The peripheral xylem of grapevine (Vitis vinifera). 1.
Structural integrity in post-veraison berries

David S. Chatelet¹, Thomas L. Rost¹, Kenneth A. Shackel²,* and Mark A. Matthews³

¹ Section of Plant Biology, University of California, Davis, CA 95166, USA
² Department of Plant Sciences, University of California, Davis, CA 95616, USA
³ Department of Viticulture and Enology, University of California, Davis, CA 95616, USA

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Abstract
During the development of many fleshy fruits, water flow becomes progressively more phloemic and less xylemic. In grape (Vitis vinifera L.), the current hypothesis to explain this change is that the tracheary elements of the peripheral xylem break as a result of berry growth, rendering the xylem structurally discontinuous and hence non-functional. Recent work, however, has shown via apoplastic dye movement through the xylem of post-veraison berries that the xylem should remain structurally intact throughout berry development. To corroborate this, peripheral xylem structure in developing Chardonnay berries was investigated via maceration and plastic sectioning. Macerations revealed that, contrary to current belief, the xylem was comprised mostly of vessels with few tracheids. In cross-section, the tracheary elements of the vascular bundles formed almost parallel radial files, with later formed elements toward the epidermis and earlier formed elements toward the centre of the berry. Most tracheary elements remained intact throughout berry maturation, consistent with recent reports of vascular dye movement in post-veraison berries.

Key words: Tracheary element, vasculature, vessel, water movement.

Introduction
In fleshy fruits, water movement in xylem and phloem is dependent upon the developmental stage of the fruit (Mattheus and Shackel, 2005). In tomato (Ho et al., 1987), kiwifruit (Dichio et al., 2003), apple (Lang and Ryan, 1994; Dražeta et al., 2004), and grape (Greenspan et al., 1994, 1996), the amount of water entering the fruit via the xylem decreases as fruit matures. Growth of many fleshy fruit, including Prunus sp. and Vitis sp., exhibits a double sigmoid pattern (Coombes, 1976) in which there are two periods of growth (stages I and III) separated by a lag phase (stage II). In grape, the transition from xylemic to phloemic water is apparently rapid, occurring at the transition from stage II to stage III (Greenspan et al., 1994). In their model of vascular function during growth and maturation of grape berries, Coombe and McCarthy (2000) have presented the xylem as becoming non-functional upon the transition to stage III (called veraison).

The Coombe and McCarthy model was deduced from the microscopic observations of Düring et al. (1987) and Findlay et al. (1987) that were interpreted as showing that the peripheral xylem was stretched to failure during veraison, and from passive dye uptake studies with several grape varieties: Muscat Gordo Blanco (Findlay et al., 1987), Riesling (Düring et al., 1987), Pinot noir, and Merlot (Creasy et al., 1993). Before veraison, apoplastic dye was passively absorbed through a cut pedicel into the axial and peripheral xylem of a berry, whereas after veraison its uptake was limited to the basal few millimetres of vascular tissue (Findlay et al., 1987; Creasy et al., 1993; Rogiers et al., 2001). This change has been interpreted as being due to the inability of the lignified xylem to increase in length during stage III growth and that growth itself causes a physical disruption in xylem continuity (Lang and Düring, 1991; Coombe and McCarthy, 2000).

However, Bondada et al. (2005) showed that by artificially establishing an appropriate hydrostatic gradient in post-veraison berries, apoplastic dye was transported from...
the pedicel to the stylar end through the axial and peripheral xylem. In addition, when post-veraison growth and subsequent potential physical failure due to stretching was prevented, apoplastic dye still did not move into the peripheral xylem without an artificial gradient. They speculated that the decrease in xylem water flow at veraison was due to a reduction in the hydrostatic gradient between the xylem in the pedicel and the xylem in the fruit flesh, and not to physical disruption of xylem continuity. There is little information, however, on berry xylem anatomy. Description of berry xylem structure apparently originated when Kroemer (1923) and de Villiers (1926) described the ventral, embryonic, and peripheral vascular bundles present in the berry flesh. This was reviewed briefly in Pratt (1971) who reported that the xylem elements are exclusively tracheids. Despite the many physiological studies published in which the interpretations are structural, there is essentially no information published on the details of the xylem structure during grape berry development. Therefore, this study was conducted to describe xylem conduit structure in pre- and post-veraison berries, looking specifically for evidence of physical integrity or loss thereof. Since Bondada et al. (2005) were able to induce dye uptake in post-veraison berries of many varieties (Chardonnay, Cabernet Sauvignon, Pinot noir, Shiraz, Muscat Gordo, and Grey Riesling) with similar results, berries of the Chardonnay variety were chosen as the model as this variety was easily available.

