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Ultrastructure Study of Apple Meristem Cells during Cryopreservation

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ABSTRACT

The ultrastructure of apple (*Malus x domestica* Borkh.) meristem cells was studied before and after cold acclimation (CA) and after sucrose pretreatment, PVS2 exposure and liquid nitrogen (LN) exposure steps of the PVS2 vitrification. protocol We compared cells of *in vitro* grown shoots of two cultivars, 'Grushovka Vernenskaya' and 'Voskhod'. Cells of the two cultivars were similar in size in all treatments. The control cells and cells recovered after LN exposure were significantly smaller than the CA-, sucrose- and PVS2-treated cells. Cells of both cultivars increased in both length and width with CA, sucrose and PVS2 treatments, and then decreased after rewarming due to cell division. Electron microscopic examination showed intense starch accumulation inside plastids of 'Grushovka Vernenskaya' after three weeks CA while 'Voskhod' plastids had small starch grains and two types of plastoglobules. Two types of small vacuoles were noted in acclimated meristem cells of 'Grushovka Vernenskaya'; one was electron-transparent with many vesicles and sediments, and the other had electron-opaque contents. There were interwoven membranes and occasional dark flecks in the vacuoles of acclimated 'Voskhod' cells. Sometimes large globules with the density and structure similar to polyphenolic compounds were observed. Overall the treatments in the protocol caused the cell size to increase when compared to the untreated cells and cells that were regrown after cryopreservation. This study contradicts the conventional thought that cryoprotective treatments cause meristem cells to decrease in size, thus allowing greater survival.

Keywords: cold acclimation, light and electron microscopy, liquid nitrogen, *Malus domestica*, vitrification
Abbreviations: BA, 6-benzylaminopurine; **CA**, cold acclimation; **EM**, electron microscopy; **ER**, endoplasmic reticulum; **IBA**, indole-3-butyric acid; **LM**, light microscopy; **LN**, liquid nitrogen; **MS**, Murashige and Skoog medium; **PVS2**, plant vitrification solution # 2

INTRODUCTION

Kazakhstan is known as one of the centers of origin and diversity for apples (genus *Malus*) (Vavilov 1930). Natural wild apple forests in mountain areas, where *Malus sieversii* (Ledeb.) M. Roemer is the predominant species, contain genetic diversity for important traits such as disease and insect resistance, fruit quality, growth attributes, dates of flowering and fruit maturation. Unfortunately, these unique genetic resources are under threat of extinction as a result of human activity and climate change. Conservation of genetic resources is now underway in Kazakhstan in field gene banks, *in vitro* storage, and cryopreserved collections of shoot tips and seeds. Cryopreservation in liquid nitrogen is a reliable technique for long-term storage of this valuable germplasm (Reed 2001).

Reports are available describing apple shoot tip cryopreservation by various methods and the results vary with the cultivar and the technique (Chang *et al*. 1992; Niino *et al*. 1992; Niino and Sakai 1992; Zhao *et al*. 1995; Wu *et al*. 1999; Zhao *et al*. 1999; Paul *at al*. 2000) Many of these cryopreservation techniques use cold acclimation (CA) as a tool to improve the recovery of shoot tips. CA differentiates chilling-sensitive plants from chilling-tolerant species and separates the freezing-sensitive species from freezing-tolerant ones (Guy 1990). Short daylengths can induce limited cold hardiness in apples while low temperatures are required for deep CA (Howell and Weiser 1970). CA induces multiple metabolic and morphological changes in plants (Thomashow 1998). Changes in membranes, especially their stabilization due to changes in lipid composition, are particularly important (Uemura *et al*. 1994). Protein content increases as cold hardiness increases (Chen *et al*. 1983). Genes induced by CA improve freezing tolerance through the production of proteins found in the cytosol and the nucleus, and stabilize macromolecules or cellular components during the dehydration imposed by freezing (Thomashow 1999). Soluble sugars, proline, and ABA increase in the first phases of CA (Bravo *et al*. 1998). Sugars protect cells against freezing injury by osmotic, metabolic, and cryoprotective means, and improve glass formation (Chen *et al*. 1998). During CA sucrose content can increase 10-fold, possibly by starch to sucrose conversion (Levitt 1980). Cold hardiness of *in vitro* shoots of 5 apple cultivars was assessed using LT_{50} testing to indicate the temperature lethal to 50% of the shoots (Kushnarenko *et al*. 2009). LT_{50} ranged from -6.7 to -9.3°C for non-acclimated shoots. These LT_{50} values resembled the natural cold hardiness of field-grown plants and allowed 10-12% regrowth after cryopreservation. Acclimated *in vitro* plantlets had LT_{50} values of -12 to -15°C after 1 to 3 weeks of CA, with 65% regrowth after 3 weeks of CA and cryopreservation.

The effects of CA were studied in several cell types. Light microscope studies of cold-acclimation in sugar beet apical dome cells showed starch grains, decreased chlorophyll, and changes in cell wall staining indicating an accumulation of sugars (Vandenbussche and De Proft 1998). Immunofluorescence techniques allowed visualization of microtubule polymerization of acclimated and non-acclimated spinach cells (Bartolo and Carter 1991). Dogwood xylem ray cells collected in winter and exposed to freezing temperatures exhibited contracted protoplasm while summer cell protoplasm fragmented (Ristic and Ashworth 1994). Immunocytochemical electron microscopy showed

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that WAP27 protein (a group 3 LEA protein) accumulated in vesicular ER in winter mulberry buds and cortical parenchyma in winter but not in the summer cisternae form of ER (Ukaji 2001).

Changes in cells during and after cryopreservation were studied for both shoot-tips and suspension culture cells (Bagniol *et al*. 1992; Gnanapragasam and Vasil 1992; Gonzalez Arnao *et al*. 1993; Helliot *et al*. 2003; Zeng *et al*. 2005; Xu *et al*. 2006). These studies showed a range of cellular alterations after various steps involved in cryopreservation. Ultrastructural studies of cryopreserved *Panicum maximum* cells found that pregrowth on osmoticum (mannitol) reduced the vacuolar volume by forming smaller vacuoles (Gnanapragasam and Vasil 1992). Papaya shoot tips exhibited plasmolysis during pretreatment and dehydration and some cells were damaged. Cryopreserved cells appeared normal after 3 days on recovery medium (Zeng *et al*. 2005). Vitrification of *Actinidia chinensis* resulted in damage to the walls, plasmalemma and nucleus of shoot tip cells. Plasmolysis increased and recovery improved after preculture and PVS2 exposure (Xu *et al*. 2006). Cells of date palm apices which survived in liquid nitrogen showed characteristics of cells under osmotic stress (Bagniol *et al*. 1992). The entire apical zone of cryopreserved sugarcane shoot tips remained alive while cells of the basal part were severely damaged or destroyed (Gonzalez Arnao *et al*. 1993).

The objective of this study was to characterize the cellular structure of apple meristem cells from two cultivars at each step of the PVS2 vitrification cryopreservation protocol: before and after CA, following sucrose pretreatment, after PVS2 exposure, and after LN exposure.

MATERIALS AND METHODS

Plant material

Micropropagated shoots of two apple (*Malus domestica* Borkh.) cultivars, 'Voskhod' and 'Grushovka Vernenskaya', were cultured in Magenta GA7 boxes on MS medium (Murashige and Skoog 1962) with 30 g/l sucrose, 0.5 mg/l N^6 benzyl amino purine, 0.01 mg/l indole-3-butyric acid, 3.75 g/l agar, 1.25 g/l Gelrite, pH 5.7. Shoots were grown at 25°C with a 16-h light (40 $\mu E \cdot m^{-2} \cdot s^{-1}$)/8 h dark photoperiod. Isolated shoot tips (0.8-1.0 mm) were recovered on the same MS medium.

Cold acclimation

Three-week old shoots were acclimated at alternating temperatures: 22°C with 8 h light (10 $\mu E \cdot m^{-2} \cdot s^{-1}$)/-1°C 16 h (darkness) for 3 weeks (Reed 1990). 'Voskhod' is very cold hardy and has an LT_{50} of -15.6°C after 3 wks CA while 'Grushovka Vernenskaya' is less hardy with a LT_{50} of -11.6°C (Kushnarenko *et al*. 2009).

