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Cell death in grape berries: varietal differences linked to xylem pressure and berry weight loss

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Abstract. Some varieties of *Vitis vinifera* L. can undergo berry weight loss during later stages of ripening. This defines a third phase of development in addition to berry formation and berry expansion. Berry weight loss is due to net water loss, but the component water flows through different pathways have remained obscure. Because of the very negative osmotic potential of the cell sap, the maintenance of semipermeable membranes in the berry is required for the berry to counter xylem and apoplast tensions that may be transferred from the vine. The transfer of tension is determined by the hydraulic connection through the xylem from the berry to the vine, which changes during development. Here we assess the membrane integrity of three varieties of *V. vinifera* berries (cvv. Shiraz, Chardonnay and Thompson seedless) throughout development using the vitality stains, fluorescein diacetate and propidium iodide, on fresh longitudinal sections of whole berries. We also measured the xylem pressure using a pressure probe connected to the pedicel of detached berries. The wine grapes, Chardonnay and Shiraz, maintained fully vital cells after veraison and during berry expansion, but began to show cell death in the mesocarp and endocarp at or near the time that the berries attain maximum weight. This corresponded to a change in rate of accumulation of solutes in the berry and the beginning of weight loss in Shiraz, but not in Chardonnay. Continuous decline in mesocarp and endocarp cell vitality occurred for both varieties until normal harvest dates. Shiraz grapes classified as high quality and sourced from a different vineyard also showed the same death response at the same time after anthesis, but they displayed a more consistent pattern of pericarp cell death. The table grape, Thompson seedless, showed near to 100% vitality for all cells throughout development and well past normal harvest date, except for berries with noticeable berry collapse that were treated with giberellic acid. The high cell vitality in Thompson seedless berries corresponded to negative xylem pressures that contrasted to the slightly positive pressures for Shiraz and Chardonnay. We hypothesise that two variety dependent strategies exist for grapevine berries late in development: (1) programmed cell death in the pericarp and loss of osmotically competent membranes that requires concomitant reduction in the hydraulic conductance via the xylem to the vine; (2) continued cell vitality and osmotically competent membranes that can allow high hydraulic conductance to the vine.

Additional keywords: berry shrivel, berry weight loss, cell death, grape berry development.

Introduction

There has been much interest recently in the water budget of the developing grape berry (Bondada *et al.* 2005; Keller *et al.* 2006; Rogiers *et al.* 2006; Thomas *et al.* 2006; Tilbrook and Tyerman 2006; Zhang *et al.* 2006*b*). This is for two reasons. First, the berry is a good model for other fleshy fruits and can provide some generalisations of how water movement into and out of the fruit is regulated through changes in solute partitioning, phloem and xylem transport, and transpiration. Second, the water content of the harvested berries and the way in which water is retained in the berry has a large impact on quality; whether this be via concentration of soluble solids and flavour molecules in wine grapes, or through crispness (turgidity) of the fruit in the case of table grapes. Yield is also determined largely by water content since water makes up the major component of berry mass. Loss of water from the berry can be substantial in some varieties and this can reduce yield by over 25%, for example in Shiraz (McCarthy 1999; McCarthy and Coombe 1999).

It is generally agreed that before veraison during the first phase of berry development (berry formation, phase 1, Fig. 1), the berry transpires and water inflow occurs via the phloem and the xylem (Lang and Thorpe 1989; Greenspan *et al.* 1994; Dreier *et al.* 2000; Rogiers *et al.* 2004, 2006). After a lag in expansion and just after the berry softens, sugar and water accumulation rapidly increase via phloem import, here referred to as phase 2 (Fig. 1). After the berry reaches maximum weight a third phase is apparent in some varieties, when berry weight loss begins (Sadras and McCarthy 2007). This can occur before grape flavour development for winemaking is evident (Coombe and McCarthy 1997), and sugar concentration can be further increased by a combination of volume decrease of the berry and further sugar import, although further sugar import to the berry seems to be plastic between seasons (Sadras and McCarthy 2007). Fig. 1 shows these phases of berry development in context with other changes in xylem function and berry water relations taken from the literature and our own data. It should be noted that phase 1 incorporates Stages I and II by Coombe (1992).

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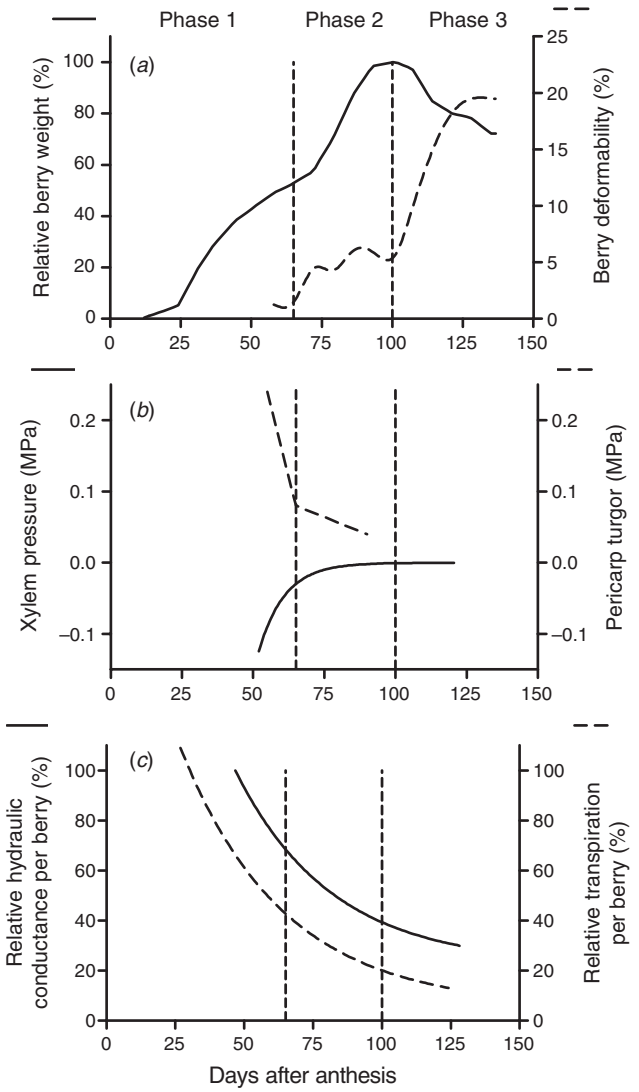

Fig. 1. Summary of the development of grapevine berries with various physiological changes associated with berry water relations. Three phases can be identified based on changes in the rate of change of berry weight (see also Sadras and McCarthy 2007). These phases are delineated by a vertical dashed line in each panel. (*a*) Berry weight and deformability. Shown are fits to data for Shiraz (J. Tilbrook, unpubl. data). (*b*) Xylem equilibrium pressure and turgor pressure of berry pericarp cells. Shown are fits to data sourced from Tyerman *et al*. (2004) (xylem pressure) and Thomas *et al*. (2006) (turgor). (*c*) Hydraulic conductance (relative to maximum in pre-veraison berries) of the xylem pathway into Shiraz berries (fitted curves by Tyerman *et al.* 2004; and J. Tilbrook, unpubl. data), and Shiraz berry transpiration relative to the maximum in pre-veraison berries (Rogiers *et al*. 2004).