Materials and methods

Plant material

Berries were obtained before and after the onset of fruit ripening (veraison) from 1-year-old grapevines (Vitis vinifera L. cv. Chardonnay), that were planted in 7.5 l plastic pots filled with a mixture of GrowCoir™ (Greenfire Co., Ltd, Sacramento, CA, USA), clay pellets, and perlite (4:1:1 by vol.) and grown in a greenhouse at UC Davis (30/20±3 °C; 40/70±10% relative humidity; natural light with a daily maximum of ~1200 μmol photons m⁻² s⁻¹ PAR). The vines were pruned to two shoots, and the shoots were vertically trained to ~2 m. Vines were fully watered daily with a modified Hoagland’s nutrient solution (in mM: NO₃⁻, 6.85; NH₄⁺, 0.43; PO₄³⁻, 0.84; K⁺, 3.171; Ca²⁺, 2.25; Mg²⁺, 0.99; SO₄²⁻, 0.50; and in μM: Fe²⁺, 28.65; Mn²⁺, 4.91; BO₃³⁻, 24.05; Zn²⁺, 1.83; MoO₄²⁻, 0.17; Cu²⁺, 2.52) with EC 1.00 dS m⁻¹ at pH 5.75. Berries were collected before 10 am, placed in a plastic bag, and transported to the laboratory within 15 min. The developmental age of the berries was determined by the number of days after anthesis (DAA) and by the concentration of soluble solids in the berry flesh. This was reviewed briefly in Pratt (1971) who reported that the xylem elements are exclusively tracheids. Despite the many physiological studies published in which the interpretations are structural, there is essentially no information published on the details of the xylem structure during grape berry development. Therefore, this study was conducted to describe xylem conduit structure in pre- and post-veraison berries, looking specifically for evidence of physical integrity or loss thereof. Since Bondada et al. (2005) were able to induce dye uptake in post-veraison berries of many varieties (Chardonnay, Cabernet Sauvignon, Pinot noir, Shiraz, Muscat Gordo, and Grey Riesling) with similar results, berries of the Chardonnay variety were chosen as the model as this variety was easily available.

Dye uptake

Clusters of pre- and post-veraison berries, with a Brix of ~5 and 16, respectively, were harvested from the plant, and the peduncle was immediately immersed in a 0.1% aqueous (w/v) acid fuchsin solution. After 5 h, the clusters were brought to the laboratory and prepared for dye uptake (basic fuchsin, 0.1% aqueous solution). The xylem was observed cross-section after 24 h, and prepared slides were observed with an Olympus Vanox-AHBT (Olympus America, Melville, NY, USA) epifluorescent microscope under bright light and under UV light (dichroic mirror DM400, exciter filter=UG1, barrier filter=L435). Digital images of macerated xylem were taken with a Pixera 600ES digital camera (Pixera Corporation, Los Gatos, CA, USA).

Scanning electron microscopy of individual elements

From the macerated solutions, a drop of solution was dropped on scanning electron microscope (SEM) stubs and left to dry. The dry samples were coated with gold (Denton Vacuum Desk II Cold Sputter-Etch Unit; Denton Vacuum, Moorestown, NJ, USA) and observed under SEM (Hitachi S-3500N; Hitachi, Japan) at an accelerating voltage of 5 kV.

Plastic embedding: cross-section and longitudinal section

Pieces of berry containing peripheral vascular bundles from the brush, midsection, and the style, the style, and most of the mesocarp tissue surrounding the vascular bundles was carefully removed without damaging the xylem. The resulting bundles were fixed in formaldehyde–glutaraldehyde fixative and embedded in glycol methacrylate (JB-4 Plus) by standard methods (Ruzin, 1999). The vascular bundles were sectioned at 2.5 μm, either transversally or longitudinally, with a Microm HM 304E rotary microtome (Microm, Walldorf, Germany). Sections were stained with 0.1% toluidine blue O (aqueous, w/v), and prepared slides were observed with an Olympus Vanox-AHBT (Olympus America) compound light microscope linked to a Pixera 600ES digital camera (Pixera Corporation).

