BIOLOGIA PLANTARUM 46 (1): 1-6, 2003

Cellular damage to the callus cells of potato subjected to freezing

M.A. ANJUM*

Department of Horticulture, Univ. College of Agriculture, Bahauddin Zakariya University, Multan 60800, Pakistan

Abstract

Callus cells of potato (*Solanum tuberosum* L.) cv. Désirée were exposed to various subzero temperatures and examined for the freezing damage. In the cells subjected to -3 °C, plasma membranes appeared to be intact, while tonoplast seemed to be damaged and organelles to be swollen. After freezing at -6 °C, the damage became severe and plasma membranes were ruptured. After exposure to -10 °C, the damage was so severe that the cell organelles could not be recognised and cytoplasm became fragmented.

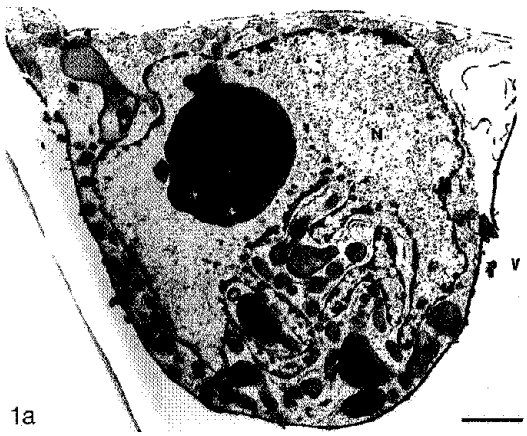
Additional key words: cell organelles, cytoplasm, electron microscopy, freezing damage, plasma membranes, *Solanum tuberosum*.

Introduction

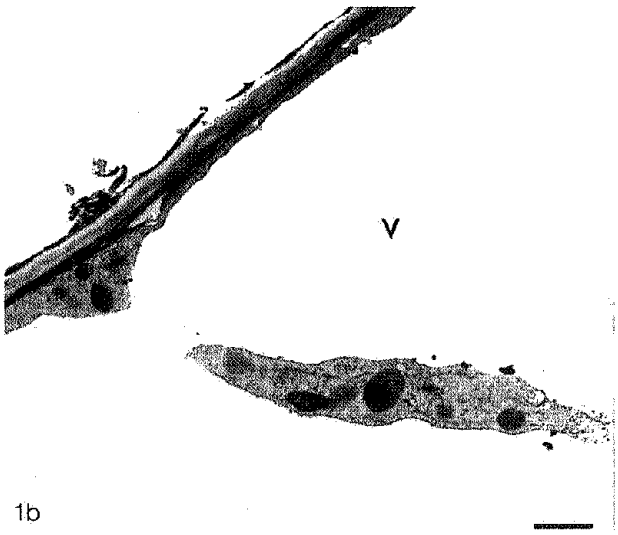
Freezing temperatures can cause injury to the plant cells through changes in the fluidity of membranes, which may disrupt ion transport, denature proteins, and induce imbalance in normal metabolism (Lindsey and Jones 1989). Freezing injury depends on the rate of freezing and thawing and the length of the freezing period. However, rapid cooling does not necessarily involve higher injury. Plants may avoid freezing stress by the ability to supercool. Therefore, in case of involved supercooling, there is less injury during rapid cooling (*e.g.* in tree buds). Rapid thawing may also affect freezing injury, although this has been observed less frequently than injury due to rapid cooling (Levitt 1980). Freezing injury itself consists of two main types; primary freezing injury and secondary freezing injury. Primary freezing injury is due to intracellular (or intra-protoplasmal) freezing, which results in rupture of the cell membranes by internal ice crystals, with the destruction of the semipermeability essential for life. However, intracellular injury has rarely been observed in nature because the rate of freezing is not too rapid. Secondary freezing injury is due to extracellular freezing as there is no direct contact between ice and cytoplasm. Damage occurring to the cell membranes during extracellular freezing is presumably a result of mechanical stress and/or freeze-dehydration (Li and Fennell 1985).

