New observations on the integrity, structure and physiology of flesh cells from fully ripened grape berry

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Abstract

The physiological/structural status of the soft ripened berry is still a matter of debate. In this paper isolated mesocarp cells from ripened berries of both wine and table varieties were studied by bright-field, fluorescence and confocal microscopy and flow cytometry to highlight the organization of berry flesh cell, function and viability. Flow cytometry analysis confirmed that protoplasting from grape berry mesocarp tissue yields a single heterogenous population of intact and viable cells. Also, the integrity of the plasma membrane and the architecture and complexity of the intracellular membranous system were shown by FM1-43 staining coupled to confocal microscopy imaging. The observed incorporation of the fluorescent glucose analogue 2-NBDG suggests that endocytosis is involved in the transport and intracellular compartmentation of apoplastic sugars. Neutral Red staining confirmed the intricate organization, size, diversity and integrity of the vacuolar apparatus that is probably related to the multifaceted roles of the vacuoles in the developing fruit.

Keywords

Mesocarp cells, protoplasts, cell viability, cell compartmentation, grape berry ripening

Introduction

Grape berries import and accumulate water, minerals, sugar, amino acids, organic acids, and synthesize precursors of flavour and aroma compounds. They undergo several modifications in size, composition, colour, texture, flavour and pathogen susceptibility during development and maturation. These structural and biochemical modifications have been extensively studied because of both the uniqueness of such processes to plant biology and the importance of grapes as a significant component of the human diet and wine industry (Coombe et al. 1987; reviewed by Conde et al. 2007a).

The grape berry shows a biphasic pattern of growth, where the first growth phase is separated from the second growth phase by a period of relatively little growth (Coombe 1992). The transition between both phases, which is called *véraison*, corresponds to the start of berry softening, sugar accumulation, and colour development in pigmented varieties (Conde et al. 2007a, Krasnow et al. 2008, Choat et al. 2009). As a result of this complex process, highly vacuolated flesh cells assume an important role in solute accumulation during berry ripening.

Fruit softening was initially correlated with loss of compartmentation of the berry mesocarp. Lang and Thorpe (1989) stated 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, and Lang and Düring (1991) proposed that the decline in firmness is due to a decline in turgor caused by a substantial loss of compartmentation of the berry mesocarp cells. Assessing membrane integrity and cell viability by fluorescein diacetate (FDA) fluorescent staining of the berry pulp, and confocal microscopy imaging, Krasnow et al. (2008) clearly demonstrated that mesocarp cells stay viable throughout development and ripening of grape berries. This conclusion was further

substantiated by the post-*véraison* expression of membrane-associated proteins, such as transporters (Krasnow et al. 2008 and references therein).

In the present study, individualized cells were isolated from pulp tissue of fully ripened grape berries through enzymatic digestion. Flow cytometry and bright-field, epifluorescence and confocal microscopy confirmed that they are viable, complex, structurally intact and physiologically active, being able to incorporate fluorescent sugars.

Material and Methods

Protoplast isolation from grape cells

Berries of a white wine variety (Vitis vinifera L. Loureiro) and of a red table variety (Red Globe) collected 14 weeks after flowering (harvesting) were used. Grape berry mesocarp tissue was enzymatically digested and protoplasts were purified by sequential steps of differential and gradient centrifugation, as previously described (Fontes et al. 2009, 2010a). Briefly, after berries were deseeded and weighed, skins were peeled and flesh tissue was cut into discs of 3-6 mm thickness (aprox. 20 g) and washed with pre-incubation buffer (Gamborg B5, 0.3 M mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 10 mM MES, pH 5.6, 1 mM DTT, 1mg/mL NAA and BAP) under constant shaking at 4ºC. Flesh discs were then incubated with medium I (205 mM KCl, 65 mM $CaCl_2$, 1 mM DTT, 10 mM MES, pH 5.6) containing 0.03 % (w/v) cellulase Y-C and 0.003% (w/v) pectolyase Y-23. A relatively long digestion period of 12 h was used, at 22ºC under constant agitation (15-25 rpm). The resulting protoplasts were gently collected, filtered through 0.5 mm mesh pore size and then purified. Protoplasts were subsequently separated from enzymes and cell debris by sedimentation, at 150 x *g* for 8 min. The sediment was washed with medium A (400 mM sucrose, 30 mM K-gluconate, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 10 mM MOPS-Tris, pH 7.2), followed

