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Metabolic Changes of Malvasia Grapes for Wine Production during Postharvest Drying

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Malvasia (*Vitis vinifera* L.) grapes were harvested at 17.8% of soluble solids content (SSC) and placed inside an innovative dehydration room where temperature, relative humidity, and air flow were maintained, respectively, at 15 °C, 40%, and 1−1.5 m s^{-1}. Weight loss of bunches reached ∼33% in 29 days. SSC increased inversely proportionally with the weight decrease, reaching at the end of experiment 23%. Abscisic acid (ABA) increased rapidly from around 29 to 80 μg g^{-1} of dry weight at 11.7% of bunch weight loss and then declined gradually. Lipoxygenase (LOX) showed the same behavior as ABA, whereas alcohol dehydrogenase (ADH), read in the way of ethanol oxidation, increased continuously when the weight loss reached ∼19.5%. In parallel with the activity of LOX, C6 compound [hexanal, hex-1-enol, (*E*)-hex-2-enal] concentrations reached a peak at 11.7% of weight loss, whereas ethanol and acetaldehyde increased with the increase of ADH and successively decrease and ethyl acetate increased. Proline increased initially as ABA and successively with the increase of ADH, 5.3-fold increase versus 4.2-fold increase of proteins. Postharvest dehydration of Malvasia grapes shows a biphasic pattern: a first metabolic stress response up to 11.7% of bunch weight loss and a second stress response beyond 19.5% of weight loss. The metabolic mechanism of these postharvest water stress responses is discussed.

KEYWORDS: Water stress; ABA; LOX; ADH; proline; volatiles

INTRODUCTION

Dehydration or water loss is a stressing event that induces significant modifications in the metabolism of fruits and vegetables (*1*). Dehydration begins when the saturation water vapor (SVP) inside a plant cell (turgor pressure) and the outside water vapor (VP) differ; this difference is known as the vapor pressure deficit (VPD). Usually, during the postharvest of fruits and vegetables, operators try to keep this deficit as small as possible, except in certain cases such as onions and garlic, to reduce the water loss from commodities, which negatively affects saleable quality. In the case of wine grapes, the dehydration process is sometimes promoted to increase sugar content by concentration and obtain dessert wine with residual sugars or dry wine with a special aroma (Amarone wine). The rate of water loss and the effects on the cell metabolism of postharvest fruits and vegetables have been reported by Hsiao (*2*). With 0.5% water loss, cell wall enzyme activity is already increased, and a further increase of water loss accelerates respiration and ethylene production, together with the loss of volatiles. To achieve sugar and proline accumulation, the cell wall potential must be between 10 and 20 bar.

In a recent paper, we showed some significant changes in white and red wine grape varieties, especially in relation to volatile compounds and polyphenols, by using an accurate innovative technology for grape drying (*3*). It has been postulated that an initial stress occurs when grapes (whole bunch) lose 10−15% of their weight and that subsequently there is a change of metabolism from aerobic to anaerobic. Shrinkage is recognized as an important consequence of fruit drying that has to be accounted for, because it modifies the shape and dimension of products, which in turn affect the mass transport phenomena (*4*). Cellular shrinkage of Ruby grape berries at different temperatures caused cell shrinkage without showing changes in cell structure, with a rate of change related to the temperature increase (*5*). Because the fruit surface dries much more quickly than its core, internal stress develops and the fruit interior becomes cracked and porous (*6*). Nonvolatile compounds migrate with the diffusing water, precipitate on the product surface, and form a crust that keeps the fruit dimensions thereafter. Consequently, at higher drying velocities the overall degree of fruit shrinkage is smaller. Wang and Brennan (*4*) observed this phenomenon through microscopy during potato-drying experiments. Ramos et al. (*5*) did not find the same result on grape berry quarters, but a change in superficial cell architecture (reduction of intercellular space and cell squeezing) should provide a barrier to gas diffusion and induce a partial modified atmosphere.

