EFFECT OF VINE NITROGEN STATUS ON GRAPE AROMATIC POTENTIAL: FLAVOR PRECURSORS (S-CYSTEINE CONJUGATES), GLUTATHIONE AND PHENOLIC CONTENT IN *VITIS VINIFERA* L. cv. SAUVIGNON BLANC GRAPE JUICE

EFFET DE LA NUTRITION AZOTÉE DE LA VIGNE SUR LE POTENTIEL AROMATIQUE DU RAISIN: PRÉCURSEURS D'ARÔMES (S-CONJUGUÉS À LA CYSTÉINE), GLUTATHION ET COMPOSÉS PHÉNOLIQUES DANS LE MOÛT DE *VITIS VINIFERA* L. cv. SAUVIGNON BLANC

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Abstract: Vine nitrogen status influence on S-cysteine conjugate precursors of volatile thiols, glutathione and phenolic content of the berries of *Vitis vinifera* L. cv. Sauvignon blanc have been assessed. Despite an increase in berry weight, the increase in nitrogen supply in the vine leads to higher cysteine precursor levels in grape juice. We also show that a late addition of nitrogen at berry set leads to a lower level of phenolic compounds in white grapes and to higher glutathione levels. Therefore, in white varieties, and particularly Sauvignon blanc, improving the nitrogen supply of the vine clearly increases its aromatic potential. However, an excess of nitrogen supply would increase the grape sensitivity to *Botrytis cinerea* and therefore decrease its quality.

Résumé: L'effet du statut azoté de la vigne sur le potentiel aromatique de raisins de *Vitis vinifera* L. cv. Sauvignon blanc a été évalué. Sur une parcelle carencée en azote (modalité N), à cause d'une faible teneur en matière organique dans le sol, un supplément d'azote équivalent à 60 u/ha a été apporté (modalité L + N). Le stade tardif de l'apport, la nouaison, visait à limiter l'effet de l'azote sur les paramètres de la vigueur. Sur les deux modalités, l'alimentation en eau de la vigne était similaire et non limitante (potentiels tige > -0,65 Mpa tout au long de la saison). Il était important de vérifier l'absence de contrainte hydrique, car il a été montré que l'alimentation en eau influe sur la teneur en précurseurs aromatiques du raisin. Sur la modalité L + N, le rendement était équivalent à L et la surface foliaire primaire était similaire, mais la surface foliaire secondaire et le poids des bois de taille étaient plus forts. L'apport d'azote a provoqué une augmentation du poids des baies et de l'acidité totale du jus de raisin, à cause d'une plus forte teneur en acide malique, et une très forte augmentation de la teneur en azote assimilable. La teneur en sucres réducteurs n'était pas significativement modifiée. Les raisins des vignes, qui avaient reçu un apport d'azote, contenaient plus de précuseurs S-conjugués à la cystéine, plus de glutathion et moins de composés phénoliques. Cette constitution permet d'élaborer des vins avec une plus forte expression variétale. Il n'est cependant pas souhaitable d'avoir une alimentation en azote excessive des vignes, à cause d'une plus grande sensibilité des raisins à *Botrytis cinerea.*

Key words : nitrogen, water status, fertilization, stem water potential, vine, *Vitis vinifera*, Sauvignon blanc, S-cysteine conjugates precursors, volatile thiols, phenolic compounds, glutathione

Mots clés : azote, état hydrique, fertilisation, potentiel tige, vigne, *Vitis vinifera*, Sauvignon blanc, précurseurs S-conjugués à la cystéine, thiols volatils, composés phénoliques, glutathion

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INTRODUCTION

Vine nitrogen status has a strong influence on vine vigor, resulting in higher must acidity and reinforced sensibility to *Botrytis* (BRANAS, 1974). Vine nitrogen status affects tannin and anthocyanin content in Merlot and Cabernet-Sauvignon (CHONE *et al.*, 2001a; TREGOAT *et al.*, 2002). DELAS *et al.* (1991) reported that tannin concentrations in the skins of Merlot grapes were higher in vines fertilized with 0 versus 100 kg N/ha. So far, little data about the influence of vine nitrogen status on aromatic compounds or precursors in grapes have been available and information about the effect of vine nitrogen status on varietal aroma in wine is scant. DOUGLAS *et al.* (1993) discussed the influence of vineyard nitrogen fertilization on monoterpene concentrations in Riesling wines but all the concentrations reported were below the established sensory threshold.

