EFFECT OF VINE NITROGEN STATUS, GRAPEVINE VARIETY AND ROOTSTOCK ON THE LEVELS OF BERRY *S*-GLUTATHIONYLATED AND *S*-CYSTEINYLATED PRECURSORS OF 3-SULFANYLHEXAN-1-OL

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Abstract

Aim: To determine the effect of vine nitrogen status in interaction with grapevine variety and rootstock on vine development and on the synthesis of 3-sulfanylhexan-1-ol precursors (Glut-3SH and Cys-3SH) in grape berries produced in controlled conditions.

Methods and results: Potted Sauvignon blanc (SB) and Cabernet-Sauvignon (CS) vines, grafted on two different rootstocks (110R and RGM), were irrigated with two nutritive solutions varying only by their nitrogen content (N(-) and N(+)). Vine nitrogen status of N(-) treatment was significantly different from that of N(+) treatment. Secondary leaf area was higher in N(+) treatment and ripening was delayed because of increased vigor. High N status increased Glut-3SH content in berries, while it did not impact Cys-3SH level. Moreover, the concentrations of 3SH precursors were higher in SB berries compared to CS and their synthesis was enhanced in berries produced by vines grafted onto RGM under N(+) treatment.

Conclusion: Glut-3SH content was mainly determined by plant N status. Grapevine variety and rootstock/N treatment interaction also had a significant, although more limited, impact. Cys-3SH level was dependent on berry developmental stage and grapevine variety, but not on vine N status.

Significance and impact of the study: A better understanding of the effect of terroir components on the biosynthesis of the precursors of volatile thiols is gained.

Key words: 3-sulfanylhexan-1-ol, *S*-glutathionylated precursor, *S*-cysteinylated precursor, nitrogen, *Vitis vinifera*, grape berry, variety, rootstock

Résumé

Objectif : Déterminer l'effet du statut azoté de la vigne en interaction avec la variété et le porte-greffe sur son développement et sur la synthèse des précurseurs du 3-sulfanylhexan-1-ol (Glut-3SH et Cys-3SH) dans le raisin issu d'une culture en condition contrôlée menée sur des vignes en pots.

Méthodes et résultats : Deux variétés de *Vitis vinifera*, Sauvignon blanc (SB) et Cabernet-Sauvignon (CS), greffées sur deux porte-greffes (110R et RGM) ont été irriguées avec deux solutions nutritives ne différant que par leur contenu en azote (N(-) et N(+)). Le statut azoté de la modalité N(-) a été significativement différent de celui de la modalité N(+). Les plantes de cette dernière modalité sont caractérisées par une surface foliaire secondaire plus importante et par un retard de la maturation des baies. Une augmentation du contenu en Glut-3SH dans les baies est observée dans la modalité N(+) alors qu'aucun effet de l'azote n'est observé sur la teneur en Cys-3SH. En outre, les concentrations des précurseurs du 3SH sont plus élevées dans les baies de SB par rapport au CS, et leur synthèse a été stimulée dans les baies des deux variétés greffées sur RGM sous le traitement N(+).

Conclusion : Le contenu en Glut-3SH est principalement déterminé par le statut azoté. Le cépage et l'interaction porte-greffe/modalité ont un effet plus limité. La teneur en Cys-3SH est dépendante du stade de maturité des baies et de la variété, mais elle n'est pas influencée par le statut azoté de la vigne.

Signification et impact de l'étude : Ouverture vers une meilleure compréhension de l'effet des composantes du terroir viticole sur la synthèse des précurseurs des thiols volatils.

Mots clés : 3-sulfanylhexan-1-ol, précurseur glutathionylé, précurseur cystéinylé, azote, *Vitis vinifera*, baie de raisin, variété, porte-greffe

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INTRODUCTION

The concept of terroir in viticulture is defined as an ecosystem in which vine development and grape ripening are influenced by the environment surrounding the vine. The quality of the wine can partly be explained by the terroir effect (Seguin, 1986; Falcetti, 1994; Vaudour, 2003; van Leeuwen and Seguin, 2006). Terroir factors include the climate, the soil type as well as the grapevine variety and the rootstock (genetic factors). Human factors also play a role through the choice of viticultural and oenological techniques. A harmonious interaction between these components maximize wine quality and typicity (Seguin, 1986; van Leeuwen and Seguin, 2006).

The soil is a major terroir factor. It influences vine physiology and grape ripening through its physical structure (Dry and Coombe, 2005; van Leeuwen and Seguin, 2006; Reynolds *et al.*, 2007; White *et al.*, 2007; Cahurel, 2007;), the water and mineral supply (Seguin, 1986; Choné *et al.*, 2001; Peyrot des Gachons *et al.*, 2005), its color (Witbooi, 2008), its microbiology (Bourguignon, 1995) and *via* the temperature at the root zone (Gladstones, 1992; Carey, 2001).

Among nutriments which vines pick up from the soil, nitrogen (N) has a major impact on vine development, shoot growth, yield and sensitivity to fungal diseases such as *Botrytis cinerea* (Bell and Henschke, 2005). Moreover, this element influences the synthesis of primary metabolites, i.e. sugar and organic acids (Keller and Hrazdina, 1998; Maigre, 2002; Rodriguez-Lovelle and Gaudillere, 2002), as well as that of secondary metabolites, i.e. amino acids, total phenolics, flavonoids (Hilbert *et al.*, 2003; Soubeyrand *et al.*, 2014) and aroma compounds such as volatile thiols and their precursors (Choné *et al.*, 2006; Lacroux *et al.*, 2008).