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Thus, it is presumed that alcohol dehydrogenase (ADH) in berries can be activated at a certain level of water loss as has been observed in *Arabidopsis* (*7*), taking into account that freshly cut grapes show an anaerobic metabolism (*8*), which is reflected in the production of ethanol, CO_2, and fermentation byproducts (*9*), affecting the volatile compounds' composition of the grapes. In *Arabidopsis* roots, de Bruxelles et al. (*10*) have found that the ADH gene was induced by exogenous treatments with absciscic acid (ABA) even in ABA-insensitive mutants *aba1* and *abi2*, and in the case of dehydration the induction of the ADH gene was ABA-mediated. Respiration and ethanol production measurements in off-vine berries revealed the partial conversion of glucose and malic acid into ethanol and CO_2 via pyruvic acid (*9*, *11*). During ripening, the grape berry can change its metabolism from aerobic to anaerobic, which is reflected in the production of ethanol, CO_2, and fermentation byproducts (*8*, *12*). Isobutanol, benzyl alcohol, 2-phenylethanol, 5-methylfurfural, γ-butyrolactone, and γ-hexalactone concentrations increased significantly in sun-dried Pedro Ximenes grapes, and these compounds are related to the anaerobic metabolism of sugars and the presence of ethanol (*13*).

Increases in alcohol dehydrogenase and glutamate oxalacetate transaminase activities caused by strong thermal shocks in postharvest grapes have been described (*14*). Before that anaerobic metabolism occurs, cell wall and plasmalemma are modified by the turgor loss, and this activates all oxidative enzymes involved in cell protection. Lipoxygenase (LOX) is an important oxidative enzyme involved in lipid oxidation, and its activity causes the formation of C6 volatile compounds, (*E*)-hex-3-en-1-ol, (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol, and the aldehyde (*E*)-hex-2-enal, which give a herbaceous (grass) taste to fruit and, in the case of grape, to the wine. The destruction of cell structure during prefermentative treatments induces strong oxidation by oxidative enzymes (*15*). In the dehydration process and the dramatic cell structure changes, it is presumed that LOX can be liberated and show the same behavior as for grape crushing.

Together with biochemical indicators, we were interested in studying the behavior of two well-known water stress markers, ABA and proline. ABA is a plant hormone implicated in multiple physiological processes such as stomatal control, adaptation to stress, dormancy, or photosynthesis (*16*). In grape berry, an accumulation of ABA has been observed during ripening (*17*), and it seems to be imported from the leaves (*18*) and in the water deficit stress (*19*). In a recent work, Pan et al. (*20*) have shown the role of ABA on the induction of grape acid invertases, soluble acid invertase (SAI), and cell wall bound invertase (CWI). It has been reported that the source of ABA in pericarp can also be the pericarp itself (*21*). It has been shown that ABA enhances skin pigmentation by mediating the physiological state of this tissue (*22*). This might explain the presence of ABA in the skin of colored varieties. In any case, the white Sémillon cultivar showed the same concentration of ABA in the skin (*23*). ABA formation occurs from mevalonic acid via the carotenoid pathway, with violaxanthin as precursor (*24*), and LOX is possibly involved in this process (*25*).

Another role of ABA is to stimulate the accumulation of a set of putatively protective proteins during water stress and in tissues undergoing desiccation (*26*). It has been seen in peaches that an exogenous ABA treatment increases sugar accumulation with a simultaneous rise of sorbitol oxidase activity, which metabolizes sorbitol to glucose (*27*). Increases of ABA, sorbitol, sucrose, and total sugars in peach fruits of trees subjected to moderate water stress were even observed by the same authors (*28*). Proline is a well-known water stress marker (*29*, *30*). The accumulation of free proline in plants may be part of a general adaptation to water stress (*31*). Free proline has been suggested as a metabolic measure of drought and is suggested to play an important role as an organic osmolyte. Proline can also detoxify free radicals (*32*). For this reason, it is likely that, in fruit, proline accumulates with ripening and senescence (*33*, *34*). According to Ribéreau-Gayon et al. (*15*), the drying process causes a concentration in sugars and has a lesser effect on acids (it even decreases their content); however, the effect of this process on the volatile fraction of the grapes has not been studied. We have seen that during the postharvest drying of grape berry under controlled thermohygrometric conditions, change in most volatiles is not only due to concentration but even to change of metabolisms (*3*). For this reason, the objective of this paper is to study some metabolisms that are involved in the postharvest grape-drying process, to modulate the induced water stress, thus influencing grape characteristics and the produced wine (*35*).