The varietal aroma of Sauvignon blanc wines is associated with the presence of volatile thiols (DARRIET *et al.*, 1995; TOMINAGA *et al.*, 1998a):

- 4-mercapto-4-methylpentan-2-one (4MMP);
- 4-mercapto-4-methylpentan-2-ol (4MMPOH);
- 3-mercaptohexan-1-ol (3MH).

These compounds also participate in the aroma of other wine varieties such as Sémillon, Manseng and Riesling (TOMINAGA *et al.*, 2000). The volatile thiols are not present in grape berries but are released during fermentation by the action of yeast (MURAT *et al.*, 2001) from odorless precursors whose structure has recently been identified as being S-cysteine conjugates (TOMINAGA *et al.*, 1998a). 4MMP, 4MMPOH and 3MH are thus released respectively from S-4-(4-methylpentan-2-one)-L-cysteine (P-4MMP), S-4-(4-methylpentan-2-ol)-L-cysteine (P-4MMPOH) and S-3-(hexan-1-ol)-L-cysteine (P-3MH). A method has been developed to assay these precursors in must (PEYROT DES GACHONS *et al.*, 2000). Consequently, by assaying S-cysteine conjugates, it is possible to study the effect of the nitrogen supply from the vine on one of the factors of the aromatic potential of grapes, i.e. the cysteine conjugate precursors.

The aromatic potential of grapes and wines also involves the levels of glutathione and phenolic compounds of the must and wines. In fact, the reactivity of varietal thiols with quinones is one of the main reasons for the instability of varietal aroma in Sauvignon wines. By limiting the formation of quinones during the various stages of making Sauvignon blanc wines, it is possible to stabilize their varietal aroma. Consequently, it is essential to minimize the level of phenolic compounds in musts and to protect them from oxidation. Glutathione in musts and wines, which is very reactive to quinones, plays an important role in protecting varietal volatile thiols from oxidation (DUBOURDIEU *et al.*, 2001).

MATERIALS AND METHODS

I - LOCATION, VINE MATERIAL AND EXPERIMENTAL SET UP

This study was conducted in 2000 in a French vineyard (Château Reynon, Bordeaux area, France). *Vitis vinifera* L. cv. Sauvignon blanc blocks grafted on *Vitis riparia* planted in 1981 were used. The vines were spaced 3 x 1 m apart and double guyot pruned. The top of the canopy was pruned mechanically and identically within the block. Shoots were vertically positioned and the height of the leaf curtain was 1.3 m. This block was chosen because 25 % of its area exhibited a severe nitrogen deficiency, whereas the remaining part exhibited a high nitrogen status, resulting in a rather high vigor. The nitrogen deficiency was explained by very low soil organic matter content (0.5 %) in that part of the block. Texture was silty-clay for top soil and clay silt for the sub soil. Root zone depth ranged from 180 cm to 200 cm. According to the texture, soil water holding capacity was estimated up to 200 mm. In June 2000, 10 days after the end of bloom, the equivalent of 60 kg per hectare of nitrogen (in the form of ammonium nitrate) were applied to vines exhibiting nitrogen deficiency. Hence in this experience, two level of nitrogen status were studied:

- L: low nitrogen status;
- L+N: low nitrogen fertilized after bloom.

Three replicates of vine vigor measurements and grape sampling were performed in each zone.

II - VINE WATER STATUS

Seasonal vine water status was assessed by means of midday stem water potential (BEGG and TURNER, 1970; CHONÉ *et al.*, 2001b) in a pressure chamber (SCHOLANDER *et al.*, 1965). On sunny days, primary leaves on the shaded side on the row were bagged one hour prior to stem water potential measurement. Values of midday stem water potential are the mean of six measurements.

III - NITROGEN STATUS

Vine nitrogen status was estimated at harvest by determining the assimilable nitrogen content of must. Must total nitrogen content and must assimilable nitrogen are highly correlated, and both provide a good estimation of vine nitrogen status (VAN LEEUWEN *et al.*, 2000). Assimilable nitrogen content of the musts was estimated by the formal method of Sörensen (AERNY, 1996) modified as followed : 50 mL of must, equilibrated to pH 8.5 were added with 25 ml of formaldehyde adjusted before hand to pH 8.5. The resulting must acidification was then titrated to pH 8.5 with a solution of NaOH 0.1 N (MASNEUF *et al*., 2000).