Volatile thiols contribute largely to the aromatic potential of wines produced by varieties like Sauvignon blanc, Semillon, Riesling, Pinot gris and Colombard (Tominaga *et al.*, 1996; Moreira *et al.*, 2002; Swiegers and Pretorius, 2007). Their contribution to wine flavor depends on their content and their interaction with other molecules and compounds (Francis and Newton, 2005). Among volatile thiols, 3-sulfanylhexan-1-ol (3SH) is a major compound that plays an essential role in the aroma of many wines. 3SH is responsible for flavors reminding grapefruit and passion fruit, which are usually appreciated by tasters (Tominaga *et al.*, 1998a; Peyrot des Gachons *et al.*, 2002). This volatile thiol is not present in the berry and the must; it is liberated during the alcoholic fermentation from odorless and non-volatile precursors synthesized in the berry. It was demonstrated that these precursors are *S*-conjugates to glutathione (*S*-3-(hexan-1-ol)-glutathione; Glut-3SH) and to cysteine (*S*-3-(hexan-1-ol)-cysteine; Cys-3SH) (Fig. 1) (Tominaga *et al.*, 1998b; Peyrot des Gachons *et al.*, 2002; Kobayashi *et al.*, 2010; Thibon *et al.*, 2011). The impact of vine N status on the concentration of 3SH precursors has been the subject of numerous studies (Choné *et al.*, 2006; Lacroux *et al.*, 2008; Peyrot des Gachons *et al.*, 2005). However, the influence of vine N status in interaction with other terroir factors on the content of these compounds was never investigated. This experimentation, which was conducted on potted plants in controlled conditions in 2014, assesses for the first time the effect of N status in interaction with grapevine variety and rootstock on vine development and on the 3SH precursors of berries. The impact of the combination of these factors was determined for *Vitis vinifera* cv. Sauvignon blanc (SB) and Cabernet-Sauvignon (CS) grafted on two different rootstocks (110R and RGM) under two N conditions in order to determine their influence on plant behavior and berry composition.

MATERIALS AND METHODS

1. Vine material and experimental set-up

The study was conducted in 2014 on Sauvignon blanc (clone 108) and Cabernet-Sauvignon (clone 169) grafted in 2011 onto two contrasted rootstocks: RGM (Riparia Gloire de Montpelier, clone 1) and 110R (110 Richter, clone 152). The experiment was carried out in out-door weather conditions in 10-L pots containing loam, sand, and perlite (50, 30 and 20%, respectively). Vines were irrigated during the whole season. Plants were pruned to two spurs of four eyes (eight buds per vine) and after bud break, only buds 3 and 4 were retained while buds 1 and 2 were removed on each spur. Viticultural practices like basal leaf removal and trimming were close to those commonly implemented in commercial vineyards and were similar among all treatments.

Nutrient solutions (1 to 1.6 L per pot per day depending on evaporative demand) were provided by an irrigation system, three times daily, from bud break to harvest. All plots were supplied with these nutrient solutions varying only by their nitrogen content. Two levels of nitrogen were applied: a strongly limiting nitrogen treatment (N(-); solution containing 11 mg L^{-1} of nitrogen) and a non limiting nitrogen treatment (N(+); solution containing 50 mg

L^{-1} of nitrogen). Each combination variety/rootstock/N modality consisted of 10 pots randomly distributed within the experimental set-up. In order to have three biological replicates, plants of each combination were divided into three groups of three plants each.

For each combination variety/rootstock/N modality, fifty fresh berries were randomly sampled from the three plants at two developmental stages: mid-ripening, MR (33 and 30 days after mid-veraison for SB and CS, respectively) and ripeness, R (43 and 48 days after mid-veraison for SB and CS, respectively). Mid-veraison was determined at the time when 50% of the berries were soft and/or red. Berries were collected in liquid nitrogen immediately after being removed from the plants and then stored at -80°C. They were then weighed and ground in liquid nitrogen to a fine powder until analyses.

2. Vine water status

Vine water status was determined by measuring the $\delta^{13}C$, which corresponds to the ratio of carbon isotopes $^{13}C/^{12}C$. This measurement is used to assess vine water status during the period between veraison and harvest (Gaudillère *et al.*, 2002; Tregoat *et al.*, 2002). The $\delta^{13}C$ (in ‰) varies between -20 ‰ (severe water stress) and -28 ‰ (no water stress) (van Leeuwen *et al.*, 2001). The $\delta^{13}C$ was measured on grape sugars at harvest (Rodriguez-Lovelle and Gaudillere, 2002; van Leeuwen *et al.*, 2001).

3. Vine and berry nitrogen status

Nitrogen status was determined by analyzing nitrogen content of leaf blades at mid-veraison (Navarro et *al.*, 2008; Romero *et al.*, 2013) and by assessing the yeast available nitrogen (YAN) in grape must at harvest. The analysis of leaf blade nitrogen content was carried on the primary leaf facing the first cluster at mid-veraison (Romero *et al.*, 2010). Leaf blades were rinsed with deionized water then dried at 70°C for three days and ground to a fine powder. The determination of leaf blade nitrogen content was carried out using the Dumas method (Buckee, 1994) at the «Plant Interactions Soil Atmosphere laboratory» (ISPA UMR 1391, INRA, Villenave d'Ornon, France).

YAN was assessed in grape juice obtained by pressing of approximately fifty berries collected at ripeness stage (van Leeuwen *et al.*, 2000). The juice was analyzed with a Fourier Transform Infra-Red spectrometer (FTIR, WineScan FOSS®, Nanterre, France) (van Leeuwen *et al.*, 2000; Destrac *et al.*, 2015).

4. Vine vigor

Vine vigor was determined by the assessment of primary and secondary leaf areas at shoot growth cessation and by measuring pruning weights. Leaf areas were determined according to the method published by Mabrouk and Carbonneau (1996). A correlation curve was established between the length of the shoots and their corresponding leaf area using a LI-3100 LICOR leaf area meter (Lincoln, Nebraska, USA). Calibration curves were set up separately for primary and secondary leaf area. Subsequently, the length of all primary and secondary shoots of three vines per replicate was measured and primary and secondary leaf areas were deduced from the calibration curves.

Pruning weights were measured for each vine (four primary shoots per vine and the corresponding secondary ramifications) and averaged per biological replicate.

5. Must composition at harvest

Grape juice was obtained by pressing fifty berries sampled the day before harvest. Sugar level, total acidity, pH, malic acid content and YAN level were determined using an FTIR spectrometer (WineScan FOSS®, Nanterre, France) (Destrac *et al.*, 2015).