Leaflets of *S. tuberosum* show freezing injury at -3 °C but can be supercooled to -4 °C for several days with no apparent injury. Several other *Solanum* species can also survive supercooling for shorter periods below their freeze-killing temperatures (Rajashekar *et al.* 1983). After a damaging freeze-thaw cycle, the first sign of freezing injury in potato is a water-soaked appearance, which is due to infiltration of intercellular spaces with liquid and loss of turgor. It is assumed that this infiltration of the intercellular spaces is the result of rupture or loss of semipermeability of the cell membranes (Li and Fennell 1985). When *S. tuberosum* leaflets were frozen to -3 °C and examined in an electron microscope, the cell membranes, chloroplasts, mitochondria and nuclei appeared to be normal when compared with those of unfrozen cells, despite 75 % leakage of cellular ions. When the temperature was further decreased, to a point below that initiating damage, swelling of cytoplasm, including chloroplasts, and mitochondria, and finally physical damage was observed in both *S. tuberosum* and *S. acaule* (Palta *et al.* 1982). The plant cells in various parts are highly organised and the growing conditions also influence the plant development. This may result in variable cell wall thickening affecting the rate of freezing and ultimately the cellular damage. Therefore, in the present studies, the callus cells were selected to observe the freezing damage in potato.

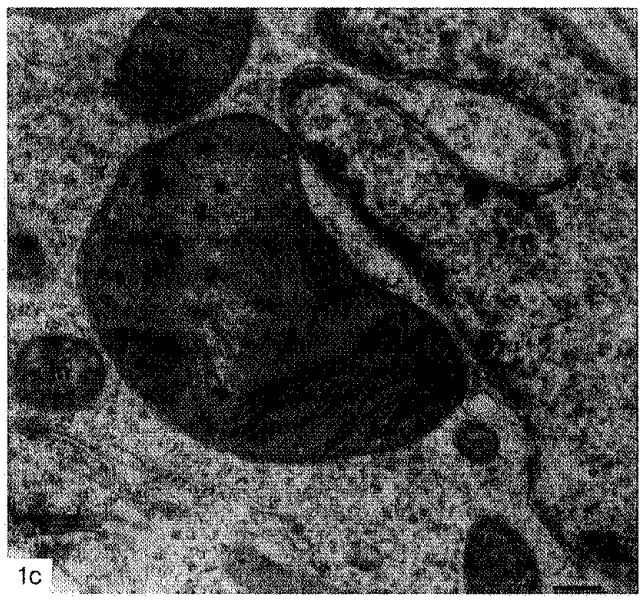
Received 20 April 2001, *accepted* 9 January 2002.
Acknowledgements: The author wishes to thank Prof. T.A. Villiers, Department of Biological Sciences, University of Salford, UK, for his valuable advice and is also grateful to the Ministry of Education, Government of Pakistan, Islamabad for financial support.
*Present address: Laboratory of Horticulture, Department of Life Sciences, Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan; fax: (+ 92) 61 9210098, e-mail: anjumbzu@yahoo.com



