Plant Cell Rep (2008) 27:241–250
DOI 10.1007/s00299-007-0467-8

CELL BIOLOGY AND MORPHOGENESIS

Vitrification–cryopreservation, an efficient method for eliminating *Candidatus Liberobacter asiaticus*, the citrus Huanglongbing pathogen, from in vitro adult shoot tips

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Received: 2 August 2007 / Revised: 1 October 2007 / Accepted: 5 October 2007 / Published online: 23 October 2007
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Abstract Huanglongbing disease (HLB), caused by *Candidatus Liberobacter asiaticus*, constitutes a most serious problem for the Chinese citrus industry. In this work, the use of vitrification-cryopreservation for eliminating *Ca. L. asiaticus* from naturally infected plants of several citrus species was investigated. Proliferating meristems were produced in vitro and excised tissue clumps were cryopreserved through vitrification using a plant vitrification solution 2. The health status of regenerated in vitro plants was checked by nested PCR. The putative HLB bacterial-free materials were subsequently re-tested after greenhouse acclimatization. Up to 98.1% of the plants obtained by cryopreservation were free from HLB bacterium, as compared with a sanitation rate of 25.3% yielded by conventional meristem tip culture. Light and electron microscopy observations of the meristem tips showed that the majority of the meristematic cells were injured either during the freezing/thawing step or during the osmotic dehydration step with plant vitrification solution 2. Only small areas of the meristematic dome survived the cryopreservation process, thereby increasing the probability of regenerating cells free of *Ca. L. asiaticus*. Large cells with big vacuoles and high water content, which are more likely to be infected by *Ca. L. asiaticus*, apparently cannot survive freezing in liquid nitrogen (LN). By contrast, small cells with dense cytoplasm located in the top layers of the meristem are more likely to escape invasion by *Ca. L. asiaticus* and can survive freezing in LN.

Keywords Citrus · Cryopreservation · Electron and light microscopy · HLB bacterium · Meristem culture · Vitrification

Abbreviations

BA	N6-Benzyladenine
DMSO	Dimethylsulfoxide
HLB	Citrus Huanglongbing
IBA	Indole-3-butyric acid
MT	Murashge and Tucker
PCR	Polymerase chain reaction
PVS2	Plant vitrification solution 2, mixture of glycerol, ethylene glycol, DMSO and sucrose in MT medium

Introduction

Citrus is one of the most economically important fruit crops and is widely grown in the tropical, subtropical, and

Communicated by H. Judelson.

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even temperate zones of the world. Today, the growing areas of citrus account for more than 7.5 million hectares throughout the world, thus making it one of the most widely grown fruit crops worldwide. Diseases constitute a major hindrance to the development of citrus industry. Citrus Huanglongbing (HLB), previously known as "greening", is one of the most severe diseases and widespread. The HLB agent in Asia is *Candidatus Liberobacter asiaticus* (Jagoueix et al. 1994, 1997), a phloem-limited, noncultured, gram-negative bacterium (Garnier et al. 1984) that infects different citrus species, leading to poor fruit quality, reduced productivity, decline and shortening of tree life (Bové 2006). To date, although HLB is controlled primarily through the production of healthy plants, reports on its elimination by laboratory methods are few. In practice, shoot-tip grafting (Zhao et al. 1986; Song et al. 1999) and heat treatment combined with antibiotics (Zhao et al. 1981; Luo 1991) have been successfully used in the past for HLB therapy. However, with shoot-tip grafting, the success of graft take is proportional to the size of the excised meristem tip whereas the pathogen's eradication rate is inversely proportional to the size of the explant (Faccioli and Marani 1998), generally about 0.1–0.2 mm for citrus. Thermotherapy is time-consuming and due to heat damage frequently has only a low success rate (Goheen 1988; Leonhardt et al. 1998).

