**Pre-harvest berry shrinkage in cv 'Shiraz' (Vitis vinifera L.): Understanding sap flow by means of tracing**

# PRE-HARVEST BERRY SHRINKAGE IN CV 'SHIRAZ' (*VITIS VINIFERA* L.): UNDERSTANDING SAP FLOW BY MEANS OF TRACING.

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## Summary

- The berry shrinking physiopathy in cv Shiraz (*Vitis vinifera* L.) is to date much debated. Currently, the critical points in Shiraz pre-harvest shrinkage are: a) the role of the xylem during post-véraison; b) the existence and timing of xylematic back flow and c) the functionality of the phloem.
- In order to try to resolve these issues, we traced the xylematic flows from the vine to the berry and *vice versa* by using the fuchsin acid as a xylematic tracer. At berry maturity, in order to verify also the phloematic functionality, we used the fluorescent tracer 6(5)-carboxyfluorescein diacetate (CFDA).
- The results showed clearly that the vine gradually loses the ability to deliver water to the berries *via* pedicel during ripening. The xylematic back-flow is active in the pre-véraison but not in the post-véraison berries. Furthermore, the CFDA experiments showed the absence of flow from the plant to the berry and *vice versa* at berry maturity.
- In cv Shiraz véraison seems to be the crucial point in the berry dehydration understanding: in pre-véraison there is a 'plant/berry' and 'berry/plant' water communication, whereas in post-véraison this seems to cease.

**Key words:** berry shrivel; phloem; phloematic tracer; physiopathy; véraison; xylem; xylematic tracer;

## Introduction

Winegrape growing has worldwide economic importance and grape characteristics of different varieties are continuously investigated with the general objective to improve wine quality.

Morphological and physiological disorders can cause impairment in terms of quantity and quality of production. One of the most common disorders that can occur in grapevine genotypes is the shrinking of berries during ripening. This has four different origins (Krasnow *et al*., 2010): a) 'sunburn'; b) 'late-season dehydration'; c) 'bunchstem necrosis' and d) 'sugar accumulation imbalances'.

The late season berry dehydration was well described in Shiraz by McCarthy (1999). McCarthy & Coombe (1999) suggested that at véraison, in correspondence with the xylem disruption that was observed in Muscat Gordo Blanco (Findlay *et al*., 1987), Riesling (Düring *et al*., 1987), as well as in Pinot noir and Merlot (Creasy *et al*., 1993), phloem sap is the unique source of water and solutes for the berry, until maximum berry weight is attained. At this point (around 90 days after flowering at about 20 °Brix), in cv Shiraz, phloem sap flow also becomes impeded and finally, 2-3 weeks later, blocked. The continuation of berry transpiration and isolation of the berry from vascular transport pathways, thus lead to shrinking of the berry and solute concentration (Hunter *et al*., 2014).

Coombe & Bishop (1980; cvs Muscat Gordo Blanco and Doradillo), Greenspan *et al*., (1994; 1996; cv Cabernet Sauvignon) and Hunter *et al*., (2014; cv Shiraz), reported that berries are more sensitive to vine water relations during pre-véraison than during post-véraison. During the pre-véraison period (phase I, cell division stage; Coombe, 2001), the berry is still actively expanding and reactive. Berry transpiration has a significant role in berry water loss in both pre- and post-véraison berries and, as the berry enters the ripening stage, the 'phloem water pathway' becomes dominant compared to the 'xylem water pathway' (Coombe & Bishop, 1980; Greenspan *et al*., 1994, 1996). The mechanism of late season berry dehydration in cv Shiraz is not clear. Water arrives to the berry *via* xylem (water, minerals) and phloem (water, minerals, amino acids, sugars). Water balance (maintenance of water relations and turgor) is most likely determined by growth in volume, soluble solids, transpiration and return of water to the plant through the xylem (Lang & Thorpe, 1986), the latter that may occur on a hot day when, *e.g.*, leaves transpire excessively, thereby surpassing water absorption by the roots and leading to what is known as 'xylem back-flow'.

The dilemma in understanding late season berry dehydration apparently lies at the stage of véraison and involves the question of xylem interruption or not and whether xylem back-flow occurs in post-véraison berries. Findlay *et al*., (1987), Creasy *et al*., (1983) and Greenspan *et al*., (1994), showed that at the start of the second growth cycle, flow of xylem sap into the berry becomes impeded, whereas Düring *et al*., (1987), in Riesling berries, showed that at véraison peripheral xylem flow ceases while axial xylem flow continues. In a figure shown by Ollat *et al*.,

(2002) it can be observed that fluorescent dye that was circulated through the xylem was present in the whole vascular network before véraison, but that it was restricted to the brush region after véraison. Zhaosen *et al*., (2014) demonstrated that after phase III, water translocation efficiency of the xylem decreased and some xylem vessels appeared indistinct and broken. Xylem breakage in maturing grape berries of cv Cabernet Sauvignon was also observed anatomically (Ollat *et al*., 2002). Chatelet *et al*., (2008a) found that most tracheary elements remained intact throughout berry maturation of the cv Chardonnay. Rogiers *et al*., (2001) observed no dye solution movement along vessels in post-véraison berries. They concluded that xylem flow into Shiraz berries must have continued beyond véraison. Chatelet *et al*., (2008b) reported that new tracheary elements continued to be differentiated within existing vascular bundles during berry development of cv Chardonnay. It was understood that xylem vessel stretch occurred in some vascular tissue (Coombe & McCarthy, 2000). Measuring xylem and phloem flow in berries of Cabernet Sauvignon, Ollat *et al*., (1998, reviewed in Ollat *et al.*, 2002) found that a xylem flow reduction occurred simultaneously with a phloem flow increase during ripening. Bondada *et al*., (2005) and Keller *et al*., (2006) showed that dye uptake in post-veraison berries is possible if the required uptake gradient is applied and concluded that a xylem disconnection does not occur in post-veraison berries. These studies indicate that maybe, according to varietal behaviour, there is no xylematic isolation in post-véraison berries and water movement from plant to berry *via* the xylem is likely impeded by a decline in hydraulic conductance to the berry during and after véraison, as suggested by Tyerman *et al.,* (2004). Keller *et al*., (2006) concluded that sugar accumulation in the berry by apoplastic phloem unloading can reduce xylem water influx into ripening berries. Coombe & McCarthy (2000) hypothesized that around 90 days after flowering, when Shiraz berries reached their maximum weight, flow of phloem sap became impeded.

