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Malate as substrate for catabolism and gluconeogenesis during ripening in the pericarp of different grape cultivars

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Abstract

Malate is accumulated in grape pericarp until the start of ripening and then it is dissimilated. One aim of this study was to determine if the potential contribution of stored malate to the substrate requirements of metabolism in ripening grape pericarp is dependent on the cultivar. Two *Vitis vinifera* L. cultivars which accumulated different amounts of malate and had ripening periods of a different length were compared. The potential contribution of stored malate over the whole period of ripening was around 20 % in the cv. Sagrantino and 29 % in the cv. Pinot Noir. The contribution was higher in Pinot Noir because it contained more malate and had a shorter ripening period. A second aim of this study was to evaluate the contribution of gluconeogenesis to the amount of sugar accumulated in the pericarp. If all the dissimilated malate was utilized by gluconeogenesis, then the maximum contribution of stored malate to the total amount of sugar accumulated in the pericarp over the whole period of ripening was around 2.4 % in Sagrantino and 2.9 % in Pinot Noir. However, the actual contribution was only about 0.1 - 0.6 % in both cultivars because the majority of stored malate was not utilized by gluconeogenesis. However, it is likely that the actual contribution is much lower. This suggests that the function of gluconeogenesis is not to support accumulation of sugars in the fruits, but probably it plays other roles.

Additional key words: fruit, respiration, sugars, *Vitis vinifera*.

Introduction

The malate content of many fruits increases until the start of ripening and then decreases (Ruffner 1982, Famiani *et al.* 2005, Sweetman *et al.* 2009). This decrease can be brought about either by the metabolism of stored malate or by a dilution arising from an increase in the volume of the fruit (Famiani *et al.* 2005, 2015 Sweetman *et al.* 2009). In grape this decrease is brought about largely by metabolism of stored malate (Ruffner 1982, Famiani *et al.* 2014a). In the flesh of grape, cherry, and tomato, radiolabelling studies have shown that a part of the malate is converted to sugars by gluconeogenesis (Farineau and Laval-Martin 1977, Ruffner 1982, Halinska and Frenkel 1991, Leegood and Walker 1999). This suggested that the amount of malate dissimilated is in excess of the tissues requirements to use it in processes other than gluconeogenesis. In grape a widely held view is that during ripening malate rather than sugars provides the bulk of the substrate used by metabolism (Peynaud and Ribéreau-Gayon 1971, Ruffner and Hawker 1977, Ruffner 1982, Kanellis and Roubelakis-Angelakis 1993, Sweetman *et al.* 2009). Recently, we have shown that in the pericarp of ripening berries of the cv. Cabernet Sauvignon, the contribution of stored malate to the amount of CO_2 released by the berry is dependent on the stage of development (Famiani *et al.* 2014a). Stored malate can provide a large contribution only for a short period just after the start of ripening, and after this, its contribution is relatively low. However, in different cultivars of grapes, the amount of stored malate present in the pericarp at the start of ripening can differ as well as the length of the ripening period (Kliewer *et al.* 1967, Diakou *et al.* 1997).

This paper is an extension of our previous study (Famiani *et al.* 2014a). One aim of the present study was

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Abbreviations: AFB - after full blossom; RQ - respiration quotient.
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to determine whether the contribution that stored malate makes to the amount of CO_2 released by the berry is dependent on the cultivar. To evaluate this, we used Pinot Noir and Sagrantino because they differed in their content of malate at the onset of ripening and in the length of their ripening period. In grape pericarp, the bulk of the CO_2 released by the berry arises from the oxidation of pyruvate by the Krebs cycle (Ruffner 1982). However, in addition to providing pyruvate for the Krebs cycle, malate is used in biosynthetic processes such as gluconeogenesis. The second aim of this study was to evaluate the contribution of gluconeogenesis from stored malate to the amount of sugar accumulated in the berry.