by resuspension in 4 vol of the same medium. A discontinuous gradient was prepared by overlaying 1/2 vol of medium B (500 mM sorbitol, 30 mM K-gluconate, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 10 mM Mops-Tris, pH 7.2) on top of the diluted protoplasts. After centrifuging at 500 x *g* for 8 min, protoplasts were recovered from the interface of the gradient, resuspended in 2 vol of medium I and sedimented for 8 min at 150x*g*. The pellet was then resuspended in the same medium and stored at 4ºC.

Protoplasts from *V. vinifera* suspension-cultured cells (CSB, *Cabernet Sauvignon Berry*) were prepared as described by Fontes et al. (2010b).

Microscopy and image acquisition

Fluorescein diacetate (FDA) staining was applied to estimate cell viability (Jones and Senft 1985). A concentrated stock solution of FDA (500 µg/µL, Sigma, St. Louis, MO) was prepared in dimethyl sulfoxide (DMSO) and stored at -20ºC. For the staining protocol, protoplasts (1 mL) were incubated with 10 µL of FDA stock solution, in the dark, for 10 min at room temperature. The amphipathic styryl chromophore FM1-43 was used as described earlier (Emans et al. 2002, Conde et al. 2007b). This fluorochrome is readily soluble in water but essentially nonfluorescent until bound to membranes. A concentrated stock solution of FM1-43 (1 mM, Molecular Probes, Eugene, OR, USA) was prepared in water, and stored at -20ºC. For the staining protocol, grape protoplasts (1 mL) were incubated with 5 µL of FM1-43 stock solution in the dark at room temperature. For nuclear staining, a stock solution (1mg/mL; 1.8 mM) of the cell permeable blue fluorescent dye Hoechst (Sigma, St. Louis, MO) was prepared in water and stored at -20ºC. Protoplasts were incubated with the dye at 30 nM (final concentration), for 5 min, in the dark, at room temperature. A concentrated stock solution of the cell-permeant MitoTracker Red probe (1 mM, Molecular Probes, Eugene, OR, USA), prepared in DMSO and stored at -20ºC, was used to label the mitochondria.

Protoplasts were loaded with 200 nM MitoTracker Red for 5 min at room temperature in the dark. To perform the triple staining, protoplasts were pre-incubated with FM1-43 overnight, prior to Hoechst and MitoTracker Red loading. Stock solution of the membrane-permeable fluorescent calcium probe Fluo-4 AM (455 μM, Molecular Probes, Eugene, OR, USA) was prepared in DMSO and stored at -20ºC. To determine the entry of the fluorescent glucose analogue 2-NBDG, berry-derived protoplasts were washed twice, after incubation, in a modified medium without sugar. A concentrated stock solution of 2-NBDG (100 mM, Molecular Probes, Eugene, OR, USA) was prepared in water. Neutral Red, a lipophilic phenazine dye (Sigma, St. Louis, MO), was used for the staining of the grape berry mesocarp vacuolar system. A stock solution of 4 mM was prepared in 500 mM mannitol, pH 7.0, prior to its use, and protoplasts were loaded at a final concentration of 4 μM. During the assays, the stock solutions were kept on ice in the dark to minimize degradation.

A Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings was used and images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. Grape protoplasts stained with FM1-43, Hoechst and MitoTracker Red were also visualized using an Olympus Fluo View FV1000 laser scaning confocal microscope, with appropriate filter settings.

