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RESEARCH PAPER

Evidence for substantial maintenance of membrane integrity and cell viability in normally developing grape (*Vitis vinifera* L.) berries throughout development

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Abstract

Fluorescein diacetate (FDA) was used as a vital stain to assay membrane integrity (cell viability) in mesocarp tissue of the developing grape (*Vitis vinifera* L.) berry in order to test the hypothesis that there is a substantial loss of compartmentation in these cells during ripening. This technique was also used to determine whether loss of viability was associated with symptoms of a ripening disorder known as berry shrivel. FDA fluorescence of berry cells was rapid, bright, and stable for over 1 h at room temperature. Confocal microscopy detected FDA staining through two to three intact surface cell layers (300–400 μm) of bisected berries, and showed that the fluorescence was confined to the cytoplasm, indicating the maintenance of integrity in both cytoplasmic as well as vacuolar membranes, and the presence of active cytoplasmic esterases. FDA clearly discriminated between living cells and freeze-killed cells, and exhibited little, if any, non-specific staining. Propidium iodide and DAPI, both widely used to assess cell viability, were unable to discriminate between living and freeze-killed cells, and did not specifically stain the nuclei of dead cells. For normally developing berries under field conditions there was no evidence of viability loss until about 40 d after veraison, and the majority (80%) of mesocarp cells remained viable past commercial harvest (26 °Brix). These results are inconsistent with current models of grape berry development which hypothesize that veraison is associated with a general loss of compartmentation in mesocarp cells. The observed viability loss was primarily in the locule area around the seeds, suggesting that a localized loss of viability and compartmentation may occur as part of normal fruit development. The cell viability of berry shrivel-affected berries was similar to that of normally developing berries until the onset of visible symptoms (i.e. shrivelling), at which time viability declined in visibly shrivelled berries. Berries with extensive shrivelling exhibited very low cell viability (15%).

Key words: Apoplast, berry shrivel, compartmentation, DAPI, FDA, fluorescence, fruit ripening, locule, propidium iodide.

Introduction

The development of many fleshy fruits typically involves the processes of expansive growth, sugar (typically hexose) accumulation, and softening. Many fruits also exhibit a biphasic pattern of growth, and, for grape (*Vitis vinifera*) berries, the first growth phase (Stage I) is separated from the second growth phase (Stage III) by a period of relatively little growth (Stage II) (Matthews *et al*., 1987; Coombe, 1992). In grape, the transition from Stage II to Stage III is known as 'veraison', and it includes the commencement of berry softening, sugar accumulation, and colour development in pigmented varieties. Some researchers have described the development of the berry as having only two stages, the early stage of berry growth and the post-veraison ripening stage (Coombe and McCarthy, 2000), but it is clear that softening, which is also associated with the process of ripening in the vast majority of fleshy fruits, is coincident with, or slightly precedes the resumption of growth at veraison (Coombe and Bishop, 1980).

One hypothesis to account for berry softening at veraison is a reduction in turgor of the berry cells, but because the osmotic potential of the fruit also declines (i.e. solutes become more concentrated) at this time (Matthews *et al*.,

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1987), if the solutes were accumulated by cells then an increase in cell turgor might be expected. Lang and During (1991) found that the osmotic potential of xylem exudate obtained with a pressure chamber was similar to the osmotic potential of the berry juice, and that xylem exudate from a cultivar that produced anthocyanins in the mesocarp cells was pink. They interpreted both of these observations as indications that the decline in firmness was due to a decline in turgor caused by a substantial loss of compartmentation of the berry mesocarp cells. Earlier, Lang and Thorpe (1989), referring to the post-veraison berry, stated: 'Were the cell membranes to be in good order the tissues would be extremely turgid and the berries hard, as indeed is the case just prior to the onset of ripening. We suggest that after the onset of ripening a grape berry is probably more accurately thought of as a small bag of sugary water rather than as a heterogeneous and complex plant tissue.' This loss of compartmentation hypothesis was one of the two alternatives presented more recently by Tyerman *et al.* (2004) to explain post-veraison berry hydraulic properties.

It is difficult to reconcile the loss-of-compartmentation hypothesis with the observed maintenance of growth and metabolism, particularly the reported post-veraison expression of membrane-associated proteins such as transporters (Fillion *et al.*, 1999) and aquaporins (Picaud *et al.*, 2003). Nunan *et al.* (1998) reported that the amounts of the major wall polysaccharides, cellulose and galacturonides, did not change appreciably after veraison on a mol percentage basis, but that the amount of arabinogalactan I (a component of pectic polysaccharides) decreased significantly during ripening. These authors also found that the protein content of the cell wall increased 50% (as measured by the percentage of wall weight) during the ripening period, especially hydroxyproline-rich proteins, suggesting continued metabolism in berry cells. Recently, Thomas *et al.* (2006) also reported that membrane osmotic integrity, as measured by the response of cells to manipulation of cell volume with the pressure probe, is maintained post-veraison, and hence a large-scale loss of membrane integrity in the mesocarp of the post-veraison grape berry appears questionable. Matthews and Shackel (2005) have suggested that solutes in the apoplastic space of ripening fruit may not indicate a loss of compartmentation, but rather a mechanism to regulate fruit cell turgor, particularly to down-regulate the high turgors that could result from accumulating high levels of solutes in the symplasm. A switch from symplastic to apoplastic phloem unloading has also been suggested as occurring in grape (Sarry *et al.*, 2004; Zhang *et al.*, 2004, 2006) and tomato (Ruan and Patrick, 1995) when these fruits begin to accumulate high levels of solutes, and such a mechanism would involve the presence of solutes in at least some of the fruit apoplast.