Cryopreservation of shoot tips offers long-term storage capacity, maximum stability of phenotypic and genotypic characteristics of stored germplasm, and minimal storage space and maintenance requirements. Thus, besides representing an ideal means for the long-term conservation of germplasm (Engelmann 1997), cryopreservation protocols have been developed for a continuously increasing number of plant species. Cryopreservation requires the use of shoot tips and therefore might function as a method for pathogen elimination, and therefore has attracted attention as a possible method for virus elimination. Brison et al. (1997) were the first to eliminate successfully *plum pox poty virus* (PPV) from an interspecific Prunus rootstock, using cryopreservation of shoot tips. Later, Helliot et al. (2002) reported the successful elimination of *cucumber mosaic virus* (CMV) and *banana streak virus* (BSV) from *Musa* spp. by cryopreservation. Wang et al. (2003) also successfully eliminated *grapevine virus* A from in vitro-grown shoot tips of *Vitis vinifera* L by cryopreservation. Here, we report on the elimination of *Ca. L. asiaticus* from in vitro adult citrus shoot tips by cryopreservation and investigate the structural changes in meristem tips that occur during cryopreservation by light and electron microscopy. To our knowledge, this is the first published study on the elimination of the HLB bacterium by cryopreservation.

Materials and methods

Plant material

Five citrus accessions, naturally infected by *Ca. L. asiaticus* collected in Guangzhou (Guangdong province, China), were used in this study: Hongjiang and Luogang sweet oranges (*Citrus sinensis* Osb.), Beijing lemon [*C. limon* (L.) Burm.], Ponkan (*C. reticulata* Blanco) and Shatianyou pomelo (*C. grandis*).

Production of citrus adult axillary shoot-tips

The citrus species naturally infected by HLB bacterium were maintained in greenhouses. To produce more axillary shoot tips, green twigs from citrus with at least two axillary buds were collected. After sterilization, axillary buds were excised and maintained on modified MT solid medium (Murashge and Tucker 1969) supplemented with 2.2 μM N6-benzyladenine (BA), 1 μM indole-3-butyric acid, 3% sucrose and 0.75% agar. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All explants were maintained at a temperature of 24 ± 2°C under a 16 h photoperiod with a light intensity of 45 μE m^{-2} s^{-1} provided by cool-white fluorescent tubes. Axillary shoot tips, about 0.5–2.0 mm in length, consisting of an apical dome with 1–3 leaf primordia were excised from the stem of 25-day-old plantlets (3–5 cm in height) that had developed from excised buds and used for cryopreservation.

Cryopreservation

Preculture

Axillary shoot tips were transferred onto the preculture medium, i.e. a MT medium containing 0.4 M glycerol and 0.3 M sucrose. These cultures were maintained for 3 days.

Loading

Pre-cultured axillary shoot tips, 0.5–2.0 mm in size were excised and kept for 50 min at room temperature in a loading solution of 2 M glycerol and 0.4 M sucrose dissolved in MT, pH 5.8. Then, the loading solution was replaced by ice-cooled and filter-sterilized PVS-2 solution (Sakai et al. 1990) containing 30% (3.26 M) glycerol, 15% (2.42 M) ethylene glycol, 15% (1.9 M) DMSO and 0.4 M sucrose dissolved in MT medium (pH 5.8). Shoot tips were immersed in the PVS-2 solution for 60 min at 0°C, transferred to 2-ml cryotubes and immersed into LN for a minimum of 1 h.

Thawing and deloading

Cryotubes containing shoot tips were rapidly thawed in a warm water bath (40°C) for 90 s. The PVS-2 solution was replaced by two changes of the filter-sterilized deloading solution (1.2 M sucrose dissolved in MT medium, pH 5.8), with each lasting 10 min at room temperature.

Recovery

Control shoot tips were loaded, dehydrated and deloaded but not frozen, and frozen shoot tips were removed from the deloading solution and placed in 9-cm plastic petri dishes onto two sterile filter papers to absorb the excess deloading solution, then transferred onto regeneration MT medium containing 2.2 μM N6-benzyladenine (BA). The first week of culture was always in the dark, with later cultures (for survival assessments) maintained in the light.

Meristem tip culture

Meristems tips with one to three leaf primordia were excised from 3-week-old stock cultures and grown in a 9-cm Petri dish containing 30 ml of solid MT supplemented with 2.2 μM N6-benzyladenine (BA). The Petri dishes were maintained in the dark at 24°C for 2 days, and then transferred to the light conditions for further growth. To investigate the effect of meristem tip size on survival and HLB bacterium elimination, explants of three different sizes (0.1, 0.3, and 0.5 mm) were used. Six weeks later, shoots longer than 3 mm were transferred to phytohormone-free solid MT, to regenerate whole plants.

Plant regeneration

Survival was estimated as the percentage of the total number of shoot tips that showed a green color after 3 weeks culture. Growth was evaluated by measuring shoot elongation and the number of fully opened leaves after 4 weeks. After survival assessment, elongated shoots longer than 5 mm were grafted in vitro onto citrange seedlings that had been prepared previously. After 4 weeks, grafted plantlets were re-grafted to citrange seedlings and grown under greenhouse conditions.