MATERIALS AND METHODS

Experimental Conditions. The artificial drying tunnel was manufactured by Europanel SpA, Laives, Bozen, Italy (patent MI2001A 002198). The principle of this technique is to reduce the relative humidity (RH) and temperature simultaneously and, in addition, increase the air movement over and through the grape rachises. We used the same pilot plant (capacity = 300 kg of grapes) that we used in our previous studies (*3*, *36*). During the drying test, thermohygrometric atmosphere conditions were monitored with a HYGROclip model probe (Rotronic AG, Bassersdorf, Switzerland) connected to HYGROwin software to record the data. The air speed was measured by means of a Terman hot-wire anemometer (LSI spa, Milan, Italy). The temperature, RH, and air speed surveys were carried out at specific critical points inside the tunnel. Grape berries (*Vitis vinifera* L.) var. 'Malvasia del Lazio' were harvested on September 11 with a soluble solids content (SSC) of 17.8 °Brix. The grape rachises were picked carefully and immediately placed in perforated plastic boxes (wall height = 15 cm) to reach a weight of 6 kg (± 500 g). The plastic boxes were immediately placed inside the tunnel, and the drying process was started. Tunnel maintenance conditions were as follows: temperature, 15 °C (± 1 °C); RH, 40% (± 5%); air speed, $1-1.5$ m s^{-1}. The treatment for Malvasia grapes lasted until October 10.

Quality Analyses. During the drying process, the following measurements were performed using samples collected from the grape boxes inside the tunnel. Every few days, 100 berries randomly picked from the rachises were squeezed separately and the juice was used for SSC measurement. The rest of the berries were frozen in liquid nitrogen and stored at −75 °C for the enzymatic assays of LOX and ADH activities, ABA and proline determinations, and gas chromatographic (GC) analysis of the volatile compounds. SSC was detected with a model RL-2 table refractometer (Abbè, Officine Galileo, Florence, Italy) calibrated at 20 °C. Eight plastic boxes were weighed daily to measure the weight loss. Some rachises, sampled from the same boxes, were used for the respiration rate (CO_2) measurement, monitoring the headspace of a 5 L jar using an Oxycarb (Isolcell, BZ, Italy).

Lipoxygenase Activity. LOX was measured by applying Bonnet and Crouzet's (*37*) method, with some modifications. All of the reagents without any specific references are from Sigma Aldrich Co.: 10 g of powder, obtained from frozen and ground grape berries, was suspended in 10 mL (1:1) of 0.5 M Tris-HCl buffer (pH 8.0) containing 1% w/v ascorbic acid, 1% w/v EDTA, and 1% w/v PVPP. The homogenate was centrifuged at 3500*g* for 15 min at 4 °C. A 1 M $CaCl_2$ solution (2% v/v) of supernatant was added for 2 h to induce pectic substance precipitation. The mixture was centrifuged at 3500*g* for 20 min, and the supernatant was desalted using a PD-10 Sephadex G-25 M column (Pharmacia Biotech AB, Uppsala, Sweden) previously equilibrated with the extraction buffer. All of the operations were carried out following the cold chain at 4 °C. The assay mixture consisted of 100 μL of crude extract in 2.7 mL of 0.2 M buffer phosphate (pH 6.5) and 0.3 mL of

incubation substrate prepared with 1 mL of 0.1 N NaOH, 5 μL of Tween 20, and 10 μL of linoleic acid regulated to the final volume of 25 mL with bidistilled water. The reaction mixture was incubated at 37 °C in a water bath for 10 min. Enzyme activity was measured at 234 nm, with a Lambda 3B UV−vis spectrophotometer (Perkin-Elmer Instruments Ltd., Seer Green, Beaconsfield, U.K.), the rise in absorbance during 2 min due to the hydroperoxide formation (*38*). One unit of enzyme is defined as the change of 0.001 Abs_{234nm} for 1 min at 20 °C (*39*).

Alcohol Dehydrogenase Activity. ADH was measured by applying Longhurst et al.'s (*40*) method, with some modifications. Five grams of powder, obtained from frozen and ground grape berries, was suspended in 10 mL (1:2) of 1 M Tris-HCl buffer (pH 7.4) containing 5 mM DTT, 1 mM EDTA, and 1% w/v PVP Polyclar-AT. The homogenate was centrifuged at 15000*g* for 10 min at 4 °C and the supernatant used for the assay. The assay mixture consisted of 0.6 mL of crude extract in 2.7 mL of incubation substrate containing 0.1 M L-glycine (pH 9.6), 0.33 M NAD^+, and 0.2 mL of 0.3 M water solution of ethanol. Enzyme activity was measured at 20 °C by reading the rise in absorbance at 340 nm due to the NADH formation following the ethanol oxidation (*41*). Results are expressed as micromoles of NADH generated per minute per fresh weight.