Fluorescein diacetate (FDA) is permeable to cell membranes in its native state, and after entry into a living cell the acetate moities are cleaved by cytoplasmic esterases, producing fluorescein, which is membrane impermeable and brightly fluorescent under blue light (Jones and Senft, 1985). For highly vacuolate plant cells, such as those typical of fleshy fruits, if active cytoplasmic esterases are present and cell and vacuole membranes are functional, then the fluorescein should be trapped in the cytoplasm and allow the cells to be visualized as a continuous bright zone adjacent to the cell wall. Hence, accumulation of fluorescein in the cytoplasm is a measure of two independent processes, membrane integrity and the presence of active esterases, and, as such, represents a potentially powerful and robust indication of cell viability. FDA was one of the first fluorescent stains used in biology (Heslop-Harrison and Heslop-Harrison, 1970) and has been used to study cell viability in a wide range of systems such as mammalian cells (Jones and Senft, 1985), algal cells (Shibata *et al.*, 2006), fungi (Soderstrom and Erland, 1986), and plant cell cultures, protoplasts, embryos, and seeds (Pritchard, 1985; Halperin and Koster, 2006; Raghupathy *et al.*, 2006), but to date there have been no detailed studies reported for fleshy fruit tissue. For some systems, such as human lymphocytes, FDA fluorescence was found to decline within a short time after staining, and that this decline was proportional to the internal fluorescein concentration and variable from cell to cell in culture, presumably indicating a substantial but variable leakage of the fluorescein from these cells (Rotman and Papermaster, 1966). Rotman and Papermaster (1966) also found that treatments expected to disrupt membranes or cell integrity, such as mechanical injury (puncture with a micropipette) and freezing/thawing, caused complete lack of fluorescence. Propidium iodide (PI) has also been used to study cell viability as it is excluded from living cells and intercalates into the DNA of damaged cells; thus the appearance of stained nuclei indicates loss of membrane integrity (Trost and Lemasters, 1994). However, disappearance of PI-staining nuclei over time has also been interpreted as indicating the loss of viability, presumably due to the breakdown of DNA (Chen *et al.*, 2006). In this study, FDA was used as a cell viability stain to test the post-veraison loss-of-compartmentation hypothesis in normally developing berries and berries exhibiting a ripening-related disorder known as berry shrivel (BS). FDA was compared with two other fluorescent stains, 4′,6-diamidino-2-phenylindole (DAPI) and the vital stain PI, for their utility in examining cell viability.

Materials and methods

Plants and growth conditions

Greenhouse-grown plants were Chardonnay clone 5 grown in 7.6 l (15.24 cm×40.64 cm) tree pots in 1/3 peat, 1/3 sand, 1/3 redwood compost, with 1.0 kg m^{-2} dolomite lime. Pots were drip irrigated

with water supplemented with calcium (90 ppm), magnesium (24 ppm), potassium (124 ppm), nitrogen as NH_4 (6 ppm), nitrogen as NO_3 (96 ppm), phosphate (26 ppm), sulphate (16 ppm), iron (1.6 ppm), manganese (0.27 ppm), copper (0.16 ppm), zinc (0.12 ppm), boron (0.26 ppm), and molybdenum (0.016 ppm), at pH 5.5–6.0. The irrigation schedule was 4 min four times a day at a drip rate of 7.6 l d^{-1}. The plants were grown under a natural photoperiod with temperatures ranging from 20 °C to 24 °C.

Field Chardonnay plants were clone 29 on 101-14 rootstock planted on a VSP trellis with 2.5 m vine spacing and 3.7 m row spacing. Nebbiolo vines were clone 1 on 3309C rootstock planted on a VSP trellis with 3.1 M vine spacing and 3.1 M row spacing. Both the Chardonnay and Nebbiolo vines were grown in the Davis experimental vineyards in Davis, CA. The greenhouse Chardonnay berries and field-grown Chardonnay and Nebbiolo berries were collected in the 2006 growing season.

Cabernet Sauvignon grapes were from the UC Davis Oakville Experimental Vineyard in the Napa Valley and were collected in the 2005 growing season. They were Cabernet Sauvignon clone 8 on VSP trellises planted on several different rootstocks in a vine and row-spacing trial. Located in this trial were vines which consistently exhibited a ripening-related disorder of unknown cause called 'berry shrivel' (BS), in which berries appear to develop normally until midway through ripening, at which time they exhibit various degrees of desiccation/shrivelling, with no apparent necrosis of the pedicel or rachis tissues. It is important to distinguish that the berry shrivel described here (BS) is not the late season shrivel described by many groups (McCarthy, 1999; Tyerman *et al*., 2004; Tilbrook and Tyerman, 2006) in the Shiraz cultivar. BS occurs much earlier in the season, and the affected berries do not develop normal colour or reach high levels of soluble solids, as shrivelling Shiraz berries do. Each vine that had consistently exhibited BS was paired with a nearby vine with normally developing fruit on the same rootstock and with the same vine and row spacing. Berries were randomly sampled from each type of vine before visible symptoms were apparent, but, after symptoms developed, only symptomatic berries were sampled from BS vines.

Collection of berries

Berries were collected over development as measured by the number of days after anthesis (DAA). Berries were carefully trimmed off the cluster at the pedicel with a sharp pair of scissors and placed into individually labelled small zip-top bags. Care was taken to treat the berries as gently as possible to avoid physical damage. The berries were placed on ice and transported to the laboratory for subsequent sectioning, staining, and visualization. Collection, sectioning, and staining were done within a 6 h time period, although preliminary experiments indicated that berries could be stored refrigerated for many days with no apparent change in staining (data not shown).

Ten vines each of Chardonnay and Nebbiolo were used for the vineyard study. One cluster per vine and two berries per cluster for each sampling date were analysed. Five ungrafted greenhouse Chardonnay vines were used. One cluster per vine and two berries per cluster for each sampling date were analysed.