Maceration

Cross-sections ~2 mm thick were made with a razor blade near the pedicel, at the berry equator, and near the style. The axial xylem was removed to leave only the peripheral xylem. Cross-sections from each location were then immersed separately in capped vials (Wheaton Glass 20 ml Scintillation Vials, Fisher Scientific, USA) containing 15 ml of a maceration solution (1:4:5 by vol. 30% hydrogen peroxide:distilled water:glacial acetic acid) and placed in an oven at 57 °C. After 12 h or 24 h, samples were removed and washed with water several times, strongly shaken to loosen the tissues, and several drops of 0.1% aqueous Safranin O (w/v) were added to the solution. After several hours, several drops of 0.1% aqueous (v/w) Calcofluor White 2MR were added, and the vials were shaken again. When the tissues settled at the bottom of the vial, a drop was pipetted out, deposited onto a slide, and scanned with an Olympus Vanox-AHBT (Olympus America, Melville, NY, USA) epifluorescent microscope under bright light and under UV light (dichroic mirror DM400, exciter filter=UG1, barrier filter=L435). Digital images of macerated xylem were taken with a Pixera 600ES digital camera (Pixera Corporation, Los Gatos, CA, USA).
After being macerated for xylem bundles radial files with the latest elements toward the epidermis bundle and the phloem on the outer side (Fig. 2A, B). The (Mauseth, 1988), with the xylem on the inner side of the The vascular bundles had a typical collateral organization and extensive in pre-veraison berries.

**Results**

**Dye movement in pre- and post-veraison berries**

Acid fuchsin and basic fuchsin were taken up through the cut pedicel and into and throughout the peripheral xylem of pre-veraison berries using the pressure membrane, the wicking, or the passive dye infusion methods (Fig. 1A). In post-veraison berries, however, the dye did not move into the peripheral xylem using the passive dye infusion method (data not shown), but the stain was taken up into the xylem when using the wicking system or the pressure membrane technique (Bondada et al., 2005). The water was then progressively replaced by a 1:1 water:commercial bleach solution [Clorox Regular-Bleach (sodium hypochlorite= 6.15%) The Clorox Company, Oakland, CA, USA]. After a few seconds, the vascular bundle started to decolorize and as a reaction between the basic fuchsin and the bleach, some gas, possibly oxygen, appeared within several tracheary elements. As the de-colorization continued, more gas was produced and its movement within the tracheary elements was observed with the microscope until the bundle was completely bleached.

**Anatomy of a typical vascular bundle**

The vascular bundles had a typical collateral organization (Mauseth, 1988), with the xylem on the inner side of the bundle and the phloem on the outer side (Fig. 2A, B). The tracheary elements were more or less linearly arranged in radial files with the latest elements toward the epidermis and the earliest toward the centre of the berry (Fig. 2A–C).

**Maceration and plastic longitudinal sections of xylem bundles**

After being macerated for ~12 h, long branched vascular bundles showed some nicks (Fig. 3A, arrows), but were never completely broken. At each of these nicks, there were always intact vessels adjacent (Fig. 3A, arrowheads). Incidentally, these nicks were also observed on vascular bundles observed in situ, carefully dissected from the berry. When macerated for ~24 h, the bundles were further separated into groups of tracheary elements appearing as a unicellular sheet (Fig. 3B, C) that presumably resulted from the separation of the radial files from one another. These elements presented a spatial arrangement reflecting the ontological development of the xylem. Initially, elements with annular secondary wall differentiated (Fig. 3B, C, arrows), followed by elements with a secondary wall forming a helix with widely spaced coils. Also, as new elements continued to differentiate, their secondary wall formed a helix that became more and more tightly coiled (Fig. 3B, C, arrowheads). The majority of the elements that were observed had helical secondary wall thickenings. Therefore, it was difficult to differentiate between proto- and metaxylem because both types can have helical thickenings. In addition, in other organs, such as leaf, stem, and roots, the protoxylem is found in elongating and sometimes in mature tissues, while the metaxylem is exclusively found in non-growing tissues. However, in the case of the berry, the growth by cell expansion is more or less continuous until late in development. As a result, the tracheary elements could be all protoxylem or some of them may be considered as metaxylem.

