Changes in the activities of enzymes involved in nitrogen and sulphur assimilation during leaf and berry development of *Vitis vinifera* 1)

by

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Introduction

Changes in enzyme activities in *Vitis vinifera* associated with berry development or ripening have been adequately described for oxidas (IVANOV and IVANOVA 1968), proteolytic enzymes (CORDONNIER and DUGAL 1968), for enzymes involved in CO₂ fixation (HAWKER 1969), organic acids and sugar metabolism (HAWKER 1969, RUFFNER and KLIWER 1975, RUFFNER and HAWKER 1977) and Krebs-Henseleit cycle (ROUBELAKIS and KLIWER 1978). Changes in the isoenzyme patterns have been also reported (DAL BELIN PERUFFO and PALLAVICINI 1975, SCHAFFER 1982, 1983). Recently, nitrate reductase activity has been detected (PEREZ and KLIWER 1978) and characteristics of glutamate dehydrogenase, glutamine synthetase and glutamate oxalacetate transaminase in leaves and roots of cv. Chenin blanc have been described (ROUBELAKIS-ANGELAKIS and KLIWER 1983 a, 1983 b, 1984).

Despite of the importance of nitrogen and sulphur in the nutrition of plants and the interactions between the two elements (DIJKHOORN and VAN WIJK 1967, STEWART and PORTER 1969, BOLTON et al. 1976, RENDIG et al. 1976, FRIEDRICH and SCHRADER 1978, PASSERA and GHISI 1982), there is little or no information on changes in enzyme activi-

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ties involved in the reduction and assimilation of nitrate and sulphate during leaf and berry development. Distribution of activities of these enzymes between leaves and berries has not yet been reported.

Therefore, the activities of ATP sulphurylase (EC 2.7.7.4) and O-acetylserine sulphydrylase (EC 4.2.95.9), the first and the last enzyme in the reaction chain of sulphate assimilation (SCHWEN and TREBST 1976), and the activities of nitrate reductase (EC 1.6.6.1), glutamine synthetase (EC 1.4.7.1) and glutamate dehydrogenase (EC 1.3.1.2), enzymes concerned with the reduction and assimilation of nitrogen (MIFLIN and LEA 1980), were followed in leaves and berries of Vitis vinifera L. cv. Merlot, from 2 weeks prior to anthesis up to the harvest of fruit.

**Materials and methods**

Leaf and berry samples of Vitis vinifera cv. Merlot were taken weekly from a 14-year-old vineyard of the Experimental Institute of Viticulture, Conegliano Veneto. Clusters and their opposite leaves of 4th internodes were utilized during the period extending from May 10th to September 12th, 1983 and from June 1st to September 30th, 1982. Significant differences were not observed among patterns of the considered parameters obtained in 1982 and 1983. This paper reports data of 1983.

Total nitrogen and amino nitrogen contents were determined according to LEGGET BAILEY (1962), nitrate to CATALDO et al. (1975) and soluble protein content as reported by LOWRY et. al. (1951)

All determinations of enzyme activities were carried out homogenizing 1—2 g leaf samples and 2—8 g berry samples.

To determine ATP sulphurylase activity, leaf or berry samples were homogenized in 10 ml of a solution containing 100 mM Tris-HCl buffer, 10 mM cysteine and 50 mM MgCl₂ at pH 8.0. The homogenate was strained through four layers of muslin cloth and centrifuged at 30,000 g for 15 min. The precipitate was discarded and 0.2 ml of supernatant were used to measure enzyme activity according to WILSON and BANDURSKY (1958).

To determine O-acetylserine sulphydrylase, leaf or berry samples were homogenized in 10 ml of a solution containing 100 mM Tris-HCl buffer, 0.1 mM pyridoxal-5-phosphate, 10 mM β-mercapto-ethanol and 15 % (v/v) glycerin at pH 8.0. The homogenate was strained through four layers of muslin cloth and centrifuged at 10,000 g for 30 min. The supernatant was utilized for the determination of enzyme activity except for the composition of the reaction mixture (50 µmol Tris-HCl, pH 8.0, 5 µmol DTT (dithiothreitol), 12.5 µmol OAS, (O-acetylserine) 2.5 µmol Na₂S and 0.1 ml of the extract, in a final volume of 0.5 ml), the procedure of PIENIAZEK et al. (1973) was followed.