Control treatment

Controls consisted of individual axillary shoot tips of the same size as those used for cryopreservation, which were excised under the light microscope and then grown and regenerated under the same conditions as the shoots that survived cryopreservation.

Detection of HLB pathogen by PCR and Nested-PCR

The health of the plant materials at different stages was determined by PCR and nested PCR protocol. The primers used for the detection are listed in Table 1.

At the beginning of the experiments, stock cultures from which shoot tips had been excised for cryopreservation and meristem tip culture were tested by PCR to ensure that only those buds confirmed to be infected with the HLB pathogen were selected for experiments (Fig. 2). The health status of plantlets recovered from cryopreservation and meristem-tip culture was initially checked at the stage of in vitro plantlets by nested PCR, which in previous investigations (Ding et al. 2004, 2005) had proven 10^4 times more sensitive than single-step PCR. The putative HLB bacterial-free plantlets were re-tested 2 months after their establishment in greenhouse conditions (Fig. 3), with rates of bacterial elimination expressed as percent of total plantlets tested.

Light microscopy and electron microscopy observation

The following samples were prepared for microscopy: (a) axillary shoot-tips from stock cultures; (b) axillary shoot-tips grown for 3 days on the MT medium supplemented with 0.3 M sucrose and 0.4 M. glycerol (pre-culture); (c) pre-cultured shoot tips loaded with 2 M glycerol and 0.4 M sucrose, followed by 1 h dehydration with PVS-2 (Sakai et al. 1990); (d) same as (c), subsequently exposed to LN for 1 h; (e) cryopreserved shoot tips after a 1 week recovery on regeneration MT medium containing 2 μM N6-benzyladenine (BA).

Shoot tips were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 under vacuum (three exposures of 5 min each), kept overnight at 4°C and post-fixed with a 1% osmic acid in 0.1 M phosphate buffer, pH 7.2. Samples were dehydrated through graded ethanol (50, 70, 80, 90 and 100%) in 0.85% saline (1 h on ice for each ethanol dilution) and kept in 100% ethanol overnight at 4°C. Embedding was in Epon 812. Sections for light microscope observations (Olympus AX 70) were cut with a LKV-Uct microtome (LKB Bromma) with glass knives and stained with toluidine blue. For electron microscopy, thin sections were collected on Formvar-coated 300 mesh grids (Maxtaform HR 25, Cu/Rh), post-stained with uranyl acetate (2% in 50% alcohol) for 20 min followed by lead citrate for 2 min (Reynolds 1963) and observed with a JEM-1010 electron microscope.

Table 1 Primer sequences used in this work, designed on GenBank sequence M94319

Single-step PCR primers/nested PCR out primers	F1:5′-TGAATTCTTCGAGGTTGGTGAGC-3′	Homologous with nt 39–61
	R1:5′-AGAATTCGACTTAATCCCCACCT-3′	Complementary with nt 573–551
Nested PCR inner primers	F2: 5′-GCGTTCATGTAGAAGTTGTG-3′	Homologous with nt 134–153
	R2: 5′-CCTACAGGTGGCTGACTCAT-3′	Complementary with nt 533–514

Fig. 1 In vitro culture of adult citrus axillaries infected by HLB bacterium. **a** Luogang sweet orange, **b** Hongjiang sweet orange, **c** Beijing lemon, **d** Ponkan, and **e** Shatianyou pomelo

Results and discussion

Axillary shoot-tips culture and PCR detection of HLB bacterium

In primary culture, all shoot tips from the five citrus accessions grew well. There were up to 3–4 axillary buds on each explant (Fig. 1a–e), which were tested by single step PCR for the presence of HLB bacterium (Fig. 2). Buds from primary cultures, confirmed to be infected with HLB bacterium were used for cryopreservation and meristem-tip culture. Figure 3 shows the health status of Hongjiang sweet orange plantlets obtained from meristem-tip culture or cryopreservation tested 2 months after their establishment under greenhouse conditions.

