Plant Physiology and Biochemistry 82 (2014) 270–278

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

# Metabolic changes of *Vitis vinifera* berries and leaves exposed to Bordeaux mixture

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ARTICLE INFO

*Article history:*
Received 5 May 2014
Accepted 25 June 2014
Available online 3 July 2014

*Keywords:*
Bordeaux mixture
Copper
Grape berry
Metabolomic profile
*Vitis vinifera*

ABSTRACT

Since the development of Bordeaux mixture in the late 1800's, copper-based fungicides have been widely used against grapevine (*Vitis vinifera* L.) diseases, mainly in organic but also in conventional viticulture; however their intensive use has raised phytotoxicity concerns. In this study, the composition of grape berries and leaves upon Bordeaux mixture treatment was investigated during the fructification season by a metabolomic approach. Four applications of Bordeaux mixture till 3 weeks before harvest were performed following the regular management practices of organic viticulture. Results showed that the copper-based treatment affected the content in sugars, organic acids, lipids and flavan-3-ols of grapes and leaves at specific developmental stages. Nonetheless, the levels of sucrose, glucose and fructose, and of tartaric and malic acids were not significantly affected in mature grapes. In contrast, a sharp decrease in free natural amino acids was observed, together with a reduction in protein content and in mineral nitrogen forms. The treatment with Bordeaux mixture increased by 7-fold the copper levels in tissue extracts from surface-washed mature berries.



## 1. Introduction

The control of grapevine fungal diseases has been a great challenge to winegrowers worldwide, downy mildew being one of the most dangerous diseases in the European viticulture (Loureiro et al., 2012). In response to these demands, several fungicides have been developed, pioneered by the Bordeaux mixture. This contact fungicide, containing copper sulphate and slaked lime, was discovered by chance in October 1882 in the Médoc region of France, and is still being applied by many grape growers (Moutinho-Pereira et al., 2001), mainly in organic farming since copper is a natural (non-synthetic) element, proven to be more effective than several alternative treatments, particularly against downy mildew (Spera et al., 2003; La Torre et al., 2008). Also, the protective effect of the application of this broad-spectrum fungicide against the scorching of clusters has been reported, associated to decreased leaf temperature, ultimately modulating grapevine vigour and development (Moutinho-Pereira et al., 2001; Greer and Weedon, 2012). The use of Bordeaux mixture has early been extended to other crops including citrus, apple and potato (Speare, 1922; Moore, 1930; Large, 1945). However, controversy regarding the effects of copper on plant health and soil contamination has risen, and impacts of copper on grapevine vigour and physiology have been investigated. Excess copper has been associated with decreased root and shoot growth, reduced leaf number, altered photosynthetic and transpiration rates, alterations in the levels of chlorophylls, carotenoids, and in the content of mineral elements, sugars and lipids in the vegetative parts of the plant (Romeu-Moreno and Mas, 1999; Moutinho-Pereira et al., 2001; Toselli et al., 2009).

Several compounds are essential in grape berry quality, including sugars, phenolics, organic acids, amino acids and mineral

*Abbreviations:* BSA, bovine serum albumine; DW, dry weight; ETAAS, electrothermal atomization atomic spectrometry; GC-TOF-MS, gas chromatography-time of flight-mass spectrometry; HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; PCA, principal component analysis; PTFE, polytetrafluoroethylene; PVPP, polyvinylpolypyrrolidone; TCA cycle, tricarboxylic acid cycle.

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http://dx.doi.org/10.1016/j.plaphy.2014.06.016

elements. The major forms of stored sugars in the grape berry are glucose and fructose which may reach concentrations of 1.5 M (Agasse et al., 2007). Tartaric and malic acids are essential for stabilizing the wine pH and contribute to the overall taste of fruits (Ford, 2012). Phenolic compounds also contribute greatly to the colour, flavour, aroma and astringency of grape and wine and many of them present powerful antioxidant properties (Conde et al., 2007; Castellarin et al., 2012; Teixeira et al., 2013). During vinification, the specific conditions of fermentation, ageing and storage modify or degrade compounds accumulated in the grape berry, giving rise to the wine's alcohol content, colour and secondary and tertiary aromas.

Previous studies showed that grape cells are able to accumulate copper intracellularly (Martins et al., 2012b), and that Bordeaux mixture induces a reprograming of the expression of grapevine Ctr-type copper transporters (Martins et al., 2014a,b), but the metabolite alterations in grape berries in response to copper-based fungicides remained elusive. In the present study, we aimed at characterizing the effects of Bordeaux mixture on the metabolite profile of grape berries and leaves, but particular emphasis was given to fruit composition at the mature stage. Results showed that the copper-based treatment during the fructification season alters the content of key solutes of the grape berry, including sugars, acids and flavan-3-ols, but in the mature fruit major differences were only observed in the levels of both mineral and organic forms of nitrogen.