Considering the above, the critical points in Shiraz pre-harvest shrinkage seem to concern: a) the role of the xylem during post-véraison; b) the existence and timing of a xylematic back-flow; and c) the functionality of the phloem.

To assess xylem functionality in the berry-pedicel interface, several researchers employed dye solutions such as eosin (Creasy *et al*., 1993), fuchsin acid (Rogiers *et al.,* 2001; Chatelet *et al*., 2008) or basic fuchsin (Zhaosen *et al*., 2014) that are able to stain xylem vessels. To describe phloematic water movement in the plant-berry network at berry maturity the phloematic tracer 6(5)-carboxyfluorescein diacetate (CFDA) has previously been used (Viola *et al*., 2001; Zhang *et al*., 2006; Zanon *et al*., 2015).

In this study, specific dye tracers were used to monitor plant-berry hydric flows *in vivo* under field conditions in order to avoid any natural system perturbation of the plant. The aims of the

study were to: a) understand the xylematic flow towards the berry from the post-fruitset to the ripe-overripe berry stage; b) understand the xylematic flow from the berry towards the plant from post-fruitset to the ripe-overripe berry stage (in other words verify the existence of a xylematic 'back-flow'); and c) clarify the hydric phloematic flow from the plant towards the berry and *vice versa* at berry ripeness.

## Materials and Methods

### Plant materials

The experiments were carried out in 2015 in South Africa and in Italy, respectively, in different growth seasons: from February to April in South Africa, and from July to October in Italy.

In South Africa, experiments were performed in a Shiraz (clone SH 9C)/101-14 Mgt experimental vineyard situated at the Robertson experiment farm of ARC Infruitec-Nietvoorbij (Stellenbosch) in the Breede River Valley, Robertson (33°5'S/19°54'E/159 m a.s.l.), South Africa. The region is semi-arid (hot and dry) with a mean annual temperature of 17.8ºC and an average rainfall per annum of 290 mm, mainly during winter (Hunter & Bonnardot, 2011). The vineyard was planted in 2003 on a flat *terroir* with clay-loam soil. Vines were spaced 1.8 m x 2.7 m, spur pruned and trained to a vertical trellis (VSP) with a cordon wire and four sets of movable wires. Canopies had approximately four layers of leaves (from side to side) and were uniformly managed (by means of shoot positioning and apical topping). The trials of this paper were done in the parcel with North-South row orientation. Three replications were used, each comprising fifteen plants.

The experiments performed in Italy were carried out in the experimental vineyard of DiSAFA, University of Turin, located in Grugliasco (45°4'N/7°34'E/293 m a.s.l.). The vineyard was planted in 2008 on a flat *terroir* with sandy soil and plant density equal to 4400 vines/hectare (0.9 m x 2.5 m). Three parcels of Shiraz/420 A were identified, each comprising twelve plants. Vines were trained to a vertical shoot positioned (VSP) system in North-South oriented rows and cane pruned (Guyot system), with a bud load of 12 per vine. The canopy had an average of three to four leaf layers. Canopy management included shoot positioning, leaf removal in the fruit zone, and apical shoot trimming. According to the soil texture, vines were well irrigated. During 2015 the main agrometeorological parameters recorded (Source: Regione Piemonte Settore Fitosanitario - Sezione Agrometeorologica) were: a) mean annual temperature equal to 14.3 °C; and b) total rainfall per annum equal to 949.2 mm, mainly concentrated during spring and autumn. Grugliasco is located on the border of the humid subtropical climate and oceanic climate

zones and on the East side of the Alps; this aspect makes the climate drier than on the West side according to the presence of a so-called föhn wind (a dry, warm, down-slope wind).

**Berry growth measurements**

Phenological stages were assessed according to the International BBCH scale (Lorenz *et al*., 1994); to do this, thirty bunches per each replication were observed as follows: ten bunches on the East side, ten bunches inside and ten bunches on the West side of the canopy. The date of flowering was also noted in order to express growing of the berry as 'days after anthesis' (DAA). At each dye application point, the weight of 100 berries was assessed and the ripeness level recorded by measuring total soluble solids (TSS, % Brix) and total titratable acidity (TTA, g $L^{-1}$ as tartaric acid) of 200 berries.

**Berry dye loading**

The central point of this research concerned the protocol of techniques to observe the presence of xylematic flow in the interface between berry and plant during berry ripening. Furthermore, at berry maturity, phloematic flow in the interface between berry and plant was also studied. In order to reach these aims, dye solutions were used and loaded into vegetative tissues.