IV - VINE VIGOR

To establish leaf area, 20 primary and 20 secondary shoots were randomly sampled. Their lenght was measured and their leaf area was determined with a LICOR leaf area meter. One correlation was established between the lenght of the primary shoots and their total leaf area and another correlation was established between the lenght of the secondary shoots and their total leaf area. The lenght of all primary and secondary shoots of 8 vines per replicate was measured. Primary and secondary leaf area was deduced from a correlation between shoots length and leaf area. Average total cluster mass per vine was measured on 4 vines per replication on the day of harvest, just before berry sampling. Average pruning mass per vine was determined on 8 vines per replication in December.

V - MUST: MAJOR COMPOUNDS

Harvest of the three blocks was performed on 3 September 2000. In the field and immediately after determining the total cluster mass per vine, two 1 kg samples were taken per replication with fragments of clusters. In the laboratory, one sample was frozen while 400 berries of the second were counted and weighed to determine average fresh berry mass. Next, the entire sample was pressed (4 and 6 bars). 100 ml were immediately frozen after adding 200 mg SO_2 per liter in order to determine total phenolic content and glutathione. The remaining juice was gently centrifuged to remove suspended solids. Sugar content was measured by refractometry and titratable acidity was measured manually with NaOH 0.1 N and bromothymol blue as color indicator. Malic acid was determined using an enzymatic kit (Boehringer, Mannheim). Assimilable nitrogen was assessed on the remaining juice after a second centrifugation.

VI - S-CYSTEINE CONJUGATE PRECURSORS OF FREE VOLATILE THIOLS AND GLUTATHIONE

The three volatile thiols (4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercaptohexan-1-ol) were released enzymatically from their precursors by percolating the must through an immobilized tryptophanase column and catalyzing an α,β-elimination reaction on the S-cysteine conjugate (TOMINAGA *et al.*, 1998a). The volatile thiols were analyzed by GC-MS. Glutathione in its reduced form was assayed by capillary electrophoresis (HP 3D Capillary Electrophoresis System) on a silica capillary (65 cm; 75 µm) with a phosphate buffer (pH=7.5; 0.05M) and at 25KV voltage. Detection was performed by fluorescence (λ excitation 365 nm; λ emission 465 nm) with a method adapted from that of NOCTOR and FOYER (1998) using specific derivatization of SH functions by monobrobimane (MBB). Samples were prepared in the following conditions. To 200 µL of pre-filtered wine were added 10 µL dithiotreitol (DTT; 10 mM), 145 µL CHES buffer (0,5 mM, pH 9,3; preparation: 2,58 g 2-(N-cyclohexylamino) ethanesulfonic acid (CHES), in 25 mL distilled water; pH is adjusted to 9,3 with NaOH). After 15 min reaction in the dark, the sample was injected in the hydrodynamic mode (5 s, 50 mbar).

VII - TOTAL POLYPHENOL CONTENT IN MUST

5 ml of the must sample was first centrifuged at 4 000 rpm (2 400 g). A C18 SPE cartridge (0.5 g of adsorbent, Supelclean LC18, Supelco Ref 57054, Saint-Quentin Fallavier, France) was activated by 5 ml of MeoH followed by 5 ml of purified water (MQ water, Millipore, Guyancourt, France). 1 ml of the must sample was then deposed on the column (elution under partial vacuum). To remove polar compounds like sugars and nitrogen compounds, two successive washings with 15 ml water and 15 ml acidified water (0.1 % v/v HCl) were performed. The phenolic compounds were then eluted with 10 ml of MeoH/ Water (9/1 v/v). The MeoH/ Water solution of polyphenols obtained was then placed in 1 cm quartz cuvette and the absorbance at 280 nm (A280) measured against pure MeoH/ Water (9/1 v/v). The total polyphenol index of must (TPIm; RIBÉREAU-GAYON, 1970) is then calculated by multiplying the absorbance by 10 (factor of dilution): TPIm = A280*10.

The statistical significance of differences was verified with the Student's-test means comparison.