## 2. Material and methods

### 2.1. Vineyard treatments and sample collection

Field experiments performed during 2012 were conducted with eight-year old grapevines cv. “Vinhão” from a commercial vineyard established in the north of Portugal [coordinates: 41°25′14.06″ latitude and 8°14′38.80″ longitude], in the region of ‘Vinhos Verdes’. Fungicide applications were performed as follows: “control” grapevines were treated throughout the season with a conventional triazole-based fungicide and “copper-treated” grapevines were sprayed with Bordeaux mixture (20 g $L^{-1}$ $CuSO_4$ + 20 g $L^{-1}$ $Ca(OH)_2$), following the regular vineyard management practices applied in commercial farms. Both control and copper-treated grapevines were cultivated under the same microclimate. Four copper treatments were performed throughout the season, every 15 days, the first performed at the pea size and the last performed 3 weeks before harvest. Three bunches and 9 leaves were collected from each grapevine at each of the following grape berry developmental stages: green stage (pea size, E-L number 31; Coombe, 1995), veraison stage (berry begins to colour and enlarge, E-L number 35) and mature stage (berries harvest-ripe, E-L number 38). Samples were frozen immediately in liquid nitrogen and stored separately at −80 °C.

### 2.2. Copper quantification in grape berries and in grape juice

For copper quantification studies, grape berries from copper-treated and control plants were rinsed thoroughly in milli-Q $H_2O$ to remove any residual external copper traces and dried briefly in filter paper. Copper quantification in leaf tissues was not performed because some copper remained adhered to the leaves surface, even after a careful washing. Whole berries were ground in liquid nitrogen to obtain grape berry homogenate which was lyophilized for 7 days, and a set of mature berries was manually crushed to extract grape juice which was filtered through a 0.45 μm membrane. Digestion of the juice and of the lyophilized berry homogenates was performed by high pressure microwave *digestion in a* Milestone ETHOS Plus Microwave Labstation (Milestone, Sorisole, Italy) and copper quantification was performed by electrothermal atomization atomic spectrometry (ETAAS) in a PerkinElmer Model 4110 ZL graphite furnace atomic absorption spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA), using Zeeman-effect background correction, equipped with a model AS-72 autosampler and the PerkinElmer AAWinLab™ software, version 2.5 (Catarino et al., 2005, 2010).

### 2.3. Metabolomic analysis of grape berries and leaves by GC-TOF-MS

To obtain detailed information on the metabolomic profile of copper-treated and control plants, grape berries and leaves at green, veraison and mature stages were ground in liquid nitrogen, lyophilized for 7 days, and subject to an untargeted metabolomic analysis. Metabolite extraction from the lyophilized samples and analysis by GC-TOF-MS were carried out in West Coast Metabolomics Center, UC Davis, as described by Fiehn et al. (2008). The metabolite extraction was performed in methanol, chloroform, and water in proportions of 5:2:2. After extraction and derivatization, samples were injected in split-less mode with a cold injection system (Gerstel, Germany) and analysed by GC (Agilent 6890, San Jose, USA) using a Rtx 5Sil MS column (30 m × 0.25 mm, 0.25 μm film thickness) and an integrated guard column (Restek, Bellefonte, USA). The GC was connected to a Leco Pegasus IV TOFMS spectrometer controlled with Leco ChromaTOF software v.2.32 (Leco, St. Joseph, USA). Peak detection and mass spectra deconvolution were performed with Leco Chroma-TOF software v.2.25. GC–MS chromatograms were processed following Fiehn et al. (2008). Further analysis after deconvolution was made using the semi-automated workflow of the UC Davis Genome Center Metabolomics Laboratory (Fiehn et al., 2005). Metabolite data were normalized using the dry weight (DW) of the samples.

Data transformation ($log_2$) and normalization were performed in GeneMaths XT software, in which the offset was determined by the average of relative abundance values and the scaling was defined according to the standard deviation. Heat maps and Principal Component Analysis (PCA) were performed to discriminate the grape berry and leaf metabolite profiles and to compare the alterations in metabolite levels throughout fruit development upon copper treatment. Hierarchical cluster analysis was performed to study the degree of similarity in accumulation patterns.

### 2.4. Protein quantification

Whole mature grape berries were homogenized in liquid nitrogen and total protein was extracted in cold extraction buffer (150 mM MOPS, pH 7.5). The protein concentration was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin (BSA) as standard.

### 2.5. Mineral nitrogen quantification

The concentration of nitrate, nitrite and ammonium was determined by colourimetric methods in lyophilized homogenates of mature grape berries. One hundred mg of grape berry powder (DW) were added to 8 ml of milli-Q $H_2O$ and vortexed vigorously for solubilization of intracellular contents. Polyvinylpolypyrrolidone (PVPP) was used to precipitate anthocyanins that could interfere with the quantification methods. The homogenate was centrifuged at 14,000 × g for 10 min at 4 °C and the supernatant was collected and filtered through 0.2 μm PTFE membranes. Nitrate and nitrite quantification was performed according to the method of Carvalho et al. (1998), where the reagent volumes were scaled down for processing 250 μl of sample.

Ammonium quantification was adapted from the method of Hernández-López and Vargas-Albores (2003), where the reaction volume was scaled up for processing 1 ml of sample.