During phase 2, the berry appears to become less hydraulically connected to the vine (Greenspan *et al.* 1994, 1996; Tyerman *et al.* 2004; Tilbrook and Tyerman 2006). From earlier dye uptake studies and calcium uptake into the berry (as a xylem tracer) (Findlay *et al.* 1987; Creasy *et al.* 1993; Rogiers *et al.* 2001), the hydraulic isolation was proposed to be due to discontinuity of xylem vessels in the berry. However, our previous quantitative measurements of the xylem pathway to the berry showed that the pathway remained functional, though hydraulic conductance was reduced in magnitude depending on variety (Fig. 1; Tyerman *et al.* 2004). Bondada *et al*. (2005) qualitatively confirmed that xylem hydraulic conductance continued in post veraison berries by demonstrating that dye uptake could still occur provided the appropriate driving force on water flow to the berry could be sustained. Tyerman *et al*. (2004) also showed that the xylem pressure changed from being negative to slightly positive during veraison, supporting the view that the nature of the driving force for water movement to the berry in the xylem changes during phase 2 (Fig. 1). These discoveries have focussed attention on the solute partitioning between apoplast and symplast in the berry because this will determine how the very negative osmotic potential of the berry juice, largely mesocarp cell sap, is translated into the driving force for water movement, both through the phloem and the xylem.

The demonstration by (Zhang *et al.* 2006*b*) that phloem unloading switches from symplastic to apolastic during veraison, suggests that osmotic potential of the apoplast decreases. This could drive greater flow through the phloem (Patrick 1997) and may explain the transition to slightly positive xylem pressure measured by Tyerman *et al*. (2004). An important issue remains, however, and this is whether or not the osmotic competence of the cell membranes, particularly of the large mesocarp cells, changes in the post veraison berry. This is important because normal cell membranes will show semipermeability, and for large solutes like sugars, an osmotic potential difference across the membrane is reflected by an equivalent hydrostatic pressure difference. The large negative osmotic potential of the mesocarp cells could balance only negative apoplast pressures and xylem tensions if the membranes remain semipermeable.

There is indirect evidence that cell membranes become leaky in the mesocarp of post veraison Riesling (Lang and Düring 1991; Dreier *et al.* 1998) and cell compartmentation can break down in Thompson seedless (Dreier *et al.* 1998). However, turgor at low pressures (0.05 MPa), and, therefore, membrane semipermeability and cell vitality, was maintained in cells to a depth of 1.5 mm below the cuticle until 100 days after anthesis (DAA) in several wine grape varieties, including Chardonnay (Thomas *et al.* 2006). The reduction in turgor pressure at veraison (transition from phase 1 to phase 2) of the mesocarp closely corresponds to increased deformability of the berry (Fig. 1) and suggests that the apoplast osmotic potential declines.

The turgor pressure observations, like most, do not continue into the final 2–3 weeks before harvest, because the fruit becomes very deformable (Fig. 1), and berry contents can be extremely difficult to work with. It is during phase 3, post 90–100 DAA in varieties like Shiraz, that considerable loss of weight can occur to the point where the berries may shrivel (Fig. 1). This loss of weight has been proposed to be due to a combination of reduced phloem inflow and continued transpiration (McCarthy and Coombe 1999; Rogiers *et al.* 2004; Tyerman *et al.* 2004; Keller *et al.* 2006) though backflow to the vine via the xylem may also contribute (McCarthy and Coombe 1999; Rogiers *et al.* 2004; Tyerman *et al.* 2004; Keller *et al.* 2006). Backflow was directly demonstrated by dye loading at the stylar end of post veraison berries and observing the dye in the xylem of the vine

(Keller *et al.* 2006). Though the apoplasmic water of the berry is available for movement back to the vine, Keller *et al.* (2006) conclude that the berry cell membranes remain semipermeable, thereby making it difficult for the leaves to extract water from the berry cells because of the large negative osmotic potential of the cell sap. Thus, if backflow alone were to account for a loss of up to 30% of maximum weight, as is often observed in Shiraz, there would have to be a loss of membrane semipermeability for a large proportion of the cells in the berry.

The aim of this work was to test the hypothesis that cells across the pericarp of berries maintain membrane competence and vitality until harvest maturity is achieved. It formed part of a project to ascertain the cause of weight loss in Shiraz berries. Shiraz, Chardonnay and Thompsons seedless fruit were compared, because Chardonnay does not normally show weight loss and Thompson seedless was found by us to have very different xylem pressures compared with the other varieties. We used two vital stains, fluorescein diacetate (FDA) and propidium iodide (PI). These dyes are a well documented and reliable method of determining cell vitality in cell suspensions and flow cytometry (Jones and Senft 1985). FDA is a non-polar, non-fluorescent molecule that crosses cell membranes. Once in the cytoplasm, esterases cleave the acetate groups from the molecule and it becomes highly polar and fluorescent green. If a cell does not have an active metabolism, it will not fluoresce. PI is a membrane impermeant dye that enters cells only when cell membranes are disrupted or not intact. It binds non-covalently, intercalating in a stoichiometric manner with single and double stranded DNA and RNA. When intercalated it fluoresces red (Cosa *et al.* 2001; Bernas *et al.* 2004; Kral *et al.* 2005). The loss of cell membrane integrity in plants is considered to mark the end of homeostasis and indicates cell death (Noodén 2004). Using these vital stains we examined the vitality of cells in medial longitudinal sections of fresh berries through development. The data were analysed to provide a new insight into cell vitality and cell membrane competence through berry development that we relate to measurements of xylem pressure using the pressure probe attached to individual berry pedicels.

Materials and methods

Fruit material

Experimental fruit (*Vitis vinifera* L.) used in the time course experiments was from the Coombe vineyard (Shiraz BVRC12 and Chardonnay I10V1, 12 years old) and Alverstoke vineyard (Thompson seedless M12, not treated with giberellic acid, 4 years old) on the Waite Campus of the University of Adelaide. Bunches of fruit were labelled individually when an estimated 50% of the flower caps from that bunch had dehisced. The day this occurred was designated as 'anthesis'. Anthesis was complete over 1–2 days. Premium quality Shiraz (unknown clone, ~30 years old) was obtained on the day of harvest courtesy of Hardys, McLaren Vale from a McLaren Flat site. Thompson seedless fruit treated with giberellic acid and showing signs of berry collapse was from CSIRO Merbein, courtesy of Mike Treeby and Tori Nguyen. All data from vineyard fruit was obtained during the 2005–06 season. The pre-veraison Shiraz BVRC12 berries in the fluorescent dye control experiment were from glasshouse grown vines at the Plant Research Centre in the Waite Campus precinct.

