HEXOSE EFFLUX FROM THE PEELED GRAPE BERRY

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Abstract:

Context and purpose of the study - After the onset of grape berry ripening, phloem unloading follows an apoplasmic route into the mesocarp tissue. In the apoplast, most of the unloaded sucrose is cleaved by cell wall invertases, and imported into the cells as glucose and fructose. Alternatively, sucrose can be imported directly from the apoplast and cleaved into glucose and fructose, either in the cytoplasm or vacuoles. In low-sucrose cultivars, such as Shiraz, glucose and fructose are the dominant sugars in vacuoles. Transport of sugars across the plasma membrane and tonoplast is a complex process, not fully understood. Some of the elements of the sugar transport mechanism may work in a reverse mode. The purpose of this study was to indirectly observe the nature of the transport mechanism by creating conditions inducing hexose efflux from a peeled berry.

Material and methods - Potted plants of cv. Shirazwere grown in a glass-house (25/16°C), from the end of anthesis onward. The experimental method was derived from the "berry-cup" technique: a peeled berry, still attached to the plant, was immersed in a MES buffer (2-(*N*-morpholino)ethanesulfonic acid, pH 5,5)**)** solution that was collected every 30 minutes over a 3 hour period. The experiment was repeated weekly during the ripening phase. Additionally, during the period of intensive sugar accumulation (one to three weeks after veraison), three treatments were imposed: (i) a comparison of sugar unloading from berries detached or attached to the vine, (ii) the addition of the membrane-impermeant sulfhydryl-specific cytotoxin p-chloromercuribenzenesulfonic (PCMBS, 1mM) to the buffer solution, (iii) exposing the berry to cold (10°C), room temperature (27°C) or warm (40°C) buffer. Collected samples were analyzed for glucose and fructose concentration.

Results - During five weeks of ripening, the rate of hexose (mg of glucose+fructose per g of berry fresh weight) efflux from the peeled berry into the buffer solution increased.There was no difference in efflux rate between attached or detached berries, however efflux rates were temperature dependent. The non-penetrating enzyme inhibitor, PCMBS, depressed glucose and fructose efflux at the first sampling date, but not two weeks later. The inhibitory effect of PCMBS on fructose efflux was different from glucose, however for both hexoses the reversible nature of PCMBS was confirmed. During ripening, the glucose to fructose ratio within the collected buffer was significantly lower than in the grape juice, and had the opposite trend. These results lead us to the conclusion that the origin of the collected hexoses was vacuolar, and that the hexose efflux mechanism is differently sensitive to PCMBS at the two stages of ripening.

Keywords: Grapevine, Sugar transport, Glucose, Fructose, Efflux, PCMBS.

1. Introduction

The accumulation of glucose and fructose into the vacuoles of grape berry mesocarp cells is one of the most integral but complex processes of berry ripening. For plants, this process serves as an important mechanism for solute potential regulation (Wada *et al*., 2008) and it also turns the fruit into a tasty seed-dispersal mechanism mediated through birds. For humans, however, sugar accumulation into the grape berry is essential to wine quality. After the onset of grape berry ripening, phloem unloading follows an apoplasmic route into the mesocarp tissue (Zang *et al*., 2006). In the apoplast, most of the unloaded sucrose is cleaved by cell wall invertases, and imported into the cells as glucose and fructose. Alternatively, sucrose can be imported directly from the apoplast and cleaved into glucose and fructose, either in the cytoplasm or vacuoles (Oparka, 1990; Sturm, 1999; Van Bel, 2003; Zang *et al*., 2006). In low-sucrose accumulating cultivars, such as Shiraz, glucose and fructose are the dominant sugars in vacuoles of the berry mesocarp cells (Devies and Robinson, 1996; Xie *et al*., 2009). Transport of sugars across the plasma membrane and tonoplast is a complex process, not fully understood. Several membrane proteins have been identified as taking part in the sugar transport mechanism, and some of them (sucrose transporters and SWEET family of 46 sugar transporters) may perform sugar transport in both directions across the membrane (reviewed by Lecourieux *et al*., 2014). The grapevine genome probably contains 20 putative hexose transporters but just a few of these have a significant role in berry hexose accumulation (Fillion *et al*., 1999; Vignault et al., 2005; Zang et al., 2008; Afoufa-Bastien et al., 2010). —

Previous studies of monosaccharide transport across the membrane of sink cells of grape berries were performed with cell suspensions (Conde *et al*., 2006; Lecourieux *et al*., 2010). Induced efflux of hexoses, were used to study the hexose-proton cotransport system in *Chlorela* (Komor *et al*., 1978) and as an indirect method for measurements of intracellular glucose in baker's yeast (Wilkins and Cirillo, 1965). In these studies, the intact peeled berry, approximated as an assemblage of cells, were immersed into a glucose and fructose free MES buffer (pH 5.5) to induce glucose and fructose efflux. The experimental technique was a derivative of the 'berry-cup' technique (Wang *et al*., 2003). The inhibiting reagent, p-chloromercuribenzenesulfonic (PCMBS), has been widely used across various plant tissues to characterize sugar transporters (M'Batchi and Delrot, 1984; Aloni *et al*., 1986; Turgeon and Gowan, 1990; Mueckler and Makepeace, 2003). This membrane-impermeant sulfhydryl-specific reagent reversibly blocks the sugar carrier but not proton extrusion (Delrot *et al*., 1980; M'Batachi *et al*., 1985). The inhibitory effect of PCMBS is strongest on sucrose transport, with a lower to no effect on glucose, while fructose transport was inhibited least (Giaquinta, 1976 ; Daie and Wilusz, 1987; Aked and Hall, 1993). Additionally, this mercuric drug blocks aquaporins (Baey and Lanzavecchia, 2000). The mercury in PCMBS is linked to a bulky organic group, which limits its ability to penetrate a protein molecule and attach to the internal Cys group and disrupt the fold. Binding of mercury ions to –SH group results in a change in enzyme activity. The binding of PCMBS is electrostatic and can been reversed by adding the sulfhydryl containing amino acid, cysteine, or by washing and removing PCMBS from the medium (Casttranova and Miles, 1976).