### 2.6. Quantification of grape berry free amino acids

Whole mature grape berries from copper-treated and control plants were ground in liquid nitrogen and lyophilized for 7 days. Extraction was performed by adding 25 ml of milli-Q $H_2O$ to 1 g of lyophilized sample and quantification of natural free amino acids (excluding tryptophan) was performed in a Biochrom 30 Amino Acid Analyser with a weak acidic cation exchange resin acting as stationary phase (200 × 4.6 mm column) and a number of weak acidic Li-citrate buffers acting as mobile phase. Stepwise pH, temperature and salt concentration gradients were applied. Detection after post column derivatization with Ninhydrin (135 °C) was performed at 570 or 440 nm. For tryptophan quantification, the sample solutions were diluted in milli-Q $H_2O$ (1:10) and analysis was performed using a Beckman System Gold HPLC equipped with an Allsphere C8, 250 × 4.6 mm (stationary phase) and using a phosphate buffer/MeOH gradient (mobile phase). Detection was performed by fluorimetry, with emission wavelength set at 340 nm and excitation wavelength set at 280 nm.

### 2.7. Statistical analysis

The data presented consists of the results obtained from three independent experiments (samples from each grapevine being one biological replicate) for each treatment at each stage, and are represented as the Mean ± SE. Metabolite levels of samples collected from copper-treated plants were compared with those of samples collected from control plants by the Student's *t*-test using Prism® 5 (GraphPad Software, Inc.) and GeneMaths XT software. In figures and tables, the values are marked with asterisks to denote the significance levels as compared to control: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

## 3. Results and discussion

### 3.1. Effect of Bordeaux mixture on copper levels in grape berries and grape juice

Grape berries were collected from grapevines treated with Bordeaux mixture (+Cu) and from control grapevines (−Cu), at the green, veraison and mature stages, and were extensively washed before the determination of copper levels by atomic spectrometry, as described in Material and Methods. Results showed that copper contents decreased during berry development in control plants, from 12.7 at pea-size to 4.5 μg $g^{-1}$ DW at maturity, probably reflecting the essential role of copper in photosynthesis. In grapevines treated with Bordeaux mixture, the berries' copper content was 7–14-fold higher than in control fruits, changing from 178.4 μg $g^{-1}$ DW in the green phase, 3 days after the first treatment, to 33.3 μg $g^{-1}$ DW in the mature phase, 3 weeks after the last treatment (Fig. 1A). Copper concentrations in grape juice from mature fruits sampled from grapevines treated with Bordeaux mixture were 4-fold higher than in the control, reaching levels of 1.6 mg $L^{-1}$ (Fig. 1B). Values between 0.05 and 37.6 mg $L^{-1}$ of copper in grape juices have been reported in the literature (Eschenbruch and Kleynhans, 1974; Darriet et al., 2001; Olalla et al., 2004; Catarino et al., 2010). Copper levels in the juice may affect the fermentation kinetics with consequences in wine quality (Darriet et al., 2001; Cavazza et al., 2013).

The observed modification of copper status is likely to be related to the measured alterations in the grape berry and leaf metabolic profiles. We showed recently that the increased copper levels in the fruit after Bordeaux mixture application (Fig. 1A) induced a reprograming of the expression of grapevine Ctr-type copper transporters (Martins et al., 2014a,b). However, other effects of Bordeaux mixture, including alterations in light absorbance and photosynthesis (Moutinho-Pereira et al., 2001) may also interfere with the metabolome of leaves and berries.

### 3.2. Changes in key solutes of grape berries and leaves throughout the fructification season in response to Bordeaux mixture

A total of 229 metabolites were detected by GC-TOF-MS in grape berry samples, 75 of which were unequivocally identified (Supplementary Table SI). In leaves, from a total of 425 detected compounds, 131 metabolites were unequivocally identified (Supplementary Table SII). Principal Component Analysis (PCA) plot readily discriminated the grape berry and leaf samples, as well as the various stages of fruit development (Supplementary Fig. S1). As depicted in Fig. 2, several metabolites were found in both grape berry and leaf samples, including intermediates of glycolysis, TCA cycle and nitrogen metabolism. The relative abundance of the metabolites was affected by the application of Bordeaux mixture depending on the grapevine organ and on the fruit developmental