Longitudinal sections of plastic-embedded vascular bundles showed no apparent breaks or nicks in individual tracheary elements (Fig. 3D, E). Although the helix of some post-veraison tracheary elements seemed stretched, an intact primary wall was still present between the gyres of the secondary wall helix (Fig. 3E, arrows). No evidence of tyloses was seen within the tracheary elements of the peripheral vasculature of berries at different stages of development (120 bundles were observed).

When macerated for ~24 h, individual tracheary elements could also be observed (Fig. 4). All these elements had helical secondary wall thickenings, and it was not clear whether they had a closed end (tracheid) or a perforation plate (vessel) (Fig. 4A). When observed under UV, however, openings corresponding to perforation plates were visible in 90 tracheary elements out of 100 observed under the microscope (Fig. 4B). The presence of a scalariform perforation plate was further demonstrated using an SEM (Fig. 4C, D). However, in ~10% of the elements, the presence of perforation plates was not conclusive; therefore, some tracheids may be present.

**Continuous xylem**

When post-veraison berries were allowed to absorb acid fuchsin passively, the dye moved into the pedicel, the receptacle, and ~1 mm into the brush (Fig. 5). When viewed under a light microscope at ×4 magnification, the...
bundles were completely stained for ~1 mm into the brush, followed by intermittent staining from 1–2 mm and no staining beyond ~2 mm. Because of this staining pattern, the vascular bundles appeared to be broken in several places (Fig. 5A, arrows). However at a higher magnification (×10), vessels were found to be present...
around and through the apparently broken portions of the vascular bundle (Fig. 5B, arrows).

After post-veraison berries were stained with basic fuchsin using the pressure membrane system, vascular bundles were separated from their surrounding fleshy tissues and observed at 310 magnification under a microscope. Due to the staining, the bundle was dark red and appeared to be partially broken on one side only (Fig. 6A). A higher magnification (×40) revealed that some of the xylem elements with loosely helical secondary walls were damaged and probably non-functional (Fig. 6B, arrows) and that the berry tissues located next to the damaged elements were also stained, seemingly due to the leaking of the dye outside of the elements. This intermittent partial breaking of the bundles appeared similar to the intermittent staining of the bundles of post-veraison berries absorbing the dye passively (Fig. 5 and previous paragraph). However, in that case, the leaking is limited to the brush area, possibly because of the loss of driving forces.

When bleach was allowed to diffuse in the water surrounding red-stained bundles, the progressive decolorization of the bundles revealed the secondary cell wall thickenings of the tracheary elements. In addition to removing the red colour, gas formed inside the water-filled tracheary elements. The water-filled elements were pale brown and it was difficult to distinguish their walls, but the gas-filled elements were dark grey with distinct walls (Fig. 6C), presumably due to the contrast in refractive index between the gas and the liquid. The gas progressively filled the tracheary elements over a period of several minutes, eventually reaching the cut end of the bundle, and forming bubbles outside the bundle (Fig. 6D). Bubbles also formed along the length of the bundle, but only on one side. Indeed, when observed at high magnification (×60), the gas exited only from the tracheary elements having the loosest helical thickenings and, within those, only where the distance between secondary walls was wider and abnormal compared with the rest of the elements (Fig. 6E, F). At a still higher magnification (×60), the primary wall between these widely spaced secondary wall thickenings was observed to be torn apart, allowing the gas to escape (Fig. 7).
The results of this study show that tracheary elements of the peripheral xylem in the grape berry contain primarily vessels and few tracheids. Vessels were clearly predominant because in only a few elements were open and scalariform end walls not observed. This was a surprise as the current view (Coombe and McCarthy, 2000; Rogiers et al., 2001; Ollat et al., 2002; Bondada et al., 2005; Keller et al., 2006), based mostly on one review article by Pratt (1971), is that the xylem conduits of the pedicel and the fruit are comprised exclusively of tracheids. Because the present study used only one grape variety (V. vinifera cv. Chardonnay), the results cannot be generalized for all grapes. However, caution should now be used when employing the terms tracheid and vessel in the description of the grape berry xylem.