6. Extraction and quantification of Glut-3SH and Cys-3SH

The method was adapted from Luisier *et al.*, (2008) and performed as described below. A mix of 1 mL of grape juice obtained by defrosting of 2 g of frozen berry powder in the presence of sulfur dioxide (200 mg L^{-1}), 1 mL of water and a final concentration of 50 µg L^{-1} of the internal standard solution containing a deuterated form of the glutathionylated *S*-conjugate ((3-*S*-hexan-1-ol)-glutathione-d_3) was percolated through a conditioned SPE column (LC-18 500 mg 6 mL, Supelco, Saint Germain-Laye, France). Impurities adsorbed on SPE columns were eliminated by ultrapure water and precursors were than eluted with 3 mL of water/methanol (70/30; v/v) in hemolysis tubes. The flow-through was subsequently evaporated using a RapidVap Vertex Dry Evaporator (Labconco, Kansas City, MO, USA). Residues were dissolved in aqueous formic acid solution (0.1%) and filtered with a 0.45-µm membrane before being analyzed by C18-RP-UHPLC-HRMS (Thermo Scientific, Illkirch, France).

The separation was performed on a Synchronis aQ column (100×2.1 mm i.d., 1.7 µm, Synchronis aQ,

Table 1 - Multi-factor analysis of variance: percentage (%) of variance explained by grapevine variety (V), rootstock (R), developmental stage (S), nitrogen treatment (N) and their interactions on vine water status, nitrogen status, vigor, berry composition at ripeness and 3SH precursors in berries

	V	R	N	S	V x R	V x N	R x N	V x R x N	Residual
Water status									
$\delta^{13}C$ (‰)	24.81 ***	6.49 ***	59.26 ***	NA	0.21 ^{ns}	0.07 ^{ns}	0.16 ^{ns}	1.42 ^{ns}	7.58
Nitrogen status									
Leaf blade N (% DW)	6.92 *	0.13 ^{ns}	73.07 ***	NA	0.02 ^{ns}	1.55 ^{ns}	0.19 ^{ns}	0.12 ^{ns}	18
YAN (mg L^{-1})	2.69 *	0.03 ^{ns}	74.46 ***	NA	4.27 *	1.71 ^{ns}	2.95 ^{ns}	0.54 ^{ns}	13.36
Vigor									
Primary leaf area (m^2/vine)	8.21 ^{ns}	1.04 ^{ns}	2.3 ^{ns}	NA	30.37 **	3.04 ^{ns}	4.92 ^{ns}	0.24 ^{ns}	49.88
Secondary leaf area (m^2/vine)	39.35 ***	1.48 *	47.00 ***	NA	0.01 ^{ns}	1.16 ^{ns}	3.3 *	0.43 ^{ns}	7.27
Pruning weight (kg/vine)	5.62 *	0.01 ^{ns}	0.37 ^{ns}	NA	0.16 ^{ns}	0.63 ^{ns}	0.22 ^{ns}	0.38 ^{ns}	92.6
Berry composition at ripeness									
Sugar (g L^{-1})	0.74 ^{ns}	1.07 ^{ns}	63.81 ***	NA	3.13 ^{ns}	1.22 ^{ns}	2.06 ^{ns}	1.3 ^{ns}	26.67
Total acidity (g L^{-1} tartaric acid)	46.57 ***	6.67 ^{ns}	9.60 ^{ns}	NA	28.21 **	5.67 ^{ns}	8.85 ^{ns}	0.01 ^{ns}	44.41
pH	21.62 ***	3.85 ^{ns}	2.52 ^{ns}	NA	32.77 ***	5.29 ^{ns}	1.83 ^{ns}	0.94 ^{ns}	31.19
Malic acid (g L^{-1})	48.83 ***	0.14 ^{ns}	4.84 ^{ns}	NA	7.84 *	0.46 ^{ns}	3.51 ^{ns}	1.29 ^{ns}	33.08
3SH precursors									
Glut-3SH (µg L^{-1})	19.44 ***	0.95 ^{ns}	36.27 ***	0.09 ^{ns}	1.00 ^{ns}	1.16 ^{ns}	9.78 ***	3.77 **	15.97
Cys-3SH (µg L^{-1})	7.08 *	0.36 ^{ns}	0.01 ^{ns}	18.06 ***	11.25 **	10.77 **	13.23 ***	0.05 ^{ns}	24.02

***, **, * indicate significance at *p* value ≤ 0.0001, 0.001, 0.05, respectively. ns, not significant (p value > 0.05). NA, not available.

Thermo Scientific, Bremen, Germany) with a flow rate of 300 µL min^{-1} of solvent A (0.1% aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile). The gradient for solvent B was as follows: 0 min, 9%; 0.8 min, 9%; 5 min, 40%; 5.2 min, 90%. The column was equilibrated with 9% of solvent B for 1 min prior to an injection. The ion source was operated in the positive ion mode at 3.5 kV. The vaporizer temperature of the source was set at 300°C, the capillary temperature at 350°C, the nitrogen sheath gas at 80, and the auxiliary and sweep gas at 5 (arbitrary units). A mass range of 100-500 was acquired in full scan MS mode. The resolution setting was 25000 (m/Δm, fwhm at m/z 400).

For each combination variety/rootstock/N modality, the analyses were carried out on the three biological replicates.

In order to quantify the metabolites in samples, standards were prepared at the same time as the berries samples, by adding 50 µg L^{-1} of the internal standard to solutions of the synthetized metabolites (mix of 1 mL water and 1 mL grape juice), with individual 3SH precursors at concentrations of 0, 110, 275, 550, 825 and 1100 µg L^{-1} for Glut-3SH and 0, 10, 25, 50, 75 and 100 µg L^{-1} for Cys-3SH.

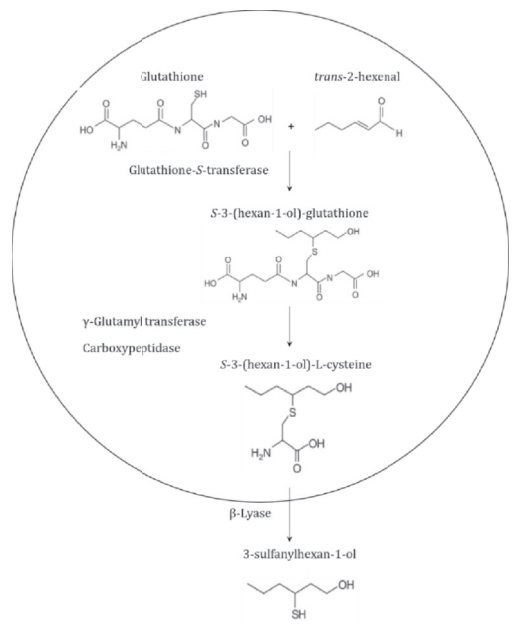

Figure 1 - Pathway of the glutathionylated (Glut-3SH) and cysteinylated (Cys-3SH) precursors in grape berries as described by Kobayashi et al. (2010) and Thibon et al. (2011). 3-Sulfanylhexan-1-ol (3SH) is produced by the yeast during the alcoholic fermentation.