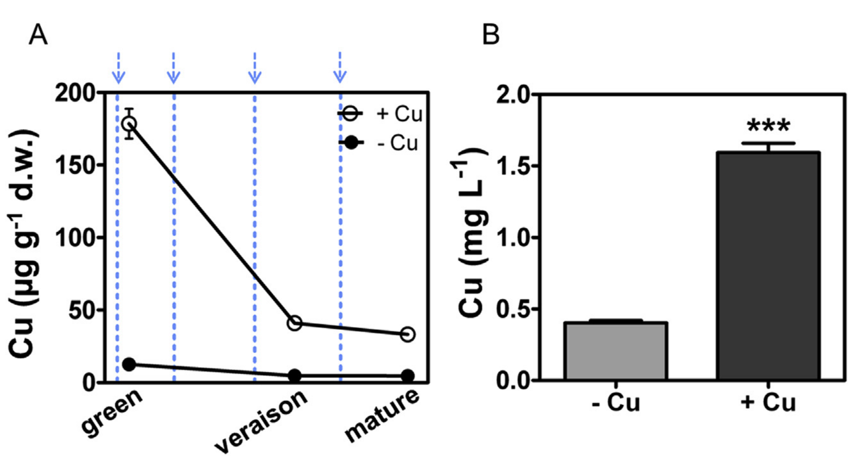


**Fig. 1.** Copper levels in grape berries cv. "Vinhão" (A) and in grape juice (B). Fruits were collected from plants treated with a triazole-based fungicide (control, −Cu) and from plants treated with Bordeaux mixture (+Cu), at the green, veraison and mature stages, and grape juice was extracted from mature fruits. Dotted lines indicate the temporal application of the Bordeaux mixture in the vines. Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control: ***$P \leq 0.001$.

stage. Due to their importance in fruit and wine quality, particular attention was given to changes in the content of key solutes of the grape berry, namely sugars, organic acids and some secondary metabolites.

Several sugars were detected in grape berries and leaves from both copper-treated and control plants, including sucrose, glucose, fructose, rhamnose, levanbiose and lyxose (Supplementary Tables SI, SII). Consistent with photoassimilate transport from source to sink organs, the sucrose levels detected in leaves were higher than in berries (Fig. 3) since this disaccharide is imported to fruits in the phloem and is mostly converted to fructose and glucose by cell wall-bound invertases, sustaining the berry growth and development (Agasse et al., 2007; Conde et al., 2007; Davies et al., 2012). Accordingly, the levels of glucose and fructose exceed those of sucrose in fruits (Fig. 3). The treatment with Bordeaux mixture did not affect significantly the levels of sugars in mature grape berries. However, a 2-fold increase was observed in the fructose content at veraison stage. In leaves, the levels of these sugars also remained mostly unaffected by Bordeaux mixture application, with the exception of the observed increased sucrose levels at the green stage. Previous studies performed in tissue-cultured grapevines showed that sucrose levels in leaves remained unchanged in conditions of excess copper in the substrate (Romeu-Moreno and Mas, 1999).

The massive accumulation of sugars in the berry from veraison onwards was accompanied by a sharp decrease in the levels of tartaric and malic acids throughout the season (Fig. 4) as previously reported (Davies et al., 2012; Ford, 2012), and the treatment with Bordeaux mixture did not significantly alter these profiles. In addition to tartrate and malate – the major organic acids in the berry -, other organic acids, including citrate, fumarate and succinate were detected in all samples (Supplementary Tables SI, SII).