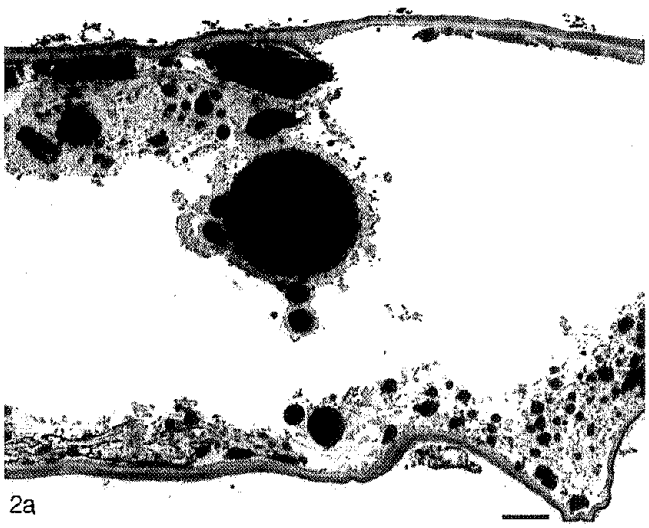
1a



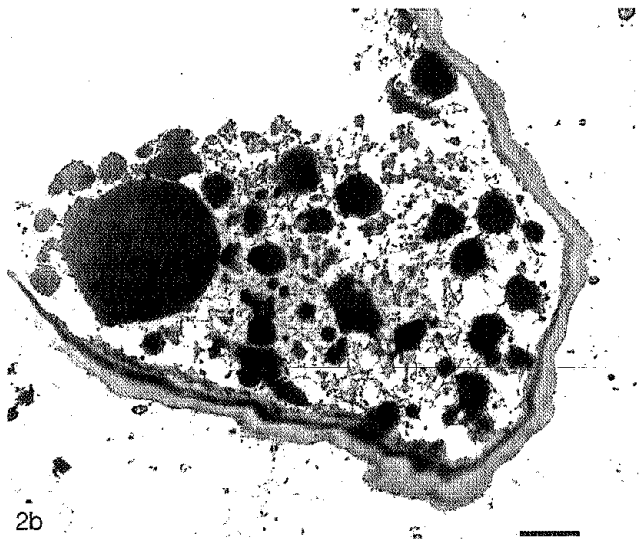
1b



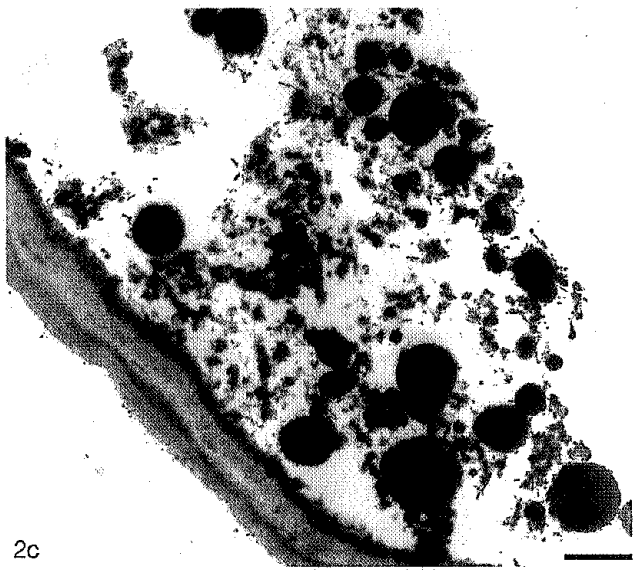
1c



2a



2b



2c

Materials and methods

Callus was initiated from leaf explants of potato (*Solanum tuberosum* L.) cv. Désirée on MS medium (Murashige and Skoog 1962) supplemented with 30 g dm^{-3} sucrose, 8 g dm^{-3} agar, 3 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3 mg dm^{-3} kinetin as described earlier (Anjum and Amjad 2000). To study the freezing damage to the cells, callus was frozen as 200 mg pieces in closed tubes and care was taken to prevent damage to callus structure (van Swaaij *et al.* 1987). To avoid supercooling, samples were cooled at -1 °C for 1 h and inoculated with a small piece of crushed ice to initiate freezing. Then the temperature was decreased at the rate of 2 °C h^{-1} to various subzero temperatures (-3, -6 and -10 °C) and samples were exposed to each test temperature for 1 h. Samples were removed at each test temperature and thawed overnight at 4 °C (Lee *et al.* 1992). Freezing damage was examined by observing through an electron microscope.

The samples of frozen and normal callus were fixed in a double fixative glutaraldehyde-osmium, dehydrated through a series of 30, 50, 70, 90 and 100 % acetone and embedded in epoxy resin. The sections were cut in an *LKB Ultratome* using glass knives and stained in uranyl acetate and lead citrate as already described (Anjum 2001). The stained sections were observed in a *Cornith 500* transmission electron microscope and selected areas were photographed.