Abscisic Acid Determination. ABA concentration in the berries' peel was measured by applying the immunoenzymatic technique (ELISA) described in Lafuente et al. (*42*).

Total Protein and Proline Determinations. Protein assay was carried out following Bradford's method (*43*), whereas proline determination was carried out using Bates et al.'s (*29*) method.

Gas Chromatographic Analyses. Volatile compound analysis was performed by applying the SPME technique GC method described in our previous work (*36*), slightly modified. Grape berry juice (5 g) was added to 5 mL of saturated $CaCl_2$ (1:1 w/v) or wine (10 mL) and homogenized with 200 μL of standard solution (2-penten-3-one). The homogenate was collected in a 25 mL glass miniflask (Supelco, Sigma-Aldrich Co.) and sealed with a Teflon silicone septum. The sample, grapes or wine, was exposed to a solid-phase microextraction fiber, respectively, for 30 and 15 min, in a Thermo Haake DL30-V15B water bath (ENCO Spinea, Ve, Italy) maintained at a temperature of 20 ± 2 °C. The fiber, 100 μm PDMS (Supelco Inc., Bellefonte, PA), was conditioned in the GC injection port at 250 °C for 2 h prior to use. After the selected extraction time, the SPME fiber was transferred to the injection port and thermally desorbed at 220 °C for 7 min. The splitless injector was mounted on a Trace GC, ThermoFinnigan UltraGC (ThermoFinnigan Inc., San Jose, CA) equipped with a fused silica capillary column impregnated with a polar phase of Carbowax 20M (Alltech Associates Inc., Deerfield, IL), 60 m long × 0.25 mm i.d. and 0.25 μm film thickness. Helium was used as the carrier gas (27 cm s^{-1}). The temperature was maintained at 40 °C for 7 min and then programmed to reach 230 °C at a rate of 3 °C min^{-1}, with a final isotherm of 30 min. A high-sensitivity flame ionization detector (FID) at 250 °C was used. The signal was recorded and integrated by a Mega Series integrator. Quantification was performed using individual calibration curves for each identified compound, and data were expressed as micro or nanograms per milliliter of must, taking into account the dry weight of the berries at each sampling time. Compound identification was achieved using a Shimadzu 17A GC-MS and a Shimadzu QP 5050A MS and matching against the NIST 107 and NIST 21 libraries and by matching GC retention times against standards.

Data Analysis. Enzymatic assay values are expressed as the mean of three replicate determinations, each one from 20 berries. ANOVA was performed for enzymatic and chemical analyses; mean separation was carried out with the LSD test. Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Grape bunches maintained under controlled conditions (tunnel) lost ∼33% of their weight in 29 days (**Figure 1**), with a daily weight loss that was stable between 1.1 and 1.4% (data not shown). The R^2 indicates the strict correlation of the dehydration with time, which is unexpected as usually the dehydration rate slows with time. This result is due to the constant thermohygrometric and ventilated condition. Cutting berries with pedicels from rachises of six bunches maintained under the same dehydration conditions, we measured the weight loss difference of berries and the green part. When weight loss was between 10 and 15%, ∼70% of water loss was from berries and 30% from the rachis; with the progressive weight loss, water loss from berries increased and that from the rachis decreased and, at the end of the experiment, it was 100% only from berries. Water loss caused a sugar concentration, which increased from the initial value of ∼18% SSC to 23%, an increase of 5% of sugars strictly correlated to the water loss. This result confirms what we observed in our previous work (*3*), where sugar increase during dehydration is due only to concentration, without any kind of gluconeogenesis. After a small initial decrease, the respiration rate (CO_2 production) increased slightly until reaching 10% of the weight loss and subsequently rose much more from 14 to 20 mL kg^{-1} h^{-1}(**Figure 2**). This increase could be induced by the progressive water stress, as well as by an anaerobic metabolism.

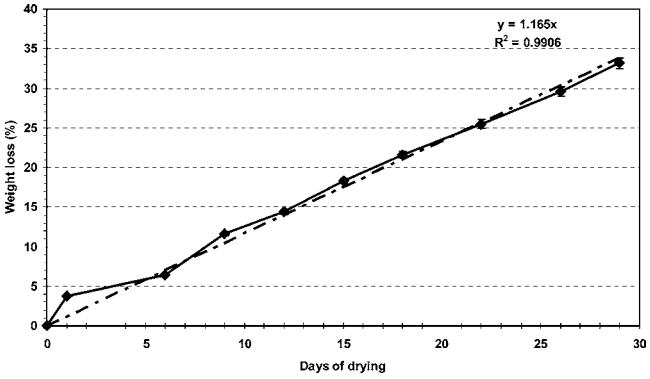

Figure 1. Total weight loss measured, in the Malvasia grape samples, throughout the 29 days of the drying process. Data are the mean of eight different boxes. Vertical bars indicate SD.