Berry weight, °Brix and osmolality

Berry weight and °Brix data reflect whole vineyard berry development. Weights are the means of 50 berry samples. For Shiraz, samples of five berries from proximal, mid and base of two random bunches on separate vines from each of five replicate panels in separate rows that formed part of a randomised block rootstock trial were collected. For Chardonnay, five berry samples from proximal, mid and base of 10 random bunches on separate vines in a row of 24 adjacent vines. These data were not collected for Thompson seedless as insufficient experimental fruit was locally available. Berries collected for weight samples were crushed, juice collected and briefly centrifuged to settle any solids. °Brix of the juice was measured using a temperature compensated digital refractometer (Model PR101; ATAGO, Tokyo, Japan). A water vapour pressure osmometer (Model 5500; Wescor, Logan, UT, USA) was used to measure juice osmolality, which was converted to osmotic potential ($\Psi_\pi = -RTC$, where $r = 8.3143$ J/mol.K and T = absolute temperature in K). Solutes per berry (g) was approximated by the product of berry weight and °Brix/100 (McCarthy and Coombe 1999).

Vital and non-vital staining of pericarp tissue

Bunches (all clones of Shiraz and Chardonnay) or clusters (Thompson seedless) were cut from within the vine canopies, placed in plastic bags on ice and taken to the laboratory (~500 m). Berries were sectioned longitudinally between the seeds (where present). One-half of each berry was pooled and crushed for juice to measure °Brix and to calculate osmotic pressure (as above). The FDA section of the method was developed from that kindly shared by Professor Ken Shackel, UC Davis. From a 4.8 mM FDA (Sigma-Aldrich, St Louis, MO, USA) in acetone stock solution, an aqueous 4.8 μM FDA solution (Oparka and Read 1994) was prepared to a similar (to within 10%) osmotic pressure as the berry juice with sucrose, then applied to excess on the cut surface of the half berries. After 15 min incubation, sectioned berries were viewed with a Leica M-Z FL111 (Leica, Wetzlar, Germany) dissecting microscope at minimum magnification under ultraviolet light with a green fluorescent protein filter in place. Images were promptly obtained with a Leica DC 300F camera and Image Pro Plus 5.1 (MediaCybernetics, Bethsda, MD, USA) with consistent settings and exposure times.

The FDA solution was blotted from the cut berry surfaces, then a counterstain of aqueous 190 μM PI solution (Sigma-Aldrich) (Oparka and Read 1994) freshly prepared from an aqueous stock solution and made to a similar (to within 10%) juice osmotic pressure with sucrose. It was applied to excess, incubated for 10 min, viewed and imaged as before. To confirm that the method was a reliable indicator of cell membrane competence and cell vitality, fresh and microwaved pre-veraison berries were prepared and imaged according to the described method (Fig. 2). The pre-veraison fruit for the set of control sections was prepared as above for the fresh sections or microwaved for 15 s at 650 W before the dyes were applied. Exposure times were consistent for the set. The fresh, sectioned berries showed a vivid fluorescent green response to FDA indicating that the cells across the pericarp had competent

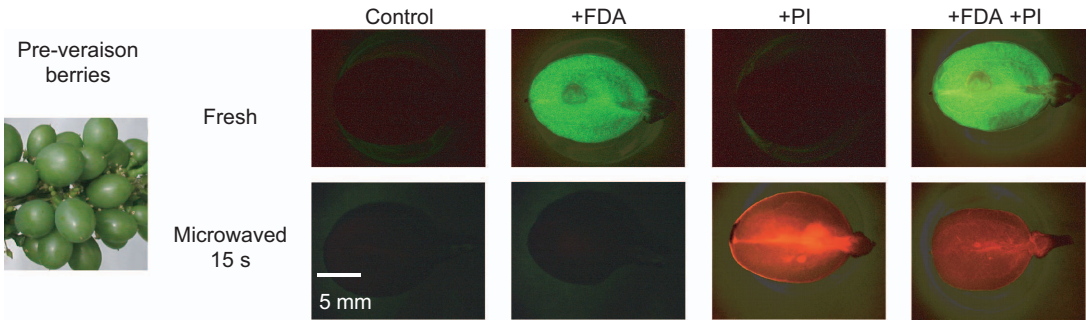

Fig. 2. Pre-veraison berries sectioned longitudinally and either left fresh or microwaved to disrupt cell membranes. A comparison is shown between fresh and microwaved berries with no dyes applied (controls), with FDA only, with PI only, and with both FDA and PI applied. Note that no autofluorescence is visible.

membranes and living cytoplasm. Berries that had been killed by microwaving had no fluorescent response to FDA (Fig. 2). The results of the PI application to the sections were a direct contrast. No red fluorescence was visible in the fresh sections (with the exception of a sliver of seed coat), however, it was intensely visible in the pericarp cells of the microwaved, cell membrane disrupted samples. PI is known to stain cell walls, but this was not visible, nor was auto-fluorescence or artefacts at the cut surface of the pericarp using this method at low magnification.

Although we refer here to the pericarp (comprising exocarp mesocarp and endocarp), the microscopy technique used in this study prevented cellular details in the exocarp region to be delineated. The vitality observations are weighted more to the large volume of the mesocarp and endocarp. However, vitality or death of cells of the exocarp can be seen as thin green outlines or red regions in the sections.

Analysis of vitality

Pixel analysis of Shiraz and Chardonnay sections stained only with FDA was performed by Global Laboratory Image/2 version 2.5 (Data Translation, Marlboro, MA, USA). Comparative analysis of pixel blocks or line transects within images showed that pixels with arbitrary values designated as >75 were vital and reflected living cytoplasm in cells (Fig. 3). Two methods were compared: (1) the berry cuticle was outlined and pixels within the outline (i.e. the cut surface) analysed. 'Vital' pixels were expressed as a percentage of the total number of pixels; (2) line transects were taken across the distal, mid and mesial (DMM) of berry longitudinal sections and the percentage of total pixels with values >75 were averaged between the three sections. A separate transect was taken through obviously vital regions of the pericarp where cell cytoplasm surrounding large vacuoles could be delineated. The percentage of total pixels with values >75 was recorded and the ratio of the averaged DMM over the pericarp transect value was converted to a percentage. This is referred to as 'relative cell vitality'. The second method yielded qualitatively similar results to the first method, but was deemed more suitable because it could account for the expansion of living pericarp cells, which resulted in a reduced count of vital pixels due to 'dilution' by the large central vacuoles. The second method also accounted for slight variations in exposure between preparations since it was self referencing within a berry. To quantify the structural variability in location

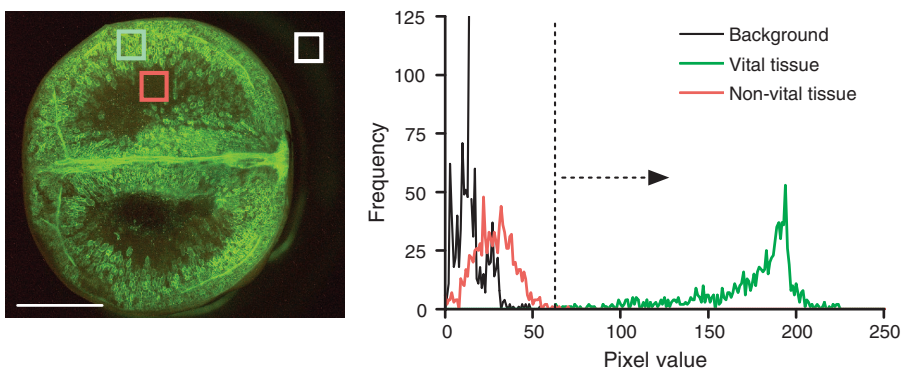

Fig. 3. Comparisons of blocks of pixels in images of berry sections stained with FDA indicate that pixels with a value of greater than 75 arbitrary units (with the highest frequencies between 150 and 200 arbitrary units) corresponded to vital, fluorescing tissue. The background of the image and non-vital tissue had pixels with values of less than 75 arbitrary units. Example shown is a post veraison Chardonnay berry. Scale bar = 5 mm.

of living and dead cells between high quality and mid-quality fruit, DMM transects were drawn and pixel values graphed for each transect.