Effects of shoot tip size used for cryopreservation on plantlet survival and HLB bacterium elimination

Unless otherwise stated, Hongjiang sweet orange was used in this experiment. Shoot tips of four different sizes (0.5, 1.0, 1.5 and 2.0 mm) were excised and their survival estimated. As shown in Table 2, the highest survival rate (about 85.2%) of cryopreserved shoot tips was obtained when they were 1.0–1.5 mm in length. Shorter or longer explants survived to a lesser extent. In contrast, the frequency of HLB bacterium elimination from regenerated plantlets was similar (94.2–98.1%), regardless of the initial size of the explants.

Effects of explants size for regeneration in culture and HLB bacterium elimination

The regeneration ability of meristem tips decreased with their size. The 0.1 mm explants were totally unable to regenerate (Table 3). Although 100% of the 0.5 mm meristem tips did regenerate, all plantlets were still HLB bacterial-positive by nested PCR. The best results with respect to HLB bacterium elimination (25.3%) and highest survival ratio were obtained with 0.3 mm explants.

Comparisons of cryopreservation and meristem tip culture on plantlet survival and HLB bacterium elimination

For cryopreservation and meristem tip culture, 1.0 mm and 0.3 mm explants were used, respectively. Survival ratio was 80.1% for cryopreservation and 72.2% for meristem tip culture (Table 4). Frequency of HLB elimination by cryopreservation was 96.3%, higher more than four times than that obtained by meristem tip culture (23.1%). Surviving shoot tips from cryopreservation showed a green color after about 14 days culture (Fig. 4a) and were more than 3 mm long a month later (Fig. 4b). Six weeks later, these explants were transferred to half-strength MT solid medium with no plant growth regulators to regenerate whole plants (Fig. 4c). Some of the survived shoot tips were grafted onto trifoliate orange seedlings for plant regeneration 1 month after cryopreservation (Fig. 4d). Normal shoots developed within 1 month (Fig. 4e), after

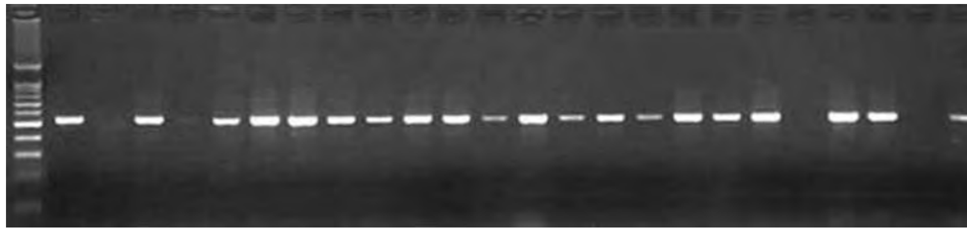


Fig. 2 PCR detection of HLB bacterium in cultured adult citrus shoot-tips. *M* 100 bp DNA ladder marker, *lane1* HLB positive control, *lane 2* negative control, *lanes 3–6* buds of Luogang sweet orange, *lanes 7–11* buds of Hongjiang sweet orange, *lanes 12–15* buds of Beijing lemon, *lanes 16–19*, buds of Ponkan, *lanes 20–24* buds of Shatianyou pomelo 172 × 40 mm

Fig. 3 Nested PCR detection of HLB bacterium in shoot tips that survived cryopreservation. *M* 100 bp DNA ladder marker, *lane 1* HLB positive control, *lane 2* negative control, *lanes 3–9* shoot tips of Hongjiang sweet orange from meristem-tip culture, *lanes 10–17* shoot tips of Hongjiang sweet orange from cryopreservation

Table 2 Effect of shoot tip size on survival and HLB bacterium elimination from sweet orange cv. Hongjiang following cryopreservation

Size of shoot tips (mm)	Survival (%)	HLB bacterium elimination from plantlets (%)
0.5	52.1 ± 5.2c	94.2 ± 4.5a
1.0	85.2 ± 1.6a	98.1 ± 3.3a
1.5	70.2 ± 4.6b	97.0 ± 3.9a
2.0	50.1 ± 5.3c	95.1 ± 6.3a

Thirty samples were used in each of three replicates. Means followed by the same letters (a, b, c) within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. Data are presented as mean ± SE

Table 3 Effect of meristem tip size on survival in culture and on HLB bacterium elimination from sweet orange cv. Hongjiang

Explants size (mm)	Survival (%)	HLB bacterium elimination from plantlets (%)
0.1	0c	–
0.3	69.2 ± 2.7b	25.3 ± 1.4a
0.5	100a	0b

Thirty samples were used in each of three replicates. Means followed by the same letters (a, b, c) within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. Data are presented as mean ± SE

which the grafted plantlets were transferred to small pots for further growth (Fig. 4f–g). Nearly 100% of the plantlets became established under greenhouse conditions. The leaf morphology of plants regenerated from cryopreserved shoot tips did not differ from that of controls.