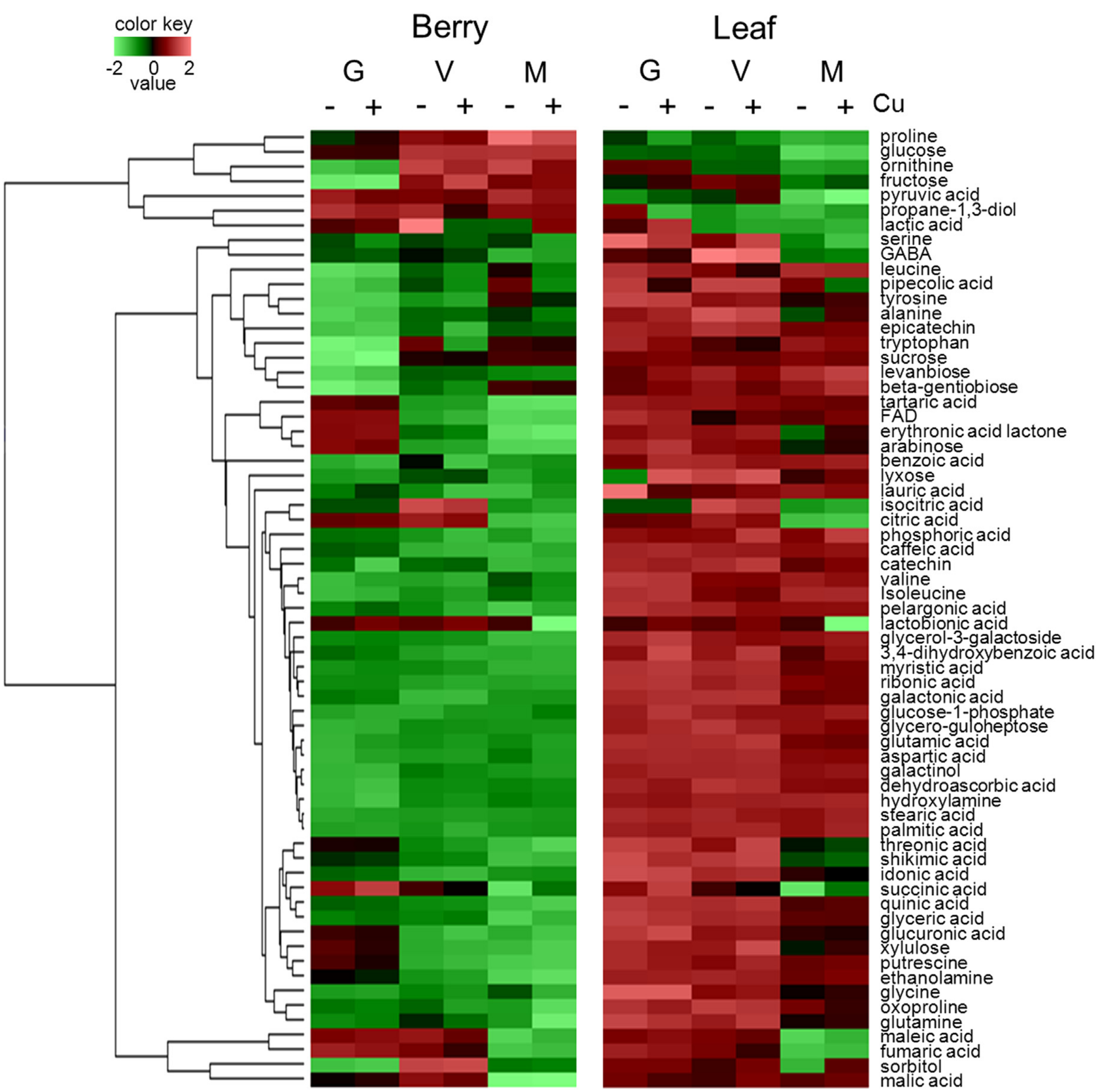


**Fig. 2.** – Heat map and clustering of metabolite changes in grape berries and leaves from vines cv. "Vinhão" treated with a triazole-based fungicide (−) or with Bordeaux mixture (+) throughout the season. Green (G), veraison (V) and mature (M) stages of fruit development. Common metabolites identified in both grape berry and leaf samples are shown. Each row represents a metabolite and each column represents the stage of berry development and the fungicidal treatment. Values were centred and scaled in the row direction to form virtual colours as presented in the colour key, in which the offset was determined by the average of relative abundance values and the scaling was defined according to the standard deviation. Metabolites that showed a similar profile were clustered together.

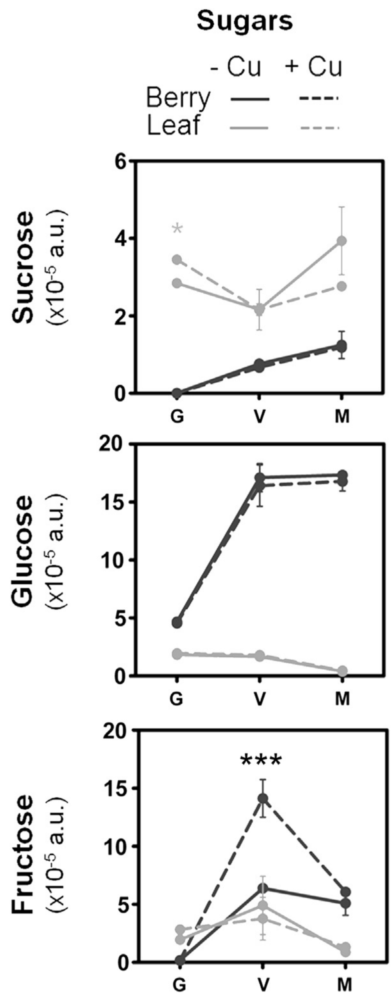


**Fig. 3.** Sugar levels in grape berries and leaves from vines cv. "Vinhão" treated with a triazole-based fungicide (- Cu) or with Bordeaux mixture (+Cu) throughout the season. Green (G), veraison (V) and mature (M) stages of fruit development. Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control samples: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

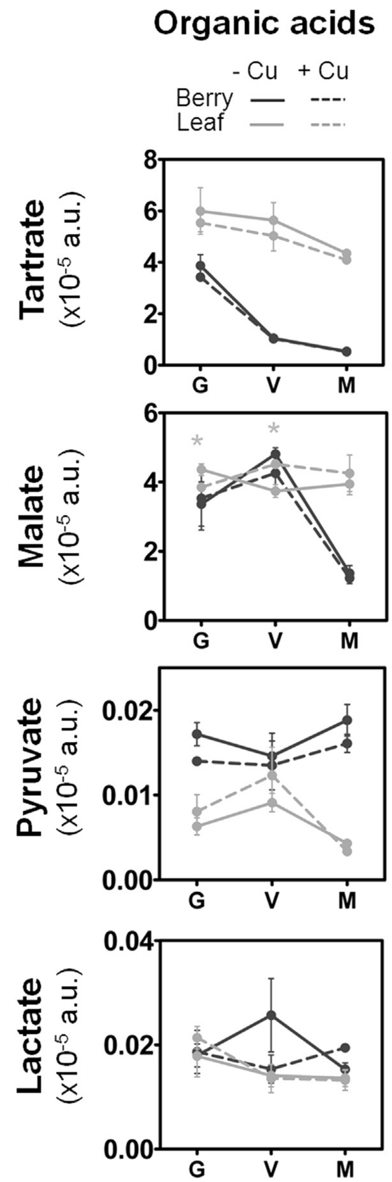


**Fig. 4.** Levels of organic acids in grape berries and leaves from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with Bordeaux mixture (+Cu) throughout the season. Green (G), veraison (V) and mature (M) stages of fruit development. Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control samples: $^{*}P \leq 0.05$.