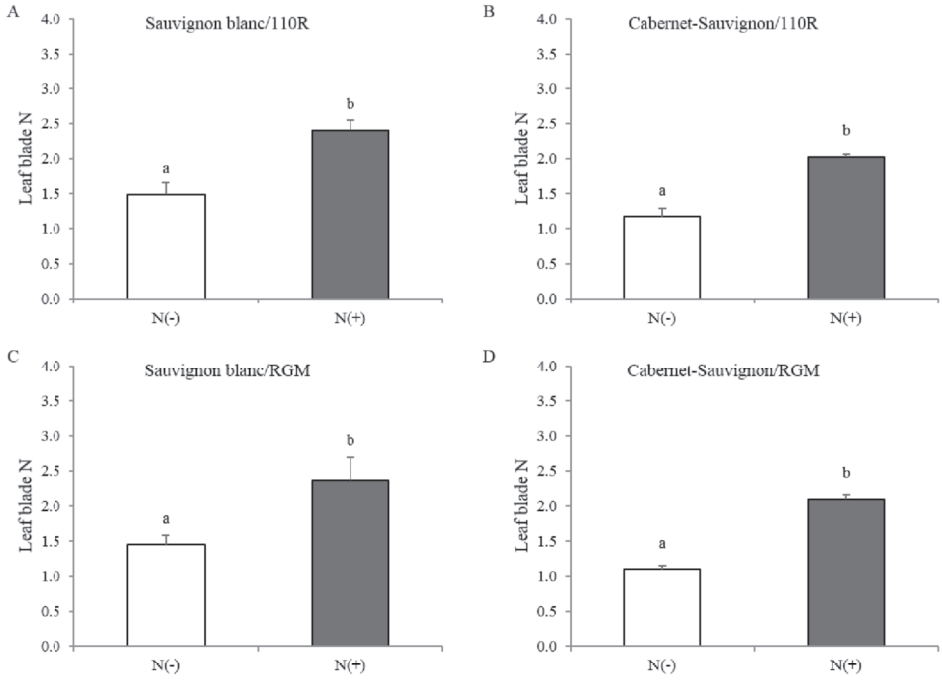

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Figure 2 - Leaf blade nitrogen (N) content (%) at mid-veraison. Each histogram is the mean of three replicates. Statistical significance was determined by Student's t test (*p* value ≤ 0.05). Error bars indicate SE. Different letters indicate significant differences.

7. Statistical analysis

Statistical analyses were conducted using the statistical package of the XLSTAT software (Addinsoft, Paris, France). All the data are expressed as the arithmetic average ± standard error (SE) from three biological replicates. Student's t test or a multi-factor analysis of variance followed by Fisher's least-significant difference (LSD) test were carried out at *p* value ≤ 0.05.

RESULTS

1. Vine water status

Despite the fact that vines were irrigated continuously during the whole season, certain treatments faced a moderate water deficit (-24.5 < $\delta^{13}C$ < -23), and others a lesser or small water deficit (-26 < $\delta^{13}C$ ≤ -24.5). Most treatments, however, did not face any water deficit ($\delta^{13}C$ ≤ -26) (van Leeuwen *et al.*, 2009). Vine water status was explained by N modality (59% of total variance explained), the grape variety (25% of total variance explained) and the rootstock (6% of total variance explained; Table 1).

Moderate water deficit was mainly observed for the N(+) treatment and can be related to high secondary leaf area in this treatment. Grapevine variety also affected vine water status (Tables 1 and 2). The $\delta^{13}C$ of CS must was higher compared to SB. Moreover, water status was affected by the type of rootstock. Significant differences in $\delta^{13}C$ values were observed between 110R and RGM. Vines grafted onto RGM faced larger water deficits compared to those grafted onto 110R, which is consistent with the literature (Tables 1 and 2) (Ollat *et al.*, 2015).

2. Plant and berry nitrogen status

Vine N status was essentially determined by the N nutrition: it was weakly affected by the grapevine variety and not affected by the rootstock (Table 1).

For SB cultivar, leaf blade N content at mid-veraison of the N(-) modality ranged between 1.45 and 1.48% of dry weight (DW), while it was higher in the N(+) modality (close to 2.4% DW) (Fig. 2A and C). YAN content also allowed clear differentiation between the two nitrogen treatments (Fig. 3A and C).

For CS cultivar, leaf blade N content of N(-) treatment was close to 1.10% DW. It increased in the (N+) treatment (values close to 2% DW), showing significant differences with the N(-) modality (Fig. 2B and D). YAN level also allowed to differentiate vine N status between the two modalities for this variety. It was higher in the N(+) treatment compared to the N(-) treatment (Fig. 3B and D).

Table 2 - Multi-factor analysis of variance of the effect of grapevine variety, rootstock, developmental stage and nitrogen treatment on vine water status, nitrogen status, vigor, berry composition at ripeness and 3SH precursors in berries