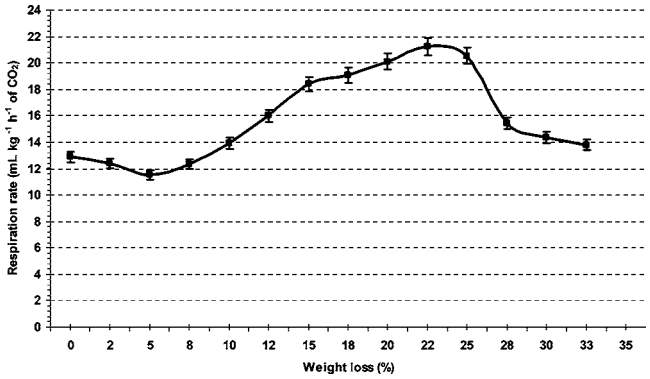

Figure 2. Respiration rate, measured in the Malvasia grape samples, expressed as CO_2 produced and plotted versus weight loss. Data are the mean of three jars. Vertical bars indicate SD.

An indication of early water stress symptoms is given by the ABA concentration in the peel, which rose rapidly from ∼29 to 80 μg g^{-1} of dry weight (dw) at 11.7% of bunch weight loss and then declined gradually during the progressive dehydration process (**Figure 3**). It is known that ABA translocation from plastids to the guardian cells is very fast under water stress conditions due to a decrease in pH (*44*). Even though grape berries have few stomata, accumulation of ABA has been found in red and white varieties such as 'Semillion' during ripening (*23*). Moreover, in peach it has been observed that ABA exogenous treatment induced sugar accumulation, mainly

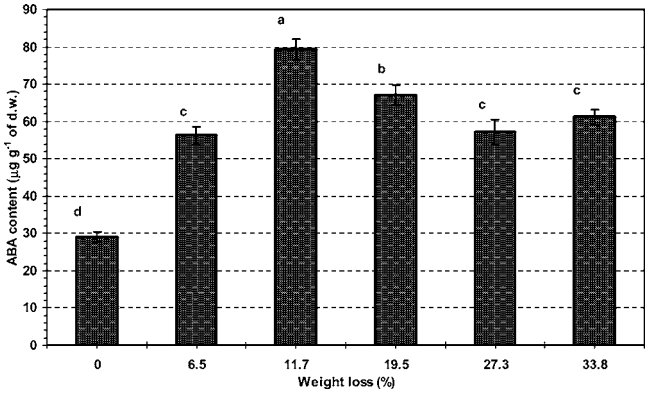

Figure 3. ABA content in Malvasia grape samples, plotted versus weight loss. Data are the means of three determinations from different berries. Results are expressed as micrograms per gram of dry weight. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters are significantly different ($P < 0.05$).

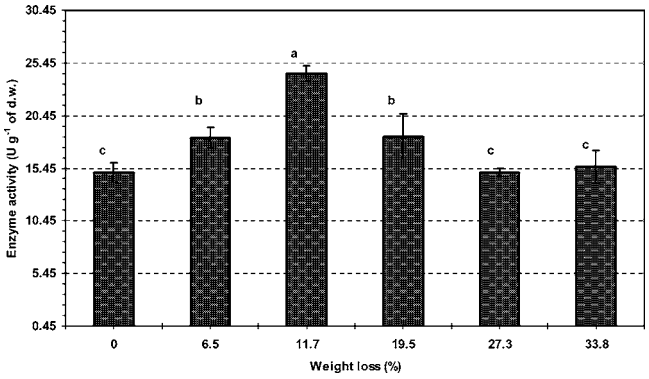

Figure 4. LOX in Malvasia grape samples, plotted versus weight loss. Data are the means of three determinations from different berries. Enzyme activity was expressed as units per gram of dry weight. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Activities with different letters were significantly different ($P < 0.05$).