**Fig. 4.** Vessel members from the xylem of a post-veraison berry showing perforation plates (arrows). (A) and (B) are the same vessels stained with Safranin O and Calcofluor White M2R, viewed under bright light (A) and UV light (B). (C) An isolated vessel member observed with SEM; and (D) the same sample as (C) at a higher magnification.

**Fig. 5.** Images of the xylem from the brush area of a post-veraison berry passively infused with acid fuchsin from the pedicel. Arrows in (A) indicate sites where the vascular bundles appear broken. However, apparently intact xylem can be seen across and around these ‘gaps’ (B, arrows). Acid fuchsin did not move past the brush.
The vascular bundles were comprised of radially contiguous groups of vessels that remained continuous and hence presumably functional during berry development. This observation confirms the works by Bondada et al. (2005) and Keller et al. (2006) on the xylem functionality in post-veraison berries, and is in contrast to the often accepted idea that xylemic water transport into berries ceases at veraison because the xylem elements in the berry become physically disrupted and dysfunctional (Düring et al., 1987; Findlay et al., 1987; Creasy et al., 1993; Rogiers et al., 2001; Dichio et al., 2003) as a direct or indirect consequence of post-veraison berry growth (Coombe and McCarthy, 2000). However, this idea has been questioned by the work by Bondada et al. (2005) and Keller et al. (2006).

The dominance of vessels rather than tracheids could be of importance in relation to movement of both water and
pathogens, such as the xylem-dwelling bacteria *Xylella fastidiosa*, a cause of Pierce’s disease in grapevine. Vessels are also more susceptible to embolism and tyloses which could block water flow. Although no tyloses were observed in the fruit, Kasimatis (1957) associated the presence of tyloses in the berry pedicel with the ‘water- • berry’ disorder in grapes. Vessels also present fewer barriers to pathogen movement than do tracheids and, in some cases, form extensive open conduits (Chatelet et al., 2006; Thorne et al., 2006), whereas movement through tracheids would require repeatedly traversing the primary cell wall.

The peripheral vascular bundles contained tracheary elements that appear, in both cross-section and upon partial maceration, as essentially linear files of 3–7 radially contiguous elements. The secondary wall thickenings of these elements appear in a specific ontogenetic pattern that indicates a progressive increase in the extent of the primary wall covered by secondary wall material. Thus, in the earliest tracheary elements, the secondary wall occurs as rings (annular thickenings); subsequent elements have helical thickenings; and as later elements differentiate the gyres become progressively closer to each other. Morphologically, the protoxylem commonly has annular and helical thickenings, and the metaxylem may have helical, reticulate, or pitted secondary wall thickenings (Esau, 1977). Since the tracheary elements of the berry had only annular and helical thickenings, and the berry growth transiently stops at veraison, the elements maturing before veraison may be considered protoxylem and those maturing after veraison considered metaxylem.

The present anatomical results do not contradict the data found in two widely cited studies (Düring et al., 1987; Findlay et al., 1987) purportedly showing direct evidence of broken xylem in the post-veraison berry, but the interpretation does. In the single published photomicrograph of Düring et al. (1987), the magnification is unknown and no details of the xylem can be observed. Hence, it is not clear whether the xylem bundle is entirely broken or not. In the course of the present study, similar observations of seemingly broken vascular bundles in fresh tissues were made. However, at a higher magnification, intact tracheary elements could be seen within and around each apparent gap. Therefore, these areas should be considered more as nicks than as proper gaps in the bundle. These observations of partially broken vascular bundles were further confirmed by observing stained vascular bundles dissected from the berry and cleared with bleach. Nicks appeared at small points along the primary wall of the early elements. Gas that formed inside these elements following the bleaching treatment (possibly chlorine or oxygen) emerged through the nicks to the outside of the conduit. This phenomenon occurred only on one side of a bundle, with the other tracheary elements, including all of the more recently developed elements, remaining intact and non-leaky. No xylem bundle was entirely broken.