- Xylematic flow

A solution of fuchsin acid was used to mark hydric flow inside the xylem, as reported by Rogiers *et al*., (2001). A 0.1 % (w/v) aqueous solution of fuchsin acid (Acid Fuchsin, Sigma – Aldrich, Milan, Italy) was prepared with distilled (Millipore) water and filtered (0.2 µm filter). In the experimental design, dye solution was used during berry ripening to 1) trace xylematic water movement from the plant towards the berry and to 2) trace xylematic water movement from the berry towards the plant. Nine bunches for each repetition (three on the East side, three inside and three on the West side of the canopy) were chosen for treatment. To study water movement towards the berry, the fuchsin acid solution was applied *via* a wing of the bunch. On each bunch, a wing was chosen and all berries removed under water (to avoid vessel embolism). The wing was then cut and immediately submerged in a glass vial containing dye solution. Shoots with treated bunches were cut after 48 hours, immediately placed in a refrigerated bag, and brought to the laboratory for observations under the microscope. At 89 BBCH stage, berries were visually divided into 'intact' and 'shrivelled'.

The fuchsin acid solution was also applied to the shoot in order to monitor movement to the berry. Half of the shoot was cut longitudinally at the attachment/insertion point on the cane. The

shoot was therefore split in the first internodium above the cane attachment. During cutting, water was sprayed on the surface in order to avoid shoot embolism. A half-cut part was immediately submerged in a Falcon tube with fuchsin acid solution and left for 48 hours.

The same dye solution as mentioned above was also employed to study xylematic flow from the berry to the vine. To reach this objective, two techniques were used. The first technique comprised injection of a dye solution into the berry by means of a 31-gauge needle attached to a 3 mL syringe (Luer slip, Once Medical co., ltd, Thailand). The injection point was immediately sealed by applying a drop of silicon used for plant grafting (Saratoga, Trezzano sul Naviglio, MI, Italy). The second technique comprised a modified method of that proposed by Tilbrook & Tyerman, (2009). A 1 mm thick slice was cut by means of a surgical knife at the stylar end of the berry and the cut surface of the berry was submerged in fuchsin acid dye solution. The dye solution was contained in a small plastic container that was sealed around the berry with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). Both techniques permitted the reaching of the desired goals. In order to apply these techniques, nine bunches for each replication were chosen, as previously described, and from each bunch five berries were chosen for implementation of the techniques. After 48 hours, shoots of the treated bunches were cut, immediately placed in a refrigerated bag and brought to the laboratory for microscopic analysis.

In the laboratory, shoots collected in the field were dissected and presence of dye solution in the shoots, petioles, leaves, rachis, pedicels and berry tissues was observed by means of an Olympus SZ-61 stereo zoom microscope coupled with a digital camera (the same microscope was used both in South Africa and in Italy). Through the observation of tissue staining, it was possible to assess and describe water movement into the xylem during berry ripening. For pictures taken directly in the field, without cutting berries, a reflex digital camera Nikon D3100 (Nikon corporation, Japan) equipped with a AF-S Nikkor 18-55 mm 1:3.5 –5.6 G lens was used.

For microscope observations samples were prepared by hand in the laboratory after collection in the field. Berries collected at phenological phases 75 and 77 BBCH were observed without removing the skin, because colouring of peripheral bundles with the fuchsin acid was very evident. Otherwise, in order to observe water movement inside the central bundles, berries were longitudinally dissected by using a surgical knife. The pedicel was also longitudinally dissected in order to show water movement towards the berry. Furthermore, the shoot and peduncle were longitudinally dissected with a well sharpened knife. Pictures were taken by means of the reflex digital camera as previously mentioned.

At the 83, 85 and 89 BBCH phenological stages, berries were carefully peeled without damaging peripheral bundles. A longitudinal cut was obtained by a surgical knife. Shrivelled berries were

handled very carefully. In order to observe fuchsin distribution in the rachis of the bunch, the rachis was carefully longitudinally dissected using a surgical knife.

- Phloematic flow

At berry maturity, phloematic flow from plant to berry and *vice versa* was evaluated. In order to observe hydric flow in the phloem, non-fluorescent 6(5)-carboxyfluorescein diacetate (CFDA - 6(5)-CFDA powder: Sigma-Aldrich, Milan, Italy) was used. The CFDA solution was prepared according to Ruan and Patrick (1995): a 2% (w/v) stock solution was prepared in acetone and stored at -20 °C until field applications. For field treatments, the stock solution was diluted with water to obtain a 0.05 % (w/v) solution.

To study water movement from plant towards berry *via* the phloem, the method proposed by Gould *et al*., (2013) in kiwifruit and adapted for grapevine applications, was used. Seven days before the experiment, the shoot tip was cut and the shoot girdled below the most basal leaf. On the day of the experiment, only one leaf was left on the shoot; this leaf was used to absorb the CFDA non-fluorescent solution: in the central lobe of the leaf a flap was created with a central vascular nerve by cutting the edges with a razor blade; this flap was submerged into a 1 ml vial with CFDA solution. After four days, treated shoots were collected, placed in a refrigerated bag and brought to the laboratory for fluorescent microscope observation. This experiment was carried out at two ripening stages: 89 BBCH stage and fifteen days later.

To observe phloematic water from berry to plant using the CFDA solution, the same technique previously described for fuchsin acid absorption by berry stylar end was used. After four days, treated shoots were cut, placed in a refrigerated bag and brought to the laboratory for fluorescence microscope observation.

In the laboratory, shoots treated with CFDA solution were separated into leaf blade, petiole, stem and berry. Each of these parts was carefully hand sectioned and examined by the Leica M205FA stereomicroscope equipped with a fluorescence module microscope. For microscope imaging the fluorophore was excited at 490 nm and fluorescence was recorded at 520 nm (standard GFP filters). These observations on shoots permitted the marking and rebuilding of water flow in the phloem at grape maturity.