Flow cytometry analysis

Flow cytometry analysis was performed in an Epics® XLTM (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass filter. For each sample, 20,000 berry-derived protoplasts were analysed at low flow rate. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on a four decades logarithmic scale. Data were

analysed by WinMDI 2.8 software.

Results and Discussion

Intact and physiologically active cells compose the flesh of the fully ripened grape berry

Isolated flesh cells from fully ripened grape berries were obtained after pulp tissue digestion and protoplast purification, and analysed by flow cytometry. The biparametric histograms, plotting log side scatter (SS) against log forward scatter (FS), of the prepared suspensions revealed a single heterogeneous cell population regarding relative size and complexity (Figure 1A). Incubation with FDA resulted in a substantial fluorescence increase (Figure 1B), suggesting that most cells are viable as confirmed by epifluorescence microscopy observation (Figure 1C). Fluorescein diacetate (FDA), a non fluorescent compound, freely diffuses across the plasma membrane and is hydrolysed to fluorescein and acetate by non specific esterases in the cytoplasm. The polar fluorescein molecule is membrane impermeable and brightly fluorescent under blue light (Jones and Senft 1985). 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 robust indication of cell viability (Krasnow et al. 2008).

Contrary to protoplasts derived from actively dividing CSB cells, which revealed a well-individualized nucleus per cell surrounded by mitochondria at the cell periphery (Figure 2) after triple staining with FM1-43 (membrane), Hoechst (nucleus) and Mitotracker (mitochondria), in grape-derived protoplasts the nucleus was never found with Hoechst. Krasnow et al. (2008) showed that DAPI fluorescence appeared to be confined to the

cytoplasm or cell wall, and DAPI-stained nuclei were rarely seen in stained berries observed under the confocal microscope.

Sucrose, the main photoassimilate exported by the leaves, is imported by the berries according to a complex pattern which depends on source/sink relationships at the plant level, competition between the different berries composing a cluster, between different clusters on a same cane, and between several competing canes, together with the remobilisation of storage carbon from the wood. The source-sink relationships may be altered by local environment and viticultural processes like defoliation (Coombe, 1987; reviewed by Agasse et al. 2009). In the berry, several lines of evidence indicate that the apoplastic pathway play a major role at late stages of grape berry development and both disaccharide transporters (DST) and monosaccharide transporters (MST) genes have been identified (reviewed by Agasse et al. 2009). In the present study the fluorescent non-metabolizable glucose analogue 2-NBDG was used to evaluate the capacity to incorporate sugars of berry-derived protoplasts. Two-deoxy-glucose and its corresponding fluorescent probe (2-NBDG) are potent competitive inhibitors of D-glucose uptake (Conde et al. 2006, Conde et al. 2007b). Sixteen h post-incubation a brightly fluorescent cytosol with several fluorescent spots, was observed (Figure 3C). Confocal microscopy (single section) showed that the analogue is confined to a particular type of vesicles, possibly vacuoles or endocytic vesicles (Figure 3C, *inset*). Also, time-lapse imaging after protoplast staining with FM1-43, confirmed a dynamic vesicle trafficking at the plasma membrane level (not shown) which may be involved in the endocytosis of apoplastic sugars and other solutes. The confirmation of these observations will have important repercussions on our understanding of the grape berry physiology after the shift to apoplastic loading at the *véraison* stage. Fluid phase endocytosis has been also suggested as a parallel uptake system for sugars in a wide variety of heterotrophic organisms, including plant cells (Baroja-Fernandez et al. 2006, Etxeberria et al. 2005a,b). These conclusions were mostly

based on the observed reduction of sugar uptake by the endocytic inhibitor wortmannin, together with confocal imaging of fluorescent probes. This vesicle-mediated mechanism parallel to plasmalemma-bound transporter-mediated uptake is thought to transport sugars and other solutes non-selectively from the cell surroundings, across the cytosol.