	Variety		Rootstock		Stage		N treatment	
	SB	CS	110R	RGM	MR	R	N(-)	N(+)
Water status								
$\delta^{13}C$ (‰)	-26.60 [a]	-25.60 [b]	-26.35 [a]	-25.85 [b]	NA	NA	-26.61 [a]	-25.01 [b]
Nitrogen status								
Leaf blade N (% DW)	1.79 [a]	1.52 [b]	1.66 [a]	1.65 [a]	NA	NA	1.30 [a]	2.24 [b]
YAN (mg L^{-1})	381.1 [a]	335.3 [b]	353 [a]	363.3 [a]	NA	NA	290.9 [a]	500.3 [b]
Vigor								
Primary leaf area (m^2/vine)	0.96 [a]	0.80 [a]	0.91 [a]	0.85 [a]	NA	NA	0.94 [a]	0.85 [a]
Secondary leaf area (m^2/vine)	0.85 [a]	0.41 [b]	0.67 [a]	0.60 [b]	NA	NA	0.36 [a]	0.95 [b]
Pruning weight (kg/vine)	0.23 [a]	0.27 [b]	0.25 [a]	0.25 [a]	NA	NA	0.26 [a]	0.24 [a]
Berry composition at ripeness								
Sugar (g L^{-1})	176.2 [a]	172.7 [a]	172.4 [a]	176.6 [a]	NA	NA	182.7 [a]	151.6 [b]
Total acidity (g L^{-1} tartaric acid)	9.48 [a]	11.14 [b]	9.99 [a]	10.63 [a]	NA	NA	10.05 [a]	10.84 [a]
pH	3.34 [a]	3.43 [b]	3.40 [a]	3.36 [a]	NA	NA	3.40 [a]	3.39 [a]
Malic acid (g L^{-1})	5.16 [a]	7.1 [b]	6.07 [a]	6.18 [a]	NA	NA	6.03 [a]	6.54 [a]
3SH precursors								
Glut-3SH (µg L^{-1})	98.20 [a]	52.14 [b]	62.59 [a]	87.76 [a]	40.60 [a]	74.34 [a]	40.60 [a]	109.74 [b]
Cys-3SH (µg L^{-1})	10.01 [a]	6.40 [b]	7.55 [a]	8.97 [a]	5.31 [a]	11.06 [b]	8.21 [a]	8.28 [a]

Statistical significance was determined by a multi-factor analysis of variance followed by Fisher's least-significant difference (LSD) test (p value ≤ 0.05). Different letters indicate significant differences. SB, Sauvignon blanc; CS, Cabernet-Sauvignon; MR, mid-ripening; R, ripeness. NA, not available.

Table 3 - Effect of vine nitrogen status on primary and secondary leaf area (m^2/vine) measured at shoot growth cessation and pruning weight (kg/vine)

	Sauvignon blanc			Cabernet-Sauvignon		
	Primary leaf area	Secondary leaf area	Pruning weight	Primary leaf area	Secondary leaf area	Pruning weight
110R						
N(-)	1.30 [a]	0.54 [a]	0.25 [a]	0.77 [a]	0.32 [a]	0.27 [a]
N(+)	1.08 [a]	1.36 [b]	0.24 [a]	0.73 [a]	0.68 [b]	0.27 [a]
RGM						
N(-)	0.85 [a]	0.41 [a]	0.23 [a]	0.86 [a]	0.19 [a]	0.30 [a]
N(+)	0.66 [a]	1.14 [b]	0.23 [a]	0.95 [a]	0.63 [b]	0.26 [a]

Each point is the mean of three replicates. Statistical significance was determined by Student's t test (p value ≤ 0.05). Different letters indicate significant differences.

Besides N treatment (73% of total variance explained), leaf blade N content also depended on the grapevine variety (7% of total variance explained). SB was characterized by higher leaf blade N content than CS. Moreover, this variable was not influenced by the rootstock (Tables 1 and 2). YAN content was mainly influenced by N treatment (74% of total variance explained) and less by grape variety (3% of total variance explained) and the scion/rootstock interaction (4% of total variance explained) (Tables 1 and 2).

These data show that N status of the N(+) treatment was higher than that of the N(-) treatment (Table 2) and that leaf blade N content and YAN were accurate indicators to account for differences in vine N status.

3. Vine vigor

Plant vigor was determined by measuring primary and secondary leaf area at shoot growth cessation and pruning weight in winter. Primary leaf area was similar between the two N treatments for all combinations variety/ rootstock/N treatment. Primary leaf area of SB ranged between 0.66 m^2/vine and 1.30 m^2/vine and that of CS between 0.73 m^2/vine and 0.95 m^2/vine. No significant differences were observed among N treatments for each variety (Table 3). However, this indicator of vine vigor was strongly influenced by the variety/rootstock interaction (30% of total variance explained) (Table 1). It seems that 110R provides a higher primary leaf area for SB than for CS. The opposite effect was observed with RGM under the N(+) treatment, where the combination SB/RGM had a lower primary leaf area than CS/RGM.

Unlike the primary leaf area, secondary leaf area was impacted by N treatment (Tables 1, 2 and 3). For SB grafted onto 110R or RGM, significant differences were observed between the two modalities. For SB/110R, secondary leaf area was 0.54 m^2/vine and 1.36 m^2/vine for N(-) and N(+) treatments, respectively, and for SB/RGM 0.41 m^2/vine and 1.14 m^2/vine for N(-) and N(+) treatments, respectively (Table 3). The secondary leaf area of CS/110R/N(+) was 2-fold higher compared to CS/110R/N(-) (0.68 m^2/vine *versus* 0.32 m^2/vine). Finally, for CS grafted onto RGM, significant differences were also observed between the two treatments (0.19 m^2/vine for CS/RGM/N(-) *versus* 0.63 m^2/vine for CS/RGM/N(+)) (Table 3).

Secondary leaf area was not only influenced by N treatment, but also by the grape variety (39% of total variance explained) (Tables 1 and 2). SB established a higher secondary leaf area than CS. The rootstock also impacted this variable (1.5% of total variance explained). It was higher for 110R compared to RGM. However, this effect was minor compared to that of N treatment and grapevine variety (Tables 1 and 2).

Pruning weight was not impacted by vine N status, nor by the rootstock (Table 3). However, grapevine variety influenced this parameter (6% of total variance explained). Pruning weight of SB was lower compared to CS (Tables 1 and 2).

Table 4 - Effect of vine nitrogen status on sugar content (g L^{-1}), total acidity (g L^{-1} tartaric acid), pH and malic acid content (g L^{-1}) in grape juice

	Sauvignon blanc				Cabernet-Sauvignon			
	Sugar	Total acidity	pH	Malic acid	Sugar	Total acidity	pH	Malic acid
110R								
N(-)	170.3 [a]	9.3 [a]	3.29 [a]	5.0 [a]	186.2 [a]	9.0 [a]	3.59 [a]	6.3 [a]
N(+)	146.9 [a]	10.6 [a]	3.31 [a]	6.4 [a]	148.4 [b]	10.9 [a]	3.50 [a]	7.1 [a]
RGM								
N(-)	193.0 [a]	9.7 [a]	3.35 [a]	5.3 [a]	181.4 [a]	11.8 [a]	3.38 [a]	7.4 [a]
N(+)	160.1 [a]	9.3 [a]	3.42 [a]	4.7 [a]	150.9 [b]	12.3 [a]	3.35 [a]	7.8 [a]

Each point is the mean of three replicates. Statistical significance was determined by Student's t test (*p* value ≤ 0.05). Different letters indicate significant differences.