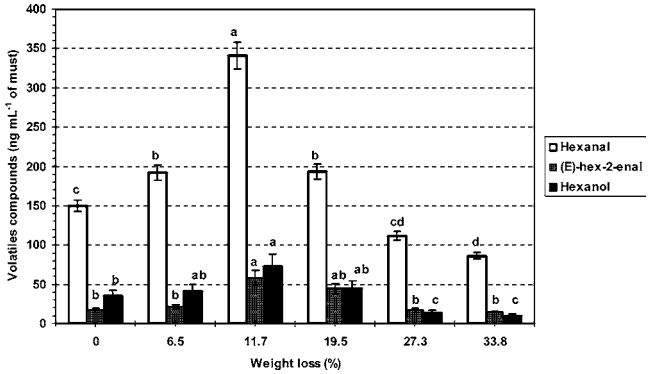

Figure 5. C6 compounds [mainly hexanal, hexan-1-ol, and (*E*)-hex-2-anal] measured in must of Malvasia grape samples, plotted versus weight loss. Data are the means of three GC runs from three juice samples obtained from different berries. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters (in the same compound) were significantly different ($P < 0.05$).

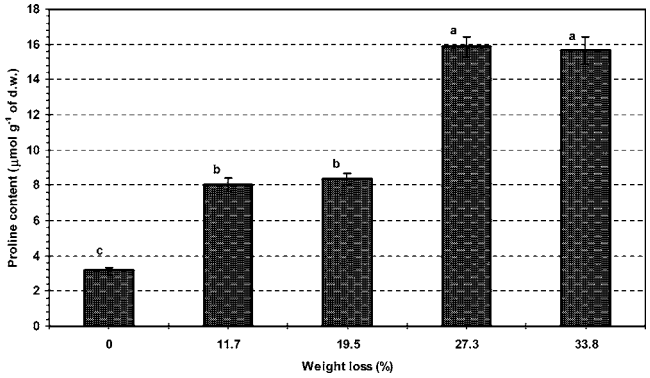

Figure 6. Proline content in Malvasia grape samples, plotted versus weight loss. Data are the means of three determinations from different berries. Results are expressed as micromoles per gram of dry weight. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters were significantly different ($P < 0.05$).

sucrose and sorbitol, by activating sorbitol metabolism (*27*, *28*). Anyway, the sorbitol increase was not due to new fruit synthesis but to the sorbitol influx into the fruit as a sink from other organs. In our case, ABA could play a role in controlling the cell turgor by closing the stomata and even facilitating the sugar accumulation. ABA is also involved in radical scavenging (*32*), and this could explain the observed rapid increase by the time water stress induces a strong oxidative metabolism. This event is confirmed by the parallel increase in LOX activity, which showed a similar ABA pattern (**Figure 4**), and it has been seen in *Arabidopsis* that the LOX gene is induced by ABA (*45*). Therefore, in our case the initial response of the berry cell to water loss is to accumulate ABA, an accumulation that could be related to stress ethylene (we have found a peak, 4 times higher, of ethylene in the first 3 days of dehydration in grape var. 'Aleatico' maintained under the same conditions), because a close connection between ABA and ethylene in peach fruit has been found (*46*); this accumulation activates LOX for the release of signaling volatile compounds. Indeed, LOX catalyzes polyunsaturated fatty acid oxidation to compounds such as C6 aldehydes and signaling molecules related to stress events and pathogenic attack (*47*), and it has been observed that in *Arabidobsis* mutants lacking in ADH, C6 aldehydes were accumulated, showing how C6 aldehyde metabolism is normally destined to C6 alcohol production (*48*).

It is interesting to observe that ABA concentration, as well as LOX activity, declines but remains at levels higher than or similar to the initial ones after the peak formation, indicating that there is a continuous synthesis as a consequence of the progressive water stress. The increase in LOX activity has determined the parallel rise in C6 compounds such as hexen-1-ol and the aldehydes hexanal and (*E*)-hex-2-enal, with a peak, approximately at the same time as the LOX peak (**Figure 5**). Undetectable contents of C6 compounds in musts from dried Pedro Ximenez grapes have been found, revealing a low LOX activity in the berry during off-vine drying and in the obtained must (*12*), but the same authors comment that it "is known that enzymes (LOX) lose their biological activity by exposure to high temperatures, to which berries are indeed exposed while drying in the sun". In our case, the temperature was much lower compared to the one used for sun-drying 'Pedro Ximenez' grapes in the Cordoba area. In other varieties such as 'Sagrantino' (red variety) and 'Pecorino' (white variety), LOX activity diminished greatly only at a very advanced level of dehydration (>35% of weight loss) (data not shown).