Sectioning of berries for FDA staining

The pedicel end of the berry was excised by a transverse cut with a razor blade in the brush region. This cut allowed the location of the seeds to be determined, and a longitudinal cut was made through the centre of the berry, between the seeds. Half of the berry was used for osmotic potential and soluble solids determination. The remaining half was blotted on the cut surface to remove cellular debris, and placed on a small cradle which held the cut surface up and horizontal in a covered, humidified Petri dish.

Determination of osmotic potential

Half of the berry was crushed in a small zip-top plastic bag and a sample of juice was taken for osmotic potential determination with a model 5100c vapour pressure osmometer (Wescor, Logan, UT, USA), calibrated with NaCl standards. The same osmometer was used to measure the osmotic potential of the sucrose solution that was used to apply the FDA. Soluble solids (°Brix) were determined on 50 µl of the juice with a temperature-corrected refractometer (Reichert AR200 digital refractometer).

Freezing of berries

Berries were half frozen by placing them on top of a metal bar immersed in liquid nitrogen. Berries were taken off the bar after the visibly frozen portion of the berry reached the equator. Berries were thawed before sectioning as described above.

FDA staining of berries

For analysis of cell viability, a 9.6 µM FDA solution was used for staining the berry cells. The staining solution was made by adding 2 µl of a 4.8 mM stock FDA solution (in acetone) to 1 ml of sucrose solution balanced to approximately the same osmolarity as the berry and, within 10 min of being made, about 250 µl of this staining solution was placed over the entire cut surface and held there by surface tension for at least 20 min to allow uptake prior to visualization. The osmotic potential of the sucrose solution was typically within ±0.3 MPa of the observed berry juice osmotic potential, although preliminary experiments indicated that larger differences (+ 0.5 to –1.0 MPa) were not associated with any visual differences in staining (data not shown).

For the comparison to PI or DAPI the FDA solution was made with the same dilution as above, but rather than using a sugar solution as the balancing osmoticum, 0.5 M phosphate buffer (pH 7.6) was used. Only pre-veraison, half frozen berries were used for this comparison. Cells were visualized at the frozen/thawed border to include both viable and non-viable cells, as indicated by staining.

DAPI staining of berries

A 36 µM DAPI solution was used for staining berry cells. This solution was made by dilution of a 360 µM solution in 0.5 M phosphate buffer (pH 7.6). One hundred microlitres of this solution was placed on the cut surface of the berry and held there for 5 min to allow uptake of the dye. The solution was then blotted off and the FDA stain added.

PI staining of berries

A 1.5 µM PI solution was used for staining berry cells. This solution was made by diluting a 150 µM solution in 0.5 M phosphate buffer (pH 7.6). One hundred microlitres of this solution was placed on the cut side of the berry and held there for 5 min to allow uptake of the dye. The solution was then blotted off and the FDA stain added.

Confocal microscopy

Berries stained with DAPI, PI, and FDA were visualized using an Olympus FV1000 laser scanning confocal microscope. The FDA excitation laser was 488 nm and the detection channel was 500–530 nm, the DAPI excitation laser was 405 nm and the detection channel was 425–475 nm, and the PI excitation laser was 533 nm and the detection channel was 560–700 nm.

Viability determination of sectioned berries

FDA fluorescence of sectioned berries was visualized with a Leica MZ12 stereomicroscope with illumination from a Leica HBO

100 mercury lamp fitted with a Leica GFP Plus Fluor filter (450–490 nm). Photographs were taken with a Leica DC 300F camera attached to the microscope. Areas of fluorescent and non-fluorescent cells and other image parameters, such as average pixel intensity, were estimated on the photographs using the Image-J software (NIH). In cases where pixel intensity was quantified, all automatic exposure features of the image acquisition software were disabled, and exposure time, gain, contrast, and gamma were set to constant values for the period of the experiment. In some cases the boundary between fluorescent and non-fluorescent areas could be traced using the automatic boundary detection feature of the software, but in most cases the boundaries were traced on the image by hand.

Results

Median longitudinal sections of grape berries stained with FDA showed clear, bright fluorescent outlines of mesocarp cells, with no staining in a frozen/thawed section of tissue (Fig. 1A), indicating essentially no staining of dead cells. An unstained berry exhibited no autofluorescence (data not shown), indicating that all fluorescence visible in samples was from FDA hydrolysis. Portions of fruit that were purposely damaged by impact or compression were similarly not stained (data not shown). Excess FDA solution occasionally ran off the cut surface of the berry during transport, or was blotted off after 20 min. Neither of these conditions produced results different from those when the solution was kept continually on the cut surface, provided there was enough time (20 min) for FDA uptake into the cells (data not shown). Areas with and without fluorescence on the cut surface of bisected berries could easily be traced and measured with appropriate image analysis software (Fig. 1B). The development of fluorescence following staining was rapid, reaching a high level in 20 min and a maximum within 50 min, and maintaining this value for at least 40 min (Fig. 2), indicating that there was little or no leakage of fluorescein from the cells in this time period. When continuously exposed to the excitation light there was some evidence of photobleaching, but this was not apparent when exposure was limited to the time required to acquire the image, even when images were acquired every minute (Fig. 2). Average pixel intensity over a similar time period for images of a fluorescence reference material (uranium glass) showed no change (data not shown), indicating that the light source and camera detector were stable.