Materials and methods

In 2004, berries were collected from adult grape vines (*Vitis vinifera* L. cvs. Pinot Noir and Sagrantino) trained to a spur pruned cordon and grown in a loamy soil type in an experimental vineyard of the Department of Agriculture, Food and Environment of the University of Perugia, Deruta, in central Italy (42° 58' N; 12° 24' E; elevation 405 m a.s.l.). The stage of the fruit development was based on days after full blossom (AFB), and the full blossom is defined as time when 50 % of flowers are open. The masses of whole berries or their pericarp (flesh + skin) were determined at several stages of development. At each time point, 3 samples of 20 healthy berries were used, and these were randomly collected from 3 groups of vines.

For each stage of development, the pericarps from the same three samples of berries were immediately frozen in liquid nitrogen. The pericarps were then ground with a pestle and mortar in liquid nitrogen. The resulting powder was used, either immediately or after storage at -80 ºC, for the determination of sugar and organic acid content. Glucose, fructose, sucrose, and malate content of the pericarp were determined using enzyme-coupled spectrophotometric methods as previously described (Famiani *et al.* 2009). For extraction of metabolites, the frozen powder (50 mg) was added to an Eppendorf tube containing 1.5 cm^3 of 80 % (v/v) ethanol and 20 % water containing 100 mM HEPES-KOH (pH 7.1) and 20 mM $MgCl_2$, incubated at 80 ºC for l h, and then centrifuged at 12 000 *g* for 5 min. A 150 cm^3 of charcoal suspension (100 mg cm^{-3}) was added to the supernatant, and then vortexed before centrifugation at 12 000 *g* for 5 min. Then the supernatant was stored at -20 ºC until required. For measurement of glucose, fructose, and sucrose in the supernatant, the assay mixture contained 100 mM HEPES-KOH (pH 7.0), 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.02 % (m/v) bovine serum albumin, 1 mM ATP, 0.5 mM NAD^+, and 3 units of hexokinase. The reaction was initiated by adding 1 unit of glucose-6-phosphate dehydrogenase. Fructose and sucrose were analysed sequentially after glucose, following addition of l unit of phosphoglucose isomerase, and 100 units of invertase, respectively, to the assay mixture. For malate, the assay mixture contained 50 mM 2-amino-2-methylpropanol (pH 9.9), 40 mM glutamate, and 1 mM NAD+. The reaction was initiated by adding 10 units of glutamate-oxaloacetate transaminase and 1 unit of malate dehydrogenase to the assay mixture.

Measurements of CO_2 release were done at the ambient temperature under darkness just before dawn (predawn) and then at 11:00 - 13:00. This was done so because CO_2 release by plant organs is lowest at predawn and highest around midday in correspondence with temperature (Grossman and DeJong 1994, Proietti *et al.* 1999). A mean value of these two measurements was used to estimate the amount of CO_2 released by metabolism over a given 24 h period. For each time point, the measurement of CO_2 release was done three times using portions of different bunches of berries (50 - 100 berries for each determination). The bunches were collected at random from the same three groups of vines that were used for the metabolite measurements. Ambient temperatures were between 11 - 19 °C (predawn) and 20 - 34 °C (11.00 - 13.00) during June and September, and between 12 - 20 °C and 26 - 36 °C in July and August. The amount of CO_2 released by the bunches of grapes detached from the vine was determined as previously described (Famiani *et al.* 2014a). Briefly, bunches of grapes detached from the vine were enclosed in the sample chamber (type *PLC-3FM*). The chamber was modified by using a sheet of Plexiglas in place of the original cup, and this was done to reduce the volume of the chamber from 2 600 to 660 cm^3. Dark conditions were produced by covering the chamber that enclosed the berries with a black cloth. The chamber was flushed with ambient air at a rate of 500 cm^3 min^{-1}, and CO_2 concentration measured using an open-system *LCA3* portable infrared gas analyzer (*Analytical Development Company*, Hoddesdon, UK). Release of CO_2 from the stalks (rachis) was also measured and this was subtracted from the value obtained for the whole bunch.

The theoretical respiration quotient (RQ) of the berry, which would arise if all the stored malate dissimilated during ripening was completely oxidised by the Krebs cycle and all NADH so produced oxidised, was calculated as RQ = [1.33 × (proportion of CO_2 potentially arising from oxidation of stored malate during each time period) + 1.00 × (1 - proportion of CO_2 potentially arising from oxidation of stored malate during each time period]; the theoretical value for the RQ that would arise if malate was the sole substrate is 1.33, and if sugars were the sole substrate, it is 1.0 (Famiani *et al.* 2014a).