The purpose of this study was to shed light on the nature of the sugar transport mechanism within the grape berry. This was achieved by creating conditions conducive to hexose efflux from a peeled berry.

2. Materials and Methods

Dormant own-rooted grapevines of cv. Shiraz (*Vitis vinifera L.)*, four years of age, were transplanted into 10 L pots and placed outdoors in a bird-proof enclosure. The vines were pruned to two short cordons, each carrying two spurs with one bud, so that there were four shoots per vine. Prior to anthesis, extra inflorescences were removed so that each shoot carried one inflorescence. Shortly after anthesis the potted vines were moved into a temperature-controlled glass-house (25/16°C) located at the National Wine and Grape Industry Centre (Charles Sturt University, Wagga Wagga, New South Wales). The vines were drip irrigated three times daily to field capacity. Twenty vines were subjected to the experiment over the 2013/2014 season.

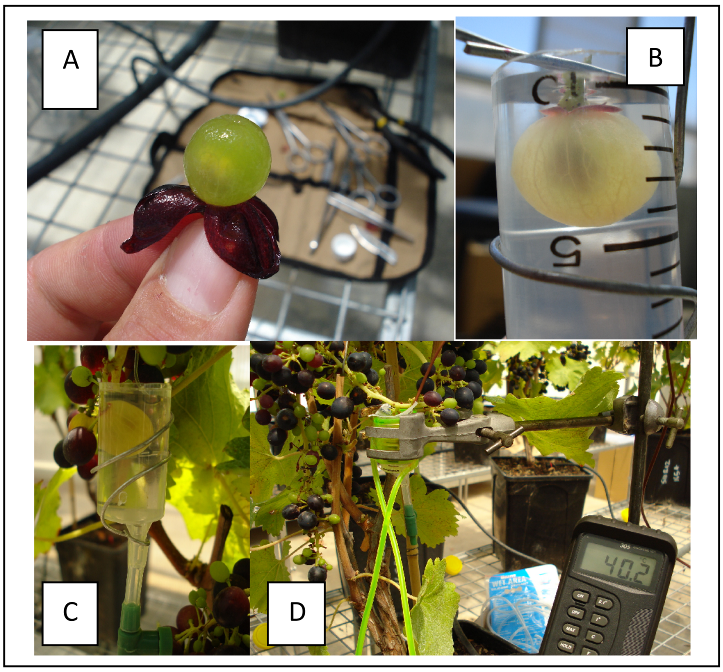

Figure 1 – «Berry-cup» experimental technique.

(A) Exocarp removal from excised Shiraz berry; (B) Excised, peeled and immersed berry showing peripheral vascular network; (C) Berry still attached to the cluster, peeled and immersed; (D) Heating of the collection medium surrounding the berry.

The 'berry-cup' technique (Wang *et al*., 2003; Lou *et al*., 2013) was originally developed for the study of phloem unloading. The removal of the berry's skin (including epidermis, epicuticular wax and hypodermal cells) exposes the peripheral network of vascular bundles. Briefly, the peeled berry (Fig. 1), still attached to the plant, is immersed in a tube with a valve to allow the drainage of its liquid contents. In our experiment, standard MES buffer (pH 5.5) was prepared by dissolving in deionised water 5mM of 2-(N-morpholino)ethanesulphonic acid (MES), 100mM of D-mannitol, 2mM of $CaCl_2$, 0.2% (w/v) of polyvinylpyrrolidone (MW40000). The pH was adjusted to 5.5 using 1M NaOH. The peeled berries were immersed into the buffer (10 ml) over a period of 3 hours with drainage and replenishment of the buffer every 30 minutes. Therefore, for every berry-cup, 6 buffer solutions were collected (samples are marked as 30, 60, 90, 120, 150 and 180 minutes). One berry per vine located in the upper third of the cluster was used. Every sampling consisted of 5 berries (five replicates).