Confocal microscopy of FDA-stained berries showed that the FDA was confined to the cytoplasm, and that clear images could be obtained from the uppermost two to three layers of intact cells below the berry cut surface (Fig. 3). On the cut surface itself it was not unusual to see small brightly stained objects that were circular in cross-section (Fig. 3A), and these were typically more numerous if the surface was not blotted after sectioning (data not shown). Three-dimensional reconstructions indicated that these objects were columnar, and appeared to be suspended in the residual fluid on the cut surface; presumably membrane-bound compartments formed as a result of the sectioning. Double staining with FDA/DAPI showed that DAPI staining was more intense in freeze-killed cells, but was also present in non-frozen cells. DAPI fluorescence appeared to be confined to the cytoplasm or cell wall, and DAPI-stained nuclei were rarely seen (Fig. 4). Double staining with FDA/PI did not distinguish between killed and living cells, and PI-stained nuclei were also rarely seen. One problem with FDA/PI double staining is the overlap between FDA emission and the detection channel for PI. Fluorescence from FDA could be observed on the PI channel unless the detection window was changed from 560–700 nm to 603–700 nm (Fig. 5A, B). Changing the detection wavelengths for the PI channel reduced the detection of FDA fluorescence, but did not allow the

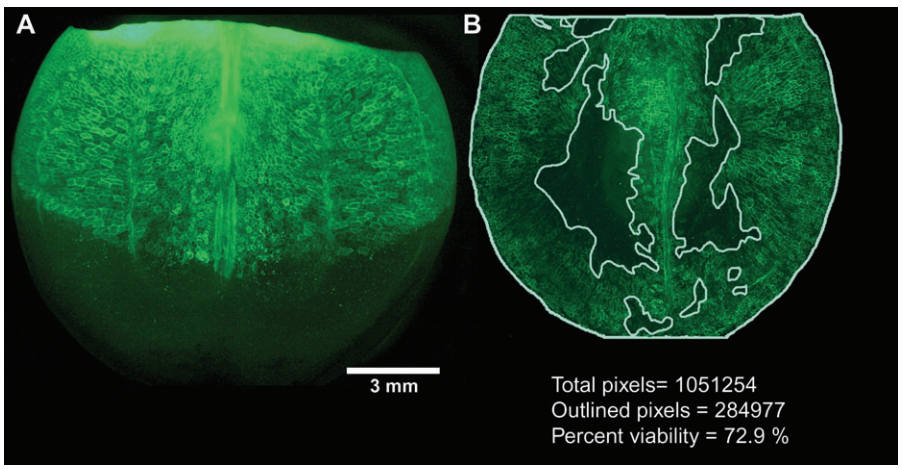

Fig. 1. (A) Median longitudinal section FDA fluorescence image of a berry that had been partially frozen (lower portion) and thawed. (B) Example of a median longitudinal section of an unmodified berry, showing areas, traced by hand, of fluorescent and non-fluorescent cells (presumably viable and non-viable areas, respectively).

distinction between living cells (non-fluorescent nuclei) and killed cells (fluorescent nuclei) (Fig. 5C, D).

Berries harvested from vines with normally developing fruit exhibited a gradual loss in viability over time after veraison, primarily in the locule region around the seeds (Fig. 6C–F), but most cells of the berry remained viable throughout development. Very late into development (131 DAA, 26.8 °Brix) overall viable area was around 80% (Fig. 6F). Throughout the season the entire mesocarp of the berries, when viewed with the dissecting scope on high magnification under white light, retained the appearance of cellularity, with no apparent cell disorganization or formation of air spaces. Both field-grown Nebbiolo and Chardonnay and greenhouse-grown Chardonnay berries in 2006 showed a similar, gradual loss in viability from about 95% around veraison (50 DAA for these varieties) to about 85% at 120 DAA (Fig. 7A). Field-grown Cabernet Sauvignon berries in 2005 showed a similar trend (Fig. 7A). There were cultivar and environmental effects on the accumulation of sugar, with the highest sugar concentrations in field-grown Chardonnay, the lowest in greenhouse-grown Chardonnay, and intermediate levels in field-grown Nebbiolo and Cabernet Sauvignon (Fig. 7B). The relationship of viability to sugar concentration was very similar for all three varieties grown in the field, but lower levels of viability were found at all levels of sugar concentration for greenhouse-grown berries (Fig. 7C). These results were obtained with pools of berries of different sugar levels, and pool size ranged from six to 108 berries. This difference in sample size was due to the fact that only a few berries reached the highest °Brix levels, while many berries were included in the lower °Brix pools.

Pre-veraison berries harvested from as early as 30 DAA from both the field and the greenhouse, unless visibly damaged, always exhibited 95–100% viability, regardless of the variety (data not shown). Before the development of BS symptoms, BS and normally developing Cabernet Sauvignon fruit exhibited essentially identical levels of viability and, at the onset of shrivel symptoms, there was a reduction in the average viability of BS-affected berries, but, more importantly, a marked increase in berry-to-berry variation (Fig. 8). For normally developing berries, there was little variation in viability and appearance on the same cluster (Fig. 9). Berries from BS-affected clusters, on the other hand, exhibited a wide range of both the degree of shrivelling as well as cell viability (Fig. 9). Normally developing berries had a smooth, spherical surface and were uniformly round in cross-section, whereas berries on BS-affected clusters were sometimes smooth with round cross-sections and sometimes wrinkled with irregularly shaped cross-sections (Fig. 9). It is interesting to note that the non-shrivelled berries from BS-affected clusters typically exhibited high viability (100%), low sugar concentration, and low pigmentation, all of these properties consistent with an apparent substantial delay in berry development.

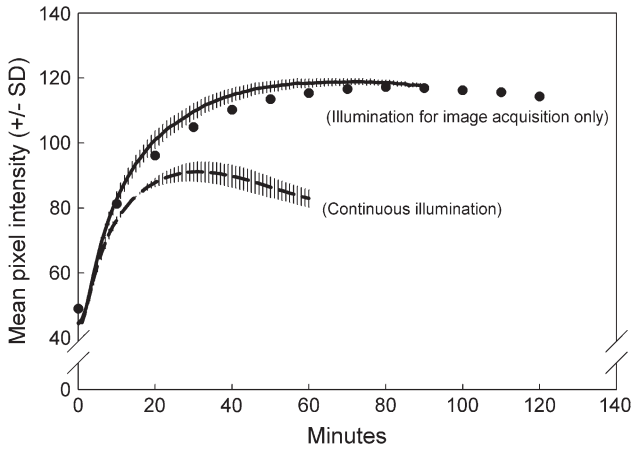

Fig. 2. Time course of average pixel intensity of FDA-stained Chardonnay berry samples (42 DAA, 5 °Brix) under continuous illumination by the excitation light, or covered between pictures and only exposed to excitation light during image capture. Lines are drawn through the mean ±SD from two berries measured at 1 min intervals and points are single measurements from one berry taken at 10 min intervals.