In the different considered periods of time during ripening, the amounts of malate that were dissimilated and of sugars that were accumulated were determined. Then, the percentage of a potential contribution that

gluconeogenesis from stored malate could make to sugar accumulation was calculated considering that all the stored malate which was dissimilated during each period was completely utilized to produce sugars (two malate molecules to give rise to one glucose molecule).

Results and discussion

For both the cultivars, the growth pattern of whole berries and their pericarps comprised two periods of a more rapid growth separated by a period of a slower growth. The period of the slower growth occurred around 55 d AFB for Pinot Noir and around 60 d AFB for Sagrantino (Fig. 1). The final mass of the berries of both the cultivars

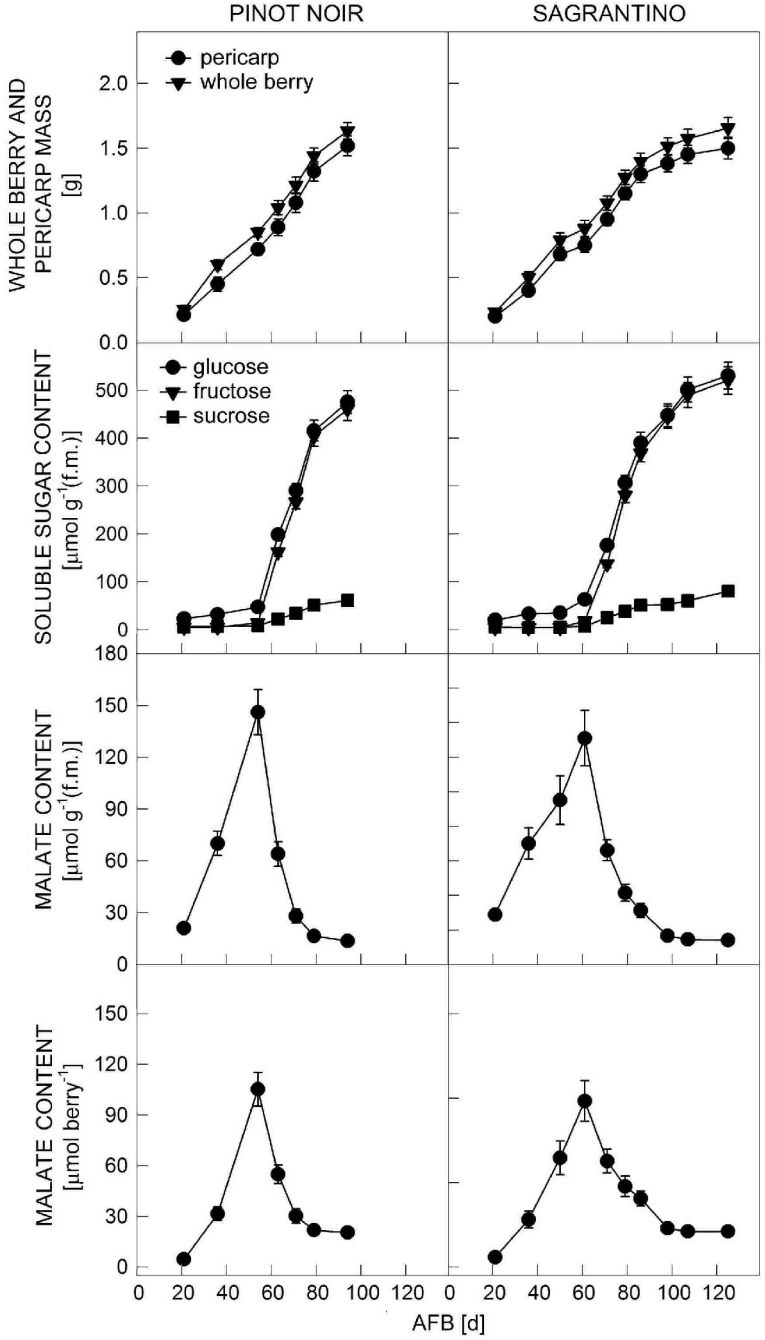

Fig. 1. Fresh mass of the whole berry and its pericarp and content of soluble sugars and malate in the pericarp at different stages of development of two grape vine cultivars Pinot Noir and Sagrantino. Means ± SE, *n* = 3. AFB - after full blossom.

was about 1.6 g. These growth characteristics are similar to those reported previously (Famiani *et al.* 2000).

