Research article

Is stored malate the quantitatively most important substrate utilised by respiration and ethanolic fermentation in grape berry pericarp during ripening?

Franco Famiani [a,*], Daniela Farinelli [a], Alberto Palliotti [a], Stefano Moscatello [b,*], Alberto Battistelli [b], Robert P. Walker [a,*]

[a] Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi di Perugia, Borgo XX Giugno, 74, 06121 Perugia, Italy
[b] Istituto di Biologia Agroambientale e Forestale, CNR, Viale Marconi, 2, 05010 Porano (TR), Italy

A B S T R A C T

A widely held view is that in grape pericarp glycolysis is inhibited during ripening, and that stored malate rather than sugars become the major substrate for respiration. In this study we determined what contribution stored malate could make to the substrate requirements of respiration and ethanolic fermentation in the pericarp of Cabernet Sauvignon berries during ripening. At a number of time points through development the amount of malate in the pericarp was measured. The change in malate content between each time point was then calculated, having first allowed for dilution arising from expansion of the fruit. The amount of CO_2 that was released by the berry in the interval between each pair of time points was measured. It was found that the contribution that stored malate could make to the substrate requirements of respiration and ethanolic fermentation of grape pericarp was dependent on the stage of ripening. At the beginning of ripening stored malate could provide a greater proportion of substrate than later in ripening, and during the latter its contribution was relatively low. Therefore, stored malate was not the quantitatively most important substrate utilised by respiration and ethanolic fermentation in the pericarp of grape berries during most of ripening. It is likely that sugars provide the bulk of the deficit in substrate. Further, the increase in the respiratory quotient during most of ripening does not arise from the use of malate as main respiratory substrate.

© 2013 Elsevier Masson SAS. All rights reserved.

1. Introduction

The growth pattern of the berry of many grapevine cultivars can be depicted by a double sigmoidal curve (Coombe, 1992). Three stages of development termed I, II and III can be distinguished. During stage I the berry increases in size. Stage II is characterised by a slowing down of the rate of increase in both the fresh and dry weights of the edible parts. During stage III ripening occurs and the rate of increase in both the fresh and dry weights of the edible parts increases (Coombe, 1992; Ollat et al., 2002). In the flesh, ripening is accompanied by compositional changes together with softening (Kanellis and Roubelakis-Angelakis, 1993; Peynaud and Ribéreau-Gayon, 1971). In addition, changes occur in berry photosynthetic capacity, respiratory rate and structure (Famiani et al., 2000; Harris et al., 1971; Palliotti and Cartechini, 2001). There is also a large decrease in both titratable acidity and organic acid content together with a substantial increase in the content of soluble sugars (Kanellis and Roubelakis-Angelakis, 1993; Ollat et al., 2002). These changes are common to most fruit species (Famiani et al., 2005, 2009, 2012; Famiani and Walker, 2009; Walker et al., 2011a,b).

At the start of ripening the respiratory quotient (RQ [RQ is defined as: amount of CO_2 produced/amount of O_2 consumed]) of grape berries increases, and this suggests that there is a major change in their metabolism (Harris et al., 1971; Kriedemann, 1968). One view is that this increase in the RQ is brought about by the utilisation of malate as a substrate for respiration during ripening (Kanellis and Roubelakis-Angelakis, 1993; Peynaud and Ribéreau-Gayon, 1971; Ruffner, 1982). Both respiration and ethanolic fermentation are responsible for the majority of the CO_2 produced in grape berry pericarp during ripening (Ruffner, 1982; Terrier and Romieu, 2001). In respiration, pyruvate derived from either sugars or malate is metabolised by the pyruvate dehydrogenase complex and the Krebs cycle. This produces CO_2 and NADH. A large proportion of this NADH is then oxidised in respiration. This consumes

* Corresponding authors. Fax: +39 075 5856255.
E-mail addresses: franco.famiani@unipg.it (F. Famiani), stefano.moscatello@ibaf.cnr.it (S. Moscatello), rob.walker@talktalk.net (R.P. Walker).

O_2 (NADH + $1/2O_2$ + H^+ → NAD^+ + H_2O) and produces ATP, and this is thought to be a major O_2 consuming process operating in the pericarp of ripening grape berries (Harris et al., 1971). A higher RQ arises when pyruvate is derived from malate rather than sugars, and this is because a molecule of CO_2 is produced in its synthesis from malate (Malate^{2-} + $1/2O_2$ + H^+ → pyruvate$^-$ + CO_2 + H_2O), and not from sugars (Glucose + O_2 → 2 pyruvate$^-$ + $2H^+$ + $2H_2O$). However, the increase in the RQ of grape berry pericarp could also be brought about by ethanolic fermentation, when either glucose or malate are used as the substrate (Terrier and Romieu, 2001). This is because this process produces CO_2 but does not consume O_2 (Glucose → 2 ethanol + $2CO_2$. Malate^{2-} + 2 H^+ → ethanol + $2CO_2$).