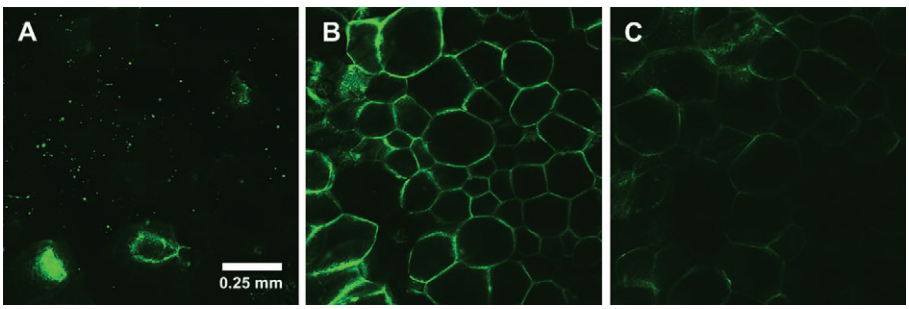

Fig. 3. Confocal FDA fluorescence images of a Chardonnay berry (46 DAA, 5 °Brix) taken at different depths into the sample: (A) surface, with some cytoplasmic faces visible (e.g. lower left); (B) 200 μM into the sample, also with cytoplasmic faces visible (upper left); (C) 400 μm into the sample.

Discussion

FDA staining appears to be a robust and useful technique to evaluate cell and tissue viability in grape berries and perhaps other fleshy fruit throughout development. The fluorescence of cells was rapid, long lasting, and could easily be observed over the entire cut surface of a berry with a fluorescence dissecting microscope. FDA staining distinguished between living and freeze-killed cells more clearly than DAPI or PI staining, with both alternative

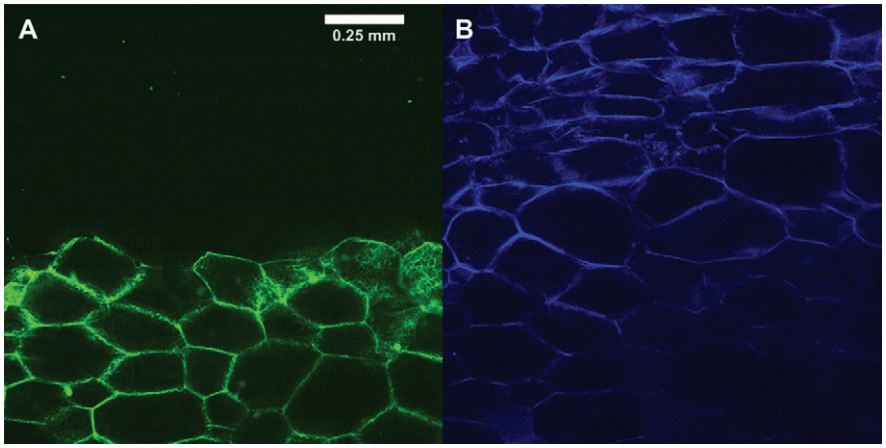

Fig. 4. Confocal FDA/DAPI fluorescence images of a partially frozen (upper portion) Chardonnay berry (46 DAA, 5 °Brix) taken at the border of the frozen area: (A) FDA detection channel; (B) DAPI detection channel.