Xylem equilibrium pressures

Xylem equilibrium pressures of berries attached to the pressure probe via the pedicel were measured as detailed by Tyerman *et al*. (2004). Briefly, whole shoots with bunches attached were cut from the field grown vines described above and were transported immediately to the laboratory with the cut end immersed in water. Once in the laboratory an individual berry and pedicel was cut from the bunch while under water. To seal the pedicel with the pressure probe a piece of tubing 15–20 mm long (Tefzel-Schlauch (Sinsheim, Germany) tubing internal diameter (ID) 1.0 mm outside diameter (OD) 1.6 mm or ID 1.6 mm OD 3.2 mm depending on pedicel diameter) was flared at one end in order to snugly fit the pedicel. The pedicel was sealed into the flared end with a cyanoacrylate glue (Super Glue TM or Loctite TM 401 or 406 adhesive with tubing treated with 770 primer). The water used to fill the tubing and probe was millipore purified water de-aerated by vacuum treatment and filtered to 0.2 µm. As the seal was tightened around the tubing the pressure was elevated in the system and was allowed to relax to a steady-state level.

Results

Pericarp cell death is evident in berries of Chardonnay and Shiraz late in development, but not Thompson seedless

At 73 days after anthesis (DAA), Shiraz and Chardonnay berries moved from the lag phase of berry development (late part of phase 1) into the rapid phase of weight and sugar accumulation (phase 2). The staining of sections of both varieties with FDA indicated that cells across the pericarp maintained vitality through veraison and continued as the fruit approached maximum weight. In Shiraz, the berries were still enlarging at 98 DAA when counterstaining with PI showed an intense red fluorescent response across the pericarp, which was repeated approaching maximum weight at 103 DAA (Fig. 4*a*). In Chardonnay the berries reached maximum weight at 108 DAA and exhibited a PI response at 110 DAA that was sustained at 117 DAA (Fig. 4*b*). Yellow fluorescence was visible where tissue showed a mixed response – living and dead cells in proximity. Although the period of sudden PI response did not occur at the same number of days after anthesis for the wine grape varieties, it was contemporaneous in terms of the calendar date. No weather, water or other stresses were noted over this period. We also observed similar death events in berries from glasshouse grown vines grown during winter of 2006 and this also did not correspond to any particular climate change. More cells adjacent to the central and peripheral vasculature maintained cell membrane competence compared with the body of the mesocarp.

Thompson seedless berries had a slight blush of PI red fluorescence at 103 DAA (Fig. 4*c*), contemporaneous with the initial wine grape PI response. This was not sustained a week later and does not appear to be linked to sugar content, number of days after anthesis or maximum weight of the berries. In complete contrast to the wine grapes, the Thompson seedless berries continued to have cells with functioning cytoplasm and competent membranes across the entire pericarp up to and beyond normal harvest date. The cell vacuoles enlarge and appear dark, particularly at maximum distance from the central and peripheral vascular bundles, but vital cytoplasm was clearly visible within each cell.

Cell vitality relative to sugar and water relations of the berry

Relative cell vitality as a function of days after anthesis is plotted in Fig. 5*a* for each of the grapevine varieties. This data is shown in the context of juice osmotic potential for all the varieties (Fig. 5*b*), and the weight and sugar accumulation in the berries of Chardonnay and Shiraz (Fig. 5*c*, *d*).

At or just after veraison all varieties showed 100% relative cell vitality. This was maintained throughout the entire measurement period for Thompson seedless and until maximum berry weight for Chardonnay and Shiraz. The measurements above 100% for Thompson seedless are ascribed to the higher density of cells adjacent to central and peripheral vascular bundles of the berry when compared with the mesocarp cells in the calculation of relative cell vitality.

The range of days after anthesis over which the PI fluorescence was observed in Shiraz and Chardonnay, are indicated on Fig. 5 as horizontal bars. These events corresponded with the onset of a linear decline in relative cell vitality measured with FDA in both varieties (inset Fig. 5*a*). The linear regressions for this phase of declining vitality indicated that Shiraz had a significantly higher rate of decline compared with Chardonnay. The initial death events also corresponded to the period just before maximum berry weight in Shiraz and just after maximum berry weight in Chardonnay (Fig. 5*c*). The death event and onset of decline in cell vitality also corresponded to a change in slope of accumulation of solutes per berry in Shiraz and a plateau in Chardonnay (Fig. 5*d*).

Xylem pressure measured using the pressure probe attached to the pedicel of individual berries is shown in Table 1. These measurements were taken in the period when cell vitality was declining. Despite the very negative osmotic potentials of the berry juice of Chardonnay and Shiraz (Fig. 5*b*) the xylem pressures of detached berries were rather small and positive (note that values given are in KPa). Contrasting behaviour was observed for Thompson seedless berries that had higher osmotic potentials (Fig. 5*b*) but which developed negative xylem pressures that would cavitate the pressure probe. In this case the measurements given in Table 1 are extrapolations of the exponential approach to equilibrium after the berry was attached to the probe (Tyerman *et al*. 2004).

Aberrant behaviour detected using the vitality assay

Transects across the pericarps of premium Shiraz fruit from 30-year-old vines in McLaren Flat, South Australia, were compared with the mid-quality fruit from 12-year-old Shiraz vines in the Coombe vineyard. The premium fruit showed a consistent and distinct pattern of vital and non-vital cells across the pericarp which can be graphically represented (Fig. 6*a*). This structural pattern was consistent in the sections