It has been proposed that there is a shift from symplastic to apoplastic phloem unloading at the onset of the ripening period (Sarry et al. 2004, Zhang et al. 2004, 2006), meaning that the flesh cells must loose plasmodesmatal connections at this time. The occurrence of simplastically isolated cells may explain, at least in part, the observed loss of fruit turgor due to the high apoplast osmolarities if large amounts of sugars are present in this compartment. The observed integrity of the mesocarp cells purified from ripened berry comforts this idea.

An intricate membranous system predominates in the cytoplasm of pulp cells

To visualise the membranous system of flesh cells, berry-derived protoplasts were stained with FM1-43 and observed under the confocal microscope. FM1-43 stained the plasma membrane immediately after incubation (not shown) and the intracellular membranous system 12 h after, providing a picture of the cytoplasm organization (Figure 3) and of the dynamics of vesicle formation/fusion at the cell periphery. 3D imaging showed a complex array of endomembrane-structures/compartments with a spherical, spheroidal or ellipsoidal shape. Confocal images of isolated vacuoles (released after mechanical rupture of the protoplast membrane by softly pressing the cover glass against the glass slide) labelled with the calcium probe Fluo-4 AM confirming they are intracellular calcium stores (Figure 3B). Calcium sequestration by the vacuoles of pulp cells confirmed previous observations in purified vacuoles from cultured cells that are able to sequester calcium through a proton-

dependent antiport system (Fontes et al. 2010b). Also, grape vacuoles may accumulate up to 1M glucose and fructose together with low amounts of sucrose (not shown).

The vacuole, as a specialized individual structure, can be differentiated into acidic and neutral types on the basis of their vacuolar acidity that depends mostly on the chemical properties of the vacuole sap compounds that in turn are determined by tonoplast transport and regulation of import. Markers for the vacuolar compartments have been proposed, but an unambiguous and a unique marker for each compartment may not exist (Bethke and Jones, 2000). In the present study the acidotropic stain Neutral Red confirmed the diversity and the acidic character of most vacuoles of the isolated cells from ripe berry (Figure 4). Neutral Red staining allowed the distinction between empty and full cell sap. The presence of violet/cresyl blue precipitates (Figure 4 a, d, f, g and h, solid arrows), indicates full cell saps, whereas the appearance of vacuoles with a colour tone similar to that found in concentrated stain solution, dull brick red, indicates empty cell sap (e.g. Fig. 4 b, c, e, dashed arrows). In addition, color tone changes can be produced by the pH differences of the 'vacuole sap', and the accumulation of Neutral Red in vacuoles can be due to interactions with phenolic substances dissolved in the cell sap (Dubrovsky et al. 2006). Grape berry vacuoles accumulate the most important fruit components such as sugars, acids, phenolics, terpenoids and ions, like potassiun, calcium, and even toxic compounds, like pesticides, supporting recent reports emphasizing the existence of different kinds of plant vacuoles (Bethke and Jones, 2000). Therefore, the observed abundance and diversity of vacuoles in pulp cells is not surprising (figures 3 and 4), but the molecular aspects of these compartmentation processes are still poorly understood. The increasing impact of proteomic studies in plant biology has generated an unexpected interest in the purification of this extremely fragile organelle and led to independent proteomic studies focused on intact vacuoles from Arabidopsis (Carter et al. 2004, Shimaoka et al. 2004, Endler et al. 2006). In our laboratory attempts to purify vacuoles

from grape berry protoplasts following the protocol used to purify intact vacuoles from grape cultured cells – derived protoplasts (Fontes *et al*., 2010b), have so far met limited success because they are very heterogeneous in size and remain tightly attached after plasma membrane rupture. This remains an important experimental avenue to be explored in a near future.