Cryopreservation procedures

The PVS2 vitrification technique (Luo and Reed 1997; Matsumoto and Sakai 2000) was used. Shoot tips of 0.8-1.0 mm were dissected from cold-hardened plantlets and from control non-acclimated shoots. Isolated shoot tips were precultured on MS medium with 0.3 M sucrose for 2 days under CA. Then shoot tips were placed in cryovials on ice with PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose, pH 5.8) for 80 min, then submerged in liquid nitrogen. Samples were warmed in a 45°C water bath for 1 min, then in 22°C water for 1 min. Shoot tips were rinsed twice in liquid MS medium with 1.2 M sucrose and placed on MS medium for regrowth.

Light microscopy

Shoot tips were fixed for light microscopy (LM) at 6 points in the process and at 5 points for electron microscopy (EM): 1) Control non-acclimated shoot tips; 2) 3-week CA shoot tips; 3) CA and 2 days culture on 0.3 M sucrose in CA; 4) CA, sucrose culture and PVS2 treatment; 5) CA, sucrose culture, PVS2, cooling in LN and rewarming; 6) After LN, rewarming and 7 days culture on recovery medium. Five shoot tips in each treatment were used for light microscopy and five for EM examination; five shoot tips of each treatment were planted on recovery medium for assessing recovery at each step. In the first steps of the protocol recovery of shoot tips was 100%, after LN recovery was 40% for 'Grushovka Vernenskaya' and 60% for 'Voskhod'.

Shoot tips were fixed in FAA (100 ml 70% ethanol, 7 ml 37% formalin and 7 ml glacial acetic acid). Sections (8-10 µm) were triple stained with Schiff reagent, Erlich hematoxalin solution and Alcian Blue (Kamelina *et al.* 1992). This triple staining reveals cell structure: cell wall (bright blue), cytoplasm (grey-lilac), nuclei (bright violet), and nucleoli (dark grey). Sections were studied and photographed with a Leica DMLS microscope with a digital video camera (Leica DC 300 F).

Electron microscopy

Shoot tips were fixed in 2% glutaraldehyde in 0.2 M cacodilate buffer (pH 7.5) for 2 h at room temperature. The fixation was followed by 4 buffer rinses (15 min each), and post fixation for 1.5 h at room temperature in 1% osmium tetroxide in the same buffer. The samples were dehydrated in an ethanol series (30, 50, 70, 80, 96, 100%), then were transferred to 100% acetone and embedded in epon-araldite which was polymerized for 48 h at 60°C. Ultrathin sections were cut using glass knives on a Reichert Ultracut Ultratome and collected on formvar coated copper grids, contrasted with alcoholic uranyl acetate and lead citrate (Reynolds 1963), examined and photographed under Jem-100B transmission electron microscope, operated at 80 kV.

Cell measurements

Cell sizes (length and width) were measured with a micrometer (MOB-1-15x) for 10 cells of the superficial zone of each of three or four meristems per genotype for each treatment (n=30 or 40). Data for cell width, cell length and width × length were analyzed by ANOVA (SYSTAT 2007).

RESULTS

Light and electron microscopic examination of meristem cells

The apical meristem is a dome located between the developing foliar primordia (**Fig. 1**). Inside the apical meristem two zones, superficial and inner zones, were apparent, with distinct cell sizes and some specialized ultrastructural

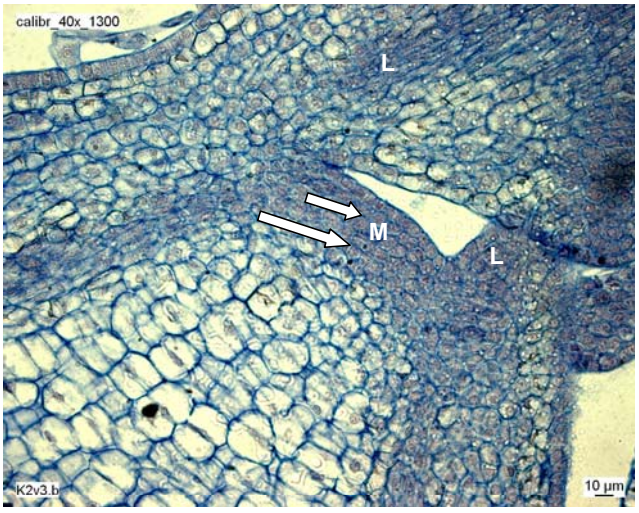

Fig. 1 Light microscopy of control shoot tip of 'Grushovka Vernenskaya'. Arrows indicate two zones of apical meristem: short arrow – superficial zone, long arrow – inner zone. Bar: 10 µm. L – leaf primordium; M – meristem.

characteristics. The cells of superficial zone (4-5 layers of cells) show the characteristics typical for meristematic cells: a high nucleo-cytoplasmic ratio, dense cytoplasm, small vacuoles. The cells of inner (basal) zones were significantly larger with lower nucleoplasmic ratio and higher level of vacuolization. Cells of the apical meristem and foliar primordia were covered with a cuticle.

Measurement of meristem cells

Cells in the superficial zone were measured to determine if treatments produced significant differences in cell size. 'Grushovka Vernenskaya' cells were slightly but not significantly larger than 'Voskhod' cells for both length and width except for differences in width after recovery (**Fig. 2**). The mean cell sizes (length and width) increased significantly ($P \leq 0.001$) with cold acclimation, remained larger with sucrose pretreatment and PVS2 treatment and then decreased significantly after LN exposure. Cells of both cultivars declined in length when measured after LN rewarming but 'Voskhod' cells retained their PVS2 width while 'Grushovka Vernenskaya' cell width declined to initial size on rewarming (**Fig. 2A, 2B**). For 'Grushovka Vernenskaya' the length × width measurements remained high for the three treatments while for 'Voskhod' the largest size was with cold acclimation but decreased slightly for sucrose treatment and showed a further non-significant decrease with PVS2 (**Fig. 2A**). When length × width was compared, the control and recovery cells were smaller than the others, and the cold-acclimated cells were the largest for both cultivars. The PVS2 and sucrose treated cells were similar in size to each other and not significantly different from the CA treated cells ($P<0.01$).