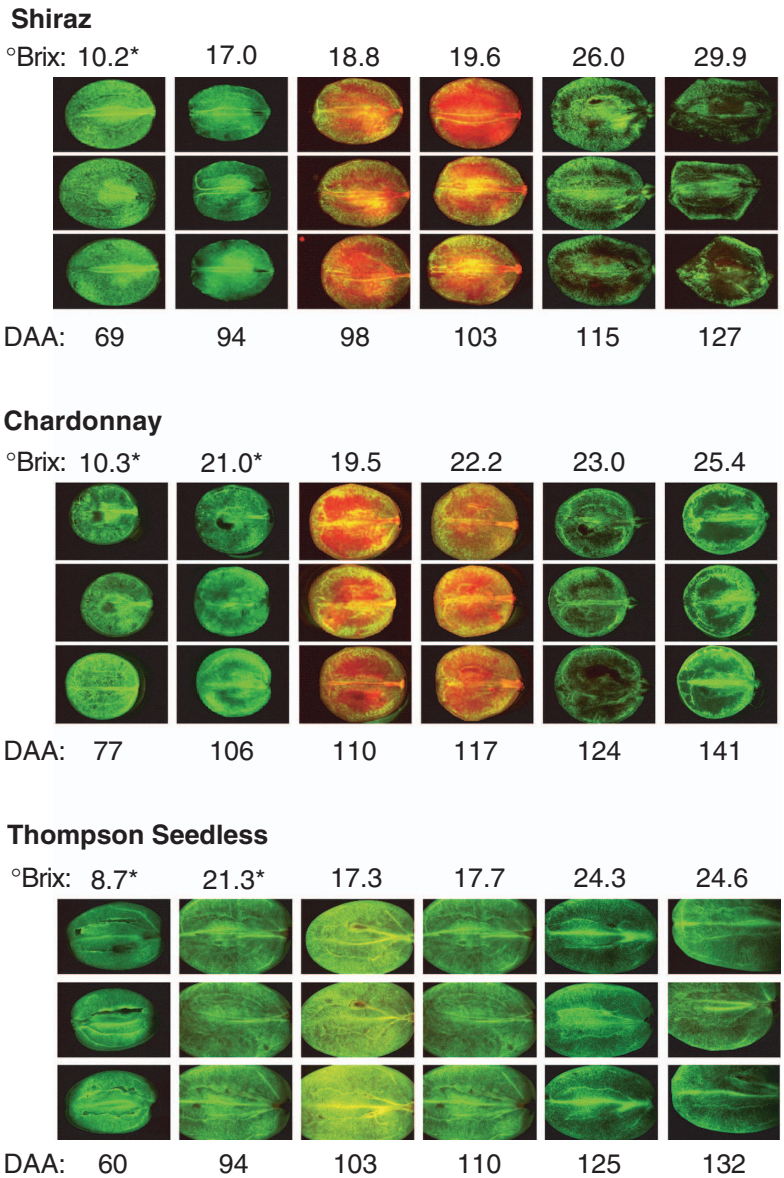

Fig. 4. Changes in berry cell vitality with development. A developmental time series where Shiraz (*a*) Chardonnay (*b*) and Thompson seedless (*c*) berries were longitudinally sectioned and stained with FDA then counterstained with PI to test vitality and membrane competence. Three separate berries from a bunch are shown at the time point indicated as days after anthesis (DAA). This is representative of a larger dataset used for Fig. 5. The total soluble solids measured at time points is shown; values with an asterix were calculated from measured osmolarity of juice using a standard curve between osmolarity and °Brix ($R^2 = 0.999$). Veraison occurred at 73 DAA for Shiraz and Chardonnay, and 78 DAA for Thompson seedless berries.

examined. At harvest maturity the cells in the premium fruit around the peripheral and central vasculature clearly maintain competent membranes but the mesocarp at maximum distance from the vascular tissue shows no response to FDA. This suggests that there are distinct regions within the pericarp where there are no vital cells at harvest. The mid-quality Shiraz showed more structural variation in the distribution of cells with and without a functioning cytoplasm compared with the premium Shiraz. The apparent loss of membrane competence in cells, or cell death, was spread across the pericarp in a less organised manner (Fig. 6*b*), although some berry sections did show similarities to the premium fruit pattern of cell death. Field-grown premium and the mid-quality Shiraz were harvested for winemaking on the same day at 29.6 and 30 °Brix, respectively. Although the apparent 'vitality' structure of the two sets of berry sections are

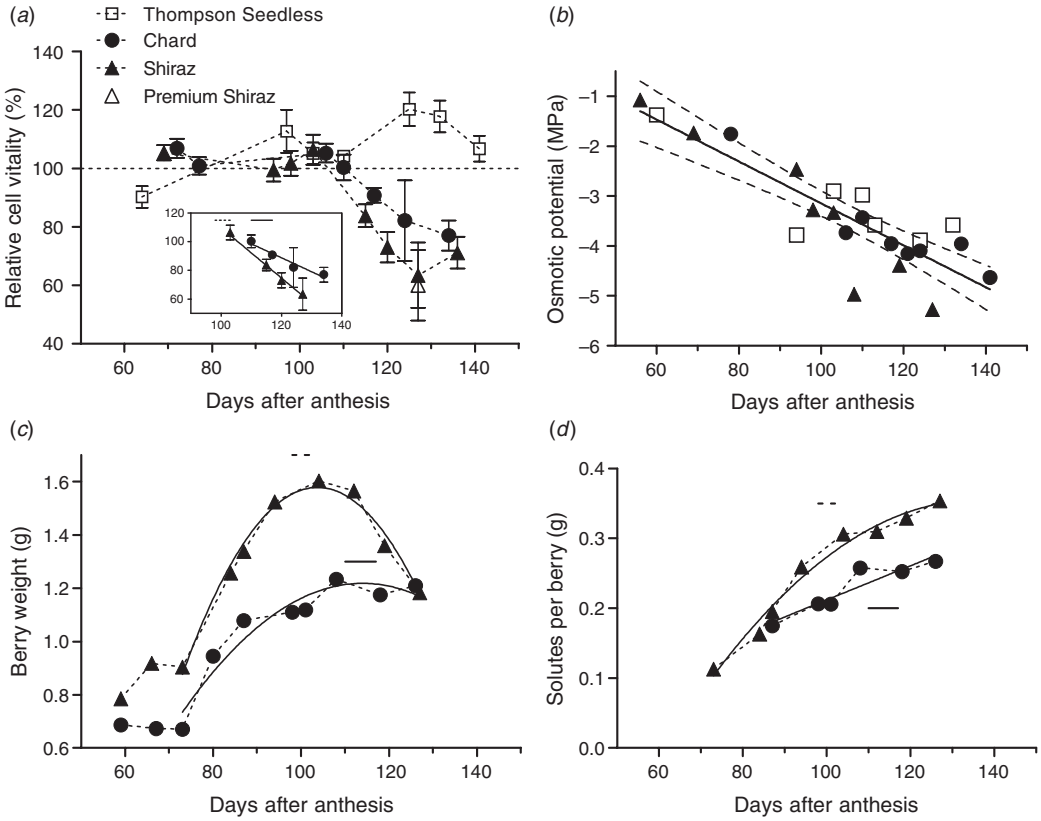

Fig. 5. Changes in berry cell vitality is associated with other developmental changes in berry physiology. (*a*) Relative cell vitality as a function of days after anthesis for each of the varieties tested, $n = 6–7$. The inset shows the linear decline in relative cell vitality in Shiraz and Chardonnay. The slopes are significantly different ($P < 0.05$). For Thompson seedless, a linear fit to the entire dataset yielded a slope not significantly different from zero. (*b*) Juice osmotic potential of the juice of pooled and crushed opposing halves of sectioned berries for each of the varieties *v.* days after anthesis. Linear fits of osmotic potential with time for each variety were not significantly different ($P > 0.05$), so a fit to the combined data is shown with 95% confidence interval. (*c*) Mean berry weight for Chardonnay and Shiraz *v.* days after anthesis. A 2nd order polynomial was fit to the data from veraison (solid lines). The fitted curves were significantly different between varieties ($P < 0.05$). (*d*) Mean solutes per berry *v.* days after anthesis for Chardonnay and Shiraz. The fitted curves (solid lines) were significantly different between varieties ($P < 0.05$). For Shiraz, the best fit was obtained with a 2nd order polynomial, and for Chardonnay, the best fit was with a straight line. Shown in each set of data are the points at which the PI response was observed as an indicator of the first sign of cell death in the pericarp (Chardonnay, solid line; Shiraz, dashed line).