Twenty vines were subjected to the experiment and all samplings were conducted across five replicates with each vine as a replicate. The experiment was divided into three components. The first component assessed hexose efflux weekly over 5 weeks of ripening. The measurements were initiated one week post-veraison, corresponding to the time when the berry skin could first easily be peeled from the distal to the proximal end of the berry. The second component compared hexose efflux from berries that were either attached (CB) or detached (DB) from the rachis. The treatments were applied at the 2nd and 4th week after verasion. Following excision and peeling, the berry was suspended by the pedicel using a wire clamp and immersed into a MES buffer (Fig.1B). The third component of the experiment consisted of three treatments: (i) immersing peeled berries into a MES buffer (Control); (ii) After initial exposure to MES buffer (30 min), the peeled berries were immersed into a MES buffer with 1mM of p-chloromercuribenzenesulfonic acid (PCMBS) over the 2nd, 3rd and 4th buffer replacements (60, 90 and 120 min). Following the 4th replacement, MES buffer without PCMBS was applied (150 and 180min); (iii) After initial exposure to MES buffer at room temperature (27°C, 30 min), the subsequent replacements were made with warm (WB, 40°C) or cold (CB, 10°C) MES buffer. The temperature treatments were applied using compact refrigerated coolant (Thermo Haake® DC10-K10) circulated through silicone tubing looped around the exterior of the cup. The treatments were applied twice, at the 2nd and 4th week after veraison. After collection, the berries and buffer aliquots were frozen at -24°C until chemical analysis. Glucose and fructose concentrations in the samples were determined using a Konelab™ 20XT (Thermo Fisher Scientific Inc., USA) with D-Glucose and D-Fructose enzyme kits (Thermo Fisher Scientific

Inc., USA). In parallel to the berry-cup sampling, five berries from the same cluster with similar diameter and maturity were collected. Average mass of these berries was used to express the results on a per gram of berry fresh weight basis. The hexose content in juice of these berries was also assessed by the same method that was used for the buffers. For the monitoring of sugar loading into berries, the approximation, suggested by Deloire (2011), was used. By this approximation, sugar content per berry was calculated by multiplying sugar concentration (mg/ml) in grape juice by berry mass (g) (Fig.2). Statistical analysis of data was carried out with STATISTICA® (TIBCO Software Inc., USA).

3. Results

Berry sugar content increased rapidly over the first three weeks of ripening (Fig.2). A fivefold increase of glucose and fructose accumulation rate during this period was followed by a negligible increase, after which content rose again.

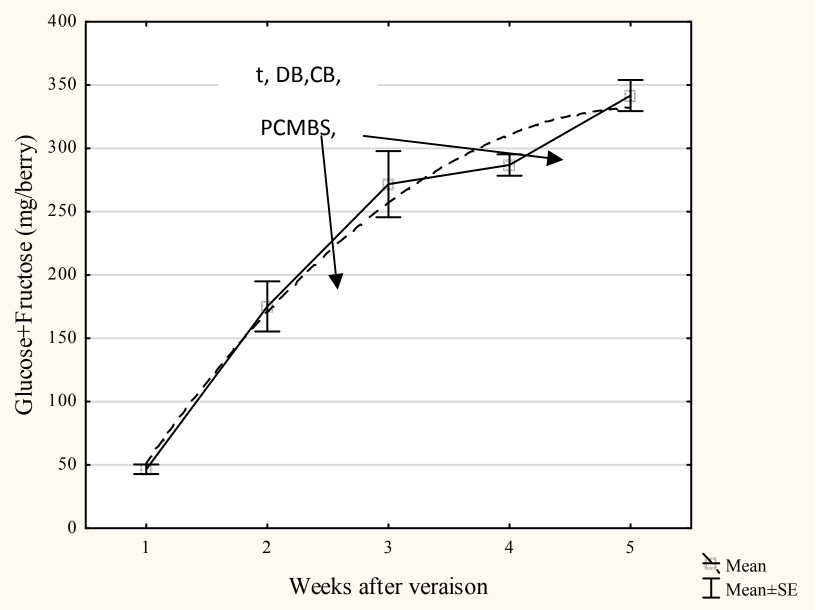

Figure 2 – Berry sugar content during ripening.

Arrows indicate treatment applications using the 'berry-cup' sampling technique: heated or cooled buffer (t), detached berry (DB), berry still attached to the cluster (CB) and MES buffer with1 mM of PCMBS (PCMBS). Veraison is referred to as the first day of softening.

During the same 5 week experimental period, glucose and fructose release from peeled berries into the buffer solutions increased with berry ripeness (Fig. 3). On all collection dates, the hexose content of the collecting medium was highest over the first 30 min interval and this subsequently declined exponentially over the following 4 collection intervals. The sugar content of the collected buffer after 30 and 180 minutes increased from 1st to 5th weeks by 5- fold and 12- fold respectively.

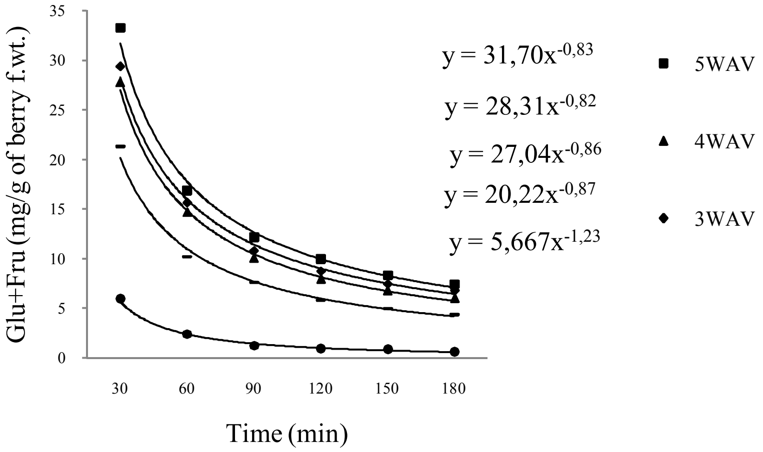

Figure 3 – Hexose (Glucose+Fructose) content in the buffer solutions following 30 min collection periods over a 3 h interval after exocarp removal.

The experiment was repeated weekly at 1 to 5 weeks after veraison. Every point represent average of 5 berries.