To determine O-acetylserine sulphydrylase, leaf or berry samples were homogenized in 10 ml of a solution containing 100 mM Tris-HCl buffer, 0.1 mM pyridoxal-5-phosphate, 10 mM β-mercapto-ethanol and 15 % (v/v) glycerin at pH 8.0. The homogenate was strained through four layers of muslin cloth and centrifuged at 30,000 g for 15 min. The supernatants were used as enzyme preparations. Nitrate reductase activity was assayed according to HEWITT and NICHOLAS (1964).

Nitrate reductase induction was carried out by floating leaf disks (Ø 0.5 cm) for 72 h on nutrient solution (HOAGLAND and ARNON 1950) enriched with KN0₃ (200 mg/l).

Glutamine synthetase extracts were prepared by homogenizing leaf or berry samples in 10 ml of extraction medium (50 mM imidazole-HCl, pH 7.2, 1 mM EDTA (ethylenediaminetetraacetate), 10 mM cysteine containing PVPP (polyvinylpolypyrrolidone; 0.5 g/ml). The homogenates were strained through four layers of muslin cloth and cen-
trifuged at 10,000 g for 10 min. The supernatants were used as enzyme preparations. Glutamine synthetase activity was assayed by the production of γ-glutamylhydroxamate. The reaction mixture contained 100 mM imidazole-HCl, 40 mM ATP, 200 mM MgSO₄, 200 mM glutamate, 40 mM NH₂OH at pH 7.2 and the extract (0.4 ml) in a final volume of 2 ml. After 20 min at 37 °C the reaction was stopped by the addition of 2 ml of colorimetric reagent (Rowe et al. 1970). The precipitated protein was removed by centrifugation and the absorbance of the solution was determined at 540 nm.

Glutamate dehydrogenase extracts were prepared according to Joy (1969) except for the addition of PVPP (0.5 g/ml). Extracts were dialyzed against the extraction medium. Enzyme activity was assayed following NADH oxidation at 340 nm. The reactions were carried out at 30 °C in quartz cuvettes and the assay mixture consisted of 80 mM imidazole-HCl, pH 7.9, 213 mM ammonium acetate, 1.67 mM ADP, 0.12 mM NADH, and 6.67 mM α-ketoglutarate. The reaction was initiated by adding α-ketoglutarate after measurement of endogenous consumption of NADH. Aliquots of 0.4 ml of leaf extracts were used.

![Graph showing berry growth](image)

Fig. 1: Time course of berry growth (g of fresh weight/berry) of *V. vinifera* cv. Merlot.

Croissance des baies (poids frais en g/baie) de *V. vinifera* cv. Merlot.

**Results**

The growth pattern of berries is shown in Fig. 1. Berry weight increased until the 4th week after veraison and thereafter declined.

Nitrate nitrogen and nitrate reductase activity were not detectable either in berries or leaves. Nitrate reductase activity was not detectable even when leaf disks were floated for 72 h in illuminated nutrient solution enriched with NO₃⁻. Therefore, our results are in contrast with those showing presence of nitrate reductase activity in leaves of grapevine (Perez and Kliewer 1978). It is pointed out that our
determinations were carried out on supernatant of leaf extracts, whereas the other researchers tested pellets of leaf extracts (Perez and Kliewer 1978).

Changes in total nitrogen, amino nitrogen and soluble protein during the development of berries and leaves are presented in Fig. 2 A and B, respectively.

Total nitrogen of leaves gradually decreased from 50 mg/g dry matter 4 weeks before anthesis to 20 mg/g dry matter at harvest. Total nitrogen pattern of berries was similar to that shown by leaves. Nitrogen content was 17 mg/g dry matter at the beginning of berry development and 7 mg/g dry matter at harvest (Fig. 2 A).

Amino nitrogen content in leaves reached the highest level in the 3rd week before anthesis; another significant maximum was noticed 3 weeks after anthesis. In berries, amino nitrogen increased with fruit development, showed a maximum 2 weeks before

Fig. 2: Changes in total nitrogen (●—●—●), amino-nitrogen (O—O—O) and protein content (+——+) (mg/g of dry matter) in developing berries (A) and leaves (B) of V. vinifera cv. Merlot.
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veraison (Fig. 2 A) and then declined. Another significant maximum in amino nitrogen was found in the 3rd week after veraison.