The soluble sugar content of the pericarp was relatively low up to about 55 and 60 d AFB in Pinot Noir and Sagrantino, respectively, and consisted mainly of glucose (Fig. 1). Throughout ripening, there was a large accumulation of similar amounts of glucose and fructose in both the cultivars. In the pericarp of the ripe berry, the content of these sugars was less in Pinot Noir (Fig. 1). This could be so because Pinot Noir has a shorter ripening period and therefore less time to accumulate sugars. In both the cultivars, the sucrose content was much lower than that of either glucose or fructose. These changes in sugars content during development are consistent with those reported in previous studies of grape vine (Coombe 1992, Famiani *et al.* 2000, 2014a,b).

In both the cultivars, malate accumulated until the start of ripening, and its final amount was 131 - 146 µmol g^{-1}(f.m.) (Fig. 1). The pattern of berry growth together with changes in the content of soluble sugars and malate indicates that veraison occurred around 55 d AFB for Pinot Noir and 60 d AFB for Sagrantino (Fig. 1). During ripening the malate content of the pericarp of both the cultivars decreased, and this was a result of both dilution (arising from an increase in size of the berry) and dissimilation (Fig. 1). The decrease in malate content per single pericarp was about 85 µmol in Pinot noir and 77 µmol in Sagrantino, and most of this decrease occurred soon after veraison (Fig. 1). Similar observations have been made previously for Pinot Noir (Famiani *et al.* 2000) and other grape cultivars (Diakou *et al.* 1997, Famiani *et al.* 2014a,b). The amount of CO_2 released per gramme of fresh mass per minute from the berries of both the cultivars decreased throughout development (data not shown). One factor that contributed to this decrease was the increase in the ratio of the vacuole to the cytoplasm of the cells that make up the pericarp. This is because respiration occurs in the cytoplasm and not in the vacuole. This decrease in CO_2 release was observed at both predawn and midday (data not shown). Of course, the values at 11:00 - 13:00 were higher than those at predawn due to a higher temperature (data not shown) The pattern of CO_2 release from the berries during development was different when it was expressed as the amount of CO_2 released per berry per min (Fig. 2). The CO_2 release per berry increased up to about 50 - 55 d AFB and then declined. These results are consistent with previous studies of grape fruits (Harris *et al.* 1971, Pandey and Farmahan 1977, Ollat and Gaudillère 2000, Palliotti *et al.* 2010, Famiani *et al.* 2014a).

The majority of the CO_2 produced in grape berry pericarp during ripening arises from the oxidation of pyruvate by the Krebs cycle (Ruffner 1982, Terrier and Romieu 2001). Over the entire ripening period, the amount of stored malate that was dissimilated in the pericarp of one berry was about 85 µmol for Pinot Noir and 77 µmol for Sagrantino (Fig. 1). The complete oxidation of a molecule of malate by the Krebs cycle produces four molecules of CO_2. Therefore, the