Table 1. Xylem equilibrium pressures of berries connected to the pressure probe

Shiraz and Chardonnay berries were measured after maximum berry weight and after the first sign of pericarp cell death. For Thompson seedless berries, pressures were determined from exponential extrapolations from the pressure equilibrium time course before cavitation was evident. Data are means ± s.e. (n). In each case the pressures were significantly different from zero (one sample *t*-test, $P < 0.05$) and were significantly different between varieties (one way ANOVA with post tests, $P < 0.05$)

Variety	Xylem equilibrium pressure (KPa)	Range; days after anthesis
Shiraz	4.7 ± 1.7 (20)	108–136
Chardonnay	11.8 ± 2.2 (15)	108–146
Thompson seedless	−24.0 ± 4.4 (5)	136

quite different on the day of harvest (Fig. 6), there was no significant difference in the relative cell vitality measurement (unpaired *t*-test, $n = 6–7$, $P = 0.49$, Fig. 5*a*). The fruit from both locations appeared similarly shrivelled.

The FDA method was applied to Thompson seedless berries (21 °Brix, unknown DAA) that had been treated with giberellic acid, and had signs of early stages of berry collapse (Fig. 7*a*). Regions of cells with no living cytoplasm were observed in the distal half of the pericarp, with adjacent distal regions appearing to show smaller areas of apparent cell death. Distinct differences were noted when this fruit was compared with fruit that was treated with giberellic acid but had no signs of berry collapse (20 °Brix, Fig. 7*b*), and untreated fruit (24.3 °Brix, Fig. 7*c*). The berries showing signs of berry collapse had elongated, semi-rectangular cells that appeared

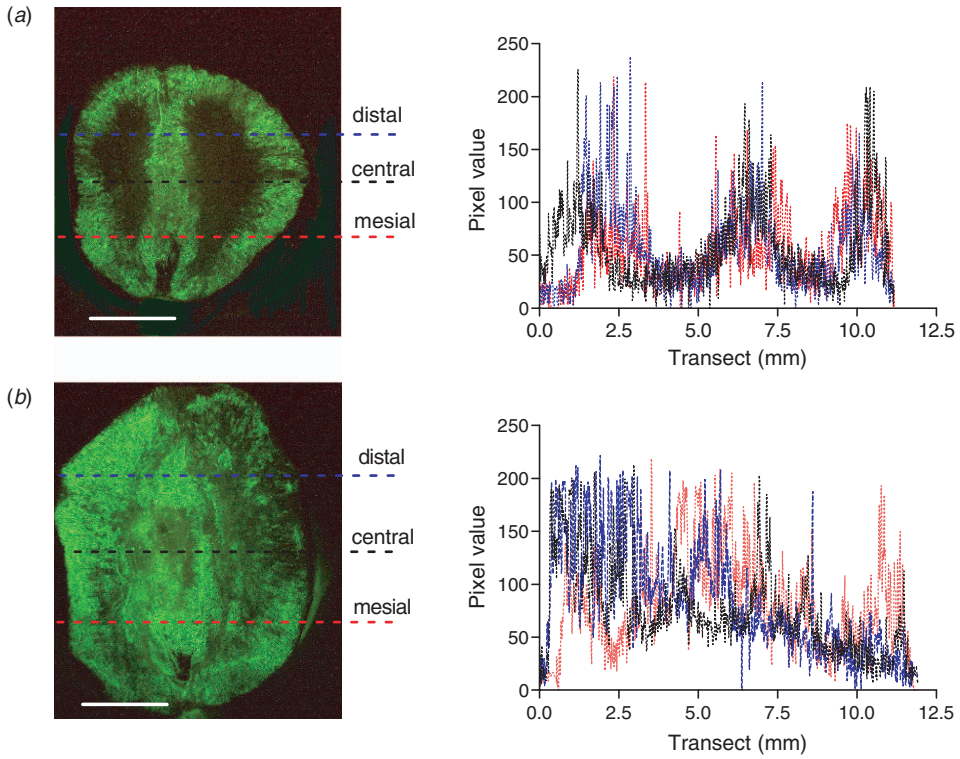

Fig. 6. The pattern of cell death differs between grades of Shiraz fruit. Premium (*a*) and mid-quality (*b*) berries stained with FDA on the day of the harvest, 30 and 29.9 °Brix, respectively. Proximal, central and distal transects were drawn and pixels analysed. The premium fruit shows a distinctive structure with vital cells being maintained adjacent to central and peripheral vascular bundles with semi-defined regions of non-vital cells in the body of the pericarp ($n = 7$). The mid-quality fruit showed varied and less organised patterns of cell death across the pericarp, $n = 6$. Although the different quality fruit showed structural differences, analysis of % vital pixels (Fig. 5) found no significant difference between the two at harvest. Scale bars = 5 mm.

to be very loosely stacked in rows across the pericarp between the central and peripheral vascular bundles, and cell death had already occurred in some regions. The central and peripheral vascular bundles appear disrupted around the regions of cell death. The giberellic acid treated fruit that was sound, and untreated berries (Fig. 7*b*, *c*) had dense, compact, irregularly shaped cells across the pericarp, with some compact elongated cells in the mesocarp between the central and peripheral vasculature. The giberellic acid treated fruit was much larger than untreated fruit, as would be expected.

Discussion

Veraison is the beginning of the ripening phase (phase 2 in Fig. 1) of grape berry development in *V. vinifera*. It is indicated by softening of the berry, a rapid accumulation of hexose sugars, and in some varieties, colour development. The fleshy, carbohydrate rich fruit is thought to have selectively evolved as a high value food reward for animals, birds in particular, that consume it and disperse the seeds widely without physical damage (Hardie and O'Brien 1988). At veraison, seeds have reached full size (Hardie *et al.* 1996) and can germinate with cold treatment (reviewed in Pratt 1971). Unpalatable phenolic compounds in berries reduce from veraison (Adams 2006) as sugars are accumulating. A second phase of softening and further sugar concentration is evident in some wine grape varieties (phase 3 in Fig. 1). The selection by human kind of cultivated varieties of grapevines for wine making or table grapes probably enhanced or reduced some of these characters over time, depending on the desired end use of the fruit. Contrasting behaviour is exhibited between the wine grape and table grape varieties observed in this work. We show here the first direct evidence of loss of membrane competence in the mesocarp in two wine grape varieties that corresponds to the phase 3 stage of development. This occurs at or near maximum berry weight and clearly defines the third phase of berry development independently of whether berry weight loss occurs or not. This third phase of development defined by loss of cell vitality was not evident in the berries of the table grape variety Thompson seedless.