It is possible to distinguish three stages in leaf protein pattern (Fig. 2 B): the first one showing the highest protein content and lasting from 2 weeks prior to anthesis up to 2 weeks after anthesis; a second stage, showing the lowest protein content, from the 4th to the 7th week after anthesis; a third stage from veraison up to 2 weeks before harvest. This last phase showed an intermediate protein content between the other two stages.

At the beginning of their growth, berries had a high content of soluble protein. Thereafter, the development of fruit was accompanied by a decrease, followed by an increase in protein concentration, reaching a maximum in the 1st week after veraison. A significant lower maximum occurred in the 3rd week after veraison. Then, protein content slowly decreased (Fig. 2 A).

In leaves, glutamate dehydrogenase activity showed two maxima: the first one at anthesis and the second on 5 weeks after anthesis. From the 6th week, activity slowly decreased (Fig. 3).

In berries, glutamate dehydrogenase showed also two maxima of activity: the first one 1 week after the beginning of berry development and the other one in the 4th week after veraison. GDH activity of berries was always higher than that of leaves (Fig. 3).

On the contrary, the levels and patterns of glutamine synthetase activity were similar in leaves and berries. In both organs three maxima of activity occurred.

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**Fig. 3**: Activity of glutamate dehydrogenase (nmol NADH oxidized \( \cdot \) mg protein\(^{-1} \cdot \) min\(^{-1}\)) extracted from developing leaves (— — — — —) and berries (— — — — —) of *V. vinifera* cv. Merlot.

Activité de glutamate déshydréogenase (nmol NADH oxydé \( \cdot \) mg protéine\(^{-1} \cdot \) min\(^{-1}\)) de feuilles (— — — — —) et baies (— — — — —) de *V. vinifera* cv. Merlot en cours de développement.
after anthesis. Like for the other tested enzymes, a maximum of GS activity also occurred at anthesis (Fig. 4).

**O-acetylsulfhydrylase** activity of berries increased up to the 3rd week after the beginning of fruit development (Fig. 5). In the subsequent weeks, there was at first a rapid decrease of activity and then a continuous slow decline until harvest.

Three maxima of O-acetylsulfhydrylase activity were found in leaves: at anthesis, in the 5th and the 7th week after anthesis, respectively. O-acetylsulfhydrylase of leaves was always lower than the activity observed in berries.

In berries, the pattern of **ATP sulfurylase** activity was similar to that of O-acetylsulfhydrylase activity (Fig. 6). However, the highest level of ATP sulfurylase activity was found 1 week after the beginning of berry development. Another maximum of activity — about 25% of the first peak — occurred 1 week before veraison. In the subsequent week, a quick reduction of activity was noticed.

Four maxima of ATP sulfurylase activity occurred in leaves: at anthesis, in the 4th, 7th and 10th week from anthesis, the third one being the highest (Fig. 6). At harvest, ATP sulfurylase activity was, similar to the other enzymes, negligible either in leaves or in berries.

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**Fig. 4:** Activity of glutamine synthetase (nmol γ-glutamylhydroxamate · mg protein⁻¹ · min⁻¹) extracted from developing leaves (———) and berries (-----) of *V. vinifera* cv. Merlot.

**Activité de glutamine synthétase** (nmol γ-glutamylhydroxamate · mg protéine⁻¹ · min⁻¹) de feuilles (———) et baies (-----) de *V. vinifera* cv. Merlot en cours de développement.
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Discussion