Conclusions

The pulp tissue of the fully ripened grape berry may be separated into a mixture of defined and viable cells. Thus, this cell population could be studied by flow cytometry, which may provide a powerful tool for toxicological assessments in grapevine (i.e. effect of fungicides, heavy metals). The intactness of the plasma membrane and of the intricate acidic vacuolar apparatus, supports that berry softening during ripening is not strictly associated with loss in compartmentation and/or membrane integrity. The abundance and diversity of the vacuoles observed in fleshy cells probably reflects their important roles in the accumulation of the complex array of organic and inorganic solutes of the ripened berry. The possibility herein demonstrated to assess by flow cytometry the structural and functional heterogeneity of flesh cell populations and of how it evolves along ripening deserves to be further explored. Although the nucleus and mitochondria were rarely visible, these cells were physiologically active, as shown by membrane trafficking and sugar uptake activity through endocytosis. Thus, these cells may be used as a model to study the biochemical and molecular mechanisms involved in sugar uptake and intracellular compartmentation throughout grape ripening. These are crucial biochemical steps during grape berry ripening as the concentration of monosaccharides (glucose + fructose) may reach 200 mg g^{-1} FW at harvest.

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Figures

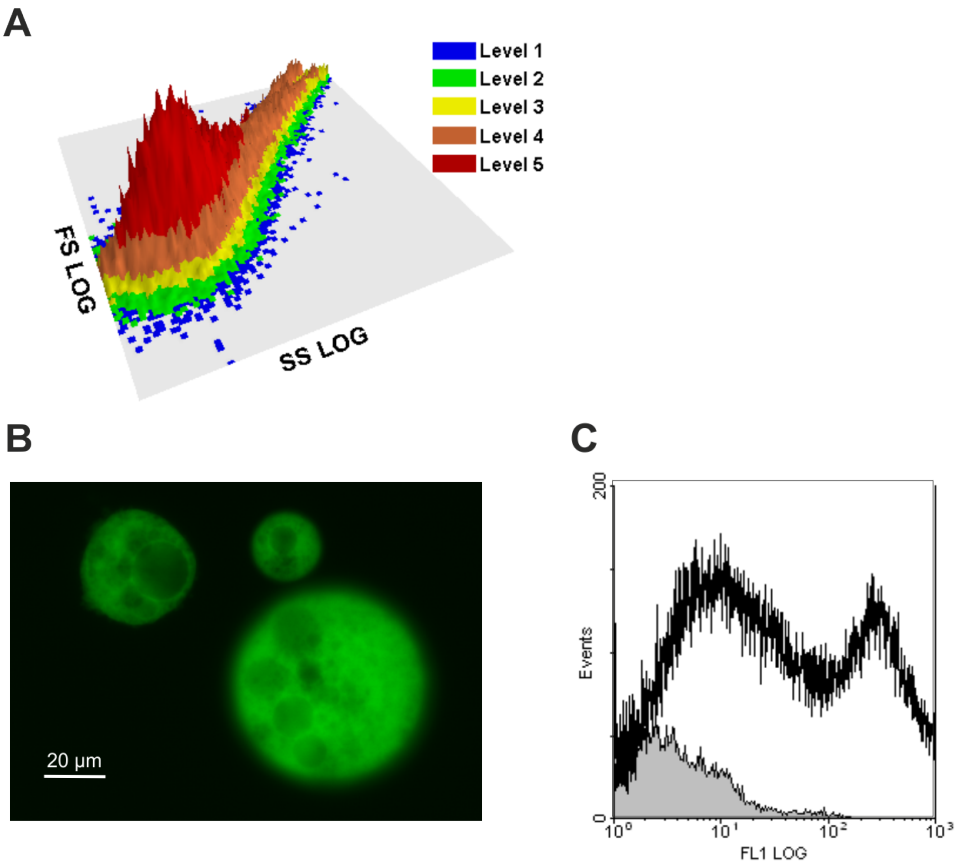

Figure 1. Flow cytometry and epifluorescence microscopy analysis of a protoplast population isolated and purified from grape berry flesh. 3D density plot of the forward angle light scatter *versus* side angle light scatter of a grape cell suspension after FDA staining (A) and overlay of green fluorescence and autofluorescence histograms of the same cell suspension (B). Isolated grape cells observed under UV light (epifluorescence microscopy, C) after FDA staining.