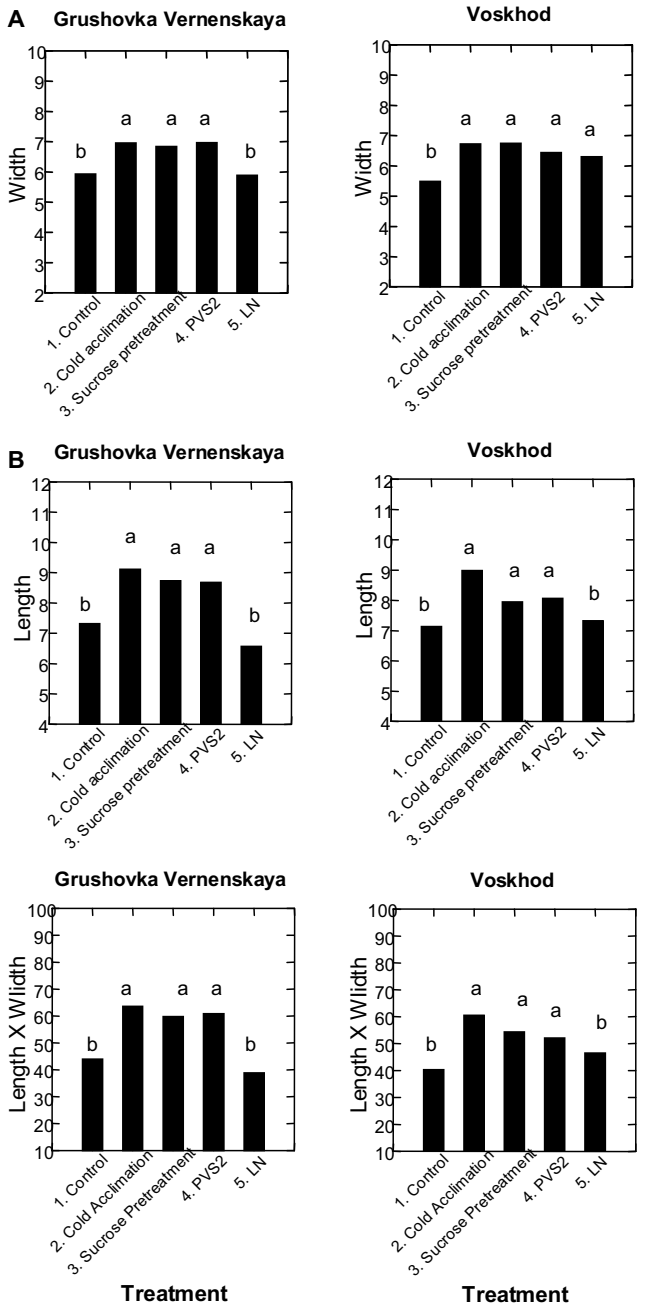

Fig. 2 Dimensions in µM of cells in the superficial zone of apical meristems of apple cultivars 'Grushovka Vernenskaya' and 'Voskhod' at each step of the PVS2 cryopreservation protocol (means of 30 cells at each step). Control, not treated; CA, cold acclimated; Sucrose, 0.3 M sucrose pretreatment; PVS2, after 20 min exposure; LN, after liquid nitrogen exposure, rewarming and rinsing. (**A**) Length, (**B**) width, (**C**) mean length × mean width (μM^2). Means separation by Duncan's multiple range test. Means for a genotype with different letters are significantly different at $P<0.05$.

Control shoot tips dissected from non-acclimated plants

Light microscopic structure of shoot tips (**Fig. 3A, 3B**) and cellular ultrastructure (**Fig**. **3C-F**) were similar for the two cultivars. The only noticeable difference was that the superficial zone of 'Voskhod' appeared thicker than that of 'Grushovka Vernenskaya'. The cells of the superficial zone contained a large nucleus (5.81 × 3.86 µm) (**Fig. 3C, 3E**). The contour of the nuclear membrane was usually wavy with multiple invaginations and projections of several sizes. The nucleolus was large (0.99 × 0.92 µm) and darkly stained. There were clusters of condensed chromatin and the nucleoli often formed significant projections. There were many ribosomes in the cytoplasm. The central vacuole was absent, but there were multiple small vacuoles. The endoplasmic reticulum (ER) was well developed and contained granular components and tubes and individual long cisternae. Plastids were electron-opaque, small, rare, and without starch grains. Mitochondria (from 0.38 × 0.26 to 0.73 × 0.60 µm) were lightly stained with narrow cristae located in the peripheries, and the center was transparent. A large central vacuole was noted in the cells of the inner zone (**Fig. 3D, 3F**). It contained intertwined membranes and some dense globules, possibly phenolic compounds.

Shoot tips dissected from 3-week cold-acclimated plantlets

Some plasmolysed cells were observed on the light sections in the basal parts of the leaf primordia and the basal part of the shoot tips (**Fig. 4A, 4B**). The nuclei in cells of the superficial zone were large (average size 5.10 × 3.88 µm) and the contour of the nuclear membrane was less twisted than the control. Nucleoplasm was characterized by the presence of small less contrasting blocks of condensed chromatin. The nucleolus was large (1.81 × 1.55 µm) and formed significant projections that were sometimes in contact with the nuclear membrane (**Fig. 4C**). The projections were up to $^1/_4$ the length of the nucleolus. Nuclei with 2-3 nucleoli were also observed (**Fig. 4F**).

In contrast with the control, the plastids were well developed in the cold-acclimated shoots. Some differences were observed between cultivars. An intense starch accumulation was noted inside plastids of 'Grushovka Vernenskaya' with 1 to 6 starch grains in each plastid (**Fig. 4C, 4E**). The linear dimensions of plastids averaged 1.69 × 1.22 µm and individual lamellae were observed in a dense matrix. In plastids of the cold-hardy cultivar 'Voskhod', small starch grains and two types of plastoglobules were observed (**Fig. 4D**).

Average sized (0.72 × 0.23 µm) but slightly extended mitochondria were typical for all of the cold-acclimated cells observed. In contrast with the control, the matrix of the mitochondria was denser and the cristae were longer.

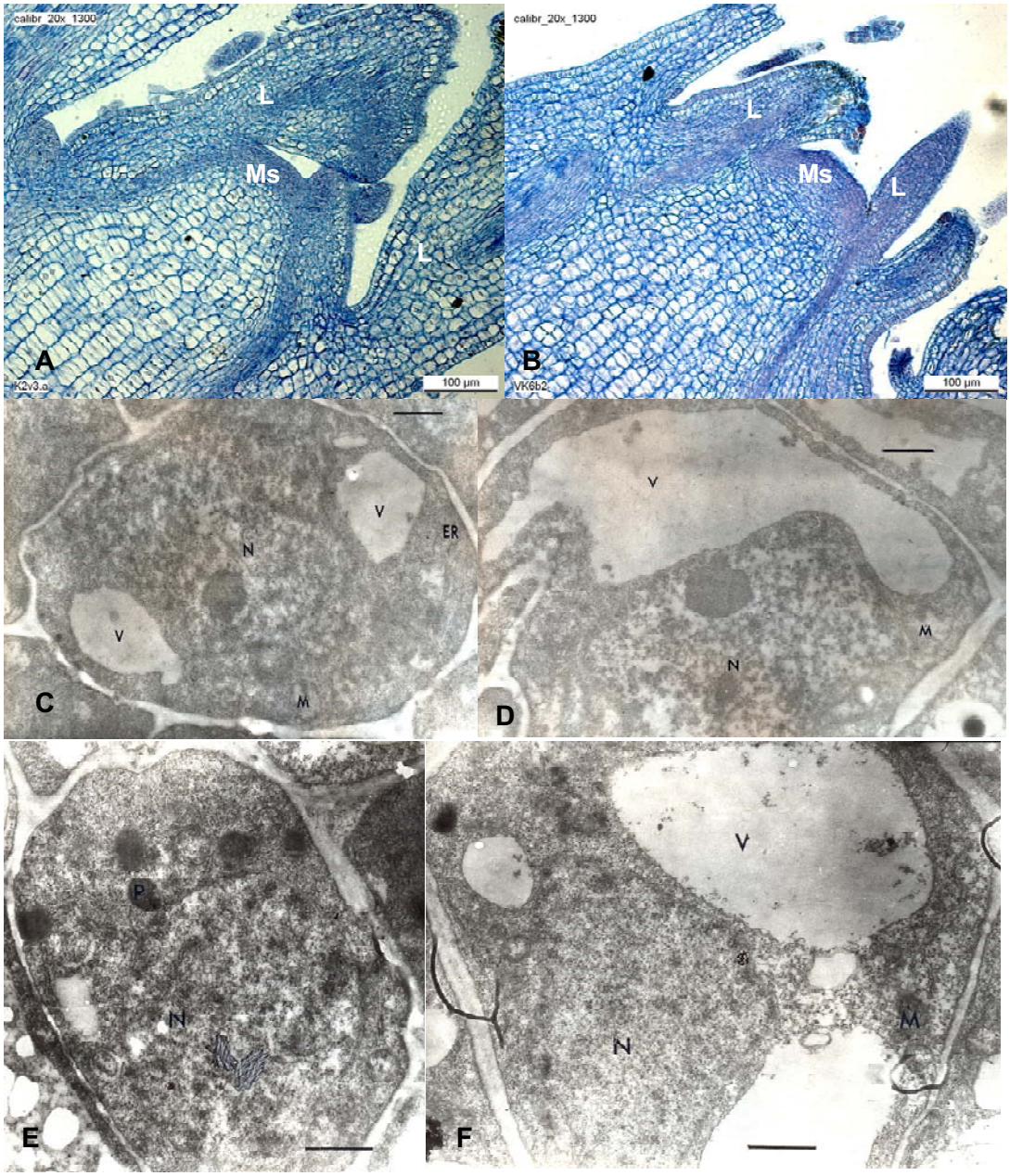

Fig. 3 Light and electron microscopy of shoot tips from control non acclimated plantlets. Longitudinal sections of 'Grushovka Vernenskaya' (**A**) and 'Voskhod' (**B**) shoot tips. Bar: 100 µm. Cells of superficial (**C**) and inner (**D**) zones of 'Grushovka Vernenskaya' meristem. Bar: 1 µm. Cells of superficial (**E**) and inner (**F**) zones of 'Voskhod' meristem. Bar: 1 µm. ER – endoplasmic reticulum; L – leaf primordium; M – mitochondrion; Ms – meristem; N – nucleus; P – plastid; V– vacuole.