Effects of genotype on plantlet survival after cryopreservation and HLB bacterium elimination

To compare the effect of different genotypes on survival rate and HLB elimination following cryopreservation, five citrus species (Luogang Sweet orange, Hongjiang Sweet orange, Ponkan, Shatianyou pomelo and Beijing Lemon) infected by HLB bacterium were examined. Shoot tips of the size 1.0 mm were subjected to cryopreservation as described previously. The results showed that the explants of the different citrus genotypes tested had fairly high survival rates, ranging from 76% (Ponkan) to about 83.4% (Shatianyou pomelo); success in HLB bacterium elimination was about the same with all the genotypes (90.9–94.3%) (Table 5).

Cell modifications in the meristematic dome of shoot tips during cryopreservation

To investigate the mode of action of cryopreservation, structural observations were made on cryopreserved highly proliferating meristem and compared with control meristem (Fig. 5). Meristematic cells were small in size (approx. 10 µm in diameter) and had high nucleo-cytoplasm ratio typical of actively dividing cells (Fig. 5a). Previous light microscopic observations revealed that this nucleo-cytoplasm proportion decreases progressively with increasing distance from the meristematic dome (Helliot et al. 2002). Observations by electron microscopy revealed the nucleolus was very distinct in the round nucleus, which was located in dense cytoplasm containing many small vacuoles. After 3 days pre-culture, slight abnormalities were observed in many cells, for example slight

Table 4 A comparison of the effect of cryopreservation and meristem tip culture on the survival and HLB bacterium elimination from sweet orange cv. Hongjiang

Methods	Survival (%)	HLB bacterium elimination from plantlets (%)
Vitrification	80.1 ± 1.8a	96.3 ± 1.9a
Meristem culture	72.2 ± 2.1b	23.1 ± 1.6b

Shoot tips used for vitrification were 1.0 mm in size. Meristems used for meristem culture were 0.3 mm in size. Thirty samples were used in each of three replicates. Means followed by the same letters (a, b, c) within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. Data are presented as mean ± SE

Table 5 Effects of citrus genotype on explant survival and HLB bacterium elimination

HLB material	Survival (%)	HLB bacterium elimination from plantlets (%)
Luogang Sweet orange	78.9 ± 3.7b	93.4 ± 3.4a
Hongjiang Sweet orange	82.6 ± 2.5a	91.7 ± 2.0a
Ponkan	76.0 ± 1.7b	92.9 ± 1.6a
Shatianyou pomelo	83.4 ± 2.3a	94.3 ± 1.8a
Beijing Lemon	69.3 ± 3.6c	90.9 ± 3.1a

Means followed by the same letters (a, b, c) within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test

plasmolysis occurred in most cells in meristematic domes (Fig. 5b). The pre-culture of proliferating meristems performed with an elevated concentration of sucrose was employed to induce tolerance to the dehydration stress caused by exposure to PVS-2 solution. This mitigates the subsequent injurious effects of the PVS-2 solution, and is necessary for successful cryopreservation (Helliot et al. 2003). The cell structure changed after loading and PVS-2 treatment before immersion in LN, because many cells, especially those of the stem axis, showed clear signs of plasmolysis (Fig. 5c). After LN immersion and thawing, plasmolysis reached its peak, and most cells showed freezing injuries (Fig. 5d–e). Except for mild plasmolysis, cells in dome remained viable for 7days after regeneration of the surviving shoot tips (Fig. 5f).