Parallel to the increase in ABA and LOX, a rapid rise in proline content from 2.6 to 8 μmol g^{-1} of dw was observed until a weight loss of 11.7%, and then a new great significant rise from 8.2 to 16 μmol g^{-1} of dw at 19% of weight loss was seen (**Figure 6**). Proline is the main amino acid, together with arginine, in grape (*49*). The initial rise could be related to the need for radical detoxification (*32*) due to strong oxidation, as emphasized by LOX activity, whereas the subse-

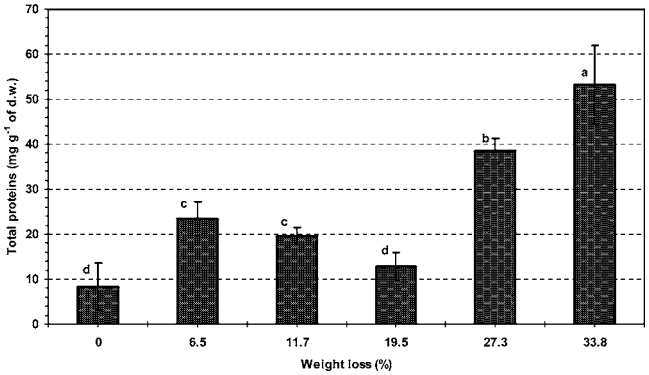

Figure 7. Total proteins in Malvasia grape samples, plotted versus weight loss. Data are the means of three determinations from different berries. Results are expressed as milligrams per gram of dry weight. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters were significantly different ($P < 0.05$).

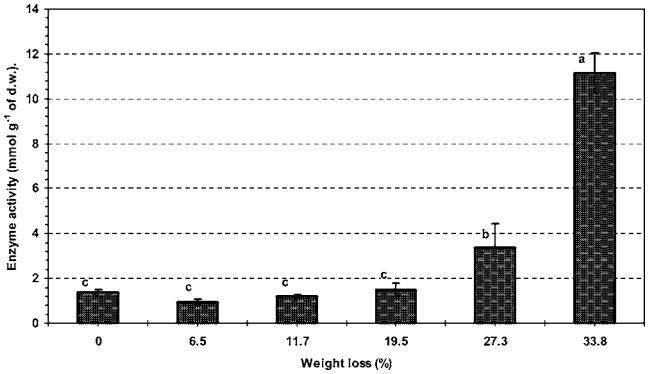

Figure 8. ADH activity in Malvasia grape samples, plotted versus weight loss. Data are the means of three determinations from different berries. Enzyme activity was expressed as millimoles per gram of dry weight. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Activities with different letters were significantly different ($P < 0.05$).

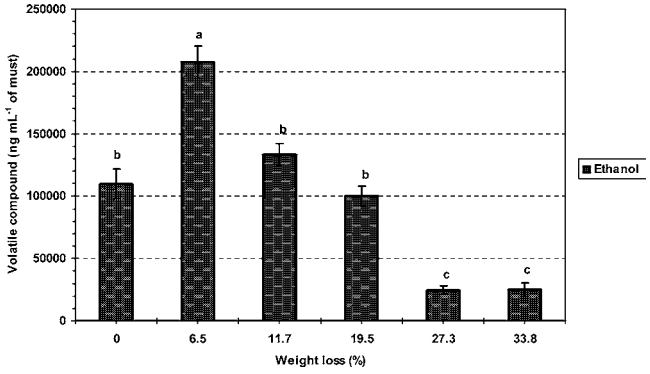

Figure 9. Ethanol content measured in must of Malvasia grape samples, plotted versus weight loss. Data are the means of GC runs from three juice samples obtained from different berries. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters were significantly different ($P < 0.05$).