In this experiment, vine vigor increased with higher N status, as shown by a greater secondary leaf area. However, primary leaf area and pruning weight were not impacted by N supply.

4. Grape juice composition at ripeness

Berry composition was assessed at ripeness by measuring sugar level, total acidity, pH and malic acid content (Table 4).

Sugar levels of the N(-) treatment were higher compared to those of the N(+) treatment for both varieties (Table 4). Though not significant in SB berries due to a high variability among biological replicates, differences between sugar concentrations (approximately 30 g L^{-1}) were relevant from an oenological point of view.

Vine N status did not impact grape juice acidity assessed by total acidity, pH and malic acid content. No significant differences were observed between N treatments. Total acidity levels of SB ranged between 9.3 g L^{-1} tartaric acid and 10.6 g L^{-1} tartaric acid, and those of CS between 9.0 g L^{-1} tartaric acid and 12.3 g L^{-1} tartaric acid (Table 4). For SB, total acidity was slightly higher compared to commercial ripeness, while total acidity for CS was much higher compared to commercial grapes at harvest. pH values were relatively close in SB/110R, SB/RGM and CS/RGM combinations (between 3.29 and 3.42). They were much higher in CS/110R combination (3.50 to 3.59; Table 4). Furthermore, it should be noted that malic acid concentrations in CS (6.3 to 7.8 g L^{-1}) were higher compared to those in SB (4.7 to 6.4 g L^{-1}).

Vine N status altered sugar accumulation without changing the other parameters (total acidity, pH and malic acid content) related to grape ripening. However, these variables were highly dependent on grape variety and grape variety/rootstock combination (Table 1). Total acidity and malic acid content were higher in CS berries compared to SB (11.14 g L^{-1} tartaric acid *versus* 9.48 g L^{-1} tartaric acid and 7.10 g L^{-1} *versus* 5.16 g L^{-1} for total acidity and malic acid content, respectively). pH was lower in SB berries compared to CS (3.34 *versus* 3.43).

In conclusion, leaf blade N content and YAN level in grape must showed that vine N status of the N(+) treatment was higher compared to that of the N(-) treatment. In addition, vines of the N(+) treatment were characterized by greater vigor (as shown by the higher secondary leaf area) and by delayed ripeness.

5. Impact of nitrogen nutrition on Glut-3SH and Cys-3SH in the berry

Berry *S*-glutathionylated and *S*-cysteinylated precursors of 3SH content was determined at two different stages: mid-ripening (MR: (v+33) for SB and (v+30) for CS) and ripeness (R: (v+43) for SB and (v+48) for CS).

Glut-3SH concentration was stable during ripening in SB berries (Fig. 4A and C). Analysis of variance showed no effect of developmental stage (Table 1). However, a strong effect of N nutrition was observed on Glut-3SH (36% of total variance explained, Table 1). Its concentration in SB berries in the N(+) treatment was 2- to 4-fold higher compared to the

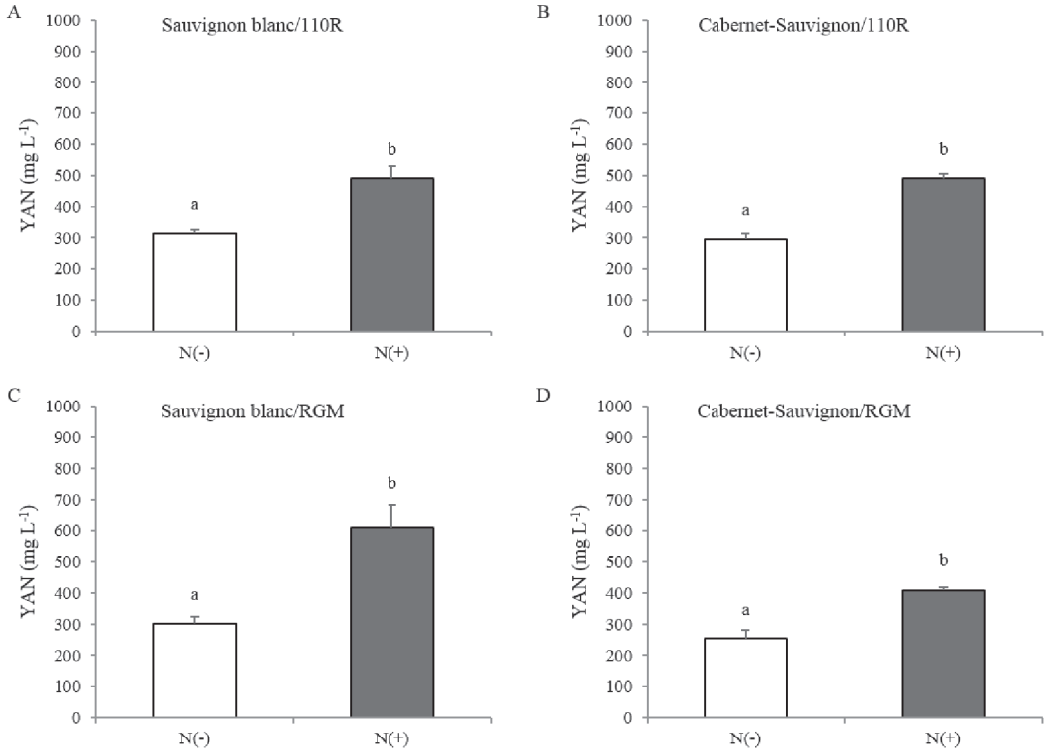

Figure 3 - Yeast available nitrogen (YAN) levels in grape must at ripeness. Each histogram is the mean of three replicates. Statistical significance was determined by Student's t test (*p* value ≤ 0.05). Error bars indicate SE. Different letters indicate significant differences.