Samples of berries, leaves and shoots treated with CFDA after been brought to the laboratory, were carefully prepared for observation by means of the fluorescence microscope. The main veins and petioles of the leaves were longitudinally dissected with a surgical knife. The surgical blade was changed after each operation. The material was then put on a glass microscope slide and observed. The shoot was transversally dissected by a surgical knife with a thicker blade.

Berries were also longitudinally dissected in order to observe CFDA internal distribution by using the same protocol as described above. Trials with CFDA were done in the Grugliasco vineyard (Italy) at 112 and 133 DAA.

## Results

The ripening parameters corresponding to the ripening stages on which experiments were carried out, are shown in Table 1. As the focus is on sap flow, data collected in both South Africa and Italy are shown in the Table, as to describe the results like an ongoing experiment and because results are referred to on the basis of ripening stage expressed as BBCH growing stage and DAA.

## XYLEMATIC TRACER

### Fuchsin acid flow from plant to berry (inflow)

At the 75 BBCH phenological stage (38 DAA: pea-sized berries), the first trial was done in order to observe dye solution movement from plant to berry (inflow). The dye solution fuchsin acid was applied to the bunch wing. Movement of dye towards the berry and its distribution inside the berry was noticed (Figure 1). Movement occurred mainly *via* the central vasculature. The epidermal staining of the berry highlights the transpirational flow that occurred at this stage.

At the 77 BBCH phenological stage (45 DAA), the Shiraz berries had a soluble solids content equal to 4.13 °Brix. At this ripening stage the fuchsin acid solution was also applied through the bunch wing in order to observe water movement towards the berry. The dye solution moved from the application point, through rachis and pedicel, to the berries. Inside the berry, the dye solution was distributed *via* peripheral as well as central vascular bundles. It is noticeable that the distribution of the fuchsin acid in peripheral bundles was not uniform at this stage, suggesting a partial disruption of some xylem vessels (Figure 2). At the same phenological stage, the dye solution fuchsin acid was absorbed by the basal part of the shoot that was partially cut in the basal internode above the cane: the other half of the shoot remained hydraulically connected to the cane while half of the shoot was longitudinally cut in the basal internode, re-cutunder water and immediately submerged into a dye solution (Figure 3). The dye solution moved upwards into the xylem and was distributed in leaves according to the transpiration flow, as well as to the bunch, with a complete distribution in the rachis and inside the berries, *via* peripheral and central vessels (similar to Figure 2). At this phenological stage, it is important to note the staining of the seeds (Figure 2).

At 83 BBCH phenological stage (véraison, 53 DAA; 9.43 °Brix), similar results to those reported for the 77 BBCH stage in terms of movement towards the berry were obtained (data not shown).

At 85 BBCH ripening stage (116 DAA; 24.10 °Brix), viability of the vessels for transport towards the berries was determined. A different situation compared to the previous scenario was recorded. At this ripeness level it was possible to follow movement of the fuchsin acid absorbed by the bunch wing and transport of dye solution inside the berry only *via* peripheral vessels (Figure 4). A berry cross section and longitudinal section did not show staining of the central bundles.

In the field experiment carried out at the 89 BBCH ripening stage (142 DAA; 27.06 °Brix), 'intact' and 'shrivelled' berries were separated. As for the previous experiment, the dye solution fuchsin acid was introduced *via* the rachis wing. Results showed that in both 'intact' and 'shrivelled' berries fuchsin acid moved towards the pedicel (Figure 5), but stopped at the receptacle/brush level (Figures 6-7). The longitudinal section showed staining of the brush, but not staining of peripheral or central vasculature of the berry. Furthermore, it is interesting to note (Figure 5) fuchsin acid movement from the introduction point to the shoot *via* the peduncle: the staining solution moved strictly downwards in the shoot to the cane, suggesting a high sap flow demand from perennial parts of the plant, including the cane (temporary), trunk and roots.

**Fuchsin acid flow from berry to plant (back-flow)**

At the 75 BBCH phenological stage (38 DAA) the berries were too small and the fuchsin acid application technique to study back-flow movement could not be optimized.

At the 77 BBCH phenological stage (45 DAA), the fuchsin acid solution was injected into berries using a syringe (Figure 8), and it was observed that: a) if the dye solution moves only in the central vasculature, it goes straight towards the seeds without going beyond the brush; b) if the dye solution penetrates the peripheral vasculature, it moves towards the brush and beyond, entering the peduncle.

At the 83 BBCH phenological stage (53 DAA), the dye solution was absorbed by the berry, instead of being injected (Figure 9). In all treated berries, the dye solution was absorbed by the berry and flow was observed inside peripheral and central vasculature bundles, crossing the brush region towards the pedicel (Figure 9). The pictures are evidence that 'xylematic back-flow'occurred.

At 85 BBCH phenological stage (116 DAA), the dye solution was injected inside the berry by the syringe as well as absorbed by the cut berry. In the injected berries, movement of the dye

solution was evident inside peripheral vessels in the whole berry without passing beyond the brush (Figure 10). In the same treated berries, movement of the fuchsin acid solution inside the central vasculature was also observed (Figure 10). In cut berries, at this phenological stage, we observed: a) diffusion of dye solution in the mesocarp; b) movement in both peripheral and central vessels; and c) interruption of dye solution flow at the brush level.

At 89 BBCH ripeness level (142 DAA), berries were treated in the same way than what was described for the previous ripening stage (85 BBCH at 116 DAA), but the treated berries were divided into 'intact' and 'shrivelled'. Although less pronounced, the same results were observed in both types of berries, identical to those of the 85 BBCH stages (116 DAA) already described (Figure 11). It is clear that at this last stage mutual attraction between mother plant and berry is rather very limited or absent.