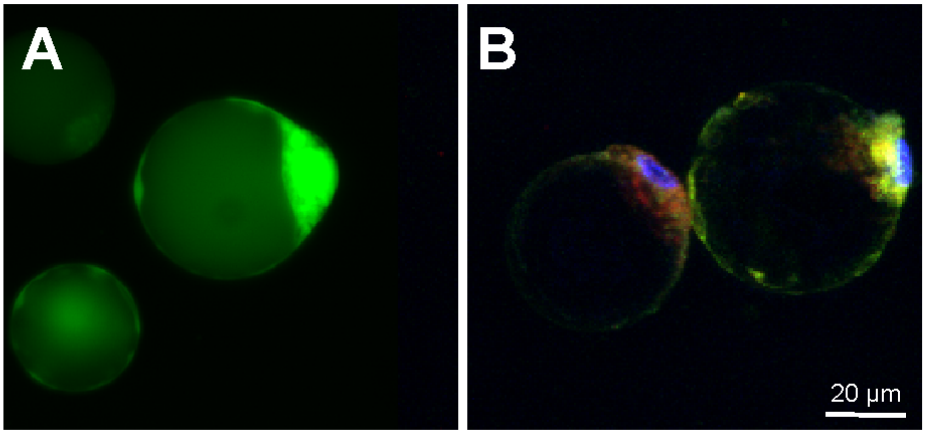

Figure 2. Protoplasts from actively dividing CSB cells (*Cabernet Sauvignon Berry*) observed under the epifluorescence and confocal microscopy. Cells were stained with FDA (A) and Hoeschst (blue, nucleus), Mitotracker Red (red, mitochondria) and FM1-43 (green, plasma membrane) (B).