Findlay et al. (1987) observed some irregular spacing of the gyres in clearings and longitudinal sections of plastic-embedded tissues from post-veraison berries. They interpreted this as the result of stretching during berry growth and as support for the hypothesis that the xylem in post-veraison berries is physically disrupted and non-functional. However, their images clearly show that not all tracheary elements are so affected. Most appear with uniform intergyre distances and hence should be regarded as intact and functional. Irregularities in gyre spacing of a few tracheary elements were also observed. In addition, it must be recognized that only elements in the same plane of focus will be clearly seen (Findlay et al., 1987). It is therefore possible that intact vessels were present below the seemingly broken ones, similar to the present observations here (Fig. 5).

The results of the present study and the recent literature demonstrate that the apparent hydraulic isolation of the berry after veraison is not simply due to a loss of peripheral xylem continuity as a result of growth-induced xylem disruption. Greenspan et al. (1994) and Rogiers et al. (2000) also produced evidence of xylem function after veraison and questioned the conventional interpretation of the previous passive dye uptake studies. Bondada et al. (2005) demonstrated that the changes in passive dye uptake at veraison were not dependent upon post-veraison berry growth and, hence, could not be due to physical disruption caused by berry growth. In the present study, even though dye always traversed the entire berry from stem to stylar end, a reduction in vascular dye movement was seen after veraison compared with pre-veraison. The nicks that developed in the early formed xylem may have influenced this process. As long as the water column in the xylem is maintained by connected and intact tracheary elements and subjected to an adequate driving force, water should be able to move from one intact tracheary element to another, either longitudinally or radially (Tyree and Zimmermann, 2002). Since each vascular bundle was comprised of a total of 10–15 tracheary elements, water transport could continue around the compromised elements. Nevertheless, the nicked tracheary elements may contribute to a reduced hydraulic conductivity of the vascular system because they represent compromised conduits. Thus, it is possible that part of the decrease in hydraulic conductance of berries that is suggested in several studies and measured in Tyerman et al. (2004) arises in part from loss of functional tracheary elements in the xylem network.

Other works point to additional, non-xylem factors in apparent hydraulic isolation. Bondada et al. (2005) speculated that the absence of passive dye uptake by post-veraison berries was the result of an absence of tension in the berry xylem possibly related to the presence
of solutes in the berry apoplast. Consistent with this interpretation, Tyerman et al. (2004) reported an equilibrium hydrostatic pressure close to zero in the berry pedicle near veraison. In addition, the possible role of a symplastic regulation of water transport (Delrot et al., 2001; Picaud et al., 2003; Tyerman et al. 2004) awaits investigation. Picaud et al. (2003) reported an increase in expression of a group of aquaporins associated with the plasma membrane (PIP1) after veraison and at harvest, but the role of aquaporins in the regulation of water transport after veraison remains to be determined. More recently, Keller et al. (2006) showed dye movement from the style of post-veraison berries back into the shoot and adjacent transpiring leaves. They proposed that the decrease in the amount of water flow into post-veraison berries may be due to apoplastic phloem unloading and solute accumulation in the berry apoplasm. They further speculated that the xylem could be used to recycle excess water from the phloem back to the shoot.

The maintenance of the physical integrity of the vascular bundles during post-veraison growth raises the question of how this growth is accommodated. Although the secondary cell wall thickenings provide support and rigidity to the xylem elements, it is presumed that xylem vessels with annular and helical secondary walls are able to stretch to a certain extent (Esau, 1965; Barnett, 1981; Mauseth, 1988). Since vascular bundles do not break as a result of berry growth, the tracheary elements may stretch to accommodate the growth, as was observed in growing leaves (Paolillo, 1995; MacAdam and Nelson, 2002).

Another mechanism that would allow the berry to grow without compromising vascular transport would be the formation of additional vessels after veraison. This could be realized by adding either new vessels in the existing vascular bundles or new vascular bundles to the xylem network, although it must also be recognized that regardless of any xylem addition, existing tracheary elements must be stretched by berry growth. The general consensus regarding vascular development in fruits is that the procambium becomes inactive upon fruit ripening (Harris et al., 1968; Considine and Knox, 1979; Gillaspy et al., 1993). However, this consensus is based only on observation of an absence of mesocarp cell divisions rather than an absence of cambial activity. The absence of breakage of the xylem by the growth of the berry leads to the formulation of two hypotheses to accommodate the growth: (i) existing vessels stretch; and/or (ii) new xylem conduits are formed after veraison. These two hypotheses will be explored in the companion paper (Chatelet et al., 2008).

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