Results

In normal callus cells, the cytoplasm is enclosed by a plasma membrane, which is surrounded by the cell wall. The cytoplasm contains different types of organelles, and among these plastids, mitochondria and microbodies are most prominent (Fig. 1*a*,*b*). Ribosomes are arranged in groups, called polysomes, in the cytoplasm. The endoplasmic reticulum appears as randomly-dispersed paired membrane sheets, which permeate the cytoplasm (Fig. 1*c*). The nucleus lies to one side of the cell surrounded by a thin layer of cytoplasm. The nucleus is bound by the nuclear envelope comprised of two unit membranes (Fig. 1*c*). Most of the nuclear volume is occupied by chromatin, which forms a dispersed network of material distributed throughout the nuclear sap, and a nucleolus. The vacuole appears to be quite large and separated from the cytoplasm by a membrane, the tonoplast (Fig. 1*b*).

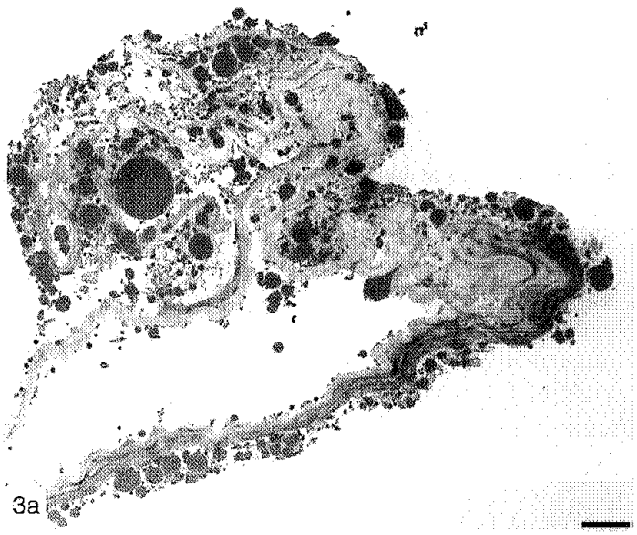
The cells subjected to -3 °C are regularly-shaped but the cytoplasm within presents a disorganised appearance (Fig. 2*a*). In addition, the cytoplasmic background in the electron micrographs appears to be changed in texture (Fig. 2*b*), and ribosomes or polysomes are not distributed in the cytoplasm in a normal fashion. Endoplasmic reticulum has also been affected and some fibrillar material has appeared in the cytoplasm, possibly coagulated protein. Most of the major organelles such as plastids and mitochondria appear more swollen and darkly-stained than those in normal, unfrozen cells. In plastids, the internal membranes are indistinct but their envelope membranes seem intact (Fig. 2*c*). Plasma membranes seem to be intact, but the tonoplast appear to be ruptured (Fig. 2*a*).

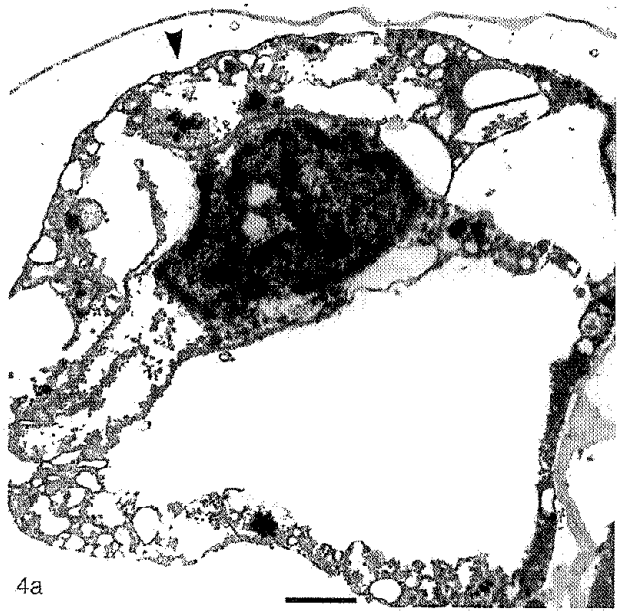
In the cells frozen to the lower temperature of -6 °C, the cell walls and cytoplasm appear distorted and collapsed, but organelles can still be distinguished (Fig. 3*a*). Plastids in some cells still could be recognised due to the presence of osmiophilic globules (Fig. 3*b*). In some cells both the plasma membrane and tonoplast seem to be ruptured (Fig. 3*c*). The nucleus also appears to be damaged, with flocculated chromatin (Fig. 3*b*).