WAV- weeks after veraison. Each curve represents an average of 5 berries.

Patterns and rates of glucose+fructose efflux from peeled detached berries (DB) were similar to those berries still connected (CB) to the cluster (Fig. 4 A and B). This was observed at both sampling dates. Absolute values of extracted sugars were higher, however, for the second sampling date, corresponding to a later phenological stage of ripening.

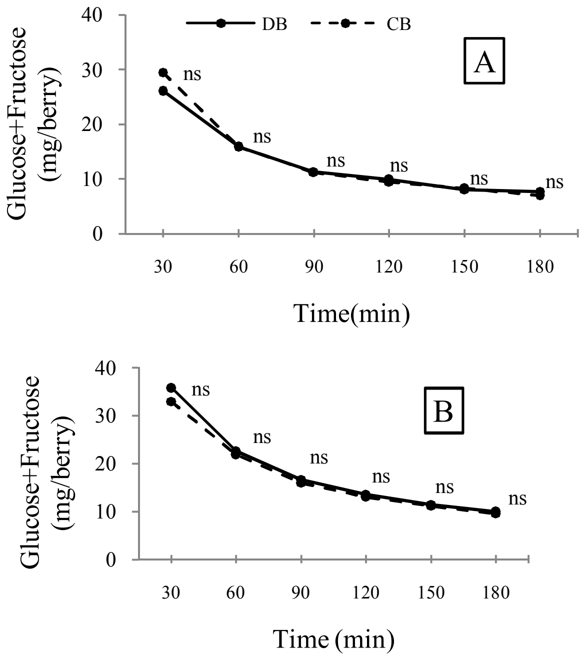

Figure 4 - Glucose+Fructose extraction from detached peeled berries (DB) and peeled berries that were connected to the cluster (CB).

Sampling dates: 2 weeks after veraison (A); 4 weeks after veraison (B); ns- non significant at 0.05 level (Newman-Keuls test).

On the basis of the results in Figure 4, it was presumed that the origin of the collected sugars in the buffer solution is the mesocarp cells. In these cells the vacuole occupies more than 90% of the cellular volume (Terrier et al. 2001). The total content of glucose and fructose in the berry mesocarp can thus be calculated using the grape juice sugar concentration and the share of mesocarp mass relative to the whole berry mass (with the approximation that 1 g of flesh represents 1 ml of grape juice). The proportion of sugars (Glucose+Fructose) diffused into the buffer, as total sugars per berry flesh (%), was obtained by using the amount of sugars collected during 180 minutes of sampling and the approximated sugars in whole berry mesocarp. Results of this approximation are shown in Figure 5. During the period of intensive sugar accumulation (Fig. 2) the proportion of extracted total sugars was significantly lower (30 %, Fig. 5). After this period, the share of extracted total sugars was relatively stable at 45-50%.

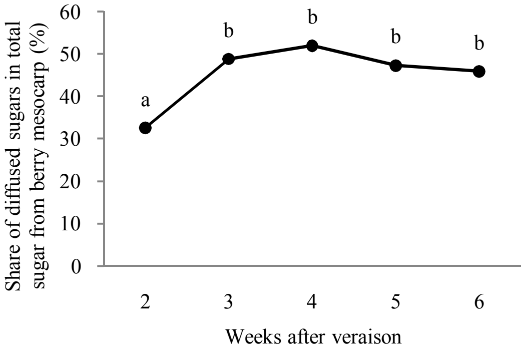

Figure 5 - Proportion of sugars (Glucose+Fructose) diffused into the collection buffer, as total sugars per berry flesh (%), during ripening.

Total sugars per berry were estimated on the basis that 1 g of berry flesh is approximately equal to 1 ml of grape juice and using the proportion of mesocarp mass within a berry (data obtained after dissection of the berries). Values with different letter are significant different at 0.05 levels (Newman-Keuls test).

The total amount of sugars (mg of Glucose+Fructose per g of berry fresh weight) collected into the buffer during 180 minutes of sampling was significantly positively correlated with the sugar concentrations in the grape juice (mg/L) (Fig. 6), as sampled weekly across the 5 weeks of ripening.

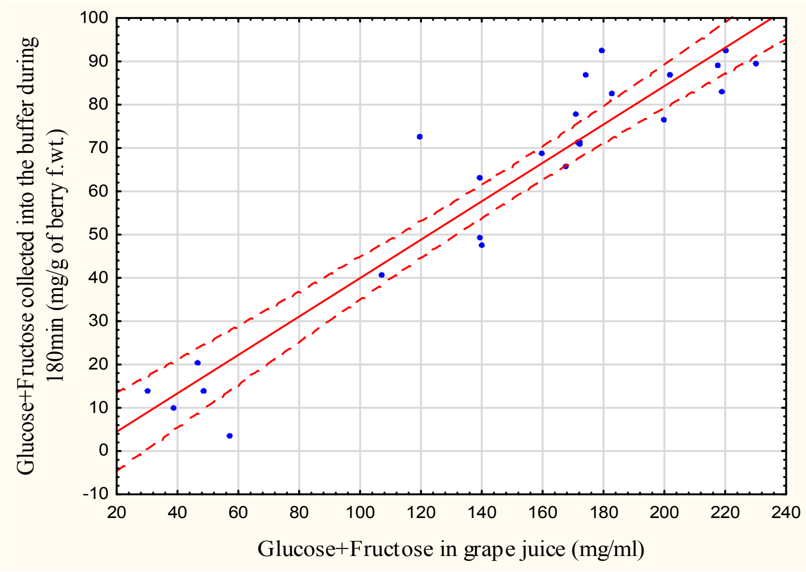

Figure 6 - Correlation between Glucose+Fructose collected into the buffer during 180 minutes, and its concentration in grape juice.