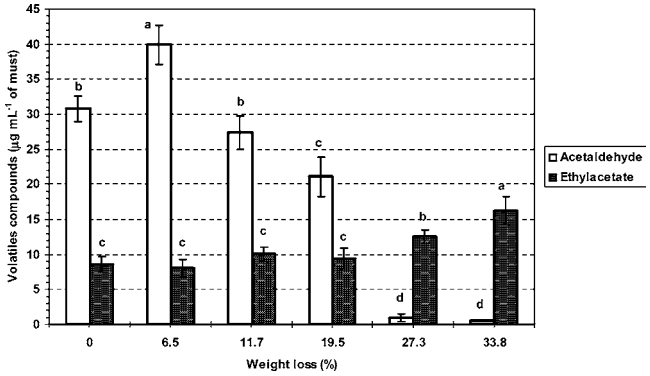

Figure 10. Acetaldehyde and ethyl acetate contents measured in must of Malvasia grape samples, plotted versus weight loss. Data are the means of GC runs from three juice samples obtained from different berries. Vertical bars indicate SD. Mean separation was performed by applying LSD test. Contents with different letters (in the same compound) were significantly different ($P < 0.05$).

quent proline increase is related to the need for osmotic protection (*31*, *50*).

Protein behavior was similar to that of proline with two significant increases, one at 6.5% weight loss and the other at 19% weight loss (**Figure 7**). This biphasic increase is due to water stress response, more than to the accumulation of proteins. In both the first and second increases, it is supposed to have de novo synthesis of proteins, mainly pathogen-related (PR) proteins such as osmotin, chitin, and thaumatin, which have been observed in high concentration in grape. It has been suggested that the partial adjustment to the rapid increase in vacuolar sugar levels may be the synthesis of proteins involved in stress management as demonstrated by several cDNAs cloned during berry ripening of grape var. 'Shiraz', which have homologues implicated in the response to water deficit in other plants (*51*). The increase in protein content in a strong water stress situation (>19% of the initial weight lost) is the consequence of ABA accumulation, which plays a role as inductor of the osmoprotective protein synthesis in some water stress and drying situations (*26*). To compare proline and proteins, from the beginning to the end of the dehydration process, proline concentration increased 5.3-fold versus 4.2-fold of total proteins, indicating that proline increase is an effect of water stress (*52*).

Parallel to the second rise in proline is the increase in ADH activity, which rose ~5.5-fold (**Figure 8**). ADH did not reach a peak, as did the LOX, but the activity continued to rise, highlighting a continuous synthesis of the protein. Because our measurement of ADH activity is from ethanol to acetaldehyde (oxidation of ethanol), it is presumed that this clear and significant ADH rise follows an accumulation of ethanol and acetaldehyde compounds, which need to be detoxified by the cell when they reach a certain amount. Indeed, ethanol and acetaldehyde rose initially until a weight loss of 6.5% and subsequently decreased (**Figure 9**), whereas ethyl acetate, as esterification of acetic acid with ethanol, increased significantly (**Figure 10**). This result will explain the increase in volatile acidity during grape drying observed in the wineries where wine from grape drying is made.

In a previous paper (*3*), we hypothesized a cell metabolism shift from aerobic to anaerobic during grape dehydration; here we confirm that this shift exists, but the cell responds not only with ethanol formation but even with its oxidation. The conclusion is that, during grape drying, ethanol increases until reaching a certain level of water loss, likely due the ABA activation (*10*), but is subsequently lost through oxidation and esterification. An increase in ADH activity was reported under dehydration stress in potato (*53*). In grapes, ADH is strongly activated during intracellular anaerobic fermentation (*54*). A few reasons can justify the shift from aerobic to anaerobic metabolism during berry water loss; first, the modification of the cell architecture alters membrane functionality (*5*, *55*), reducing gaseous diffusion (*1*, *56*), a reduction that occurs at the same time as the higher oxygen demand by the cells due to the

increase in respiratory metabolism following water stress; membrane alteration affects the functionality of membrane-bound enzymes such as ATPase, which is responsible for cell acidosis in the case of reduced oxygen availability (*57*); moreover, water stress causes organic acid concentration (pH decrease), which activates pyruvate decarboxylase (*58*, *59*). This intense fermentative activity is initially required by the cell to have a sufficient carbon flux, but subsequently the hydrolysis process of membrane lipids begins, a process that progressively increases with the consumption of fermentable sugars (*60*), which would explain the increase in ADH activity.

In conclusion, Malvasia grape berry cells undergo an initial water stress response with accumulation of ABA, proline, and LOX protein until reaching a bunch weight loss of ~10−12%; a second step of the dramatic change in metabolism is observed at >19% of weight loss, when a significant accumulation of proline and protein and an increase in ADH (ethanol to acetaldehyde way) are observed. This two-step metabolism leads initially to the formation of C6 compounds, ethanol and acetaldehyde, which subsequently decrease following the formation of ethyl acetate (volatile acidity), which, as we know, grows during grape drying in the wineries.

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