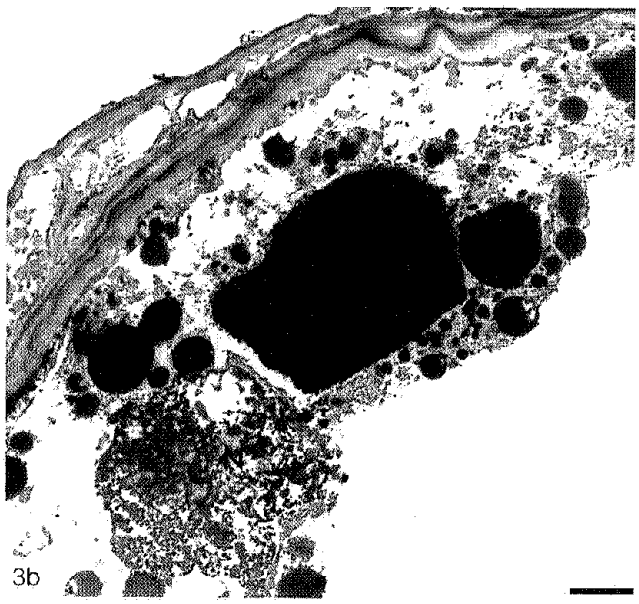
Exposure of callus cells to the lowest freezing temperature of -10 °C resulted in severe injury to the cells (Fig. 4*a-c*). The cytoplasm appears to be in a highly disorganised state, with a flocculated appearance (Fig. 4*c*). Severe vesiculation can be seen in some cases (Fig. 4*a*). Many vesicles of varying sizes have appeared in the cytoplasm, which are probably the result of swelling or disintegration of the endoplasmic reticulum system. In some cells the only organelles which could be identified were plastids (Fig. 4*a*). The damage is so severe in certain cases that no organelles can be distinguished (Fig. 4*c*). Some cells with intact cell walls, appear empty or with little, highly-damaged cytoplasmic material (Fig. 4*b*). The nucleus also appeared to be disorganised. "Frost plasmolysis" could also be seen in some cells (Fig. 4*a*).

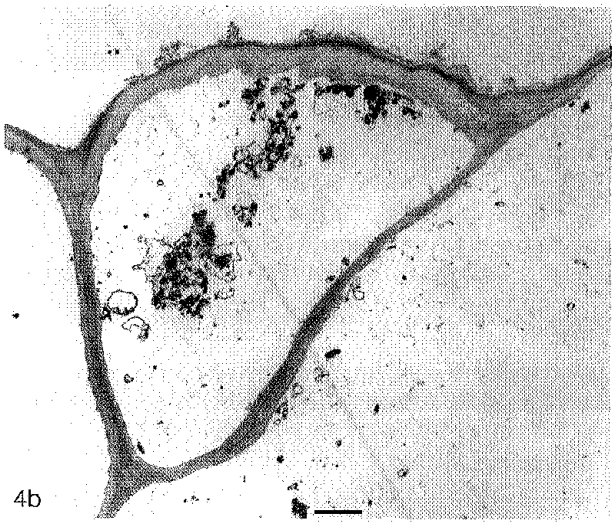
Fig. 1. Normal callus cells (without freezing) of *S. tuberosum* cv. Désirée: *a* - part of a cell showing nucleus (N) with a nucleolus and nucleolar organiser; plastids and mitochondria can also be seen in the cytoplasm (*bar* = 2 μm); *b* - section along part of a cytoplasmic strand passing across the vacuole (V), and a thin layer of cytoplasm with plasma membrane and cell wall (*bar* = 1 μm); *c* - a part of cell showing the nuclear envelope (ne) and also plastids (p), mitochondria (m), dictyosome (d) and ribosomes (r) in the cytoplasm (*bar* = 0.2 μm).