**PHLOEMATIC TRACER**

**CFDA flow from plant to berry (inflow)**

At the 85 BBCH stage (112 DAA; 19.30 °Brix) the CFDA solution was applied to the leaf main vein and distribution of the fluorescent solutions in the main and peripheral veins of the leaf, in the petiole and in the stem of the main shoot observed by means of a fluorescence microscope (Figure 12). No movement of the CFDA solution towards the rachis, i.e. towards berries, was observed. At the 89 BBCH stage (133 DAA; 20.93 °Brix) similar results were found (data not shown). Moreover, in Figure 12 C it is interesting to note the xylem staining in the shoot cross section.

**CFDA flow from berry to plant (back-flow)**

At the 85 BBCH ripening stage (112 DAA) berries were cut at the stylar end level and submerged into the CFDA solution. By means of the fluorescence microscope it was possible to assess the tracer movement inside the treated berries *via* peripheral and central bundles, without staining of the pedicel (Figure 13). Furthermore, diffusion of the fluorescent tracer in the mesocarp of the berry is noticeable (Figure 13 and Supplementary Figure 14). Similar results were obtained in the trials done at 133 DAA (data not shown).

**Discussion**

Shiraz is commonly referred to as model for 'berry dehydration' research. Although many studies have hither to try to explain the phenomenon, generating different disputes, it appears that the exact mechanisms involved are still not fully clarified. Essentially, two different deductions are generally observed in literature: a) from véraison through ripening the berry gradually attains

'vascular isolation' from the mother plant, both xylem and phloem becoming impeded; b) from véraison through ripening the berry remains hydraulically connected to the mother vine, but inside the berry the roles of the xylem and phloem change: during pre-véraison the xylem supplies water to the berry, whereas during post-véraison it is used to drain the phloem water supply surplus, because water is supplied to the berry essentially *via* the phloem during this period.

- **Xylematic flow: from plant to berry.**

In this study, data clearly showed that the vine gradually loses the ability to deliver water to the berries *via* the pedicel during berry ripening. In tomato (*Solanum lycopersicum* L.), some authors (Lee, 1989; Rancić *et al.*, 2010) observed changes in hydraulic properties of the fruit and considered them as consequences of xylem anatomical changes. Findlay *et al.*, (1987) and Creasy *et al.*, (1993) found that the peripheral xylem tracheids in grape berries stretch and break at véraison and that these phenomena can explain the water flow cessation/reduction into the berries *via* the xylem at véraison. Therefore, during the pre-véraison stages, when cell division occurs in the berries, water moves undisturbed from the plant to the berry *via* the xylem. At véraison, as also found by Zhaosen *et al.,* (2014) for the cv Kyoho, water movement towards the berry becomes limited and not all vascular bundles participate in water transport. In fact, our study showed a non-uniform distribution of water from the plant to the berry already before véraison, indicating that vessel breakage/disturbance is indeed likely promoted by the increase in berry size. For kiwifruit, Dichio *et al*., (2003) observed a drastic reduction in the number of functional bundles at around 20, 55 and 90 days after anthesis with a partial recovery between these phases; a permanent dysfunction occurred at around 120 days after anthesis in over-ripe fruits. They hypothesized that the fruit expansion promotes vessel stretching and thus breakage, coupled with new xylem formation that ceases at the overripe stage. This behaviour can explain the decreasing calcium transport into the kiwifruit during ripening, calcium being a xylem-mobile element (White, 2001). Ferguson & Watkis (1989) suggested that the imbalance between xylem and phloem, presumed by the calcium:potassium imbalance, is related to apple bitter-pit, whereas in kiwifruit, the low calcium concentration was found to be involved in premature fruit softening (Prasad & Spiers, 1991).

Etchebarne *et al.*, (2010) found that calcium transport into the berry only continued under favourable water conditions, but with a marked decrease in accumulation during the last period of ripening under both irrigated and non-irrigated conditions. This indicates a limitation in transport into the berry during late ripening that is independent of water availability in the

mother plant, as also found by Hunter *et al.*, (2014a). Dehydration in Shiraz berries resulting from berry transpiration and causing fruit softening may be an additional impacting factor positively correlated with the observed decrease in xylem support of water flow into the berry after véraison. Choat *et al.,* (2009) measured the xylem hydraulic resistance in whole berry, receptacle and pedicel in developing fruit of cv Chardonnay, and observed just for the whole berry and receptacle a significant increase in the late post-véraison stage (80 days after anthesis). However, they concluded that the fruit is not hydraulically isolated from the parent plant by the xylem, but hypothesized that xylem transport is 'hydraulically buffered' by water delivered *via* the phloem.

In this study, results showed that in some treated berries the red marker (fuchsin acid) accumulated in the brush zone without any movement into and inside berries by means of peripheral or central bundles. This is in agreement with Coombe & McCarthy, (2000) who correlated Shiraz disorder with stretching of tracheids and breakage of tracheid wall membranes, especially in the brush zone where vascular bundles enter the berry. Zufferey *et al.,* (2015) reported a decline in rachis hydraulic conductance after véraison in comparison with the pre-véraison measurements, confirming what was observed by Tyerman *et al.*, (2004). On the other hand, Chatelet *et al.*, (2008), studying the peripheral xylem structure in cv Chardonnay, found that tracheary elements remained intact throughout berry maturation, in agreement with findings of Bondada *et al.,* (2005) and Keller *et al.*, (2006) who suggested xylem functionality in post-véraison berries. It is important to note that Bondada *et al.*, (2005) applied a hydrostatic gradient, whereas the "plant to berry" water movement trials in this study were done under field conditions, without disturbing the plant-bunch system. Data suggest that lack of water movement from vine to fruit is due to a probable xylem blockage.