ER, as in the control, had granular components and short tubes and cisternae were rarely noted. Two types of small vacuoles were observed in acclimated 'Grushovka Vernenskaya' cells; one was electron-transparent with separate vesicles and granules, and the other with electron-opaque contents (**Fig. 4E**). There were pieces of membrane, interwoven membranes and occasional dark flecks in the vacuoles of acclimated 'Voskhod' cells. Sometimes large electron dense globules with density and structure similar to polyphenolic compounds were observed (**Fig. 4G**).

Some cells appeared degraded after CA while others were normal. The cytoplasm of the moribund cells became granular, plastids were damaged with only starch grains noted and the nuclei (7.50 × 4.80 µm) were conserved. The nucleolus (1.45 × 1.29 µm) was easily observed in the lightened nucleoplasm.

Cells after cold acclimation and 2 days culture on MS medium with 0.3 M sucrose

Compared to earlier cryopreservation steps, more plasmolysed cells were observed in both cultivars, especially in the large basal cells of shoot tips and in basal parts of the leaf primordia (**Fig. 5A, 5B**). In addition, large cells with granular contents were noted in this part of the shoot tips. Large nuclei (6.71 × 5.56 µm) were observed in 'Grushovka Vernenskaya' cells of the superficial zone (**Fig. 5C**). The contour of the nuclear membrane was wavy. The average nucleolus was 2.35 × 2.09 µm and projections were very rarely noted. Clusters of condensed chromatin were also very rarely observed. Plastids were small (1.26 × 1.03 µm) and most contained starch grains. The elements of lamellar systems were absent. Mitochondria were rare, and those noted were small, and slightly extended (0.67 × 0.60 µm). ER was only observed as tubes of a granular type. The small electron-transparent vacuoles were rarely seen.

Central vacuoles at several stages of development were observed in 'Grushovka Vernenskaya' inner zone cells (**Fig. 5D**). Large nuclei were clearly noted (6.20 × 4.76 µm). The nucleolus appeared with high contrast (1.71 × 1.29 µm) and projections were seldom observed. Plastids were better developed than in the cells of superficial layers, with both

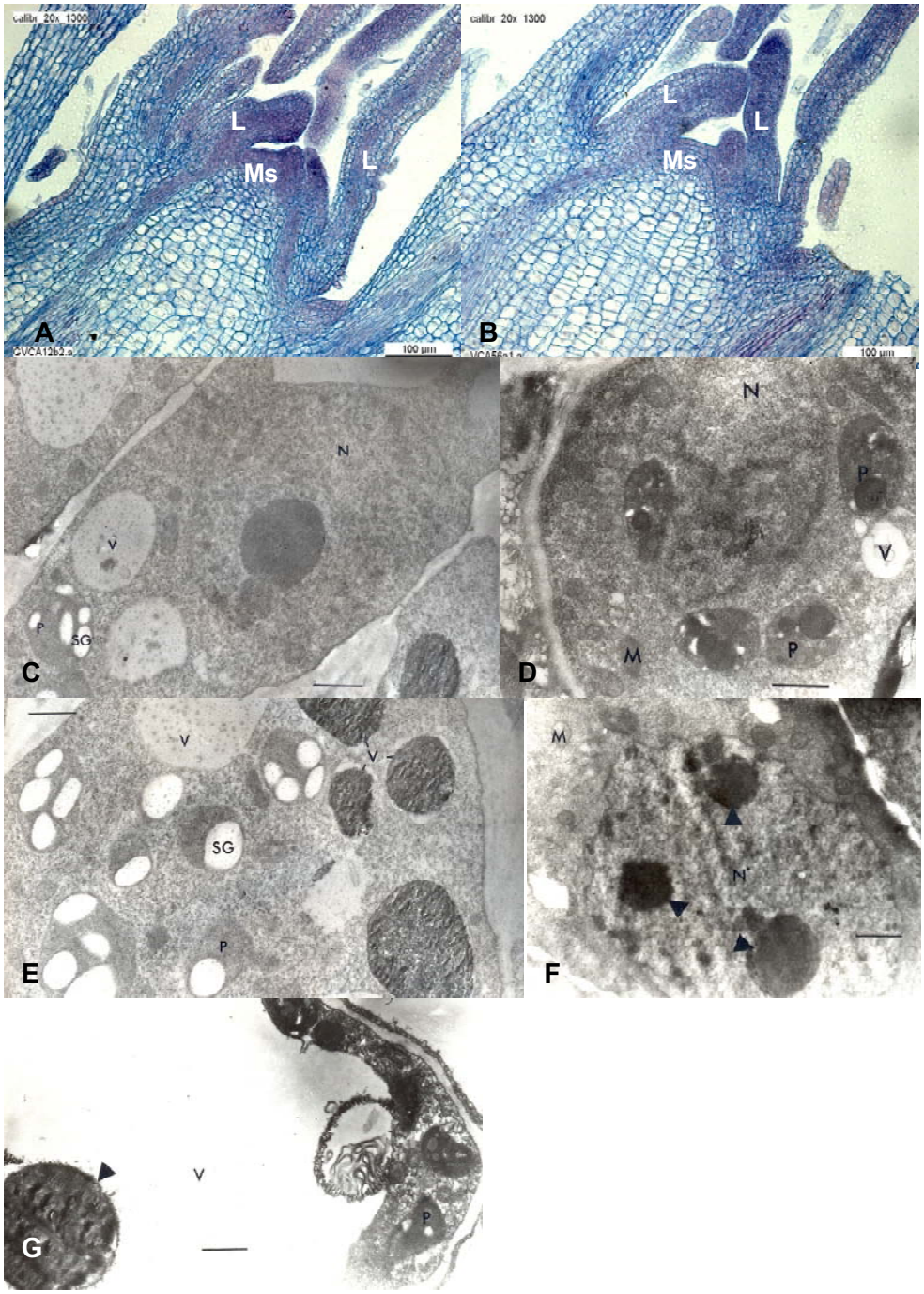

Fig. 4 Light and EM of shoot tips dissected from the plantlets following 3 weeks of CA. Longitudinal sections of 'Grushovka Vernenskaya' (**A**) and 'Voskhod' (**B**) shoot tips. Bar: 100 µm. Parts of cells from superficial zone of meristem: (**C**) 'Grushovka Vernenskaya', note protuberance of nucleolus; (**D**) 'Voskhod', note plastoglobules in the plastids. Bar: 1 µm. Parts of the cells of inner zone of 'Grushovka Vernenskaya' meristem: note the vacuoles with dense contents (**E**); the arrows indicate three nucleoli (**F**). Bar: 1 µm. (**G**) Part of the cell from inner zone of 'Voskhod' meristem with large globule (arrow) in the vacuole. Bar: 1 µm. L – leaf primordium; M – meristem; Mc – mitochondrion; N – nucleus; P – plastid; SG – starch grain; V– vacuole.

small and large starch grains (1.37 × 1.01 µm). ER was observed as granular tubes and short cisternae. The average size of mitochondria was 0.60 × 0.47 µm, however some mitochondria were very long with the length exceeded the width by 5-7 times.

'Voskhod' superficial zone cells also contained large nuclei (4.46 × 3.51 µm) (**Fig. 5E**). Some cells had two nucleoli (0.83 µm diameter) and the nucleoli sometimes formed noticeable projections, which were sometimes in contact with the nuclear membrane. In contrast with 'Grushovka Vernenskaya' the blocks of condensed chromatin were rather large and numerous. Plastids were small (0.64 × 0.38 µm) with starch grains in some. There were slightly developed elements of lamellar systems in some plastids. The ER was well developed. The rarely seen small vacuoles were electron transparent. Mitochondria were swollen (0.64 × 0.57 µm) with fewer cristae in comparison with controls and a lightened matrix in the center.