The levels of citric acid also remained unaffected by Bordeaux mixture application (Supplementary Table SI), but the levels of pyruvic acid, a central intermediate in several metabolic pathways, suffered an apparent reduction in green berries and a decreasing trend was also observed at the mature stage. In contrast, the copper-based treatment caused an apparent increase in lactic acid levels at mature stage (Fig. 4). In leaves, the levels of malic, succinic and citramalic acids were significantly affected by Bordeaux mixture application, at the green and veraison stages of fruit development (Fig. 4, Supplementary Table SII). As a whole, results showed that the sugar/acid balance in ripe grapes was not significantly affected by the application of Bordeaux mixture.

Grape lipids act as precursors in the synthesis of wine aroma compounds, including volatile fatty acids (Conde et al., 2007). In this study, several lipids including stearic, palmitic, lauric and myristic acids were identified in both grape berries and leaves. As shown in Fig. 5, leaves were richer in stearic, palmitic and lauric acids than grape berries. The treatment with Bordeaux mixture did not significantly affect the levels of these acids in ripe grape berries. However, a significant decrease was observed in the content of palmitic acid at veraison stage. In leaves, the levels of lauric acid were reduced by the copper-based treatment at green stage of fruit development, while the levels of palmitic acid were higher at the harvest stage.

Our metabolomics analysis detected only a few secondary metabolites, including well-known hydroxycinnamates, such as benzoic acid, 3,4-dihydroxybenzoic acid and caffeic acid, and flavan-3-ols, namely catechin and epicatechin (Supplementary Tables SI, SII). These flavan-3-ols are the most abundant class of phenolics in the grape berry and catechins are responsible for bitterness in wine and may also be partially associated with astringency (Adams, 2006; Conde et al., 2007). This contribution is even higher in red varieties since the grape berry skin is very rich in flavan-3-ols (Teixeira et al., 2013). As shown in Fig. 6, the treatment with Bordeaux mixture did not significantly affect the levels of shikimic and benzoic acids in grape berries. The levels of catechin in fruits decreased by 4-fold at the green stage, when it is synthesized (Terrier et al.,

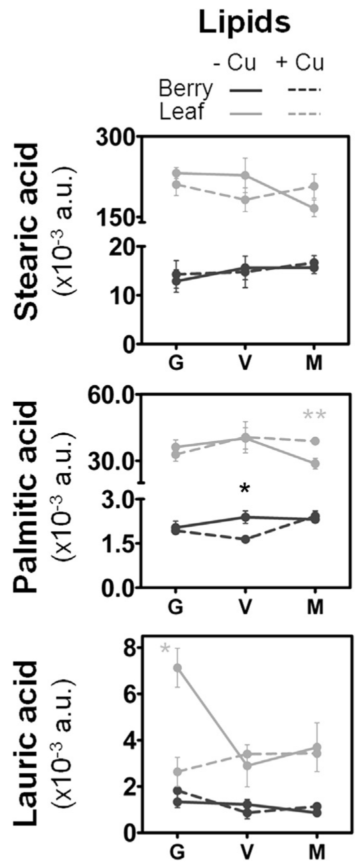


**Fig. 5.** Lipid levels in grape berries and leaves from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with Bordeaux mixture (+Cu) throughout the season. Green (G), veraison (V) and mature (M) stages of fruit development. Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control samples: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

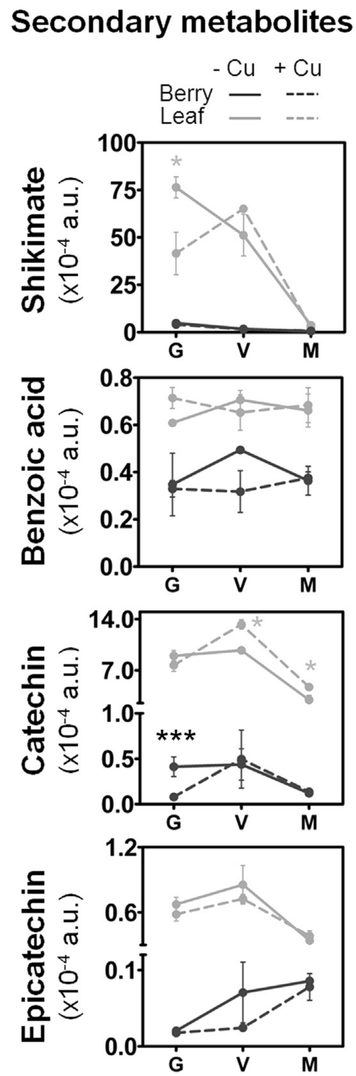


**Fig. 6.** Secondary metabolite levels in grape berries and leaves from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with Bordeaux mixture (+Cu) throughout the season. Green (G), veraison (V) and mature (M) stages of fruit development. Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control samples: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