Correlation coefficient is significant (0.949).

The diffusion of hexoses from the peeled berry was dependent on buffer temperature (Fig. 7). This was apparent at two time points: during the period of intensive sugar accumulation and also two weeks later. When the peeled berries were immersed into a room temperature buffer (point 30' on Fig. 7), differences in collected sugar, between treatments were not significant. The exception is the sampling performed two weeks after veraison with cold buffer application. This was a consequence of mechanical peeling or choosing berries that were more advanced phenologically. After the next buffer change (point 60' on Fig. 7), the amount of collected sugars decreased in all treatments. In the case of the warm buffer application (WB, Fig. 7), the decline in hexose efflux was not as severe as that of the control. This situation was maintained almost until the end of sampling (point 180', Fig. 7). At this point, there were no differences in sugar extraction between the WB and Control in both sampling dates. In the case of cold buffer application (CB, Fig. 7) the observed decrease in hexose efflux was more rapid than the Control. This situation was maintained until the end of the collection period in both sampling dates. While the Control and CB resulted in an exponential decline in sugar efflux the WB resulted in a linear decline. The total amount of collected sugars during 180 minutes of sampling was significantly higher in the case of the WB but significantly lower in case of CB, relative to the Control.

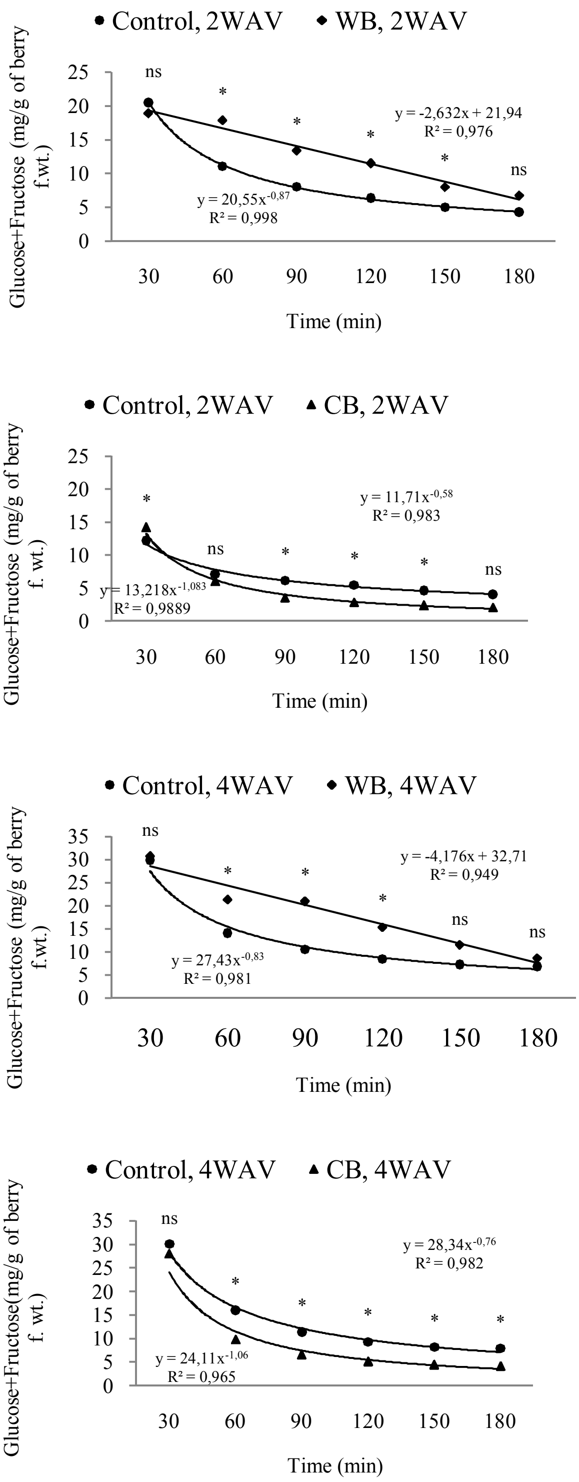

Fig.7. Influence of buffer temperature on Glucose+Fructose extraction from the peeled berry. Treatments: WB-warm buffer (t=40°C); CB-cold buffer (t=10°C); Control-room temperature buffer (t=27°C). Sampling time: 2WAV- 2 weeks after veraison; 4WAV- 4 weeks after veraison. Significance of differences between treatments for each buffer changing time (Time(min)), are represented: ns- non significant; *- significant differences at 0.05 level (Newman-Keuls test).