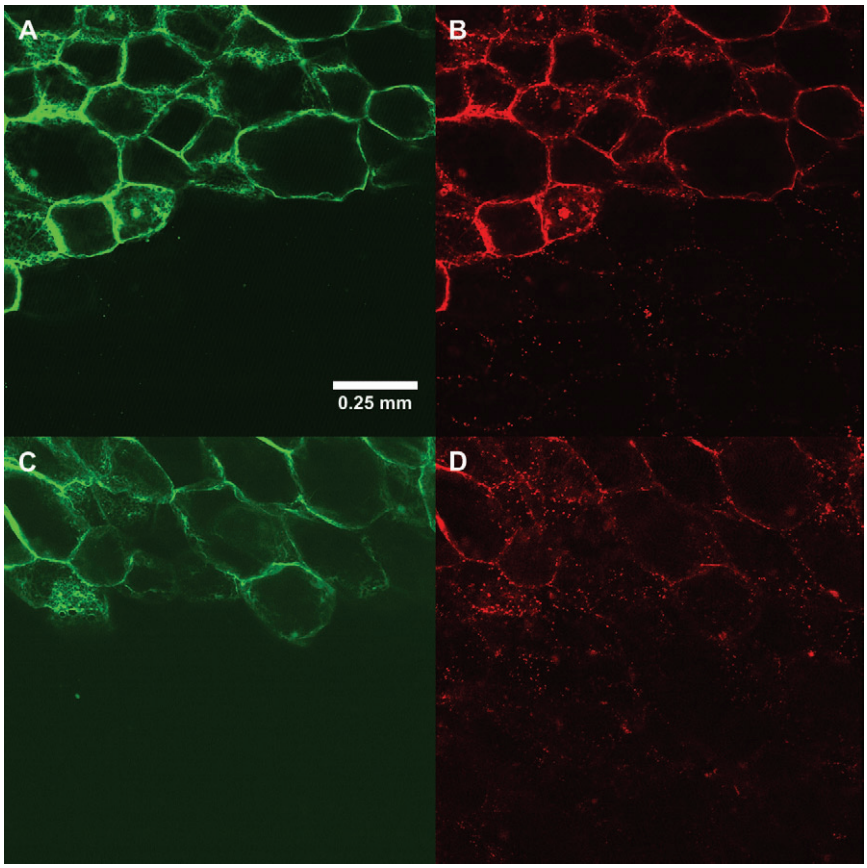

Fig. 5. Confocal FDA/PI fluorescence images of two partially frozen (lower portion) Chardonnay berries (46 DAA, 5 °Brix: A and B, berry no. 1; C and D, berry no. 2:): (A, C) FDA detection channel; (B, D) PI detection channel; (B) image with the detection window set for 560–700 nm (the default settings); (D) image with the detection wavelength restricted to 603–700 nm to reduce FDA fluorescence detected on this channel.

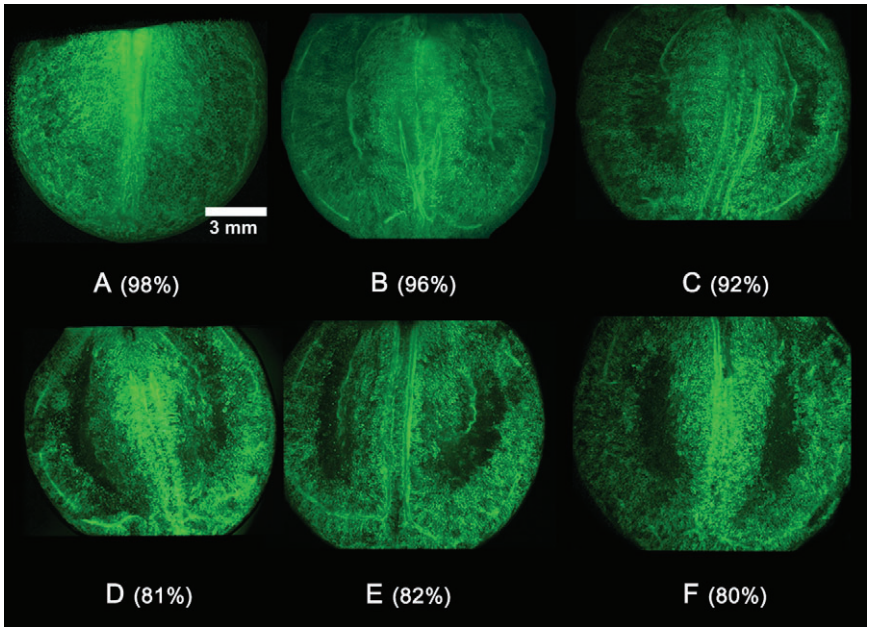

Fig. 6. FDA fluorescence images of Nebbiolo berries at various DAA: (A) 42 DAA, 4.7 °Brix; (B) 82 DAA, 16.6 °Brix; (C) 97 DAA, 23.3 °Brix; (D) 110 DAA, 23.5 °Brix; (E) 120 DAA, 26.1 °Brix; (F) 131 DAA, 26.8 °Brix. Numbers in parentheses are the fluorescent areas as a percentage of total berry area.

vital stains staining both living and killed cells. Both DAPI and PI stained the cell wall or cytoplasm of cells, and stained nuclei were rarely seen in either living or dead cells. Confocal microscopy confirmed the expected localization of FDA fluorescence to the cytoplasm, which allowed the majority of cells in a median section of a grape berry to be identified when viewed through a dissecting microscope by their fluorescent outlines. Confocal microscopy also confirmed that FDA fluorescence was present in at least the first two to three cell layers below a cut surface, and, assuming that these layers are representative of cells in the rest of the berry, non-staining areas (Fig. 1) should be a reasonable estimate of areas of cells which have lost viability or membrane integrity. It is interesting to note that berries grown in different years, in different viticultural areas, and in the greenhouse all displayed similar amounts of cell death as a function of DAA, but not of °Brix. This suggests that the progression of cell death in normally developing fruit is a function of chronological age rather than other ripening parameters, although the developmental regulation of cell viability was not specifically addressed in this study.

The results of this study, together with those of Thomas *et al.* (2006), indicate that the substantial loss in membrane integrity and compartmentation that has been suggested as occurring around the time of grape berry veraison (e.g. Lang and During, 1991; Tyerman *et al.*, 2004) may only occur for a small zone of locular tissue, and only to a substantial degree late in fruit development, much later than veraison. This lack of staining is not due to the formation of air spaces or cell disorganization during cell expansion, as the mesocarp remains cellular for the extent of ripening, at least until 126 DAA (Hardie *et al.*, 1996). Further anatomical work on this phenomenon is warranted, in view of the fact that the locular areas of other fruits (e.g. tomato) undergo 'liquefaction' as part of normal fruit development (Cheng and Huber, 1996, 1997; Atta-Aly *et al.*, 2000). Hence, this may be an important process common to the development of other fleshy fruits.