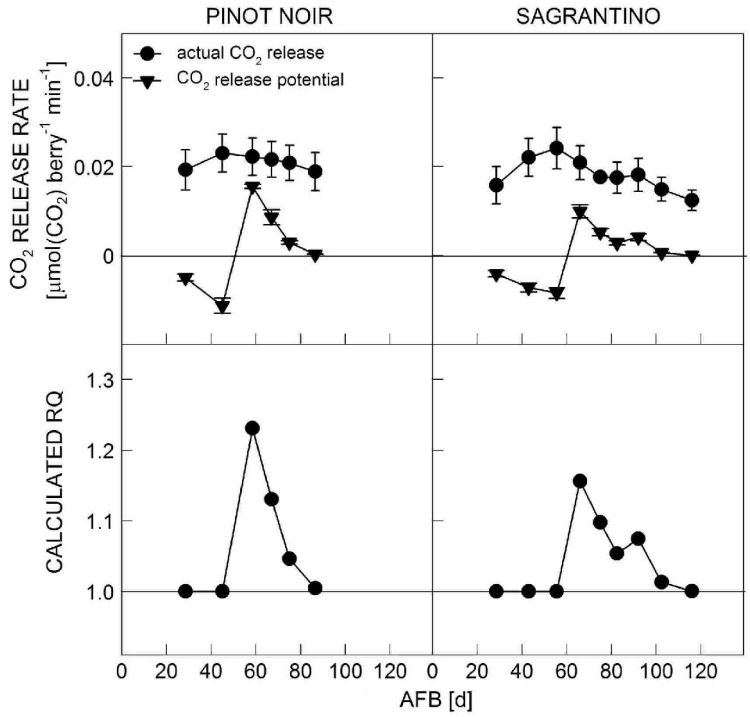

Fig. 2. The rate of release of CO_2 per berry during each considered period of time (*top panels*). Values are means of measurements taken at predawn and between 11:00 - 13:00. Potential amount of CO_2 that would release if all stored malate was dissimilated is also shown. Means ± SE, n = 3. *Bottom panels* show the theoretical respiration quotient (RQ) of the berries when all stored malate which was dissimilated during each considered period of time would be completely oxidized by the Krebs cycle. AFB - after full blossom.

maximum amount of CO_2 produced by the complete oxidation of stored malate by the Krebs cycle would be 340 and 308 μmol $berry^{-1}$, respectively.

A comparison was made between the amount of CO_2 released from the berry and the amount of CO_2 that could potentially arise from the metabolism of all stored malate. This was done for different periods of time throughout the ripening of the berry (Fig. 2). For each period of time, the amount of malate that was dissimilated was calculated and, from this, the maximum amount of CO_2 that would be produced from the complete oxidation of this malate was determined. Then the approximate amount of CO_2 that was released by the berry during this period was calculated. In each cultivar, the maximum potential contribution of malate to the amount of CO_2 released by the berry was soon after veraison, for a period of one to two weeks (Fig. 2), when such a contribution could be around 70 % in Pinot Noir and 47 % in Sagrantino. After this period, this potential contribution declined sharply in both the cultivars (Fig. 2). However, malate is also used in biosyntheses in addition to providing pyruvate that is completely oxidized by the Krebs cycle. Studies in which radiolabelled malate was injected into ripening grape pericarp have shown that at all stages of development, about 75 % of this malate is oxidized to CO_2 and 25 % used in biosyntheses (*i.e.*, gluconeogenesis, amino acid synthesis, *etc.*; Steffan and Rapp 1979). Therefore, soon after veraison, the contribution of malate to the substrate requirements of metabolism would be about 52 % in Pinot Noir and 35 % in Sagrantino. From Fig. 2 it can be calculated that the maximum contribution that stored malate could make to the amount of CO_2 released from the berry over the whole period of ripening is around 20 % of the total amount of CO_2 in Sagrantino and about 29 % in Pinot Noir (Fig. 2). These values are the potential maximum contributions that would arise if all the dissimilated malate was completely oxidized to CO_2. These results are broadly similar to those obtained for Cabernet Sauvignon (Famiani *et al.* 2014a). However, if the proportion of malate that is used in biosyntheses is considered (Steffan and Rapp 1979), the contribution of malate to the substrate requirements of metabolism would be about 15 % in Sagrantino and about 22 % in Pinot Noir. In Pinot Noir the potential contribution was much higher (+ 40 %) than in Sagrantino. This was because Pinot Noir contained more malate at the start of ripening and the duration of its ripening period was shorter (Fig. 2). This indicates that the potential contribution of malate to metabolism is dependent on the cultivar. Nevertheless, the results of our study indicate that in both the cultivars, about 78 - 85 % of the substrate utilized by metabolism in the ripening grape pericarp was not malate. The deficit in substrate is likely provided by sugars, and this is in agreement with the observation that enzymes of the glycolytic pathway are present in the pericarp (Famiani *et al.* 2000, 2014b, Terrier and Romieu 2001, Terrier *et al.* 2005, Negri *et al.* 2008, Fortes *et al.* 2011).