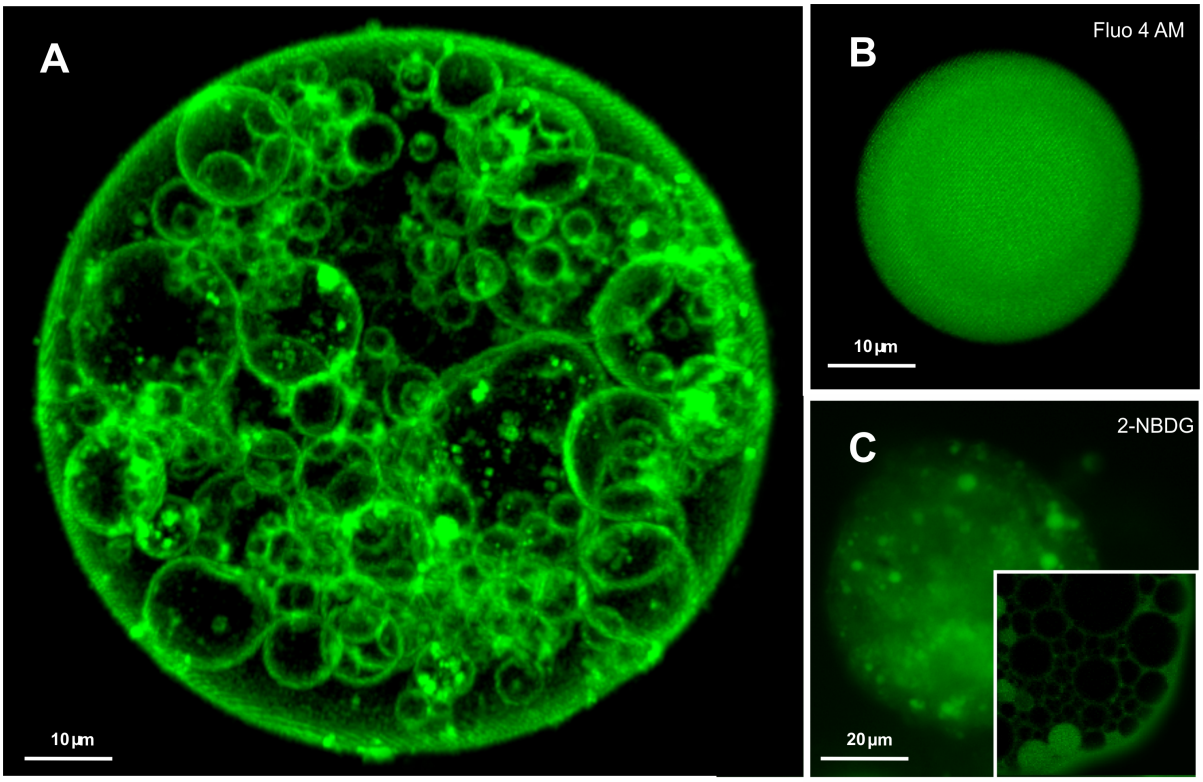

Figure 3. **Mesocarp cells from fully ripened berry observed under the confocal microscope.** Protoplasts were imaged by confocal laser scanning after being immersed overnight with the styryl dye FM1-43, and a maximum Z projection of 20 sections covering approximately 30 µm is represented (A). Intact vacuoles imaged after staining with the calcium fluorescent probe Fluo4-AM (B). Plasma membrane integrity assessed under the fluorescence microscope with the fluorescent glucose analogue 2-NBDG (16 h incubation) (C). Insert: single section of protoplast loaded with 2-NBDG observed by confocal microscopy 16 h after incubation.

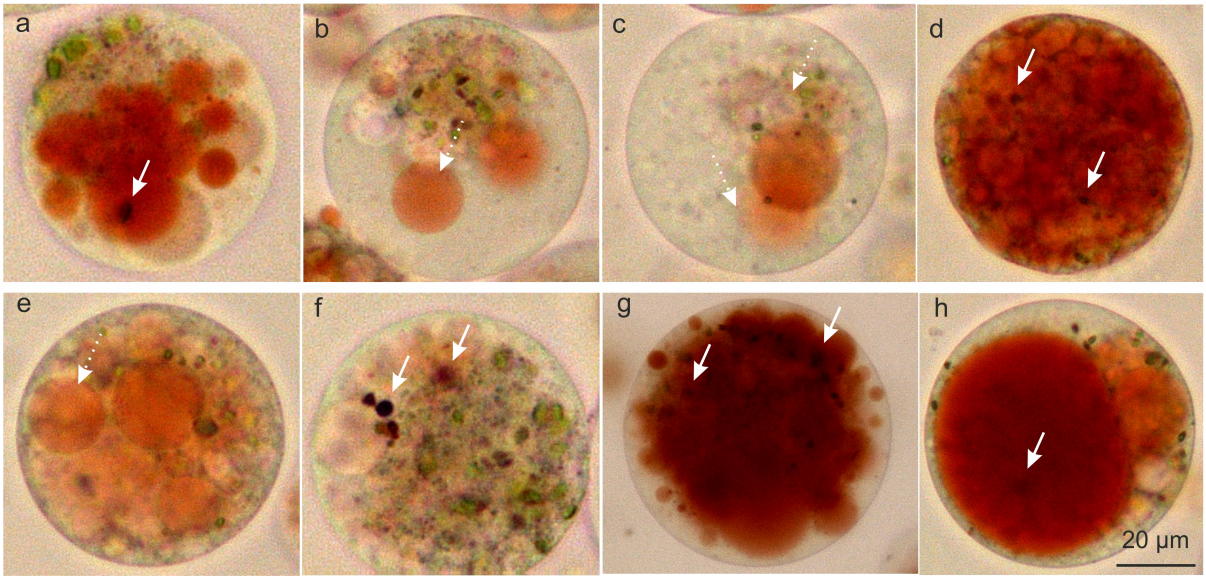

Figure 4. Diversity in size, number and vacuole sap composition/pH of berry-derived protoplasts as assessed after staining with the lipophilic phenazine dye Neutral Red. Vacuoles with precipitates (full cell saps; solid arrows) and without precipitates (empty cell saps; dashed arrows).