In grape berries there are many dramatic changes that occur at or around veraison, including an increased rate of solute (hexose) accumulation, catabolism of malate, softening of fruit, and the accumulation of pigments in red varieties. Matthews and Shackel (2005) suggested that the softening of the grape berry around the time of veraison, and the softening of other fleshy fruits during ripening, may be caused by an accumulation of solutes in the apoplast and a resultant reduction in fruit cell turgor pressure. Apoplastic solutes have been detected in post-veraison grapes (Lang and During, 1991; Wang *et al.*, 2003), but the pathway for these solutes into the apoplast has not been identified. In addition to sugars, Lang and During (1991) reported that anthocyanins, which are normally present in the vacuoles of mesocarp cells for the cultivar studied, were also present in apoplastic fluid exuded from the pedicel of these berries under pressure, and concluded that both vacuolar and cytoplasmic membrane integrity was lost. The results presented here suggest that such a loss may indeed occur for cells in the

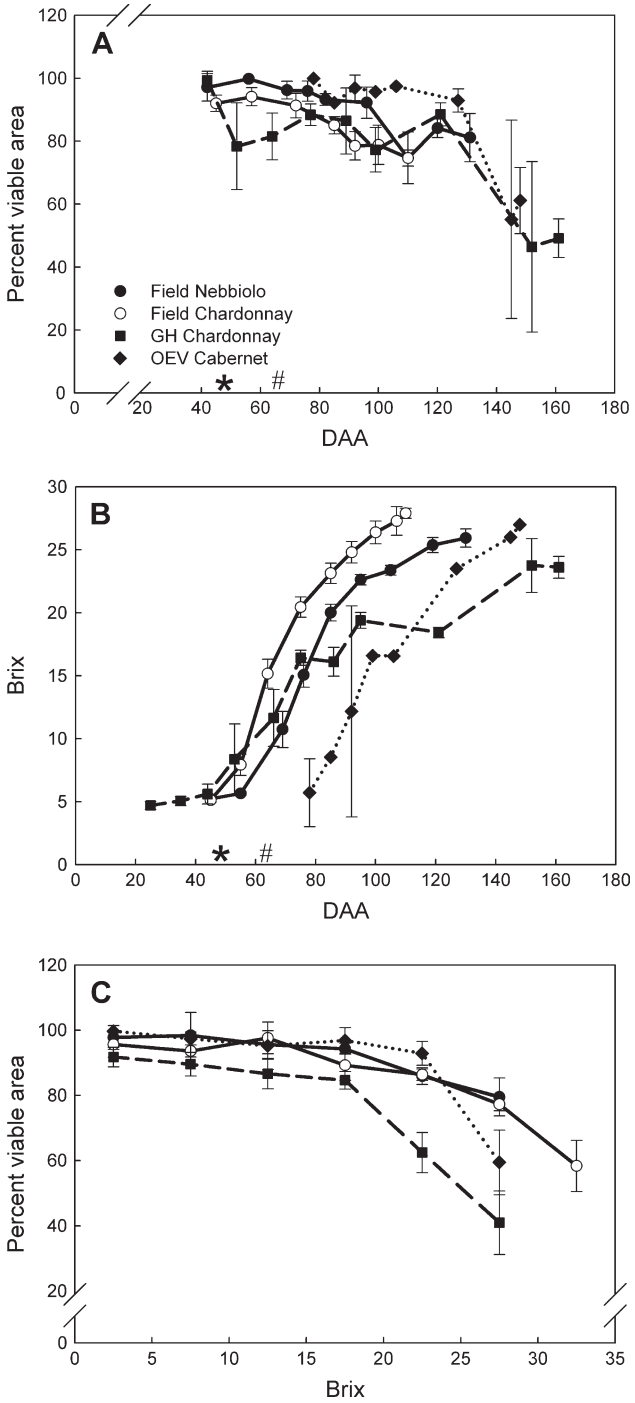

Fig. 7. (A) Percentage fluorescent (viable) area and (B) soluble solids concentration (°Brix) over time for field-grown Chardonnay and Nebbiolo berries, and greenhouse-grown Chardonnay berries from the 2006 season and Cabernet Sauvignin berries from the 2005 season. Lines are drawn through the mean of 2–20 berries ±95% confidence intervals. Approximate time of veraison is indicated by a hash sign for 2005, and an asterisk for 2006. (C) Percentage viable area as a function of °Brix for the data in (A) and (B), pooled into °Brix categories of 5 °Brix. Lines are drawn through the mean of 6–108 berries ±95% confidence intervals.

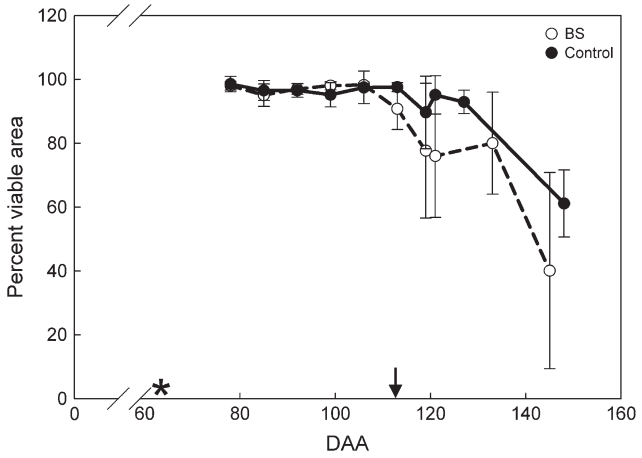

Fig. 8. Percentage viable area at various DAA for field-grown Cabernet Sauvignon berries on normally developing and BS-affected vines in 2005. The arrow indicates the first visible symptoms of BS, and the asterisk indicates the approximate time of veraison. Lines are drawn through the mean of 6–8 berries ±95% confidence interval.

locular areas of the fruit, and, since these areas are close to the central vascular bundle in grapes, compounds normally restricted to the vacuole may be expressed through the vascular tissue when the berry is pressurized. Since the apoplastic volume of the entire berry is a small fraction of the total cell volume, a loss in compartmentation of a small fraction of cells may be all that is necessary to account for a high concentration of solutes in the apoplast without the need to hypothesize a general loss of membrane integrity.