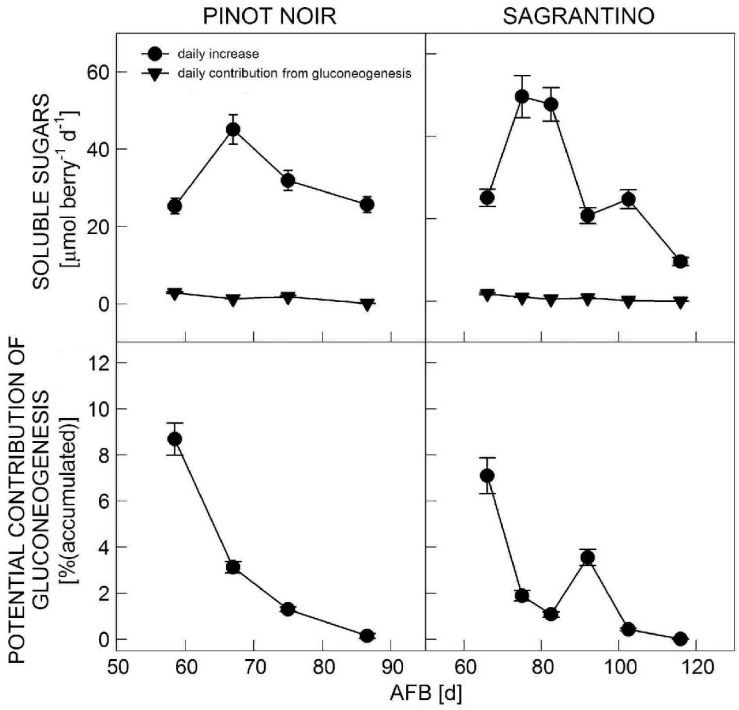

Fig. 3. Accumulation of total soluble sugars (glucose + fructose + sucrose) per berry during each considered period of time together with the maximum amount of sugars that could be produced by gluconeogenesis from stored malate (*upper panels*). Maximum percentage of sugars accumulated during each period of time that could be derived from stored malate by gluconeogenesis (*lower panels*). Means ± SE, $n = 3$. AFB - after full blossom.

For each of the above periods of time during ripening, we calculated the theoretical RQ of the berry. The value was around 1.23 for Pinot Noir and 1.16 for Sagrantino for a short period just after veraison, and then the RQ was much lower (Fig. 2). If the contribution of malate to biosyntheses is considered (Steffan and Rapp 1979), these values would become 1.17 and 1.12. Measurements of the actual RQ of grape berries have shown that before ripening it is close to 1.0 and throughout ripening it is between 1.2 and 1.5 (Kriedemann 1968, Harris *et al.* 1971). The value for the RQ that would arise if sugars were the sole substrate is 1.0, and if malate was the sole substrate is 1.33. The increase in the RQ during ripening has been proposed to arise largely from the use of malate as substrate for respiration (Peynaud and Ribéreau-Gayon 1971, Ruffner 1982, Kanellis and Roubelakis-Angelakis 1993). However, our results clearly show that other processes must have been responsible for this increase in the RQ for most of the ripening period. Two such processes are ethanolic fermentation and the use of NAD(P)H in biosyntheses, both of which can cause an increase in RQ (Romieu *et al.* 1992, Terrier and Romieu 2001, Famiani *et al.* 2014a).