The localization of damaged cells indicated that freezing injuries were generally observed in cells with more vacuolization. In fact, freezing injury results primarily from intracellular water crystallization, which occurs during freezing in LN and/or thawing. In our cryopreserved explants, small islands of surviving cells, which had the sane outward aspect of the controls, were identified in leaf primordia and in the tunica of the meristematic dome. These small areas of surviving cells, observed also by Helliot (1998) in cryopreserved meristems of plum, maintained their structural organization and capacity for active division, thus leading to the regeneration of whole plants (Haskins and Kartha 1980). Light microscopy showed that cryopreservation acted as a micro-scalpel, as it kept alive a few layers of

Fig. 4 Cryopreservation and regeneration of citrus shoot tips. **a** Normal growth of survived shoot tip after cryopreservation. **b** Shoot tip regeneration 1 month after cryopreservation. **c** Shoot tip regeneration 6 weeks after cryopreservation. **d** Shoot tip grafting on a trifoliate citrus seedling 1 month after cryopreservation. **e–f** Outcome of shoot tip grafting after 1 (**e**) and 2 months (**f**). **g** Regenerated plantlet, transplanted in a small pot

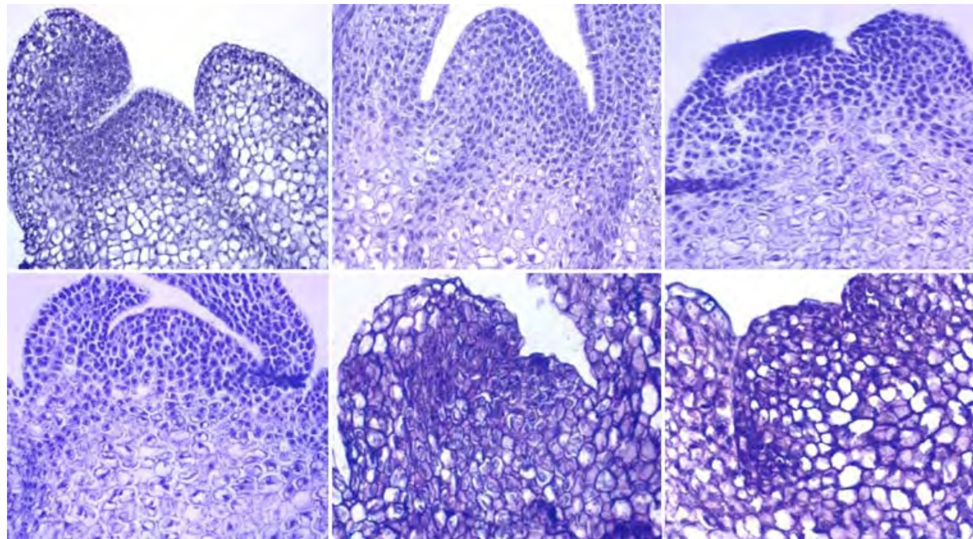


Fig. 5 Modifications of cells of the meristematic dome of citrus shoot tips following cryopreservation (×400) **a** Normal cell structure. **b** Slight plasmolysis after 3-day preculture. **c** More prominent plasmolysis after loading and PVS2 treatment, before immersion in liquid nitrogen. **d** Cell structure changes after thawing (plasmolysis more advanced). **e** Severe cell plasmolysis and death of a heavily damaged shoot tip. **f** Survived shoot tip seven days after regeneration, showing apparently normal cells in the meristematic dome

meristematic cells but killed the more hydrated parenchyma cells (Wang et al. 2003).