The diurnal water budget of the berry also changes dramatically at veraison with a shift from both a phloem and a xylem contribution to an almost exclusively phloem contribution to the berry water balance (Greenspan *et al*., 1994). The cause of this shift was previously thought to be a mechanical disruption in xylem continuity (During *et al*., 1987; Findlay *et al*., 1987; Creasy *et al*., 1993), but recent evidence has indicated that this is not the case (Bondada *et al*., 2005; Keller *et al*., 2006). The presence of high concentrations of apoplastic solutes in grapes combined with an intact xylem pathway (Bondada *et al*., 2005) poses a problem of solute loss to the parent plant through backflow in the xylem. It has been shown in *Phaseolus* that sieve elements of the phloem lose 6% of photosynthate per centimetre of stem, but that two-thirds of this loss was reabsorbed by surrounding cells and returned to the phloem (Minchin and Thorpe, 1987). Perhaps an analogous process could be occurring in the grape berry pedicel tissue, reducing the amount of solutes that would be withdrawn from the fruit through the xylem.

Symptomatic BS berries had reduced cell viability compared with normally developing berries (Fig. 9), and the extent of viability loss correlated with the visible appearance (shrivelling) of the berry. Since berries on BS

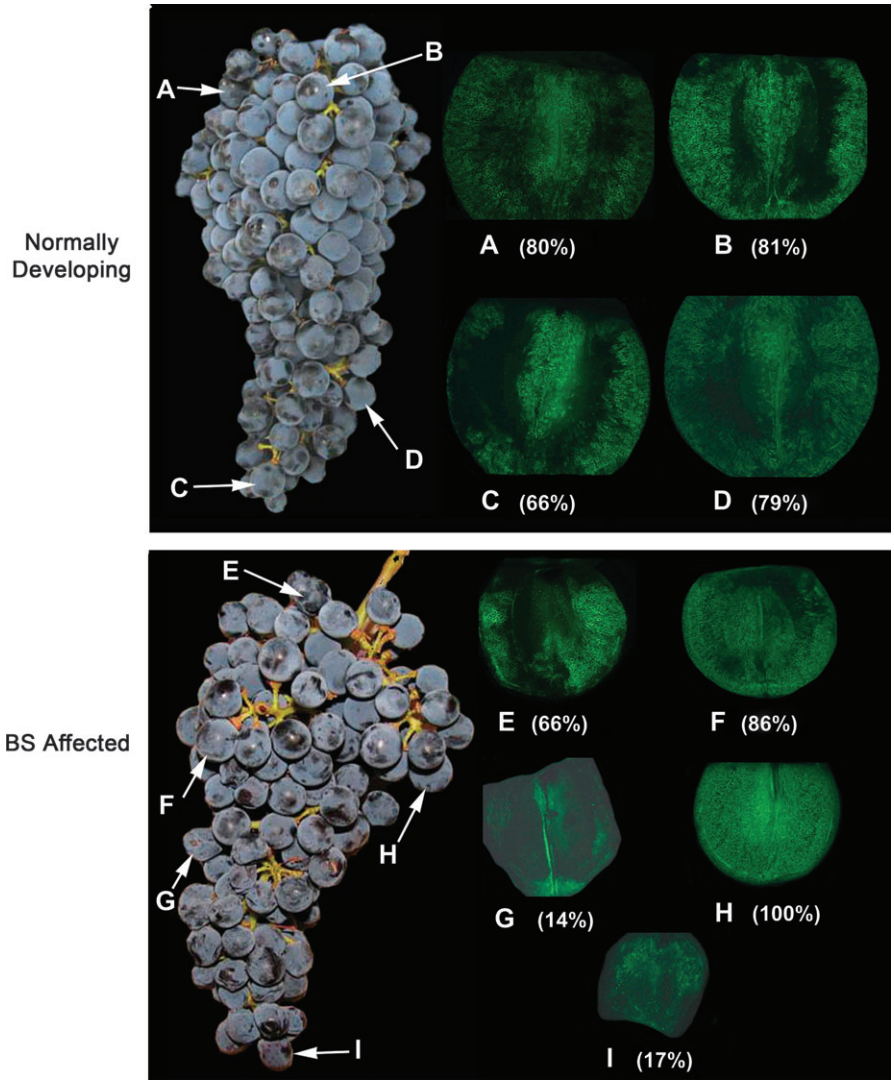

Fig. 9. Pictures of normally developing and BS-affected Cabernet Sauvignon clusters at 141 DAA, showing the appearance of the individual berries used for FDA staining, as well as the FDA fluorescence images from these berries. Numbers in parentheses indicate percentage viable area. The range in soluble solids concentration was 24–26 °Brix for normally developing berries, and 14–19 °Brix for BS-affected berries.

clusters displayed a wide range in the severity of shrivelling, there was a much wider range of cell viability in these clusters than in normally developing clusters (Fig. 9). Late-season berry shrivelling is also common in the Shiraz cultivar of *V. vinifera*, and this phenomenon has been studied recently from a water relations perspective by several groups (McCarthy, 1999; Tyerman *et al*., 2004), specifically testing the hypothesis that the shrivelling is due to loss of water from the berry through xylem backflow. It may be, however, that late season shrivelling in Shiraz berries is analogous to the cell viability loss shrivelling that was observed in BS-affected Cabernet Sauvignon berries, as may also be inferred from images using the technique developed in the authors' laboratory and presented by Tilbrook and Tyerman (2006). Berry softening, the presence of apoplastic solutes, and, to some extent, hydraulic isolation may be considered evidence for general tissue senescence, and from this perspective it is not surprising that the post-veraison berry has been described as a 'small bag of sugary water' (Lang and Thorpe, 1989). However, it is clear from this and other studies that berry softening is not simply the result of a general loss in compartmentation and/or membrane integrity. The role of localized cell viability and membrane integrity loss in fruit development and the importance of these processes to overall fruit water relations and fruit cell turgor deserve further study. This relatively simple, FDA-based method should contribute to a better understanding of the concert of processes which occur as part of fruit development and ripening, and how these processes are influenced by variety and environmental and cultural conditions.

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