From the fluorescent dye test series it is clear that autofluorescence, damaged cells on the cut surface and other artefacts do not interfere at this 'macro' level of microscopy. In the second phase of development in Chardonnay, Shiraz and Thompson seedless berries, the dye studies initially showed vital cells across the pericarp and no visible response to PI. On the same day (14 February 2006), the wine grape sections showed a dramatic, vivid red response to PI, and the Thompson seedless

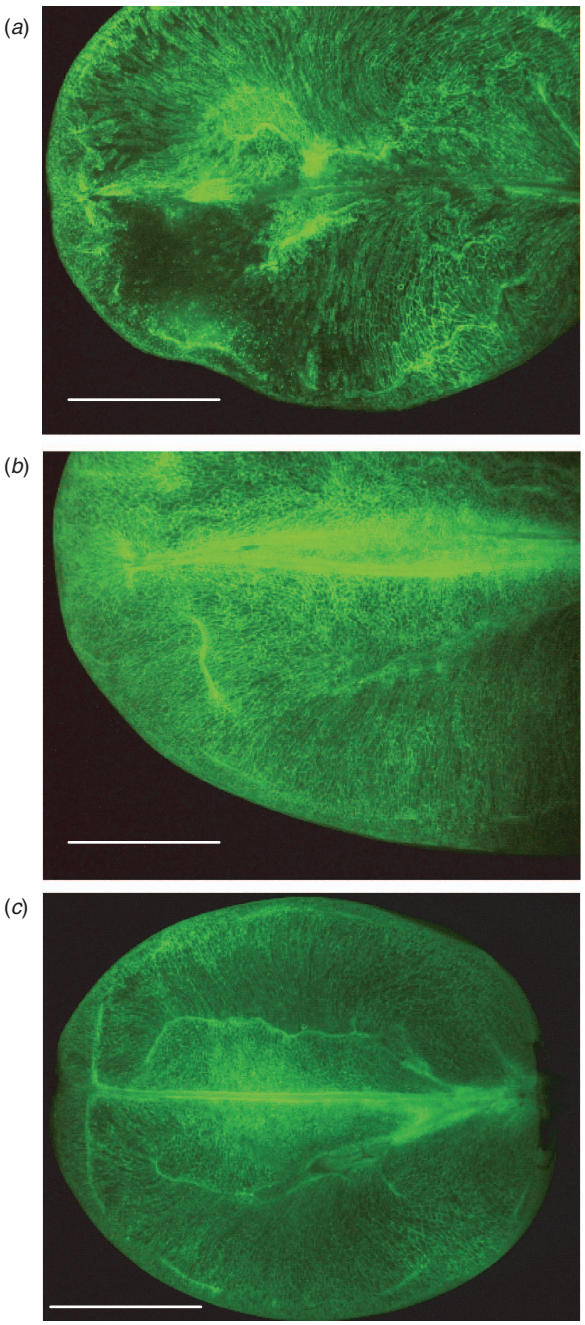

Fig. 7. Longitudinal sections of Thompson seedless berries (*a*) with and (*b*, *c*) without berry collapse, FDA applied. (*a*) Giberellic acid treated berry with visible collapse (23 °Brix, days after anthesis (DAA) unknown) shows a dark region at the distal end of the berry reflecting cell death associated with collapse, and disruption of central and peripheral vasculature. Pericarp cells appear elongated, semi-rectangular and loosely stacked through the distal two-thirds perhaps indicating the spread of berry collapse. (*b*) Healthy berry treated with giberellic acid (20.2 °Brix, DAA unknown) shows more compact, functioning cells across the pericarp and intact vasculature. (*c*) Healthy berry not treated with giberellic acid (24.3 °Brix 124 DAA) also has compact, functioning cells across the entire pericarp and intact vasculature. Remnants of the locules, one with a vestigal seed, are visible. (Fruit treated with giberellic acid courtesy of Mike Treeby and Tori Nguyen, CSIRO, Merbein) Scale bars = 5 mm.

sections had a slight blush of red fluorescence. The response was the same a week later in the wine varieties, but was not repeated in the table grape. No weather, water or other stresses were noted over this period. We believe that the contemporaneous calendar timing of the PI response was not an artefact of any treatment we imposed on the sampling regime for the following reasons: (i) the strong PI response in the wine grapes corresponded to the beginning in the decline of cell vitality using the FDA method that was observed over the following weeks. It also corresponded to other developmental changes including maximum berry weight and slowing of sugar accumulation; (ii) the same degree of cell death measured with FDA was observed in fruit sourced from a different region and vineyard, and measured at the same time after anthesis.

The reason why the PI response was not sustained in the wine varieties when the FDA studies show increasing cell death is not clear. Perhaps the nucleic acids denature to a degree that a structural change interferes with the PI intercalating into a conformation that is fluorescent. Compounds in the cytosol of other plants have been found to reduce PI accessibility of DNA when cells are lysed (Price *et al.* 2000; Noirot *et al.* 2003; Loureiro *et al.* 2006). If intercalation with RNA was responsible for the fluorescence seen, RNA is rapidly degraded during senescence (Jones 2004) and there may be insufficient quantities to fluoresce after the initial response. This needs further work, but it is likely that the strong PI response observed in the two wine grapes is part of the cell death process in the fruit because it mirrors the increasing, subsequent pattern of cell death across the pericarp indicated by the FDA data. Although PI can stain cell walls it was not evident in the control experiments at the magnification used. Total DNA in the pericarp of Shiraz berries has been found to peak at 35 DAA, then be maintained until 100 DAA on a per berry basis (Ojeda *et al.* 1999). The dataset stops several days after maximum berry weight was achieved at 95 DAA, which is probably the period where loss of membrane competence has started to occur so it cannot be related to data in this paper. Anecdotal evidence of increasing difficulty in extracting DNA and RNA as harvest maturity of berries approaches may be related to cell death and subsequent catabolism of nucleic acids. This is certainly an area of research that warrants further investigation.

Cells surrounding the central and peripheral vasculature of berry sections maintained vitality compared with the rest of the pericarp, although this reduces as harvest approaches. This pattern is highly evident in the transects of the premium Shiraz berries (Fig. 6). Although the relative vitality was not significantly different at harvest, a difference was found in the distribution or pattern of vital and non-vital cells across the sectioned pericarp of premium Shiraz fruit from 30-year-old vines in McLaren Flat when compared with the mid-quality fruit from 12-year-old Shiraz vines in the Coombe vineyard. The premium fruit showed a consistent and distinct pattern of vital and non-vital cells across the pericarp. At harvest maturity the cells in the premium fruit around the peripheral and central vasculature clearly maintain competent membranes (and living cells) but the mesocarp at maximum distance from the vascular tissue shows no response to FDA. This suggests that there are distinct regions within the pericarp

where there are no vital cells at harvest. The mid-quality fruit showed variation in pattern and less organised cell death across the pericarp.

Visible disintegration of cell membranes are described as terminal or late events in the cell death process in plants (Noodén 2004). In later stages of berry development, the mesocarp cells of Traminer have lost internal membranes and cell contents including lipids, starches and polyphenols were mixed within (Hardie *et al.* 1996). Autolysis of vacuoles is likely to be indicative of cell death (Thomas *et al.* 2003) and no functioning cytosol clearly means a cell is dead. Both mitochondria and plasma membranes appeared intact in mesocarp cells late in the ripening of fruit of the table grape *V. vinifera* × *V. labrusca* cv. Kyoho (Zhang *et al.* 1997). Mitochondrial integrity is often maintained until late in the senescence process (Noodén 2004). The continuing vitality we observed in Thompson seedless indicates that cell death is variety dependent and, therefore, vitality may also be maintained in the Kyoho grape.