RESULTS AND DISCUSSION

I - VINE WATER STATUS

Soil water holding capacity was at field capacity at the middle of spring (early May), which is usual in the Bordeaux area. Rainfalls in May, June and July 2000 were respectively 48, 34 and 70 mm. No significant rainfall occurred in August 2000. From early July (day 182) to end of August (day 246), seasonal midday stem water potential did not differ significantly between « L » and « L + N » (figure 1). Furthermore, midday stem water potential remained constantly high in both parts of the block. Values were always greater than -0.65 Mpa, which means that vines were not subjected to water deficit in this experiment (CHONÉ *et al*., 2001b; CHONÉ, 2001). It was important to monitor precisely vine water status in this experiment, because it has been shown that severe water deficits can negatively affect Sauvignon blanc aroma potential (PEYROT des GACHONS *et al.*, 2005).

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II - NITROGEN STATUS AND VINE VIGOR

On 3 September, must assimilable nitrogen content was six-fold higher in L+N than in L; this difference was highly significant (table I). Hence, this demonstrated the high ability for the vines to take up nitrogen from the soil after bloom, a finding never previously reported.

Total cluster mass, the number of clusters and primary leaf area were identical in L and L+N (table I).

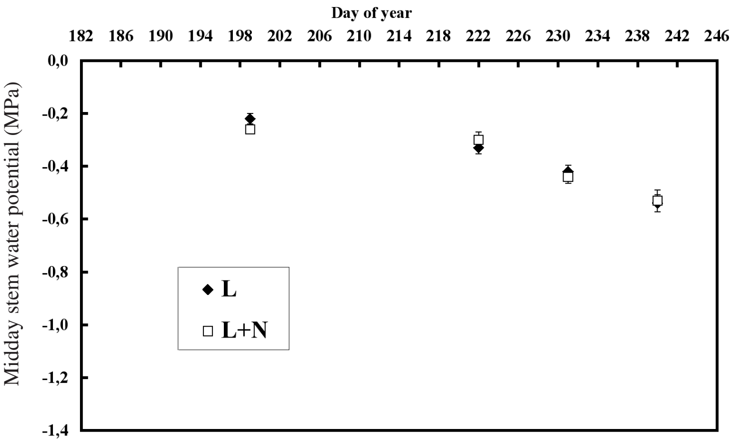

Figure 1 - Seasonal midday stem water potential on vines subject to two levels of nitrogen status: L, low nitrogen status; L+N, fertilized 60 kg/ha at fruit set.
Error bars indicate s.e. Bloom and harvest occurred on days 154 and 246 respectively.

Évolution du potentiel tige au cours de la saison de vignes soumises à deux niveaux d'azote : L, faible nutrition azotée ; L + N, fertilisé 60 kg/ha à la nouaison.
Barres d'erreur indiquent s.d. Floraison et récolte ont eu lieu au jour 154 et 246 de l'année respectivement.

Table I - Vigor indicators on vines with low (L) and high (L + N; fertilized at fruit set) nitrogen status.
(1)Values within rows followed by the same letter do not differ significantly (Student test, p<0.05).

Indicateurs de vigueur pour des vignes avec un faible statut azoté sans fertilisation (L) et avec fertilisation (L + N ; fertilisé à la nouaison).
(1)Valeurs suivies d'une même lettre ne sont pas significativement différents (Test de Student, p<0.05).

	L	L+N
Assimilable nitrogen (mg/L)	29 a (1)	174 b
Clusters per vine	11.1 a	9.9 a
Total cluster mass per vine (kg)	1.4 a	1.6 a
Primary leaf area (m^2)	2.1 a	2.3 a
Secondary leaf area (m^2)	0.4 a	1.4 b
Pruning mass per vine (kg)	0.5 a	0.8 b

Secondary leaf area and pruning mass per vine increased significantly with late fertilization in L+N versus L. The constant primary leaf area associated with significantly higher secondary leaf area in L+N versus L showed that the increase in vine nitrogen status under non-limiting water status conditions enhanced secondary shoot growth in L+N. Hence, in L and L+N, vine vigor was similar except for secondary leaf area and pruning weight, while nitrogen status was dramatically different.

III - FRESH BERRY MASS, MUST SUGAR CONTENT AND TOTAL ACIDITY

Fresh berry mass in L+N was significantly higher by 21 % than in L (table II). As observed on Cabernet-Sauvignon (CHONÉ *et al.*, 2001a), low vine nitrogen status limited berry mass. There was no difference at harvest between the must sugar contents of the two plots. Conversely, must titratable acidity in L was significantly lower than in L+N. This appeared to be mainly due to the significantly lower must malic acid content in L. In Cabernet-Sauvignon must from vines with low nitrogen status, CHONÉ *et al.* (2001a) also observed lower malic acid content compared to must from vines with high nitrogen status.