A widely held view is that glycolysis is inhibited in grape pericarp during ripening, and that stored malate rather than sugars are the major source of pyruvate (Kanellis and Roubelakis-Angelakis, 1993; Peynaud and Ribéreau-Gayon, 1971; Ruffner, 1982; Ruffner and Hawker, 1977; Sweetman et al., 2009). However, Terrier and Romieu (2001) provided evidence that glycolysis and the utilisation of sugars occurred in the flesh of grapes during ripening. Hence, there is uncertainty about what is the major substrate utilised by respiration in the pericarp of grapes during ripening. In addition, it is also unclear what contribution the use of malate as a respiratory substrate makes to the increase in the RQ of grape berries during ripening. The aim of this paper was to resolve these uncertainties. This was done by determining what contribution stored malate could make to the substrate requirements of metabolism in the pericarp of grape at different stages of ripening, which has never been done. To do this the amount of stored malate that was dissimilated in the pericarp of Cabernet Sauvignon berries at different stages of ripening, together with the amount of CO_2 released by the berry during each of these stages, were determined.

2. Materials and methods

2.1. Plant material

Grape berries were collected in 2004 from 6-year-old vines, which were trained to a spur pruned cordon, of the cultivar Cabernet Sauvignon (Vitis vinifera L.). These were grown in a loamy soil type, in an experimental vineyard of the Faculty of Agriculture of the University of Perugia, in Deruta (PG) — 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 bloom (AFB); full bloom is defined as being the time when 50% of the flowers are open.

2.2. Measurement of fresh weights

The weights of both whole berries and their flesh + skin (pericarp) were determined at several stages of development. At each time point 3 samples of 20 healthy berries collected from 10 different vines were used.

2.3. Preparation of a nitrogen powder

For each stage of development the pericarps from the same 60 berries that were used to determine fresh weights were frozen in liquid nitrogen. This was done as soon as possible after the separation of the pericarp from the seed. The pericarps were then ground in a mortar containing liquid nitrogen. The resulting powder was used, either immediately or after storage at −80 °C, for the determination of sugar and organic acid contents. The nitrogen powder was prepared in order to ensure that the samples used for metabolite measurements were representative of the pericarp of a large number of grapes.

2.4. Measurement of soluble sugar and malate contents

Frozen powder (50 mg) was added to 1.5 ml of 20% water, containing 100 mM HEPES-KOH (pH 7.1) and 20 mM MgCl$_2$, and 80% ethanol in an Eppendorf tube, incubated at 80 °C for l h and then centrifuged at 12 000 g for 5 min. One-hundred-fifty μl of charcoal suspension (100 mg ml^{-1}) was added to the supernatant, vortexed, and then centrifuged at 12 000 g for 5 min. The supernatant was stored at −20 °C until required. An enzyme-coupled spectrophotometric method was used to measure the amount of glucose, fructose and sucrose in the supernatant (Famiani et al., 2005). Also malate was measured using an enzyme-coupled method (Famiani et al., 2005). For measurement of both sugars and malate, recovery experiments were done in which a known amount of metabolite was co-extracted with the nitrogen powder of the pericarp sampled at 55 days AFB. For all metabolites the recovery was higher than 90% (data not shown).

2.5. Measurement of CO_2 release by grape berries

Measurements of CO_2 release were done at the ambient temperature under darkness both just before dawn (predawn) and also between 11.00 and 13.00 h. This was because temperatures are generally lowest at predawn and highest around noon, and CO_2 release by plant organs is lowest at predawn and highest around midday (Grossman and DeJong, 1994; Proietti et al., 1999). A mean value of these two measurements can then be calculated and this 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 (around 100 berries/measurement) for each determination. Bunches were collected at random from the same 10 vines used for the collection of samples for metabolite measurements. Ambient temperature was between 12—18 °C (predawn) and 23—30 °C (11.00—13.00 h) during June and September and between 13—20 °C and 27—34 °C in July and August. Bunches 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 2600 to 660 mL. Dark conditions were produced by covering the chamber that enclosed the berries with a black cloth. For measurement of CO_2 efflux from the berries, the chamber was flushed under darkness with ambient air at a rate of 500 mL min^{-1}. The CO_2 efflux of the berries was then determined using an open-system LCA3 portable infrared gas analyzer (Analytical Development Company, Hoddesdon, UK). CO_2 release from the stalks (rachis) was also measured and this was subtracted from the value obtained for the whole bunch.

2.6. Calculation of theoretical RQ of the berry

The theoretical RQ of the berry that would arise if all the stored malate which was dissimilated during ripening was completely oxidised by the Krebs cycle and all the NADH so produced oxidised was calculated. In the different considered periods of time during ripening, the amount of malate that was oxidised was insufficient to account for the amount of CO_2 released, and the additional substrate required to release this surplus CO_2 was assumed to be sugars. The RQ was then calculated by the equation 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 1.00. As an example, if half of the CO_2 released during