'Voskhod' inner zone cells had vacuoles which contained interwoven membranes, flecks and vesicles (**Fig. 5F**). Nuclei were large (5.10 × 3.51 µm) and the contour of the nuclear membrane was wavy. The nucleolus had a non-central position and often touched the nuclear membrane. The blocks of condensed chromatin were large and numerous. Mitochondria (0.68 × 0.57 µm) were swollen with a lightened matrix in the center and had fewer cristae than the control. Plastids (0.96 × 0.45 µm) were well developed with rare starch grains but with noticeable plastoglobules, in contrast with 'Grushovka Vernenskaya' cells. The ER was evident throughout the cells.

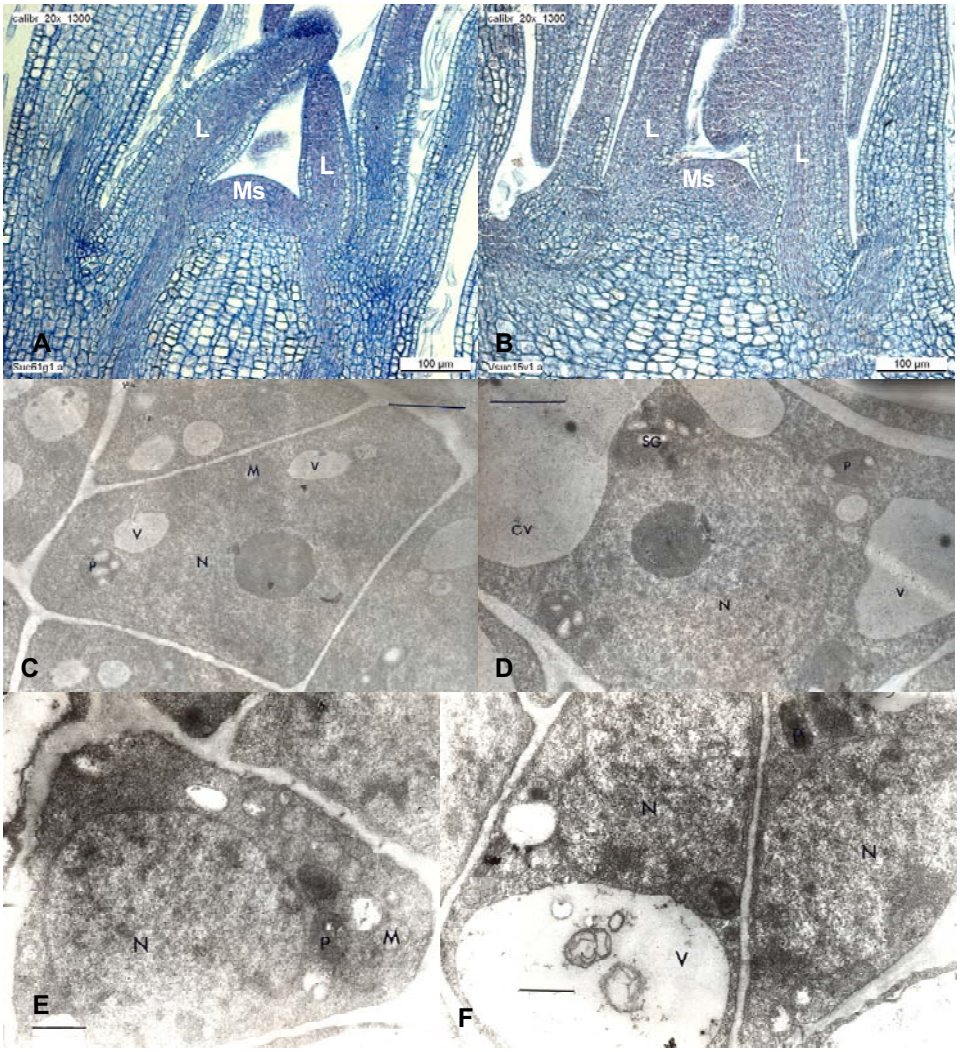

Fig. 5 Light and electron microscopy of shoot tips following 3 weeks of cold acclimation and 2 days culture on MS medium with 0.3 M sucrose. Longitudinal sections of 'Grushovka Vernenskaya' (**A**) and 'Voskhod' (**B**) shoot tips. Bar: 100 µm. Cells of superficial (**C**) and inner (**D**) zone of 'Grushovka Vernenskaya' apical meristems. Bar: 2 µm. (**E**) Part of the cell of the superficial zone of 'Voskhod' meristem (note the plasmodesmata in cell wall). Bar: 1 µm. (**F**) Two neighboring cells of inner zone of 'Voskhod' meristem. Bar: 1 µm. CV – central vacuole; L – leaf primordium; M – mitochondrion; Ms – meristem; N – nucleus; P – plastid; SG – starch grain; V– vacuole.

Cells after PVS2 treatment

Many plasmolysed cells were observed in the basal parts of shoot tips and in the leaf primordia (**Fig. 6A, 6B**). The cytoplasm of these plasmolysed cells was stained yellow-brown indicating that these cells were damaged. The majority of meristematic cells was not damaged and had blue-stained cytoplasm.

The large nucleus (5.49 × 4.02 µm) was usually located in the central position of 'Grushovka Vernenskaya' superficial cells (**Fig. 6C**). There were no clusters of condensed chromatin or projections of nucleoli (1.45 × 1.21 µm) in the nucleoplasm. Some nuclei had two nucleoli. The cytoplasm contained small electron-transparent vacuoles, rare small plastids with starch grains and long mitochondria (0.78 × 0.46 µm). Some mitochondria were cup shaped and were twice as large as normal.

In the cells of the inner zone, the nuclei (7.77 × 5.13 µm) contained up to 3 nucleoli. Nucleoli (1.93 × 1.66 µm) were usually located in a central position and showed high contrast. Plastids were long (2.59 × 1.32 µm) and contained starch grains and poorly developed lamellar systems. Some plastids were irregular shapes. Mitochondria were large (0.89 × 0.67 µm). Some vacuoles contained large globules of electron-dense material. Some cells were damaged by the PVS2 and only the nuclei remained in the cells. All 'Voskhod' cells were damaged in processing, so no EM data is available.

Cells after LN and rewarming

The best mean shoot regrowth of 'Grushovka Vernenskaya' after 3 weeks CA, sucrose pretreatment, and PVS2 vitrification was 58.3% while 'Voskhod' was significantly better at 74.2%. The majority of cells in the basal parts of shoot tips and leaf primordia were plasmolysed (**Fig. 7A**). In some shoot tips the peripheral layers of leaf primordia consisted of yellow-brown stained cells, a sign of necrosis (**Fig. 7A**). In other shoot tips almost all leaf primordia and many cells in the basal part of the shoot tip were stained yellow (**Fig. 7B**). The meristematic cells in some shoot tips were not damaged and had the normal blue cytoplasm (**Fig. 7A**). The superficial zones of both cultivars were characterized by the presence of a large nucleus of the chromonema type ('Grushovka Vernenskaya' 4.89 × 4.27 µm and 'Voskhod' 5.74 × 4.48 µm). The presence of long filaments of chromatin indicated high activity in the nucleus. The nuclear membrane was straight or slightly twisted with ribosomes on the outer surface. Many nuclei of 'Grushovka Vernenskaya' had 2-3 nucleoli (**Fig. 7C, 7D**). If a single nucleolus was present it formed projections. Most of the nuclei of 'Voskhod' had a single nucleolus without projections. A chromonema-free area was noted around the nucleolus (**Fig. 7D, 7E**).