The non-penetrating chemical modifier, PCMBS, inhibited glucose and fructose extraction from the peeled berry into the buffer at the first sampling date (Fig. 8 A). The 30' buffer solution contains contamination of the broken cellular contents resulting from the peeling process. In the second sample set (60'), sugar concentrations in the buffer solutions decreased in both treatments with the sample containing the PCMBS at significantly lower levels than the control (2 vs. 7 mg hexoses per mg of berry f.wt.).This inhibitory effect of PCMBS continued through the next two buffer changes (90' and 120'). Subsequently, and until end of experiment (150' and 180'), the PCMBS was removed and there was evidence of a recovery in sugar efflux with an increase in sugar concentrations within the buffer. At the end of the experiment, differences between treatments were no longer statistically significant. Two weeks later, at the second sampling date (Fig.8 B), PCMBS did not have an influence on sugar efflux from the peeled berry. With the exception of the initial sample (30'), representing purging of the peeled berry, there were no significant differences between Control and PCMBS for the other collection periods. The amount of sugar extracted during this later stage of ripening was almost two fold higher than the previous one. This was likely a consequence of the increased sugar content in the grape berry (Fig.2).

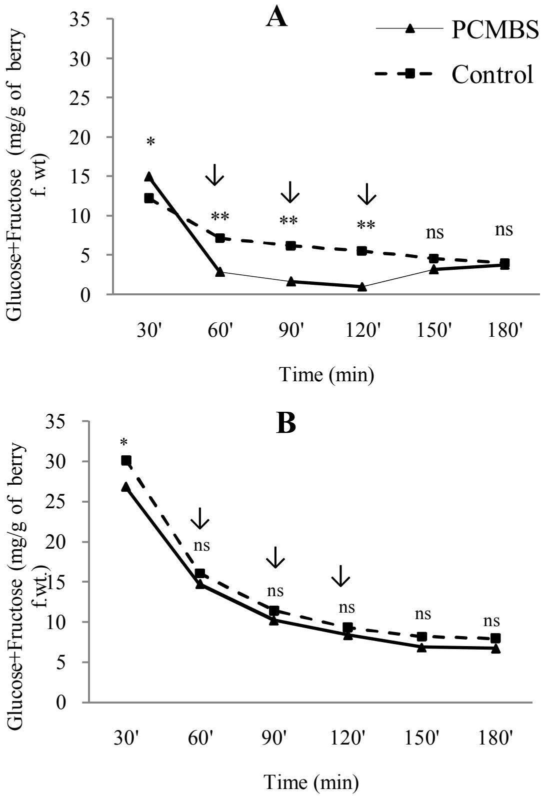

Figure 8 - Effect of p-chloromercuribenzenesulfonic acid on Glucose+Fructose efflux from the peeled berry.

First sampling date (A); second sampling date (B); ns- non significant; *, **- significant differences at 0.05 and 0.01 level respectively (Newman-Keuls test). Arrows indicate the application time of the inhibitor.

In this experiment, the observed inhibitory effect of PCMBS was not just related to the depression of sugar efflux from peeled berry (Fig.8, A) but also with type of extracted sugars (Fig.9). To quantify the inhibitory effects on glucose and fructose separately, two new parameters were calculated. The Glucose ratio represents the ratio between the amount of extracted glucose (mg/g of berry f.wt.) in the PCMBS treatment relative to the Control. The Fructose ratio was calculated similarly. The glucose and fructose ratios were almost equal at the first sample time (30'). However upon applying the inhibitor (60'), there was evidence of significantly different extraction between glucose and fructose; the extraction of glucose was more depressed than fructose by the addition of PCMBS. Over the next two collection periods (90' and 120') the glucose ration remained stable, however the fructose ratio declined and approached the level of the glucose ratio. Maximal depression of glucose extraction was reached after the first 30 minutes of modified buffer application. In the case of fructose extraction, that level of depression was reached 30 or even 60 minutes later than that of glucose. During the next two buffer changes (150' and 180'), in the absence of PCMBS, there was a recovery of glucose and fructose extraction (Fig.9). In the first 30 minutes of that period, the recovery of fructose extraction was significantly faster than glucose (Fig.9). At the end of the recovery, there was no significant difference between glucose and fructose extraction.

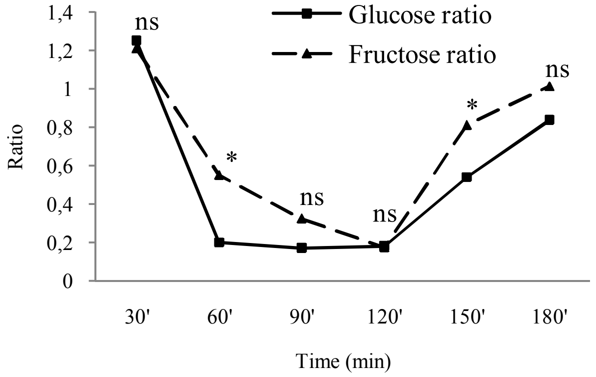

Figure 9 - Differential effect of p-chloromercuribenzenesulfonic acid on glucose and fructose efflux from the peeled berry.

The Glucose ratio and Fructose ratio represent the ratio between amounts of extracted glucose and fructose respectively (mg/g of berry f.wt.) in the PCMBS relative to the Control with each buffer change. Statistical differences: ns- non significant; * - significant differences at 0.05 level (Newman-Keuls test).

The glucose to fructose ratio in the buffer was significantly lower than in the grape juice (Fig. 10). With the progression of ripening, the glucose to fructose ratio of the grape juice and buffers have the opposite trend. Two weeks after veraison, this ratio in the buffers was <1 while two weeks later it was close to 1. In parallel, the glucose to fructose ratio in the grape juice decreased from 1.3, approaching 1.2.