IV - S-CYSTEINE CONJUGATE PRECURSORS OF FREE VOLATILE THIOLS, TOTAL POLYPHENOLS AND GLUTATHIONE

The correction of nitrogen deficiency after bloom (L + N) led to a significant increase in the concentration of cysteine precursors in grape juice compared to L (table III), despite greater berry size. This indicated that higher vine nitrogen status increased cystein precursor synthesis in Sauvignon blanc grapes. The response of the three pre-

Table II - Berry mass and major compounds in grape juice from vines with low (L) and high (L + N; fertilized at fruit set) nitrogen status.
(1)Values within rows followed by the same letter do not differ significantly (Student test, p<0.05).

Poids des baies et composition du jus de raisin de vignes avec un faible statut azoté sans fertilisation (L) et avec fertilisation (L + N ; fertilisé à la nouaison).
(1)Valeurs suivies d'une même lettre ne sont pas significativement différents (Test de Student, p<0.05).

	L	L+N
Fresh berry mass (g)	1.5 a (1)	1.9 b
Must-reducing sugar (g/L)	202 a	199 a
Must titratable acidity (g of tartrate/L)	8.0 a	9.9 b
Must malic acid (g/L)	2.72 a	4.22 b
Assimilable nitrogen (mg/L)	29 a	174 b

cursors to nitrogen fertilization was not the same. Compared to L, the levels of P-4MMP and P-4MMPOH in the must of L+N were 76 and 171 % greater, respectively, while the P-3MH level was 341 % greater (table III). Consequently, the metabolism of P-3MH might be different from that of P-4MMP and P-4MMPOH.

The level of total polyphenols in the must of L was significantly greater (30 %) than that of L+N (table III). Therefore, higher vine nitrogen status had the same depressive effect on the phenolic compound levels in white Sauvignon as in red varieties (DELAS *et al.*, 1991; CHONÉ *et al.*, 2001a). High quality potential grapes for red wine making have high levels of total phenolics, but high quality potential grapes for white wine making should have low levels. The influence of nitrogen supply on must glutathione levels showed the same tendency as that observed for cysteine precursors. Glutathione levels in L+N were seven times higher than those in L. Consequently, the increase in nitrogen supply without an increase in yield had a positive effect on glutathione levels. Higher glutathione levels in L + N will provide a better protection of volatile thiols during grape processing.

Nitrogen deficiency decreases Sauvignon blanc aroma expression, because grapes contain less aroma precursors, and more volatile thiols are lost during grape processing because of higher total phenolics and lower glutathione levels. However, excessive nitrogen supply to the vines should also be avoided. Although this aspect was not monitored in this study, vine *Botrytis* susceptibility is increased by high berry nitrogen content.

Table III - Aroma potential, total phenolics and glutathione in grape juice from vines with low (L) and high (L + N; fertilized at fruit set) nitrogen status.

(1)Values within rows followed by the same letter do not differ significantly (Student test, p<0.05).

Potentiel aromatique, composés phénoliques et glutathion dans le jus de raisin de vignes avec un faible statut azoté sans fertilisation (L) et avec fertilisation (L + N ; fertilisé à la nouaison).

(1)Valeurs suivies d'une même lettre ne sont pas significativement différents (Test de Student, p<0.05).

	L	L+N
P-4MMP (ng eq/L)	405 a (1)	715 b
P-4MMPOH (ng eq/L)	760 a	2059 b
P-3MH (ng eq/L)	3358 a	14812 b
Total Polyphenol Index	0.28 a	0.21 b
Glutathione (mg/L)	17.9 a	120 b

CONCLUSION

Despite higher berry weight, the increase in nitrogen supply in the vine leads to higher cysteine conjugate precursor levels in the berries. We also show that, as in red varieties, a late addition of nitrogen at berry set leads to a decrease in the levels of phenolic compounds in white grapes and to higher glutathione levels in grape juice. Low levels of phenolics and high levels of glutathione are important factors for preserving volatile thiols during grape processing. Therefore, in white varieties, and particularly *Vitis vinifera* L. cv. Sauvignon blanc, unlimited nitrogen supply of the vine is an important factor for optimum varietal aroma expression. However, an excess of nitrogen supply would increase the grape sensitivity to *Botrytis cinerea* and therefore decrease its quality.

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