2009), but no effect was observed at maturity. The levels of epicatechin were not affected in fruits. In leaves, the most dramatic changes were detected in the levels of shikimic acid at the green stage of fruit development, and in catechin levels from veraison onwards. The content in α-tocopherol, 3,4-dihydroxybenzoic acid and gallic acid was also significantly affected by Bordeaux mixture application in leaves (Supplementary Table SII). Recent studies have shown that the phosphate and sulphate status, as well as interactions with pathogens and epiphytic yeast, influence the accumulation of secondary metabolites in grapevine, such as the anthocyanins, and the expression of genes coding for enzymes involved in their biosynthetic pathways (Yin et al., 2012; Borges et al., 2013; Rühmann et al., 2013; Tavares et al., 2013). Further studies aiming at the targeted characterization of berry phenolics will allow a complete understanding of the effect of Bordeaux mixture in berry flavours and aromas.

### *3.3. Effect of Bordeaux mixture in nitrogen content and protein levels of ripe grape berries*

As shown by GC-TOF-MS, the levels of most amino acids increased throughout fruit development, peaking at mature stage, and Bordeaux mixture applications resulted in consistent alterations in their profile and relative amount (Supplementary Table SI). This prompted us to perform absolute quantification of free amino acids and of key mineral nitrogen forms in mature fruits. As shown in Fig. 7, the total free amino acid content in fruits from copper-treated plants was reduced by 40%. This reduction was associated to a 24% decrease in protein content.

In plants, nitrogen is firstly taken up from the soil as inorganic nitrate, which is further converted to nitrite and ammonium, the latter being the primary precursor of free natural amino acids (reviewed by Martins et al., 2012a). As shown in Fig. 8, the nitrate levels detected in control fruits (6 mg $g^{-1}$ DW) were 100-fold higher than nitrite levels, indicating that the latter is rapidly converted to ammonium. The treatment with Bordeaux mixture caused a reduction in the levels of both nitrate and nitrite, but no significant changes were observed in ammonium content.

The quantification in a Biochrom 30 Amino Acid Analyser (and by HPLC) showed a general reduction in the levels of nineteen free natural amino acids when grapevines were treated with Bordeaux mixture, which was more severe for asparagine, glutamine, proline and all the basic amino acids, namely arginine, histidine and lysine (Fig. 9). Arginine and proline were the most abundant amino acids detected in control berries and decreased by 48% and 34%, respectively.

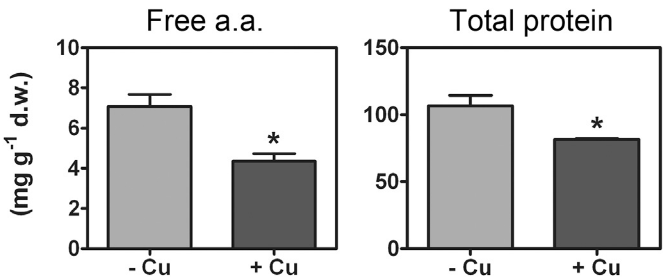


**Fig. 7.** Total protein levels and total free amino acids in mature grape berries from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with Bordeaux mixture (+Cu). Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control: *$P \leq 0.05$.

This effect may bring further consequences in grape quality since arginine participates in the biosynthesis of polyamines, guanidines and other amino acids including proline (Supplementary Fig. S2), which is likely synthesised in the fruit (Roubelakis-Angelakis and Kliewer, 1992). Moreover, the degradation of arginine by arginase in grapes and wine releases urea which reacts with ethanol under acidic wine conditions, yielding ethyl carbamate, a suspected human carcinogen (Ough, 1991). The amino acids that derive directly from ammonium, namely asparagine and glutamine (Supplementary Fig. S2), which together with arginine are the main organic carriers of nitrogen in plants (Miflin and Lea, 1990), suffered a reduction of 40% and 60%, respectively (Fig. 9). Previous studies have shown that copper excess decreases the levels of these amino acids in roots of tissue-cultured grapevines, indicating a reduction of nitrogen assimilation and the limited uptake of inorganic nitrogen (Llorens et al., 2000). Aspartic acid and glutamic acid which may also derive directly from ammonium and are indirect precursors of arginine and proline did not change significantly (Fig. 9, Supplementary Fig. S2). The changes in the content of the remaining amino acids were more subtle but still followed a decreasing trend in berries collected from grapevines treated with Bordeaux mixture. In contrast, leaf amino acids were less affected by Bordeaux mixture applications and only a few responded to this treatment (Supplementary Table SII). For instance, the levels of both phenylalanine and alanine revealed a significant increase at harvest stage.