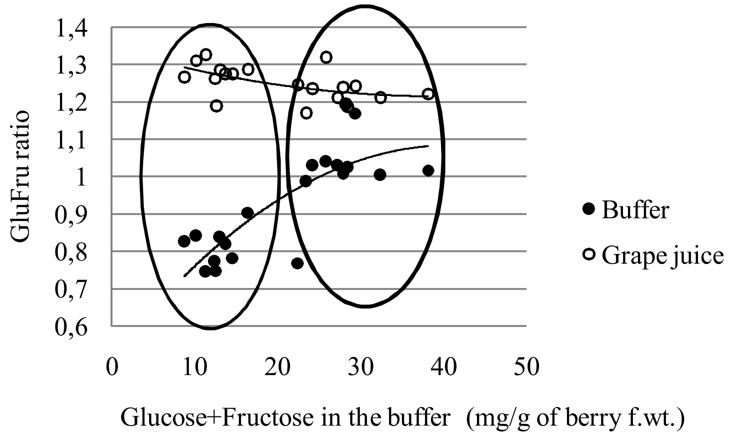

Figure 10 - Dynamics of glucose to fructose ratio.

Closed circles (●) represent glucose to fructose ratio of the buffers after the first 30 minutes (30') of collection. Open circles (○) represent glucose to fructose ratio of grape juice from berries at the same ripening stage and volume as those berries immersed in the buffer. Two sampling dates (two weeks after veraison (left oval shape) and four weeks after veraison (right oval shape)) are presented.

4. Discussion

The observed dynamics of sugar accumulation during ripening of Shiraz berries (Fig. 2) is in accordance with the cited literature (Tyerman et al. 2004; Wada et al. 2008; Castellarin et al. 2016; Abeysinghe et all. 2019). The dynamics of sugar concentration in the collection buffers is also comparable to those presented in the original paper outlining the 'berry cup' technique (Wang et al. 2003). As the authors noted, the replacement of the buffer every 30 minutes achieved a non-saturating efflux of sugars. Following the first two buffer changes, significantly lower amounts of sugars were extracted during the next four buffer collection periods (Fig. 3). Irrespective of the number of weeks post-veraison, the trend line of the hexose concentrations within the collected buffers was similar, where the kinetics of the leakage can be described as an exponential decrease (Konstantina Kocheva, personal communication). While the dynamics of efflux is similar to those of Wang et al. (2003), there are striking differences in absolute values with 1000 fold higher levels in the current work. This is despite both studies focusing on the Shiraz cultivar and using the same peeling technique. A similar difference in the order of magnitude was found by Lou et al. (2013) using the 'Fenghou' grape, but these authors have maintained the interpretation that the efflux represents phloem unloading. It is unlikely that such a significant amount of sugar can be transported by phloem over such a short period taking into account sugar import rates per berry per day, along with the sugar concentration and volume of phloem sap (Souza et al. 2005, Zang et al. 2006, Wada et al. 2008). As suggested by Iland (1984), the high efflux rate from Shiraz skin tissues in the first 15 minutes was likely a consequence of the rapid movement of compounds from the apoplast, cytoplasm and vacuoles of injured cells. Similar dynamics and absolute values of hexose efflux between attached and excised berries (Fig. 4) provides further strong evidence that the sugars were released from berry mesocarp cells rather than from the phloem sap. Comparable results were reported by de Jong and Wolswinkel (1995) in the case of sugar efflux from attached and detached seed coats of *Pisum sativum* L. They use the "empty seed coat technique", and showed that PCMBS reduced the release of sucrose and glucose from attached as well as from detached seed coats, suggesting that carrier mediated transport might be involved.

After the first week of veraison the proportion of extracted sugars from the total sugars within the berry flesh increased significantly and remained relatively stable until the end of observation period of ripening (Fig. 5). Therefore, despite increasing sugar concentrations in the grape juice during the last four weeks of the experiment (Fig. 2), the proportion of extracted sugars did not change. Brown and Coombe (1985) however found that berry skin segments released an increasing proportion of total sugars during ripening. They reported even higher values than those presented in this paper.

The altered hexose efflux dynamics in response to buffer temperature (Fig. 7) is consistent with changes in membrane function. Buffer warming (40˚C) resulted in a linear decline in efflux rates while the control and cold buffers resulted in an exponential decline, thus indicating lower rates of efflux with lower temperatures. High temperature can damage membranes, not surprising since 40°C may cause conformational changes in some proteins. In contrast, low temperature has consequences on the lipid components of plasma membrane and viscosity of the cytoplasm (Sidell and Hazel, 1987; Quinn, 1988) and thus may explain the lower efflux rates by this treatment.

This paper provides *in vivo* evidence for the inhibitory effects of the non-penetrating chemical modifier PCMBS on hexose efflux from the peeled berry into a buffer shortly after veraison (Fig. 8). This –SH group reagent has already been confirmed to inhibit sucrose transport through the plasma membrane in different plant tissues (Giaquinta, 1976; Delrot et al., 1980; M'Batachi et al., 1985; Aloni et al., 1986; Turgeon and Gowan, 1990). After phloem unloading sucrose is cleaved immediately in the apoplast or, if a small amount was transported across the plasmalemma, in the vacuole by vacuolar invertases. By employing the berry cup technique, a small amount of sucrose could potentially be recovered from glucose and fructose during its transport from the vacuoles to the outside buffer. However the absence of sucrose (or at detection thresholds, data not shown) in the collected buffers indicates that sucrose synthesis did not occur, comparable to the results of Wang et al. (2003). Hexose transporters are normally not very sensitive to PCMBS, but there was a clear change in PCMBS sensitivity to sugar efflux at the two ripening stages (Serge Delrot, personal communication). Recent discovery of SWEET transporters validated the involvement of low-affinity, high-capacity sugar transport (Hernâni Geros, personal communication). The role of those sugar uniporters in sugar efflux was apparent in the case of phloem loading (leaves), nectar secretion, and interaction between plant cells and microorganisms (reviewed by Chen, 2014). In the case of grape berry, six SWEET transporters were identified post-veraison, but further studies need to give information about its role in a sugar accumulation (Chong et al., 2014). The observations of this experiment support the notion that transport of glucose and fructose, through the plasma membrane of berry flesh cells shortly after veraison, is facilitated by membrane structures which contain an –SH group. Further support for this notion may be derived from the findings of Komor et al. (1978) which demonstrated that the –SH group is essential for the membrane protein involved in facilitated diffusion of hexose in *Chlorella.*