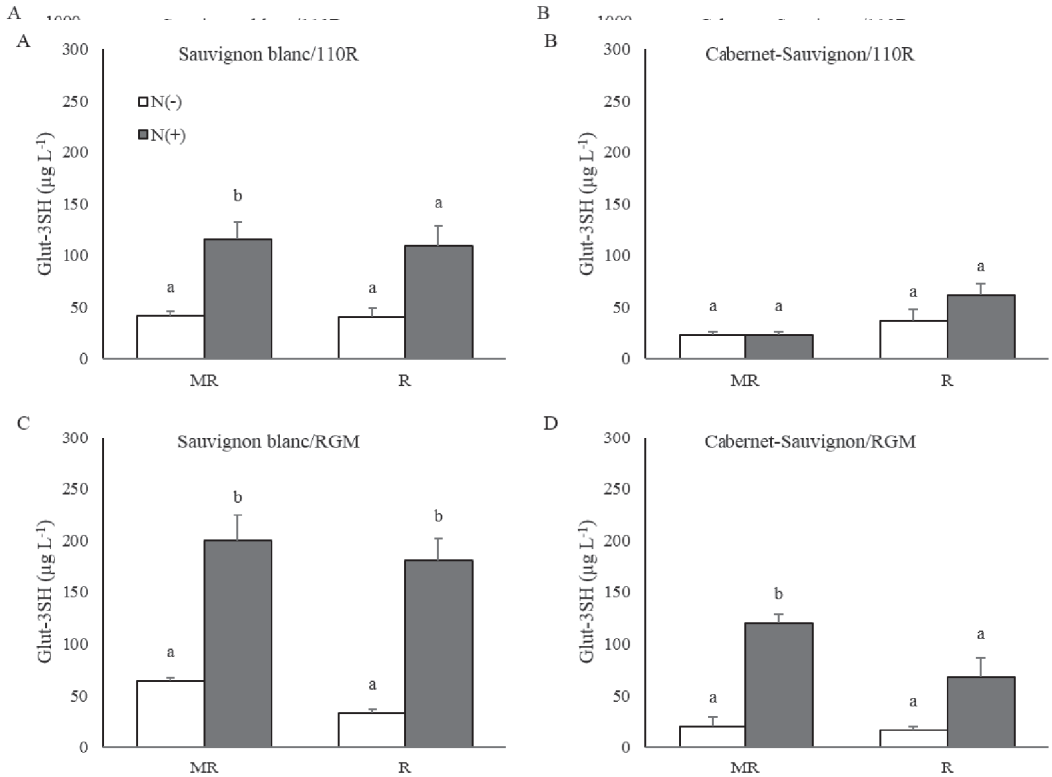

Figure 4 - Effect of vine nitrogen status on Glut-3SH level in grape berries at mid-ripening (MR) and ripeness (R). Each histogram is the mean of three replicates. Statistical significance was determined by Student's t test (*p* value ≤ 0.05). Error bars indicate SE. Different letters indicate significant differences.

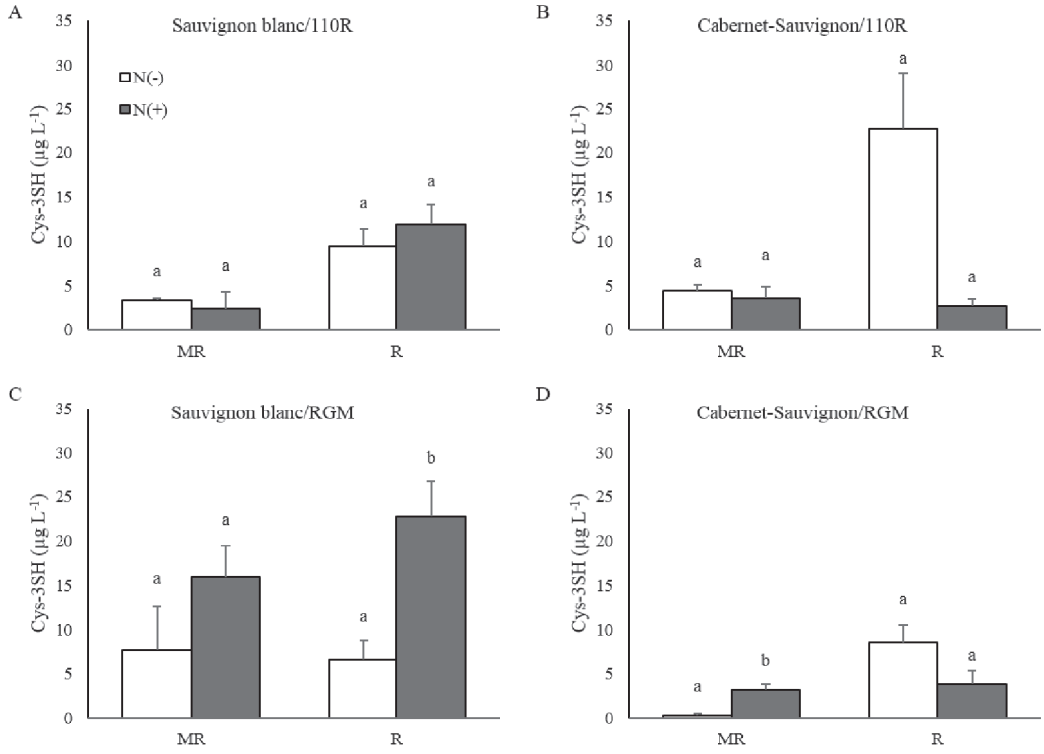

Figure 5 - Effect of vine nitrogen status on Cys-3SH level in grape berries at mid-ripening (MR) and ripeness (R). Each histogram is the mean of three replicates. Statistical significance was determined by Student's t test (*p* value ≤ 0.05). Error bars indicate SE. Different letters indicate significant differences.

N(-) treatment. For example, Glut-3SH content in SB/RGM berries at R was 33 µg L^{-1} in the N(-) modality *versus* 181 µg L^{-1} in the N(+) modality (Fig. 4A and C). Grapevine variety also impacted Glut-3SH content (19% of total variance explained). SB berries contained higher levels of this precursor compared to CS (98 µg L^{-1} *versus* 52 µg L^{-1}) (Table 2). Finally, rootstock/N treatment interaction also influenced Glut-3SH content (10% of total variance explained). Its level in berries of SB grafted onto RGM under N(+) treatment was higher compared to that of SB grafted onto 110R under the same treatment (181 µg L^{-1} *versus* 110 µg L^{-1} at R) (Fig. 4A and C). The rootstock/N treatment interaction also appeared to impact the final Glut-3SH content in CS berries (Fig. 4B and D).