Data clearly showed that after fruit-set water flowed straight to the seeds *via* central bundles, highlighting that at this stage the seeds are major sinks in terms of water/mineral/hormone uptake. On the contrary, in the consecutive ripening stages peripheral bundles seemed the preferential way by which the water entered the berry. This is in contrast to findings of Düring *et al*., (1987). This evidence suggests that seeds do not act as a predominant water sink after their growth has stopped and a switch towards berry maturity has occurred.

- **Xylematic flow: from berry to plant (xylematic back-flow).**

The 'back-flow' experiment indicated that before véraison water is able to move from the stylar end to the pedicel (plant), whereas after véraison the water continues its distribution in peripheral bundles, but without transgressing the brush zone of the berry. With this evidence it is possible

to argue that when the plant is actively growing vegetatively, communication between the vegetative and reproductive compartments regarding hydric status is very important to, *inter alia*, support the leaves in accommodating the environmental evaporative demand, but at the same time progressively supporting reproductive growth; during pre-véraison the fruit is a 'green' part of the plant, displaying some (limited) activity common to leaves, *i.e*. transpiration and photosynthesis. Indeed, vascular water influx is linked to ambient vapour pressure deficits (Measham *et al*., 2014). Livellara *et al.,* (2011) found that in apples sap flow is linked to the vapour pressure deficit. Measham *et al.*, (2014) reported that the leaf evaporative demand was the dominant driver of flow within the spur/fruit/leaf complex. It seems that after véraison, the fruit loses its ''vegetative nature'' and the goal is to spread seeds.

In a recent paper, Keller *et al.,* (2015) proposed a conceptual model that shows the destiny of phloematic water that arrives into the post-véraison berry: the surplus of this water partly evaporates from the berry surface and partly moves apoplastically to the xylem for out-flow. It is however questionable whether any of these arguments satisfy the dynamic movement of water along osmotic potential gradients. Furthermore, Keller *et al.,* (2015) confirmed that the decrease of xylem in-flow in a post-véraison berry is a consequence of the sink-driven increase in phloem inflow. From this point of view, the xylem back-flow in the berry is interpreted as a way to deliver towards the plant excess phloematic water (Rogiers *et al*., 2004; Tyerman *et al*., 2004). Again, it is doubtful whether an already senescing vine with fully ripened berries, increasing plant water potential, access to soil water, and a mechanism of berry transpiration would actively regulate berry water potential; passive flow also seems unconvincing. Also Tilbrook & Tyerman, (2009) demonstrated the movement of the water from the berry to the vine *via* the xylem, but with a varietal-linked behaviour: in cv Chardonnay xylem back-flow ceased at 97 days after anthesis, whereas in Shiraz berries there was still water movement outside the berry at 118 days after anthesis. They concluded that xylem back-flow could in part be responsible for post-véraison weight loss in Shiraz berries. However, McCarthy & Coombe (1999) attributed shrinkage mainly to the transpiration of water from each berry. They argued that the reverse movement of water from berry to vine was unlikely. Our results suggest to specify that after véraison xylematic back-flow is unlikely.

- **Berry shrivel and xylem relationship.**

The gradual 'hydraulic isolation' of the berry that we observed after post-véraison is well sustained by the behaviour of the shrivelled berries (at a more advanced maturity level: 142

433 DAA), not showing any water exchange with the mother plant: this isolation can explain the
434 shrinkage. Rogiers *et al*., (2004) also concluded that decreased vascular flow of water into the
435 berry coupled with continued transpiration promote pre-harvest berry weight loss. As reported in
436 the results, movement of the water (marked with fuchsin acid and introduced *via* the rachis wing)
437 towards the bottom part of the shoot (Figure 5) may suggest that the plant is supplying water to
438 perennial/permanent parts in order to sustain turgor balances/recuperate water relations and
439 support root growth activity during this time (Van Zyl, 1988; Hunter *et al.,* 2014 a, b). From an
440 ecological point of view, berry dehydration of cv Shiraz would require a 'plant-berry' vascular
441 disconnection. This scenario is complicated by the hypothesis that rachis phloem functionality
442 may also play a role in changing water status and soluble solid accumulation patterns of the
443 berry (Coombe & McCarthy,2000; Zufferey *et al.,* 2015).
444 Hunter *et al.*, (2014) clearly showed that rachis:berry sucrose ratio increased with ripening,
445 indicating reduced demand and restricted transport and unloading from rachis to berry, despite
446 favourable sucrose and osmotic potential gradients. The continuing shrinking of the berry during
447 late ripening, irrespective of highly negative berry water potential, was also shown by Rogiers *et*
448 *al.,* (2006) and Greer & Rogiers, (2009). Indeed, Hunter *et al*., (2004) deducted that, for Shiraz, a
449 physiological endpoint of sucrose demand by the berry seemed to occur during the later stages of
450 ripening. Hunter *et al.,* (2014) reported that rachis and berry behaviour is not concerted during
451 berry ripening, particularly during late ripening; the rachis continued to display typical
452 vegetative tissue behaviour, whereas the berry advanced with physiological and morphological
453 maturation changes/levels involving dehydration (with progressively diminishing importance of
454 hydraulic status of the mother plant), sugar concentration and physical deterioration.
455 The results of this study indicated that neither berry transpiration forces (*vid*. also Greer &
456 Rogiers, 2009) or flux velocity of phloem and xylem (with partial or full functionality) (*vid*. also
457 Lang & Düring, 1991; Greenspan *et al.,* 1994; Rebucci *et al.,* 1997; Chatelet *et al*., 2008a, b)
458 seemed to be able to sustain influx during late ripening and maintain a fully intact berry without
459 shrivelling.