The hexose efflux inhibition by PCMBS was reversible in this experiment (Fig.8 A). Upon the removal of the inhibitor, significant recovery of glucose and fructose efflux occurred, gradually approaching to the control level. This phenomenon was previously observed by M'Batchi and Delrot (1984) in a study of sucrose uptake in *Vicia faba* leaf discs.Despite the inhibitory action of PCMBS on glucose and fructose efflux in the first sampling date, by the second sampling date this was no longer apparent (Fg.8 B). Three possible explanations are discussed here. First, a depression effect of the high sugar concentration may be at play. M'Batachi et al. (1985) reported that glucose and fructose had a weak or no effect on PCMBS binding and sugar transport across the membrane in leaf tissues. However, these same authors presented evidence that sucrose was highly efficient, following maltose and raffinose. Sucrose also decreased the inhibitory effect of PCMBS on phloem unloading in *V.faba* stems (Aloni et al.; 1986). However we must consider that during the post-veraison period, the sucrose content of grape juice and the apoplast is very low or on detection threshold and significantly lower than hexoses (Wang et al., 2003; Wada et al., 2008; Xie et al., 2009; Dai et al., 2013). Efflux likely occurs directly as hexoses and the hexose transporters involved at the early and late stage of ripening have differential sensitivity to PCMBS (Serge Delrot, personal communication). The second potential explanation therefore is related to the lower expression of hexose transporters at the second sampling time. While the hexose transporters *Vv*HT2, *Vv*HT3, *Vv*HT11 had a higher expression 4 to 6 weeks after veraison (Hayes et al., 2007; Afoufa-Bastien et al., 2010), high expression and activity of *Vv*HT1 was noted in berries pre-veraison followed by a decrease and a minimum shortly after veraison (Fillion et al., 1999; Vignault et al., 2005; Conde et al., 2006; Hayes et al., 2007). This phenomenon was suggested to be a consequence of the repressive role of glucose on *Vv*HT11 expression (Conde et al., 2006). A similar situation was apparent with sucrose transporters, where the expression of *Vv*SUC11 and *Vv*SUC12 increased after veraison but expression of *Vv*SUC27 rapidly decreased during the same period of berry development (Davis et al., 1999). Finally, the third explanation as to the lack of inhibitory action of the PCMBS at the second sampling date may be related to structural differences in the hexose transporters and the accessibility of the reactive group to an inhibitor (Mueckler et al., 2004).

The inhibitory effect of PCMBS was more pronounced on glucose than on fructose transport (Fig. 9). Once the inhibitor was removed, the recovery of glucose efflux was more rapid relative to fructose. It

appears that the targeted hexose transporters had a higher affinity for glucose than fructose. This evidence agrees with the assertion that some hexose transporters located in the grape berry have a high affinity for glucose in particular (Hayes et al., 2007; Vignault et al., 2005; Afoufa-Bastien et al., 2010).
The decreasing glucose to fructose ratio and its approach to 1 in grape juice from veraison onwards is a characteristic occurrence for varieties with hexose accumulation (Souza et al. 2005). In the case of the peeled berry immersed into a buffer, the collected hexoses had a ratio <1 during the period of intensive sugar accumulation (Fig. 10). This indicates that efflux of fructose was greater than that of glucose in this period of ripening. During the latter part of ripening, efflux of both hexoses was almost equal (glucose to fructose ratio approaching to 1). Keller and Shrestha (2014) found a similar trend in the glucose to fructose ratio of the appoplast and grape juice during ripening of Merlot.

5. Conclusion

Data from this experiment has shed light on the properties of hexose efflux from an intact peeled grape berry during ripening and has also characterised the influence of various external factors on that process. During ripening, hexose efflux into the collection buffer increased with greater sugar concentration in the grape juice. There was no difference in efflux rate between attached or detached berries, however efflux rates were temperature dependent. The efflux of fructose was greater than that of glucose during the period of intensive sugar accumulation, but later once sugar accumulation slowed, efflux of both hexoses was almost equal. The non-penetrating enzyme inhibitor, PCMBS, depressed glucose and fructose efflux at the first sampling date during early ripening, but not two weeks later. The inhibitory effect of PCMBS on fructose efflux was different from glucose, however for both hexoses the reversible nature of PCMBS was confirmed. These results lead us to the conclusion that the origin of the collected hexoses was vacuolar, and that the hexose efflux mechanism is differently sensitive to PCMBS at the two stages of ripening. It can also be surmised that the berry-cup technique as a potential application to the study of phloem unloading requires further method development.

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