In the cells of both cultivars the vacuoles were small and rare. ER was well developed and consisted of granular cisternae and tubes. Plastids were small, rare, and without starch grains. In 'Grushovka Vernenskaya' cells there were

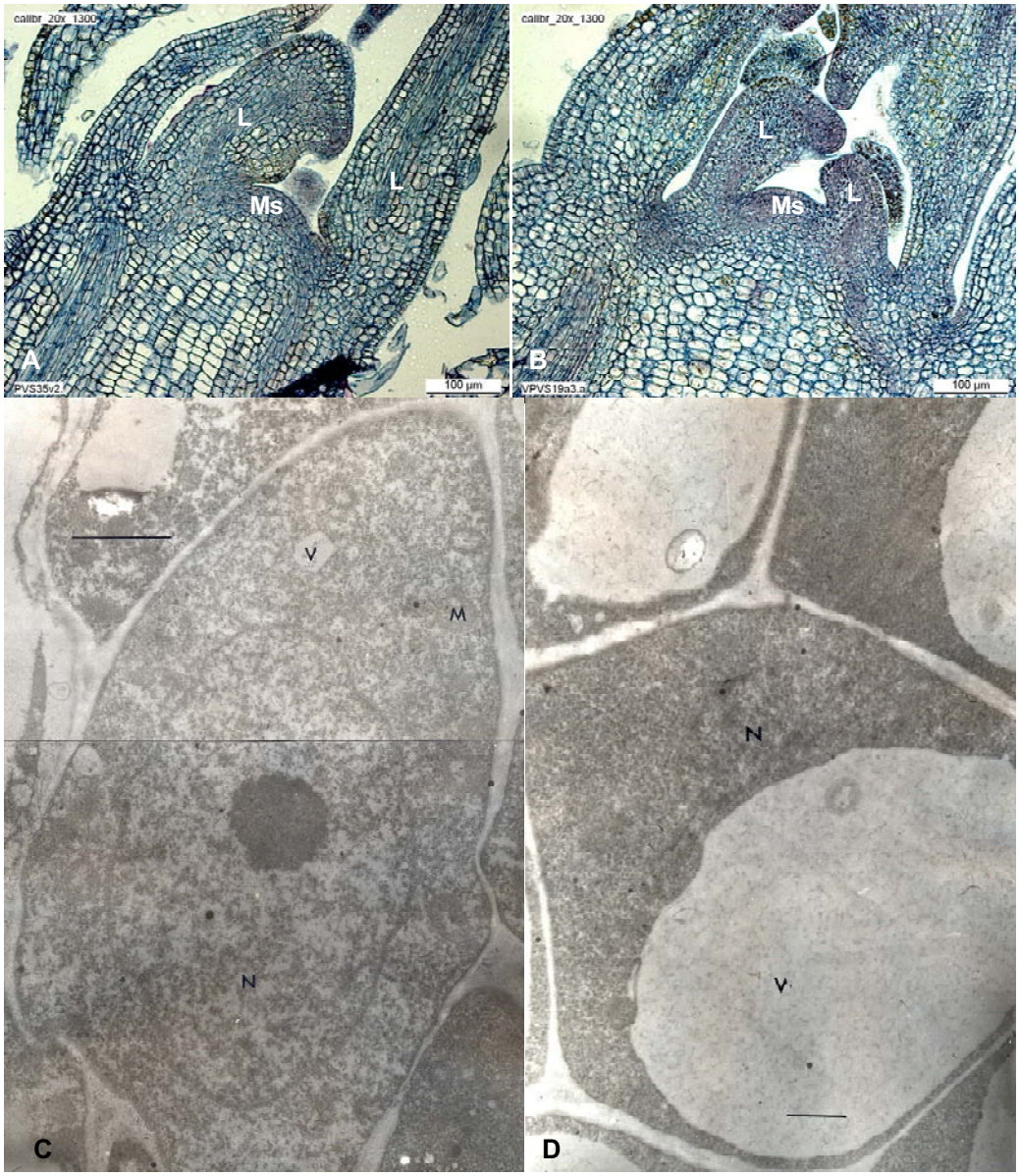

Fig. 6 Light and electron microscopy of shoot tips following PVS2 treatment. Longitudinal sections of 'Grushovka Vernenskaya' (**A**) and 'Voskhod' (**B**) shoot tips. Bar: 100 µm**.** Cells of the superficial (**C**) and inner (**D**) zones of 'Grushovka Vernenskaya' meristem. (**C**) – Bar: 2 µm; (**D**) – Bar: 1 µm. L – leaf primordium; Ms – meristem; M – mitochondrion; N – nucleus; V– vacuole.

two types of mitochondria; small (0.81 × 0.48 µm) with dense, long cristae, and large (1.13 × 0.64 µm), with short cristae and a lightened matrix in the center (sign of degradation). Mitochondria of 'Voskhod' cells were numerous and had a typical structure. Recovery of cellular activities could be observed in the superficial zone of 'Grushovka Vernenskaya' and recently divided cells were visible (**Fig. 7C**).

The cells of the internal zone were much more vacuolated than the cells of the surface zones (**Fig. 7C, 7D**). Large electron-dense globules were often observed in the vacuoles, which may be an accumulation of polyphenols. In addition to vacuoles, a significant amount of small vesicles were present in the cytoplasm. The ER and mitochondria were well developed, plastids were small and rare. Some cells of both cultivars were in various stages of degradation and some were plasmolysed (**Fig. 7F**).

Shoot tips after one week culture following LN

After one week following LN shoot tip recovery was visually evident with greening and growth. On the light sections two types of shoot tips were observed. 1) Shoot tips with necrosis where the majority of cells were plasmolysed, and the cells of meristems and leaf primordia had yellow stained cytoplasm (**Fig. 8A**). Dividing cells were not apparent. These shoot tips are likely dead. 2) Shoot tips consisted of a basal stem of pre-existing shoots and a recovering new apex (**Fig. 8B**). These parts were clearly distinguishable. Cells of the pre-existing stem were mostly plasmolysed and with yellow-brown contents, whereas cells of recovering shoot tips had light blue cytoplasm, and distinct nuclei with nucleoli. In addition many cells were in various stages of mitosis (mostly metaphase and anaphase).

DISCUSSION

Understanding the damage or changes that occur in cryopreserved cells is an important part of understanding how cryopreservation protocols affect the viability of plant cells and organized shoot tips. The steps of CA, sucrose conditioning, PVS2 cryoprotection, and liquid nitrogen exposure each impose stresses on cells of the shoot tip and contribute to their death or recovery. Determining the cellular response to these steps may contribute to improving the success of protocols. Electron microscopic examination of apple apical meristem cells demonstrated some structural and functional intracellular changes during the cryopreservation protocol.