460 • **Phloematic flow.**

461 In order to understand also the phloematic berry connection to the vine, on the basis of what was
462 reported by McCarthy & Coombe (1999), it seemed useful to investigate the phloematic sap flow
463 between berry and vine at maturity. To do this, we used 6(5)-carboxyfluorescein diacetate
464 (CFDA) as a fluorescent marker of phloem transport (Viola *et al*., 2001; Zhang *et al*., 2006;
465 Zanon *et al*., 2015). The CFDA is a membrane-permeable and non-fluorescent compound that,

when degraded to 6(5)-carboxyfluorescein (CF) in living cells, becomes a membrane-impermeable fluorescent dye. Grignon et al. (1989) reported that CF is a good tracer of long-distance translocation of phloem sap. The CFDA demonstrated the absence of flow from the berry to the plant during late ripening stages. Despite a perfect distribution inside the berry, movement towards the pedicel was not observed. Concomitantly, phloematic water movement from plant to berry was also not observed during this time. The fluorescent marker was successfully transported throughout the whole network of leaves, petiole and shoot vascular bundles, but did not enter the rachis. It is important to note the migration of CFDA into xylem vessels of the shoot: it was after all a watery substance and it was applied through an 'open channel/vein', meaning that it would also be available for transpiration by leaves, therefore also transport in the xylem. The results suggest that at this time the berry was already isolated and therefore did not act as a sink anymore, or, with the vascular bundles being physically impeded, the vine had no ability to actively deliver water to the bunch anymore. Indeed, Zufferey *et al.,* (2015) observed a significant degradation as well as a loss of functionality of primary phloem in the rachis of 'berry shrivelled' clusters. Hunter *et al.,* (2014a) stated that water relation gradients, along with photosynthetic activity, sucrose accumulation patterns and enzyme activity in leaves and berries during this time, do not support active water transport dynamics and flow to berries. Translocation studies involving $^{14}C$ showed that grape berries are the major sinks in the canopy between berry-set and véraison stages, but that this focus fades after that (Hunter & Visser, 1988). This may also be deducted from photosynthetic behaviour, sucrolytic enzyme activity and carbohydrate accumulation patterns (Ruffner *et al,.* 1990; Hunter *et al.*, 1994; Zhang *et al.,* 2006).

## Conclusions

The experiments performed in this study showed a lack of xylem flow from the plant to the berry during post-véraison, but did not allow clarifying whether this is due to a vessel/tracheid breakage or not. However, it allowed to state that the xylem back-flow in post-véraison berries is unlikely. The results further demonstrated the absence of flow from berry to plant and *vice versa* during late ripening stages. The experimental evidence showed that for cv Shiraz berry dehydration must also be interpreted from an environmental/ecological point of view, especially during late ripening. Genetic behaviour as well as environmental conditions have an impact on physiological processes that ultimately trigger and steerthe fruit ripening process in perennial plants until full maturity is reached, be it to satisfy technological/oenological or ecological/botanical purposes. These processes would logically lead to physico-chemical

changes in the fruit. Results of this study indicate that the preceding dynamics leading to fruit maturity in the grapevine are well regulated, coordinated, and responsive to environmental and cultivation influences.

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**Author Contribution**

A.C. and J.J.H. planned, designed and performed the research and wrote the paper. C.L. helped with the phloem experiment planning. A.G. helped with the fluorescence microscope. V.N., A.F. and C.L. revised the manuscript.

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**Table 1.** Grape berry maturity parameters during ripening of Shiraz in South Africa (SA) and Italy (I).

| Country | Phenological Stage (BBCH) | BBCH description | DAA | Berry Weight (g) | Total Soluble Solids (°Brix) | Sugar/berry (mg) | Titratable Acidity (g $L^{-1}$ as tartaric acid) |
|---|---|---|---|---|---|---|---|
| **I** | **75** | Berries pea-sized, bunches hang | 38 | 0.45 ± 0.03 | | | |
| **I** | **77** | Berries beginning to touch | 45 | 0.75 ± 0.05 | 4.13 ± 0.03 | 30.98 | |
| **I** | **83** | Berries developing colour | 53 | 1.08 ± 0.11 | 9.43 ± 0.98 | 101.84 | |
| **SA** | **85** | Softening of berries | 116 | 1.96 ± 0.02 | 24.10 ± 0.37 | 472.36 | 4.62 ± 0.25 |
| **SA** | **85 intact** | Softening of berries | 128 | 1.85 ± 0.10 | 26.29 ± 0.22 | 486.37 | 4.78 ± 0.10 |
| **SA** | **89 intact** | Berries ripe for harvest | 142 | 1.61 ± 0.03 | 27.06 ± 0.22 | 435.66 | 4.15 ± 0.01 |
| **SA** | **85 shrivelled** | Softening of berries | 128 | 1.79 ± 0.02 | 25.78 ± 0.74 | 461.46 | 4.99 ± 0.08 |
| **SA** | **89 shrivelled** | Berries ripe for harvest | 142 | 1.59 ± 0.02 | 27.74 ± 0.36 | 441.07 | 3.15 ± 0.14 |
| **I *** | **85** | Softening of berries | 112 | 2.47 ± 0.09 | 19.30 ± 0.35 | 476.71 | 5.17 ± 017 |
| **I *** | **89** | Berries ripe for harvest | 133 | 2.00 ± 0.12 | 20.93 ± 0.07 | 418.6 | 4.50 ± 0.29 |

**Data are expressed as mean ± standard error of the three field repetitions**

*** these ripening stages refer to the berries treated with CFDA in Italy.**

## Figure legends

**Figure 1.** At 75 BBCH stage (38 DAA): Fuchsin acid absorption by a wing of the bunch.

a) A whole berry in which water passage from the rachis to the berry *via* the pedicel is shown. The epidermal staining highlights the berry transpiration.

b) A longitudinal section of the berry to show water movement into the central vasculature.

c) Longitudinal section of the berry pedicel interface.