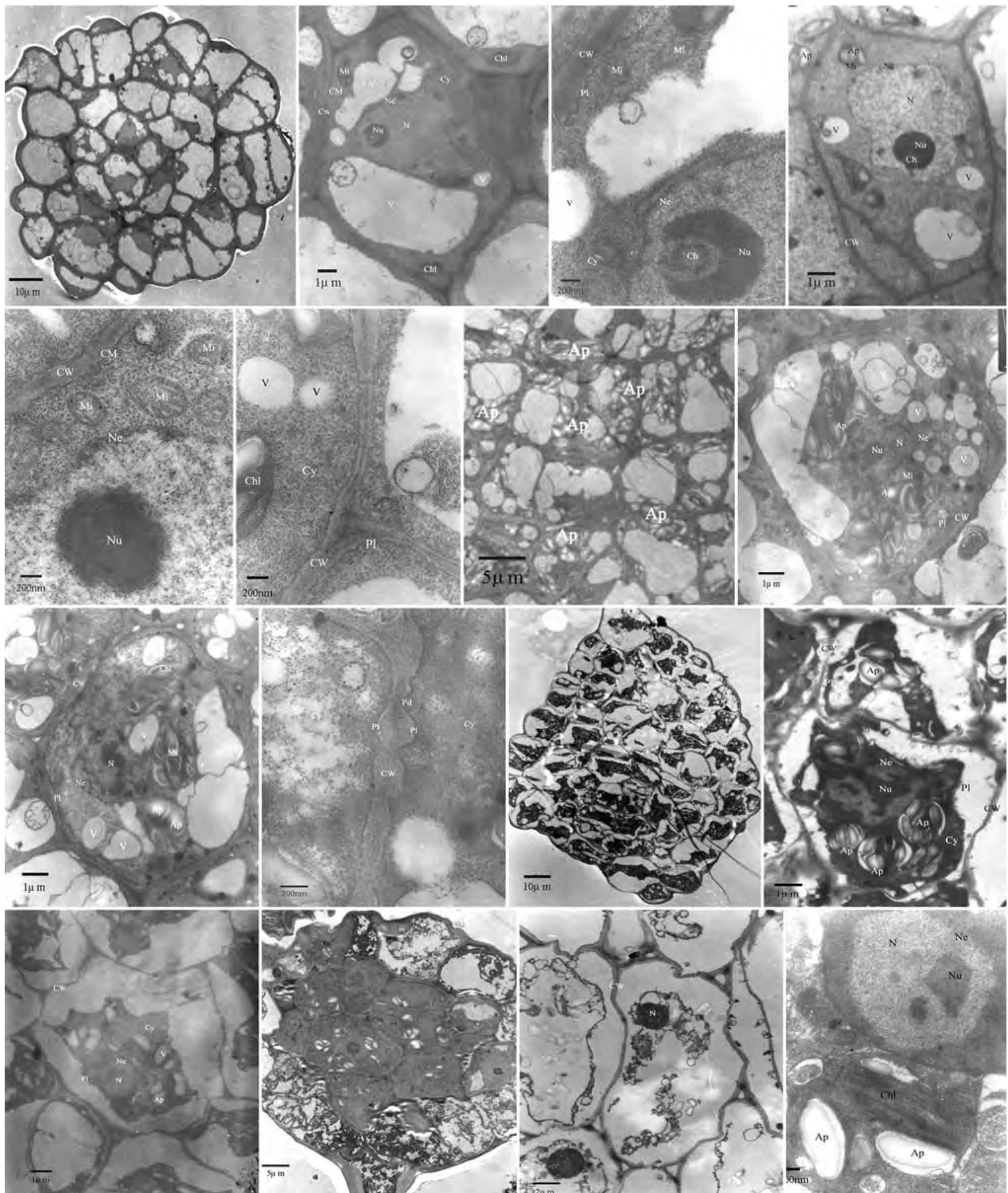
Ultrastructural changes of shoot tip cells associated with cryopreservation

In order to improve the protocol for conservation of citrus germplasm and HLB bacterium elimination, the mode of action of cryopreservation needs to be understood. Therefore, ultrastructural observations on cryopreserved highly proliferating meristems were made and compared with control meristems.

A characteristic of meristematic cells of the controls as seen under the electron microscope was their small size (approx. 20–30 μm in diameter) and high nucleo-cytoplasm ratio (Fig. 6a) that, as previously reported by Helliot et al. (2002), decreases with increasing distance from the meristematic dome. These cells had well-preserved nuclei with prominent nucleoli and small patches of heterochromatin at the periphery. The cytoplasm contained many small vacuoles and a large number of organelles (i.e. dictyosomes, proplastids and mitochondria) typical of a highly active cell metabolism (Fig. 6b–c). Proliferating meristems pre-cultured on sucrose-containing MT medium showed numerous ultrastructural changes, for example, fragmentation of large vacuoles into smaller ones, differentiation of proplastids into amyloplasts (Fig. 6d–e), and undulation of the plasma membrane caused by partial plasmolysis (Fig. 6f). In the present study, cell solute concentration was obtained in a 2 h treatment with a solution (PVS-2) containing high concentration of penetrating cryoprotectants (dimethylsulfoxide and glycerol) and osmotically active compounds (ethylene glycol and sucrose). Ultrastructural changes of shoot tips were therefore observed after loading and PVS2 treatment. Light (Fig. 6h), mild (Fig. 6i) or severe (Fig. 6j) plasmolysis of the cells of the corpus after a 2 h cryoprotection occurred in different samples. Severely plasmolysed cells had probably lost contact with neighboring cells since intercellular connections through plasmodesmata were broken (Helliot et al. 2003).