In contrast to the two wine grape varieties, membrane competence and vitality of pericarp cells in Thompson seedless berries was maintained throughout development and into the post harvest period. This may explain why Thompson seedless grapes maintain turgidity, crispness and consumer appeal post harvest. The continuing cell vitality data corresponds with pedicel xylem pressure measurements showing that this variety generates negative pressures in the pedicel xylem until harvest. This indicates a continuing, functioning xylem connection to the apoplast, and cells with osmotically competent membranes that sustain a pressure gradient across them as the berry matures. This observation is consistent with anecdotal evidence of growers severing fruiting canes of Thompson seedless from the vine to prevent water uptake and berry splitting if there is a rain event as harvest approaches. The data from Thompson seedless berries in early stages of collapse warrants further investigation using the methods described here, which provide a quick visual way of identifying cell death and patterns of aberrant cell death.

Recent work shows a change of phloem unloading from a symplastic to an apoplastic pathway at or just before veraison (Zhang *et al.* 2006*b*). Both Zhang *et al*. (2006*b*) and Lang and Düring (1991) presented direct evidence that the apoplastic solute concentration rises from 65 and 60 DAA, respectively, in a table grape and a wine grape variety. Lang and Düring (1991) found that there was no significant difference in the osmotic potential of berry pedicel xylem exudate and berry juice between 60 and 110 DAA. These data clearly separate the increase in apoplastic solute concentration in berries from the later loss of cell membrane competence that is shown in this paper. This means that the 'leaky membrane' theory described by Lang and Düring (1991) as an explanation of the rise in apoplast solute concentration at veraison is not supported, but it is apt later in berry development in Chardonnay and Shiraz. The loss of cell vitality in Chardonnay and Shiraz compared with its maintenance in Thompson seedless suggests that their senescence processes are significantly different, so care needs to be taken when applying research results from one variety to another.

The image analysis showed that Shiraz and Chardonnay had a significantly different time course of loss of cell vitality in the pericarp relative to DAA. Regions in the pericarp of Shiraz entered the senescence or cell death phase earlier in berry development compared with Chardonnay, and this difference increases significantly because relative vitality declines more rapidly in Shiraz berries. This difference is exacerbated by later harvesting for winemaking (127 DAA) compared with the Chardonnay fruit that was harvested at 117 DAA.

Our data suggests an hypothesis for the mechanism of weight loss observed in Shiraz berries that occurs generally after 90–100 DAA (McCarthy 1999; Tyerman *et al.* 2004). Since the membranes of the pericarp cells begin to loose semipermeability at this point, as judged by loss of vitality, the large negative osmotic potential of the berry sap is no longer effective in opposing the xylem tensions developed by the leaves. We have shown previously that Shiraz berries late in development maintain higher hydraulic conductance back to the vine compared with Chardonnay berries (Tyerman *et al.* 2004), therefore, significant volume may leave the berry back to the vine in Shiraz. Reduced phloem inflow and continued transpiration would all combine to cause a net loss of water from Shiraz berries. Chardonnay berries also maintain some finite conductance back to the vine (Tyerman *et al.* 2004), but the quantitative conductance is much less than in Shiraz and drops to very low levels by 90 DAA, that is, at about the same time that cell death begins to be evident in Chardonnay. Recent work of Bondada *et al*. (2005) and Keller *et al*. (2006) demonstrated qualitatively that backflow can occur, however, our previous work demonstrated large varietal differences in a quantitative measure of the pathway for this flow, the hydraulic conductance.

More generally we propose that there is a balance in development between the programmed cell death of the pericarp cells and the decline in hydraulic conductance from the berry to the vine. If the hydraulic conductance to the vine is reduced sufficiently when the pericarp cell membranes lose semipermeability (cells non-vital), backflow would be reduced. This would be the case for Chardonnay. For Shiraz, there is an apparent miss-timing where the pericarp cells loose vitality and osmotic competence earlier in development, but the hydraulic conductance remains high back to the vine later in development. For Thompson seedless where cell vitality is maintained through development, the xylem tension can be resisted by the osmotic potential of the pericarp cell sap. In this case the hydraulic conductance to the vine could be kept high. This is supported by our measurements of xylem pressure which remains negative in Thompson seedless.

The question remains how the hydraulic conductance from berry to vine via the xylem is regulated. Tyerman *et al*. (2004) showed that the largest change in hydraulic conductance through development occurred within the distal part of the berry. The dye studies have shown that the xylem remain qualitatively connected (Bondada *et al*. 2005; Keller *et al*. 2006) late in development, but it is possible that the hydraulic conductance of vessels and tracheids is still reduced. Another possibility is that the cells surrounding the xylem vessels function as an effective barrier from the apoplast of the berry to the xylem lumens. Variable hydraulic conductivity of these cells could be via regulated

activity of aquaporins. The overall semipermeability of this layer of cells, characterized by the *reflection coefficient*, would depend on the degree of development of apoplast barriers analogous to the composite transport model applied to roots (Tyerman *et al*. 2004). Our work has demonstrated that despite extensive cell death in the mesocarp and endocarp, the vascular tissue remains conspicuously vital, indicating that regulation at the membrane level in these cells is possible.

The processes of ripening and senescence are often interrelated (Jones 2004). During ripening, a variety of physical and biochemical mechanisms change the structure of cell walls in fleshy fruits with textural results ranging from crisp to melting or soft and deformable (Brummell 2006).The deterioration of plant membranes during senescence is well documented and several enzymes are involved (Paliyath and Droillard 1992). Research has focussed on the post harvest period, but interestingly, enzymes of the lipoxygenase family have been associated with membrane lipid peroxidation in ripening saskatoons (Rogiers *et al.* 1998) and volatile aroma or flavour development in tomatoes (Chen *et al.* 2004) and kiwifruit (Zhang *et al.* 2006*a*). It has been noted that aroma compounds in wine grapes increase late in ripening when sugar accumulation has slowed or stopped and non- anthocyanin glycosides rise sharply at around 100 DAA in Shiraz (Coombe and McCarthy 1997).

In this context, another important issue raised by our results is whether flavour development in Shiraz grapes can be attributed to the significant loss of cell integrity within the body of the pericarp and the ensuing degradation processes. According to this data, it is more than loss of compartmentation in the mesocarp; it is extensive lysing and mixing of cell contents. The trend for 'hang time' while the winemakers wait for 'ripe flavours' in this variety would also contribute to the loss of cell vitality by harvest. The highly organised pattern of cell death in the premium Shiraz fruit suggests that this idea should be examined.

In conclusion, the variety dependent vitality changes in the pericarp of grapevine berries late in maturity seems to be linked to the strategy of berry water balance in the particular variety. We hypothesise that there are two strategies: (1) cell death in the mesocarp and loss of osmotically competent membranes requires concomitant reduction in the hydraulic conductance of the pathway via the xylem back to the vine; (2) continued cell vitality and osmotically competent membranes that can allow continued hydraulic conductance to the vine. A miss-match in the timing of strategy 1 could cause substantial backflow to the vine and loss of berry weight. For strategy 2, there is a greater danger of berry splitting upon the vine attaining high water potentials. Our data also strongly suggests that closer examination of the cell death processes in wine grapes would contribute significantly to understanding the final phases of flavour development.

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