**Figure 2.** At 77 BBCH stage (45 DAA): Fuchsin acid absorption by a wing of the bunch.

a), b) and c) Berries in which fuchsin acid is entering *via* some peripheral bundles.

d) The entering of fuchsin acid into the berry *via* some peripheral and central bundles.

e) Staining of the peripheral vasculature.

f) Seed staining.

**Figure 3.** At 77 BBCH stage (45 DAA): Absorption of the dye solution by the shoot basal part without shoot disconnection.

a) Technique used to absorb the fuchsin acid *via* the shoot.

b) Hydric flow stained with fuchsin acid and the water clearly flowing from the bottom part of the shoot towards the bunch.

c) Staining of the leaf veins.

**Figure 4.** At 85 BBCH stage (116 DAA): Absorption of the fuchsin acid by a bunch wing.

a) Berries showing the presence of fuchsin acid in the pedicels.

B and c) Peeled berries showing fuchsin acid distribution in the peripheral bundles.

d) Longitudinal section of the berry showing the lack of fuchsin acid staining in the central vasculature.

**Figure 5.** At 89 BBCH stage (142 DAA): After absorption by the bunch, the fuchsin acid goes into the rachis (a), towards the pedicels (b), from the bunch to the shoot (c), and then towards the basal part.

**Figure 6.** At 89 BBCH stage (142 DAA): Intact berries that received the fuchsin acid *via*the pedicel. The accumulation of fuchsin acid stopped at the brush level without going into the berry or the peripheral and central bundles.

a) An intact berry with a longitudinal section of the pedicel.

b) A peeled intact berry pictured from the top.

c) and d) Peeled intact berries with longitudinal sections of the pedicel.

e) and f) Longitudinally dissected peeled intact berries.

**Figure 7.** At 89 BBCH stage (142 DAA): Shrivelled berries that received the fuchsin acid *via*the pedicel showing an accumulation of fuchsin acid at the brush level without entering the berry or the peripheral and central bundles.

a) A whole shrivelled berry with a longitudinal section of the pedicel.

b) and c) A whole peeled shrivelled berry with a longitudinal section of the pedicel.

d) Longitudinally dissected peeled shrivelled berries.

**Figure 8.** 77 BBCH stage (45 DAA): Injected berries.

a) The injection technique.

b) Fuchsin acid movement inside the central vasculature.

c) Fuchsin acid movement in peripheral bundles and entering the pedicel (xylematic back-flow).

**Figure 9.** At 83 BBCH stage (53 DAA): Fuchsin acid absorption by the berry.

a) Fuchsin acid absorption technique.

b) Visual evidence of the fuchsin acid movement from the berry towards the rachis *via*the pedicel (xylematic back-flow).

c), d), e) and f) Fuchsin acid distribution across peripheral bundles and pedicel: evidence of xylematic back-flow.

688 **Figure 10.** At 85 BBCH stage (116DAA): Fuchsin acid absorption by the berry.

689 a) and b) Whole peeled berries with fuchsin acid distribution inside peripheral bundles, but without
690 movement towards the pedicel: no xylematic back-flow.

691 c) Pedicel cross section: no trace of fuchsin acid.

692 d) Berry cross section with central vasculature stained by fuchsin acid up until the brush.

693 **Figure 11.** At 89 BBCH stage (142 DAA): Fuchsin acid absorption by intact (a and b) and
694 shrivelled (c and d) berries at 89 BBCH without xylematic back-flow evidence.

695 a) Whole peeled berry with fuchsin acid diffusion inside the mesocarp.

696 b) Pedicel longitudinal section of the intact berry.

697 c) Cross section of the shrivelled berry.

698 d) Pedicel longitudinal section of the shrivelled berry.

699 **Figure 12.** At 85 BBCH stage in Italy (112 DAA): CFDA absorption by the leaf, according to
700 Gould *et al.* (2013). Observations were made by means of a fluorescence microscope.

701 a) CFDA distribution inside the leaf veins observed with a longitudinal section.

702 b) CFDA distribution inside the petiole observed with a longitudinal section.

703 c) CFDA distribution inside the shoot observed with a cross section.

704 **Figure 13.** At 85 BBCH stage in Italy (112 DAA): CFDA absorption by the berry. Observations
705 were performed using a fluorescence microscope.

706 a) Reference of the longitudinal dissected berry.

707 b) and c) CFDA fluorescence and distribution in the peripheral vasculature and diffusion inside the
708 mesocarp in the longitudinally dissected berries.

709 d) Reference of the longitudinally dissected berry plus pedicel.

710 e) and f) CFDA fluorescence and diffusion in the berry mesocarp, without fluorescence in the
711 pedicel.

712 g) Reference longitudinally dissected berry plus pedicel.

713 h) and i) CFDA distribution in the central vasculature without fluorescence in the pedicel of the
714 longitudinally dissected berries (h).

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