The amount of sugars that could be produced if all the stored malate was converted to sugars, together with the actual amount of sugars that accumulated during a given time period is shown in Fig. 3. The maximum contribution of stored malate to sugar accumulation was dependent on the stage of development and was highest soon after veraison (Fig. 3). In the week following veraison, gluconeogenesis from malate could potentially contribute up to a maximum of about 7 and 9 % of the sugars accumulated in the pericarp of Pinot Noir and Sagrantino, respectively. After this, the potential contribution decreased sharply to very low values 0.1 - 2.0 %. The average value over the entire ripening period was about 2.9 % for Pinot Noir and 2.4 % for Sagrantino (Fig. 3). Nevertheless, the actual contribution was much less for the following reasons. Firstly, feeding radiolabelled malate to grape pericarp has shown that at most 17 % of this malate is converted to sugars by gluconeogenesis (Ruffner *et al.* 1975). A similar experiment in tomato gives a lower figure of about 5 %, and this depends on conditions used (Halinska and Frenkel 1991). Secondly, sugars and not malate were the main substrates utilized by metabolism in the ripening grape pericarp, and therefore glycolysis from sugars is necessary to produce metabolic substrates (Fig. 2). The occurrence of gluconeogenesis could arise because the supply of malate exceeds the requirements of metabolism at certain times. Most of the malate content of grape pericarp cells is located in the vacuole (Ruffner 1982, Sweetman *et al.* 2009, Etienne *et al.* 2013), and it is possible that at certain times or under certain conditions there is an increase in malate efflux from the vacuole to the cytosol. Then the products of malate breakdown exceed the demands of metabolism and hence they are used in gluconeogenesis (Walker *et al*. 2015).

Determining the actual contribution of stored malate to the amount of sugar accumulated in grape pericarp is very difficult for the following reasons. Firstly, the ratio between malate used in catabolism and that used in gluconeogenesis changes as result of environmental conditions, for example, higher temperatures increase the ratio (Ruffner 1982). Hence, the contribution of malate to gluconeogenesis is likely to be dependent on the location of the vineyard, cultivar, and season. Secondly, experimental manipulation when performing radio-labelling studies may alter the conditions within the cells of the pericarp and hence the amount of malate used in gluconeogenesis (*e.g*., feeding malate to the tissue could result in a higher concentration of malate in the cytosol). However, based on the maximum potential contribution of malate to sugar accumulation (Fig. 3) and taking into account the radiolabelling studies done on grape and tomato (Ruffner *et al.* 1975, Halinska and Frenkel 1991), it is very likely that in grape pericarp during ripening less than 1 % of stored sugars are derived by gluconeogenesis from stored malate (5 - 20 % of dissimilated malate = 0.1 - 0.6 % of the sugars accumulated during ripening; Fig. 3). Thus, even just after veraison when the dissimilation of malate is highest, the contribution of gluconeogenesis from this malate to sugar accumulation is likely to be very low (around 1.4 - 1.8 % of the sugars accumulated during that period).

In conclusion, during the ripening of the pericarp of both Pinot Noir and Sagrantino, stored malate can provide only a fairly small proportion of the total substrate used by metabolism. Thus, in the pericarp of both the cultivars, about 79 - 85 % of the substrate utilized by metabolism over the whole ripening period was not malate. The actual contribution of malate is dependent on the cultivar and is higher in cultivars such as Pinot Noir which contains more malate at the start of ripening and has a shorter ripening period. In all grape cultivars, the contribution of malate is likely to be dependent on the stage of development. This is because the decrease in the malate content of pericarp mostly occurs during a short period following the start of ripening.

It would appear that in the flesh of fruits other than grape, sugars and not stored organic acids account for the bulk of the substrate utilized by metabolism during ripening. This can be deduced by comparing the decrease in organic acid content of the flesh of these fruits and the amount of CO_2 released by the fruits (Pavel and DeJong 1993, Famiani *et al.* 2005, 2012, Walker *et al.* 2011). Given that glycolysis is necessary at all stages of development of grape pericarp, the question arises why gluconeogenesis from malate occurs. A possible explanation is that at certain times there is an increased release of malate from the vacuole, and this leads to a temporary excess of malate that is used in gluconeogenesis. Based on the amount of malate dissimilated and the amount of sugars accumulated, it can be calculated that gluconeogenesis from malate could potentially produce 2.4 - 2.9 % of the sugars accumulated in grape pericarp. However, the actual contribution will

be much less (< 1 %), and this is because most of the stored malate is not utilized by gluconeogenesis. This suggests that it is likely that the function of gluconeogenesis is not to support the accumulation of sugars in fruits, but it has other metabolic roles.

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