminant analysis (PLS-DA) performed on VOCs detected in Vermentino grapes following cooling treatment at 4 and 10 °C for 48 h and control treatment at 22°C. Cadinene, cubebene, dihydro-citronellol, isoledene and ethyl acetate levels were used as predictor variables while the different treatments and T0 were used as response variables. Each color represents different treatment with five replicates. 95% confident intervals are presented in ellipses. B) The variable importance in projection scores of PLS-DA (VIP scores). The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

rative driving force for water movement and affected by both temperature and relative humidity (Cirilli *et al*., 2012). Harvested fruits, including grape berries, already react at low WL values with metabolic changes eventually affecting grape composition (Costantini *et al*., 2006; Rizzini *et al*., 2009; Tonutti and Bonghi, 2013). In the present trial, control grapes that showed the highest WL values most likely underwent specific water stress-related reactions more pronounced than those occurring in low temperature samples. Previous studies (Bellincontro *et al*., 2009) have demonstrated that a temperature between 5 and 10°C helps to reduce weight loss and to maintain the cellular structure of the berries, with a general reduction of metabolic events. In both control samples (kept for 24 or 48 h at 22°C), high values of TSS well correlate with the loss of weight and the consequent concentration of solutes. The variability present among samples for this parameter but also for pH and TA might be the consequence of the heterogeneity of the samples, collected in a commercial vineyard.

The effects of cooling grapes before vinification on these technological parameters appear to be more clearly defined after 48 h of treatment. This appears also true concerning TPC that increased in cooled samples after 48 h. The effect of postharvest low temperature on TPC has been reported for table grapes by Maoz *et al.* (2019) who showed that storage at 0°C for 6 weeks, led to an upregulation of several genes involved in the phenylpropanoid pathway and to the accumulation of stilbenes and flavonoids. Maintaining harvested berries at 4-10°C can be considered as mild stress: it is well known that postharvest cold stress induces changes in fruit secondary metabolic pathways and compounds, including phenylpropanoids (Dixon and Paiva, 1995; Ruiz-García and Gómez-Plaza, 2013; Mencarelli and Bellincontro, 2018).

Concerning the volatile compounds, our results indicate that, as general effect, low temperature conditioning of Vermentino grapes has an impact on the volatile terpenoid content of the berries. It is well known that the presence of terpenoids significantly affects the aroma of grapes and wines (D'Onofrio *et al*., 2017), and this is particular important for wines vinificated from neutral variety such as Vermentino. Terpenoids are classified based on the number of carbons present in the chemical structure: monoterpenes (10 carbons), sesquiterpenes (15 carbons), diterpenes (20 carbons), triterpenes (30 carbons), and carotenes (40 carbons) (Yu and Utsumi, 2009; Li *et al.*, 2019). Among the different classes, a significant influence on the aroma of grapes and wine has been attributed to the monoterpene class which, in wine, is generally associated with pleasant floral notes (D'Onofrio, 2011). Along with monoterpenes, sesquiterpenes are another important subclass. To date, there has been limited research on sesquiterpenes since they are considered less volatile and aroma-active than monoterpenes (May and Wüst, 2012; Black *et al*., 2015). However, sesquiterpenes have been recently correlated with significant organoleptic characteristics of grapes (D'Onofrio *et al.*, 2017). Indeed, their concentrations in berries can be crucial for the final wine quality (Luo *et al*., 2019) since sesquiterpenes are more stable than monoterpenes and once extracted from the berry they can be retained in the finished wine (Dunlevy *et al*., 2009). It has been suggested that they provide balsamic, woody and spicy notes (Slaghenaufi and Ugliano, 2018). Based on our preliminary results, it can be hypothesized that low temperature post-harvest treatment is effective in improving specific aromatic traits of Vermentino berries and, possibly, wines. The observed increase of terpenoids could be the result of changes in specific metabolic steps of this chemical class. A key reaction is the conversion of farnesyl pyrophosphate (FPP) to sesquiterpenes, catalyzed by the different members of the terpene synthase (TPS) family (Tholl, 2006; Muhlemann *et al*., 2014). It is well known that TPS activity and so terpenoids

biosynthesis strongly depends on both endogenous and environmental factors (Robinson *et al*., 2014). Specific studies performed on grape berries in the field showed that high temperatures could reduce terpenoids biosynthesis, and also induce the degradation of thermolabile compounds (D'Onofrio, 2011). Concerning specifically post-harvest, TPS have been studied in toon buds by Zhao *et al.* (2019). They found a strong increase of transcripts related to terpenoid biosynthesis under low temperature condition, which resulted with sesquiterpenoid accumulation. Additional studies are so needed to understand if the observed increase of terpenoids in Vermentino berries is due to a low-temperature induced biosynthesis or just a maintenance of the pre-accumulated compounds.

The use of low temperature in post-harvest and food production is widespread. This preliminary study shows that cooling treatment immediately after harvest have significant metabolic effect on wine grapes. As general effect, it seems that cooling treatments are effective in improving the terpenoids-related aroma pool of the neutral variety Vermentino. This effect appears to be strongly dependent on the applied temperature as well as on the treatment duration: the specific effects of cooling and the interplay between these two parameters (temperature x treatment duration) need to be further elucidated. In fact, the time-course of events occurring in detached fruits (progression of senescence and water loss) may amplify/widen, or limit/reduce the metabolic changes induced by low temperature treatments. In addition, these preliminary results obtained on wine grape berries need to be implemented and compared with the technological and organoleptic evaluations of the resulting wines.

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