In CS berries, even if differences were not always statistically significant, Glut-3SH concentration was higher in N(+) treatment than in N(-) treatment at both stages and rootstocks, except for the combination CS/110R at MR (Fig. 4B). At the same developmental stage, Glut-3SH content was 6-fold higher in N(+) treatment compared to N(-) treatment for the combination CS/RGM (119.8 µg L^{-1} *versus* 19.9 µg L^{-1} for N(+) and N(-), respectively) (Fig. 4D)

Cys-3SH content was affected by berry developmental stage (18% of total variance explained) and grapevine variety (7% of total variance explained, Table 1). Cys-3SH levels were higher at R compared to MR (11.1 µg L^{-1} *versus* 5.3 µg L^{-1}, Table 2). SB berries contained more of this compound compared to CS (10.0 µg L^{-1} *versus* 6.4 µg L^{-1}, Table 2). However, neither rootstock nor N treatment impacted its level in berries. Nevertheless, the interactions between rootstock x N treatment (13% of total variance explained), rootstock x grapevine variety (11% of total variance explained) and grapevine variety x N treatment (11% of total variance explained) had a strong influence on the content of this precursor (Table 1). In SB berries, Cys-3SH content increased with N level on RGM (Fig. 5A and C), while it tends to decrease in CS berries, in particular at R (Fig. 5B and D).

DISCUSSION AND CONCLUSIONS

In order to gain a better understanding of the effect of plant material (cultivar and rootstock) and vine N status on the content of the *S*-glutathionylated and *S*-cysteinylated precursors of 3SH in grape berries, an experiment on potted plants in controlled conditions was carried out. Vines were irrigated with nutritive solutions containing different N levels and were

supposed no to be water stressed. However, $\delta^{13}C$ measurements carried out on grapes at ripeness showed that some of the treatments had faced weak to moderate water deficits. Because of the small size of the pots, the root system of these plants was not very much developed. Moreover, the plants were relatively young (3 years old). This can explain why water deficits appeared, despite daily irrigation.

Leaf blade N content and YAN level in grape must were higher in the N(+) treatment compared to the N(-) treatment, showing that the applied N was correctly assimilated by the vines. YAN levels were relatively high in this study compared to commercial vineyards, due to the continuous irrigation of the pots with the nutritive solutions during the season. N status did not influence primary leaf area and pruning weight but modified secondary leaf area. The latter one increased with N content in the applied solutions. Furthermore, a delay in grape ripening was observed in the N(+) treatment compared to the N(-) treatment, as is shown by the lower berry sugar content in the N(+) treatment where values ranged between 147 and 160 g L^{-1}. However, total acidity was not modified.

Glut-3SH content was mainly determined by vine N status, and to a lesser extent by the grapevine variety and rootstock/N treatment interaction. Nitrogen had a positive effect on grape berry Glut-3SH content. This was confirmed on both varieties and both investigated rootstocks. Cys-3SH level was dependent on berry developmental stage and grapevine variety, although the percentage of variance explained by these factors was relatively low (18% and 7%, respectively). Vine N status did not influence the synthesis and the accumulation of this compound: a large variability in the response of this metabolite regarding N fertilization was observed, as evidenced by rootstock x N treatment, rootstock x variety and N treatment x variety interactions. Glut-3SH and Cys-3SH contents in SB berries were higher compared to those of CS, confirming that grapevine variety also impacted their synthesis and accumulation (Cerreti *et al.*, 2015).

In this experiment, the rootstock (RGM or 110R) did not influence 3SH *S*-conjugate concentrations, although interactions between rootstock x N treatment, as well as between grapevine variety x N treatment were shown. RGM seems to enhance their synthesis under N(+) treatment more so than 110R. A possible impact of vine water deficit on Glut-3SH can be hypothesized because RGM under N(+) treatment showed more water deficit compared to all other combinations. However, this point has to be clarified in more specific experiments investigating the effect of vine water status on Glut-3SH, which was beyond the scope of this research.

Cys-3SH increased with the level of ripeness of the grapes, while Glut-3SH did not. Grape berry ripening rate was higher for SB vines grafted onto RGM compared to 110R (higher sugar levels, higher pH). This difference between rootstocks was not observed in this study for CS. Hence, the fact that Cys-3SH was higher in SB/RGM compared to 110R, but not in CS/RGM compared to 110R, might be an indirect effect of an acceleration of the ripening rate in the combination SB/RGM. However, the result of a possible effect of rootstock x grapevine variety interaction on the level of these metabolites needs to be confirmed by additional experiments.

The rootstock 110R confers an important vigor to vines, while RGM has the opposite effect. The differences between the 3SH *S*-conjugate levels between the two rootstocks could be explained by the vigor. A rootstock that confers high vigor (i.e. 110R) could limit directly or indirectly the synthesis of secondary metabolites such as Glut-3SH, while a rootstock that confers low vigor (i.e. RGM) could have the opposite effect. Glut-3SH accumulation seems to be increased in berries produced by vines grafted onto a rootstock that confers low vigor and grown under high nitrogen supply. This hypothesis requires to be verified by additional studies.

In the field, it was demonstrated that 3SH level in Sauvignon blanc wine increased with N supply (Choné *et al.*, 2006; Lacroux *et al.*, 2008). Moreover, grapevine variety was shown also to affect their levels. For example, Sauvignon blanc berries contain higher levels of 3SH precursors than Grechetto and Malvasia (Cerreti *et al.*, 2015). Studied simultaneously in this work, we showed that vine N status had a greater impact than grapevine variety on the content of these metabolites. In fact, N absorption and/or assimilation is largely determined by the genotype of the plant to which it is related (Stines *et al.*, 2000; Tomasi *et al.*, 2015). This observation highlights the importance of terroir and the interaction between its components on the synthesis of 3SH precursors.

In our study, vines were relatively young (3 years old) and since their growth and grape composition depend largely on reserves accumulated over previous years, including N reserves, experiments on older vines will be necessary. Such experiments would allow to (i) study the impact of N reserves on berry composition, (ii) understand the effect of N metabolism on 3SH precursor synthesis and (iii)

better understand the response of variety/rootstock interaction regarding N nutrition, taking into account vine water status and vine vigor.

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