At anthesis, enzymes involved in nitrogen and sulphur assimilation showed a maximum of activity. Thus, our results agree with the observation that during this stage plants are subjected to remarkable metabolic changes (LEOPOLD and KRIEDEMANN 1975). Two interpretations are possible: either enzyme synthesis occurs de novo, or existing enzymes are activated (RHODES 1980). Nucleic acid and protein synthesis (LEOPOLD and KRIEDEMANN 1975) and a high total activity of acid phosphatase (SCHAEFER 1982) during anthesis have been reported.
Our results show that sulphate assimilation occurs either in leaves or berries since both tissues showed ATP sulphurylase and O-acetylserine sulphhydrylase activities (Schwen and Trebst 1976). If the "in vitro" enzyme activity measurements reflect the picture in vivo, our results indicate in berries a higher rate of SO\textsubscript{4}\textsuperscript{2-} assimilation (from 5 to 15fold) than that observed in leaves. In addition, our results indicate that ATP sulphurylase activity of berries develops earlier than O-acetylserine sulphhydrylase. It has been reported that in leaves of S-deprived maize plants the development of ATP sulphurylase activity follows the development of O-acetylserine sulphhydrylase (Passera and Ghisi 1982). Probably, the synthesis of these enzymes in fruits is differently regulated. In addition, since activity levels of ATP sulphurylase were always higher than those observed for O-acetylserine sulphhydrylase, we suggest that in grapevine ATP sulphurylase might not be the regulatory enzyme of sulphate assimilation pathway as suggested by Hawes and Nicholas (1973) for Saccharomyces cerevisiae, and Passera and Ghisi (1982) for maize leaves. Recently, Brunold and Schmidt (1976) have suggested that adenosine-5'-phosphosulphate sulphotransferase might be the regulatory enzyme in SO\textsubscript{4}\textsuperscript{2-} assimilation in chlorophyllous tissues.

Extracts from leaves and berries exhibited glutamate dehydrogenase and glutamine synthetase activities. Therefore, both organs are able to assimilate NH\textsubscript{4}\textsuperscript{+} via glutamine synthetase pathway and reductive amination process (Miflin and Lea 1980).
Either in leaves or berries the glutamine synthetase activity was higher than that of glutamate dehydrogenase; therefore, our data confirm for grapevine the generally accepted conception that the glutamine synthetase/glutamate synthetase pathway is the main responsible for \( \text{NH}_4^+ \) assimilation (MIFFLIN and LEA 1980). In addition, the glutamine synthetase pathway does not preclude increments of the glutamate dehydrogenase activity as confirmed in our experiments (MIFFLIN and LEA 1980, ROUBELAKIS-ANGELAKIS and KLIEWER 1983 a).

The comparison among the activities of glutamine synthetase in berries and leaves indicates that the rate of nitrogen assimilation by this enzyme is similar in the two organs. On the contrary, the reductive amination process in berries appears to be twice or threefold more active than that occurring in leaves. Glutamate dehydrogenase is mainly operative when tissues are richer in \( \text{NH}_4^+ \) (MIFFLIN and LEA 1980) and in juvenile grapes most of nitrogen (50% of total nitrogen) is present as ammonia (BURROUGHS 1970). Our results show a high level of amino nitrogen in berries during the initial stages of fruit development.

The high contents of amino nitrogen and protein in berries confirm, on the other hand, the major rate of the amino acid and protein synthesis during the initial stages of fruit development. However, a possible translocation of amino acids from leaves to berries might also occur as the leaf enzymes showed a maximum of activity in the same time period (HALE and WEAVER 1962). This translocation of amino acids should be inhibited in the next stage of ripening. In fact, protein increased in leaves and decreased in berries along with the amino nitrogen content. Increases in the concentration of amino acids in grape berries during maturation have been reported (KLIEWER 1968).

**Summary**

The changes in the activities of O-acetylserine sulphydrylase, ATP sulphurylase, glutamate dehydrogenase, glutamine synthetase and nitrate reductase in extracts from leaves and berries of *Vitis vinifera* L. cv. Merlot were studied at weekly intervals prior to anthesis up to grape harvest. Total nitrogen, amino nitrogen and protein contents were also determined.

The total nitrogen content decreased from the beginning of the determinations either in leaves or in berries. Protein and amino nitrogen increments in berries matched their decreases in leaves.

Nitrate reductase activity was not detectable either in leaves or berries. On the contrary, all the other examined enzymes showed at least three maxima of activity: one at anthesis and the others between the beginning of berry development and grape harvest.

The ATP sulphurylase, O-acetylserine sulphydrylase and glutamate dehydrogenase activities of berries were always higher than those observed in leaves, whereas glutamine synthetase activity was similar in the two organs.

Our results indicate that sulphur and nitrogen assimilation occur either in leaves or berries of grapevine.

**Literature cited**


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