Cryopreservation affected severely or killed many cells as shown in Fig. 6k. Localization of damaged cells indicated, as reported by Helliot et al. (2002), that freezing injuries were linked with increased vacuolization. The key to successful cryopreservation thus lies more in the induction of tolerance towards dehydration, rather than tolerance to the freezing process itself. In this study, the expansion of the nuclear cisternal space was observed, which affected the structure of the nuclear envelope sometimes resulting in its fragmentation (Fig. 6l). Vacuole-like electron lucent areas were present in some nucleoli (Fig. 6m), which could result either from a cessation of the nucleolar activity or the formation of small ice crystals (Tannoury and Vintejoux 1997; Helliot et al. 2003).

Meristematic tissues observed 1 week after recovery from thawing comprised cells with a well-preserved structural organization (Fig. 6p) that were surrounded by layers of heavily damaged or dead cells (Fig. 6n, o). These small areas of surviving cells, previously observed by Helliot (1998) on cryopreserved plum meristems, keep their capacity for active division (Haskins and Kartha 1980), leading to regeneration into whole plants.



Results of our observations with the light and electron microscope on cryopreserved citrus explants agree with those from the literature (Tannoury and Vintejoux 1997; Haskins and Kartha 1980; Helliot 1998; Helliot et al. 2003) that the chance of explants regenerating are fairly high (up to 85%) if the right size of explants is chosen

Fig. 6 Ultrasctuctural changes of citrus shoot tip cells during cryopreservation. **a–c** Control cells. **a** Normal ultrastructure of whole shoot-tip. **b** Normal ultrastructure: Cell wall, nucleolus, nucleus envelope, vacuole and other cell organelles. **c** Nucleolus, nucleus-envelope, mitochondria. **d–f** Changes of citrus shoot-tips ultrastructures after preculture. **d** Entire cell ultrastructure: smaller vacuoles and a few amyloplasts. **e** Cell wall, nucleolus, nucleus envelope. f The undulations of the plasma membrane caused by partial plasmolysis. **g–j** Changes of citrus shoot-tips ultrastructures after loading and PVS2 treatment. **g** Proliferation of amyloplasts. **h** Ultrastructure of the cell disrupted: cell organelles in good condition. **i** Slightly plasmolysis. **J** The ultrastructure of cell hurt harder: nucleolus dispersed; other cell organelles well kept. **k–m** Changes of citrus shoot-tips ultra-structures after LN treatment. **k** Entire shoot-tip ultrastructure: obvious plasmolysis. **l** Plasmolysis of cell with entire structure. **m** Severe plasmolysis of cells. **n** Nucleolus and chloroplast of cell in good condition. **n–p** Changes of citrus shoot-tips ultrastructures after regeneration. **n** Entire shoot-tip ultrastructure: cells outside damaged; cells inside in good statue for regeneration. **o** Cell organelles broken. **p** Recovered cell ultrastructure resemble control cells. *Ap* Amyloplast, *Ch* chromatin, *Chl* chloroplast, *CM* cell member, *CW* cell wall, *Cy* cytoplasm, *Pl* plasmalemma, *Mi* mitochondria, *N* nucleus, *Ne* nucleus envelope, *Nu* Nucleolus, *Pd* plasmodesma, *V* vacuole

(1.0–1.5 mm in length) and the vitrification procedure is properly done. In terms of sanitation, regardless of the citrus genotypes tested, cryopreservation yielded a much higher rate of elimination of *Ca. L. asiaticus* (up to 98.1%) than the classical in vitro meristem tip culture (25.3%). In conclusion, this is the first report of the use of cryopreservation of adult shoot tips as a successful tool for eliminating the HLB bacterium from citrus. Relative to meristem culture, cryopreservation yields a much higher rate of viral elimination and avoids the difficulties involved in excising very small meristems and in plant regeneration. Moreover, vitrification techniques are easy to perform, facilitate the treatment of large amounts of plant material, and do not require expensive equipment. Cryopreservation appears to be a very promising procedure for sanitation of citrus germplasm from the HLB bacterium and, possibly, other infectious agents. Studies on the elimination of other citrus pathogens via the cryopreservation of shoot tips are currently underway in our laboratory.

Acknowledgments This study was supported by China Ministry of Education (No. IRT0548), National Natural Science Foundation of China (No. 30700550), Key Plan of Guangdong province (No.2003B21601), and Key Plan of Hubei province (No.2006AA2 03B05). We especially thank Prof. G.P. Martelli, University of Bari, Italy and Prof. Tom hsiang, University of Guelph, Canada, for critical reading of the manuscript.

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