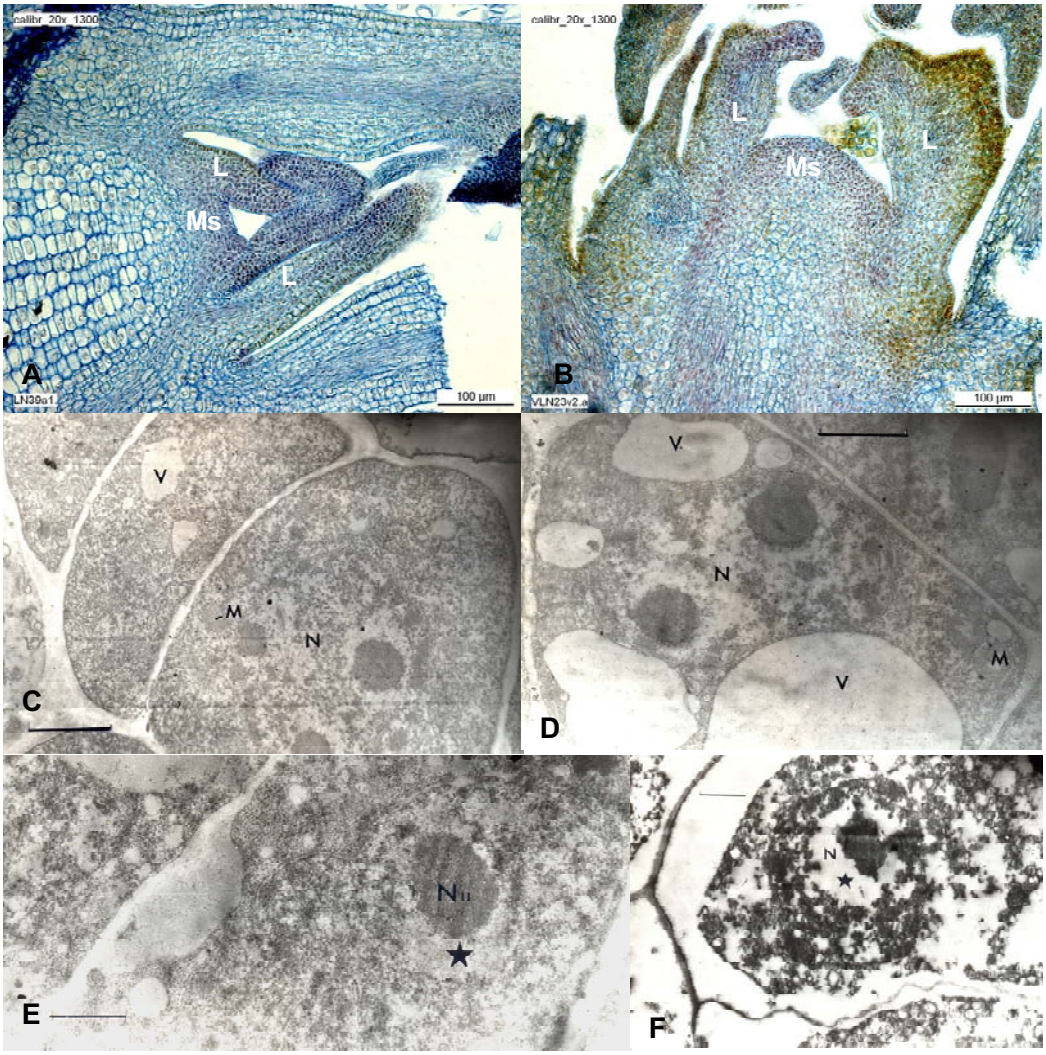

Fig. 7 Light and electron microscopy of shoot tips following LN. Longitudinal sections of 'Grushovka Vernenskaya' (**A**) and 'Voskhod' (**B**) shoot tips. Arrow indicates plasmolysed cells. Bar: 100 µm. Cell of superficial (**C**) and inner zone (**D**) of 'Grushovka Vernenskaya' meristem. Note nucleoli in nucleus. Bar: 2 µm. (**E**) Part of the cell of 'Voskhod' meristem superficial zone. Bar: 1 µm. (**F**) Plasmolysed cell of 'Voskhod' meristem inner zone. Starlet indicates chromonema-free area around nucleolus. L – leaf primordium; Ms – meristem; M – mitochondrion; N – nucleus; V– vacuole.

These were especially visible at CA, PVS2 treatment, and after rewarming.

CA and freezing responses of plant cells and tissues are documented by various types of microscopy. Immunofluorescence techniques allowed visualization of microtubule polymerization of CA and non-CA spinach cells (Bartolo and Carter 1991). Freezing caused depolymerization of microtubules in mesophyll cells of spinach in both CA and non-CA cells, however CA cells recovered fully within 1 h while non-CA cells recovered, then the microtubules depolymerized over time. Dogwood xylem-ray cells collected in winter and exposed to freezing temperatures exhibited contracted protoplasm under electron microscopic study (Ristic and Ashworth 1994). The cytoplasm appeared dense with vacuoles, and chloroplasts and mitochondria were apparent. Light gray droplets were seen in chloroplasts. The plasma membrane was appressed to the cytoplasm without vesicle formation. Occasionally pieces of broken plasma membrane were seen on the cell wall. Summer and spring ray-parenchyma cells exposed to freezing showed protoplasm contraction and fragmentation. In winter-collected mulberry buds and cortical parenchyma, immunocytochemical electron microscopy showed that WAP27 protein (a group 3 LEA protein) accumulated in vesicular ER in winter, but not in the summer cisternae form of ER (Ukaji *et al*. 2001). All these studies indicate significant changes resulting from CA.

After CA, several modifications occur in apple meristem cells. A change of cell size was evident for both genotypes (**Fig. 2**). Meristem cells of both cultivars were larger following CA than the controls. Large plastids with a well developed lamellar system and considerable accumulation of reserve compounds (starch grains or plastoglobules) appeared in the meristem cells (**Fig. 4C-E**). Increased metabolic activity was indicated by increased mitochondrial matrix density and length of cristae (**Fig. 4D**). In addition vacuoles with electron-opaque contents (**Fig. 4E**) and with electron-dense globules (**Fig. 4G**) appeared following CA of apple shoot tips. This electron-opaque content in vacuoles may represent accumulation of proteins, which were shown by Avetisova and Kadykov (1986) in vacuoles of plants from cold regions. Electron-dense globules may represent an accumulation of toxic polyphenolic compounds, which were also apparent in cells of banana meristems after LN exposure (Helliot *et al*. 2003). Apple tissues also produce large amounts of polyphenols, which oxidize as a reaction to stress. Field-collected banana buds contained plasmolysed cells and some plasmolysed cells were observed in the bases of apple leaf primordia and shoot tips (**Fig. 4A, 4B**). Increased starch and density of mitochondria, smaller vacuoles, and increased size of cells are all consistent with metabolic changes due to CA.

Increased osmoticum used as a pretreatment serves to dehydrate cells and to increase the concentration of sugars in the cells. In banana shoot tips, pregrowth on a high sucrose medium resulted in several cellular changes (Helliot *et*

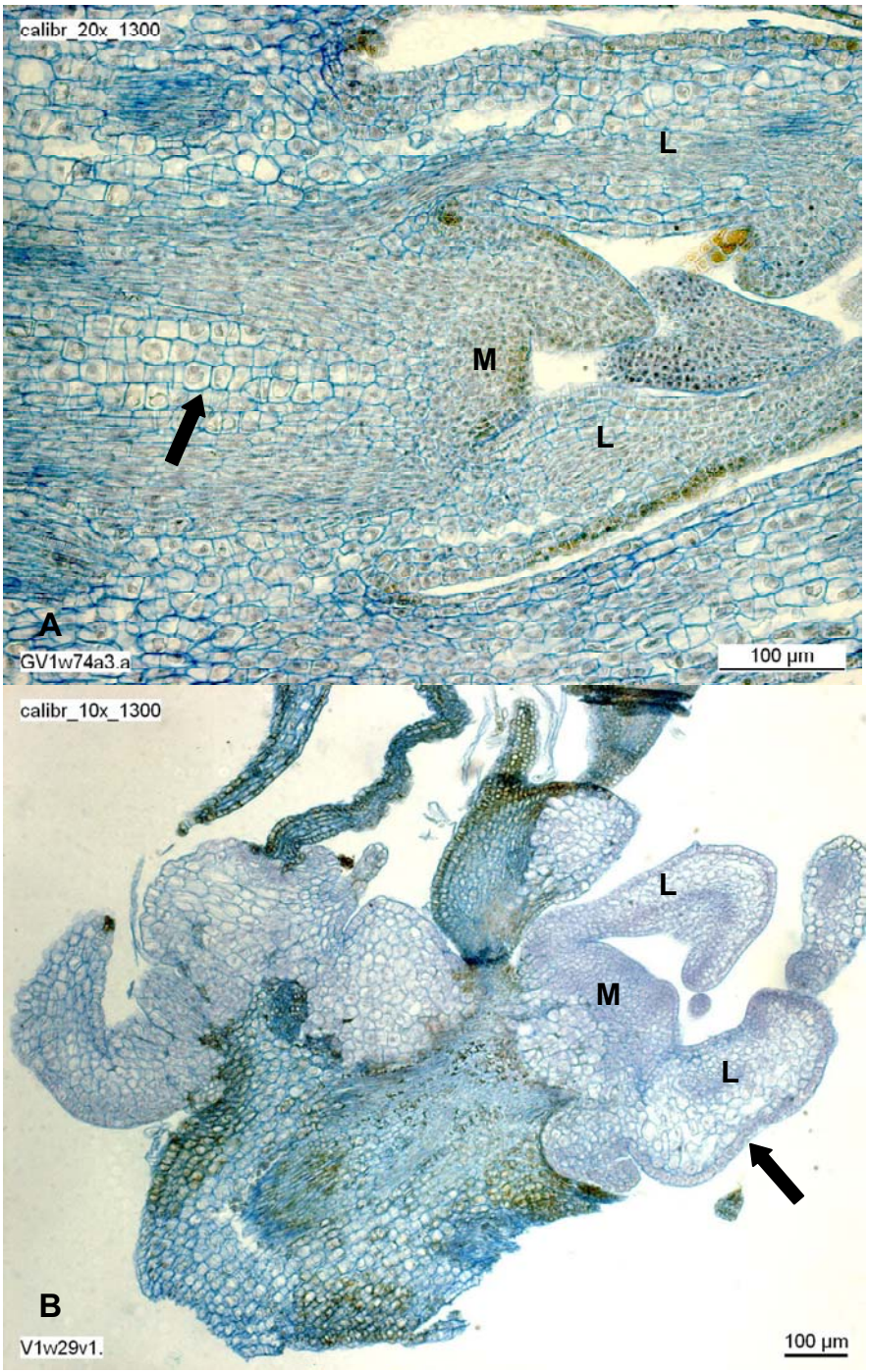

Fig. 8 Light microscopy of shoot tips after 1 week culture following LN. (**A**) 'Grushovka Vernenskaya', arrow indicates plasmolysed cells. (**B**) 'Voskhod', arrow indicates shoot regrowth. Bar: 100 µm. L – leaf primordium; M – meristem.