Nitrogen flow to clusters is predominant from bloom to harvest, and large amounts of free amino acids are transported from roots to berries towards the end of fruit ripening (Wermelinger, 1991). Bordeaux mixture application could limit the mobilization of nitrogen to the grape berries or the formation of amino acids from nitrate in the berry itself. In agreement, copper excess has been associated with a decreased activity of several enzymes of the nitrogen metabolism (Llorens et al., 2000). The observed differences in amino acid content in the berries from plants treated with Bordeaux mixture may have a great impact on fruit and wine quality since it is known that berry amino acids may account for up to 90% of the nitrogen in grape juice (Roubelakis-Angelakis and Kliewer, 1992; Conde et al., 2007). Besides affecting the colour density of the must, total tannins and anthocyanins of the berry skin, the nitrogen composition of grape juice has further implications in yeast growth and in the rate and duration of the fermentation process (Roubelakis-Angelakis and Kliewer, 1992; Bell and Henschke, 2005; Vilanova et al., 2007).

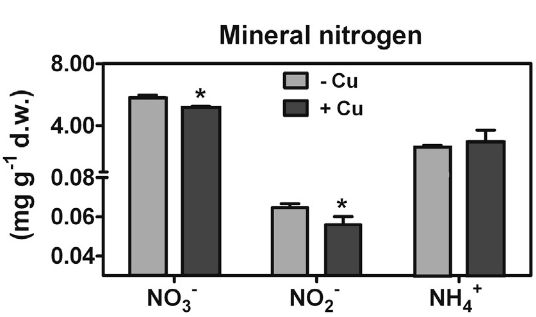


**Fig. 8.** Nitrate ($NO_3^-$), nitrite ($NO_2^-$) and ammonium ($NH_4^+$) levels in mature grape berries from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with the Bordeaux mixture (+Cu). Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control: *$P \leq 0.05$.

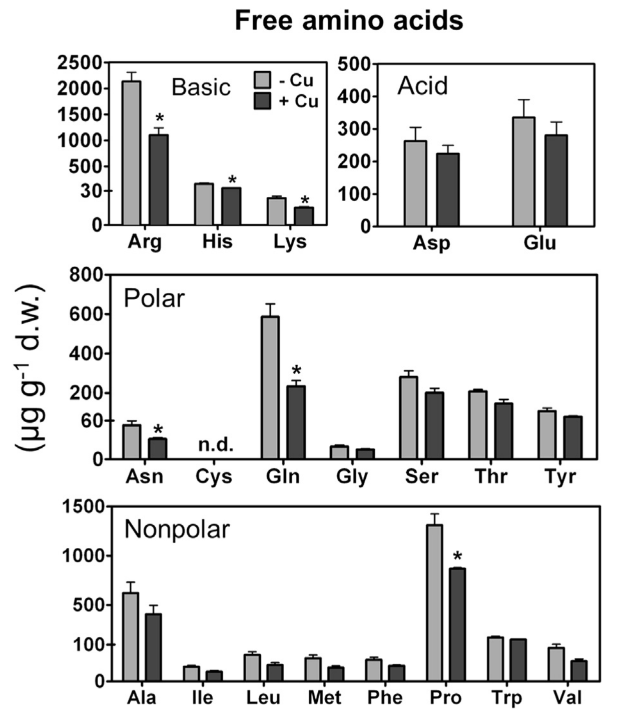


**Fig. 9.** Natural free amino acid concentrations in mature grape berries from vines cv. "Vinhão" treated with a triazole-based fungicide (−Cu) or with Bordeaux mixture (+Cu). Results indicate mean ± SE of three independent experiments (biological replicates). Asterisks denote the significance levels as compared to control: *$P \leq 0.05$.

## 4. Conclusions

Bordeaux mixture was applied in field grapevines cv. "Vinhão" following regular vineyard management practices for organic agriculture, and resulted in increased copper levels in the grape berry tissues. Although some sugars, especially fructose, organic acids and secondary metabolites were changed by Bordeaux mixture application in fruits at some point of development, a very significant decrease was observed only in the levels of free amino acids, mineral nitrogen and total protein content in the ripe berry. Understanding the effects of copper-based fungicides in grape berry composition may help vine growers to adjust the frequency/intensity of copper applications in the vineyard and winemakers to optimize the fermentation and vinification processes. Since the year of growth largely influences the transcriptome of the berry and its composition (Dal Santo et al., 2013), monitoring the changes in key solutes in berry and musts at each season may ultimately allow the production of wines of standardized quality.

## Acknowledgements

This work was supported by European Union Funds (FEDER/COMPETE-Operational Competitiveness Programme) and by portuguese national funds (FCT – Portuguese Foundation for Science and Technology) under the project FCOMP-01-0124-FEDER-022692, the research project FCOMP-01-0124-FEDER-008760 (Ref. FCT PTDC/AGR-ALI/100636/2008), the project PEst-OE/AGR/UI4033/2014, and the PhD grant no. SFRH/BD/64587/2009 to V.M. This work also benefited from the networking activities within the European project INNOVINE (ref. 311775) and the European COST ACTION FA1106 "QualityFruit". The Will W. Lester Endowment, University of California (E.B.) also supported this work.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.06.016.

## Authors contributions

VM, AT and HG raised the hypothesis underlying this work. VM, AT, E Bassil, E Blumwald and HG designed the experiments. VM and AT carried out the experiments, performed data processing and statistical analysis. VM, E Blumwald and HG wrote the article. E Blumwald and HG directed the study. All authors read and approved the manuscript.

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