al. 2003). Large vacuoles split into smaller vacuoles, and plastids developed starch grains. Many organelles became swollen. The plasma membrane became undulated and membrane structures appeared in the cytoplasm. An EM study of *Panicum* cells exposed to mannitol indicated swelling of organelles and invaginations in the tonoplast (Gnanapragasam and Vasil 1992). Due to the reduced cell volume, the plasmalemma of *Panicum* cells became invaginated, and there was swelling of organelles, especially the ER. Neither of these studies included a CA step. In our study of apple meristems there were few differences in cell structure between CA and the sucrose pretreatment and the cells were larger than untreated cells indicating that the cell volume had increased rather than decreased. CA and osmotic treatments appear to have similar effects on these cells.

Cryoprotectant exposure is an additional stress for cells and overexposure to highly osmotic cryoprotectants is a common cause of cell death. In banana, PVS2-treated cells had minor to severe plasmolysis and the nuclei were less electron dense (Helliot *et al*. 2003). Following cryoprotectant exposure of *Panicum* cells, osmophilic granules were found on the plasma membrane of dead cells (Gnanapragasam and Vasil 1992). Lethally damaged cells had disrupted organelles. Optimally cryopreserved cells had a 3X increase in ER and dilated cisternae. Intracellular changes in apple cells were evident after PVS2 treatment, most of which represented synthetic-process activation, such as the appearance of one to two large nucleoli in the nuclei, and cup-shaped mitochondria twice the normal size (not illustrated). Some damaged cells occurred in the meristems of PVS2-treated cells in which only a giant nucleus remained (not illustrated).

The final steps of cryopreservation protocols, LN exposure and rewarming, may also result in cell damage. Highly vacuolized cells of banana had lethal damage following

liquid nitrogen exposure compared to those with small vacuoles (Helliot *et al*. 2003). Osmophilic globules were noted near the cell wall and electron-dense granules observed in the vacuole were suspected to be polyphenolics. Lacunae in the nucleoli indicated a loss of nucleolar activity. Most damaged meristematic cells suffered injury during PVS2 exposure or during the cooling/rewarming step. *Panicum* cells showed little damage two days after rewarming and looked normal after 10 days. Careful dehydration and cryoprotection were required to limit cell damage (Gnanapragasam and Vasil 1992). Date palm meristems do not withstand freezing in LN as intact structures and the surviving cells are located mainly on the surface of the apex. The central cells, as well as the ones located at the base, do not withstand freezing and their cell walls were broken and the plasmalemma was detached (Bagniol *et al*. 1992). In apple, after LN exposure and rewarming, cells of the internal zone were much more vacuolated than those of the superficial zone (**Fig. 7C, 7D**). Large electron-dense globules (most probably polyphenolic compounds) were often observed in the vacuoles and many small vesicles were observed in the cytoplasm. ER and mitochondria were well developed; plastids were small and rare. Some cells of both cultivars showed stages of degradation (**Fig. 7F**) and some were plasmolysed (**Fig. 7A**). Greater activation of the nucleoli, indicated by 2-3 nucleoli with prominent projections, was observed after recovery from liquid nitrogen exposure and no starch was in the small, rarely seen plastids (**Fig. 7C, 7D**).

Following LN and one week culture on recovery medium, some shoot tips showed features of necrosis observed on light sections: numerous plasmolysed cells in all parts of shoot tips and a change in staining of cells in the apical meristem and foliar primordia (**Fig. 8A**). Other shoot tips began to recover and form new leaf primordia (**Fig. 8B**). Pre-existing parts of these shoot tips consisted of plasmolysed and brown stained cells, but meristematic cells most likely withstood LN exposure and grew new shoot tips (**Fig. 8B**). These results are similar to those of Zeng *et al*. (2005) who found that papaya shoots that plasmolysed during cryopreservation treatments appeared normal 3 days after growth on recovery medium. Cells of sugar cane in the apical zone were alive following cryopreservation, while the basal cells were damaged or dead (Gonzalez Arnao *et al*. 1993). Cells of date palm showed characteristics of osmotic stress (Bagniol *et al*. 1992). Volk and Walters (2006) showed that water content of shoot tips exposed to 0.3 M sucrose was about 4 g H_2O gdm^{-1} while treatment with glycerol and sucrose decreased water content to about 2 g H_2O gdm^{-1} and PVS2 even lower, compared to the 4 to 6 g H_2O gdm^{-1} of control shoot tips. These levels are consistent with signs of cellular osmotic stress. They propose that exposure to PVS2 does not cause massive cell shrinkage, but rather desiccates the cells to the critical moisture content needed for successful vitrification. This fits well with the physical evidence from our study of apple shoot tips. The size of the treated cells actually increased rather than decreased, yet the cells survived vitrification. The cells of 'Grushovka Vernenskaya' were only slightly larger than 'Voskhod' cells for most treatments (**Fig. 2**), so cell size could not account for the higher recovery of 'Voskhod'. The cells of 'Voskhod' with less vacuolation may be less vulnerable to the stresses imposed by cryopreservation than those of 'Grushovka Vernenskaya' with more vacuoles.

Our earlier study showed that differences in the cold hardiness of the cultivars, as indicated by $LT_{50,}$ directly relate to recovery from cryopreservation (Kushnarenko *et al*. 2009). Both cultivars had the best regrowth after reaching the lowest $LT_{50.}$ Ultrastructural differences between the cultivars were apparent with CA. After CA an intense starch accumulation was noted inside plastids of 'Grushovka Vernenskaya' (**Fig. 4C, 4E**). In plastids of the cold-hardy cultivar 'Voskhod', small starch grains and two types of plastoglobules were observed that were not present in 'Grushovka Vernenskaya' (**Fig. 4D**). These differences indicate a metabolic difference between the cultivars, where large starch grains are the only storage product produced in 'Grushovka Vernenskaya' and may be less amenable to freezing than are the smaller starch grains and lipid globules of 'Voskhod'. At recovery following LN exposure, many nuclei of 'Grushovka Vernenskaya' had 2-3 nucleoli or a single nucleolus with projections (**Fig. 7C, 7D**). Most of the nuclei of 'Voskhod' had a single nucleolus without projections and a chromatin-free area around the nucleolus (**Fig. 7E**). In 'Grushovka Vernenskaya' cells, mitochondria were either small with dense, long cristae, or large with short cristae and a light central matrix. Mitochondria of 'Voskhod' cells were numerous and had a typical structure. The light central matrix and unusual mitochondrial structure of 'Grushovka Vernenskaya' mitochondria may indicate damage.

Overall this study confirms that cells with small vacuoles and small starch grains are more likely to survive cryopreservation. The steps of the cryopreservation process affect cell size and limit the amount of freezable water in the cells. This is evident in cell structure. The largest change in cell size came with cold acclimation, and the increased size was mostly maintained by sucrose and PVS2 treatments in both cultivars. Cold-acclimated cells had condensed chromatin and well developed plastids. Small starch grains appeared to be better for survival than large grains. Sucrose treatment produced plasmolysed cells in both cultivars but plastoglobules were only noted in cells of cold hardy 'Voskhod'. PVS2 treatment plasmolysed most basal cells, but meristematic cells appeared normal. After LN exposure basal cells were plasmolysed, but most meristematic cells were healthy. Vacuoles were small and rare in both cultivars. Damaged mitochondria were large with short cristae and a light matrix while healthy mitochondria were small with dense, long cristae. After one week of culture, new growth could be observed to arise from the meristematic tissues while the older basal cells were often damaged or necrotic.

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