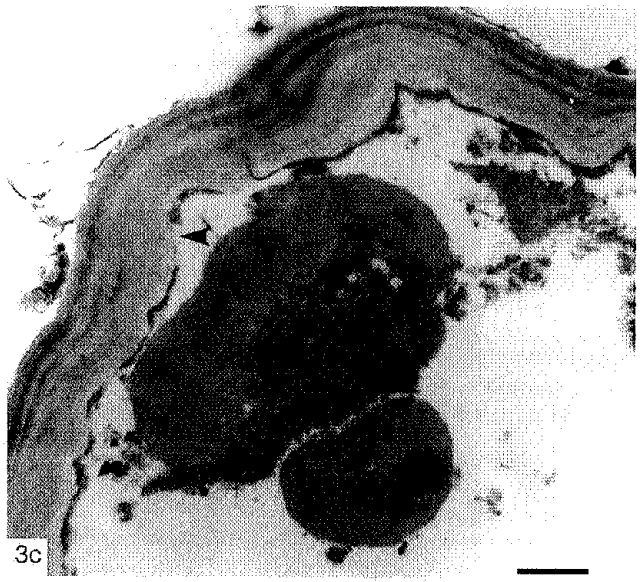
Fig. 2. Cells subjected to -3 °C: *a* - part of a cell bounded by cell wall, showing disorganised cytoplasm (*bar* = 2 μm); *b* - a part of cytoplasm showing organelles with indistinct internal membranes; ribosomes are not distributed in a normal fashion and fibrils (possibly protein) can also be seen (*bar* = 1 μm); *c* - an enlarged view of a part of cell wall and disorganised cytoplasm (*bar* = 2 μm).

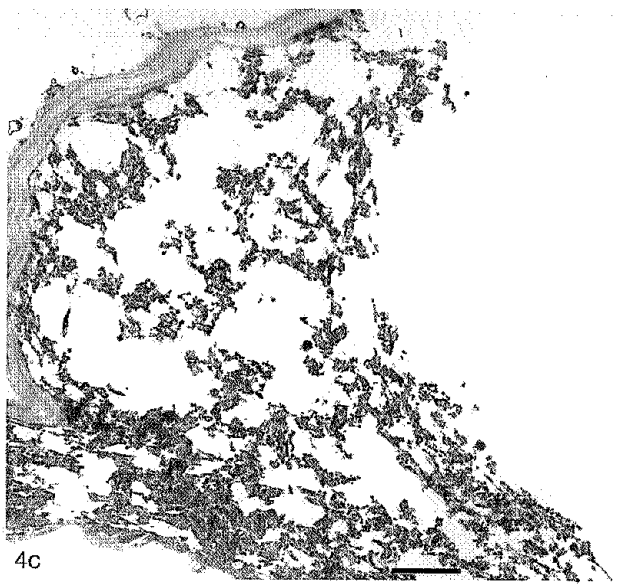

3a


4a


3b


4b


3c


4c

Discussion

The cells of normal callus (without freezing) possessed a normal range of cellular organelles, and their structure and arrangement suggested that the cells were metabolically active when fixed. A freezing temperature of -3 °C is regarded as a frost-killing temperature for *S. tuberosum*. When callus cells were frozen to this temperature, organelles appeared swollen and were probably non-functional. Plasma membranes still seemed to be intact, whereas the tonoplasts appeared damaged. The damage to cellular structure appeared irreversible and hence, at -3 °C *S. tuberosum* cells are killed. Similar damage has been reported by Palta *et al.* (1982) in the cells of *S. tuberosum* leaflets frozen to -3.5 °C. In their experiment cells frozen to -3 °C appeared to be normal and similar to the control (unfrozen). Probably this difference is due to the different nature of cells (mesophyll and callus origin) and the freezing techniques used (Tiedemann *et al.* 1998), especially rate and duration of freezing. On the other hand, it could be due to the variability for frost tolerance within the species *S. tuberosum*. Plasma membranes appeared intact in their experiment, and also in the present study, which indicates that at this temperature freezing injury was not caused by physical rupture of plasma membranes, but these might have become denaturated. According to Wang *et al.* (1982), the denaturation of membrane protein and the change in lipid-protein interaction of tonoplast and plasma membrane is the primary reaction when cells are subjected to freezing followed by a slow thawing. Radiation frosts and subsequent frost damage to potatoes have also been recorded in the temperature range of -4 to -5 °C by Fuller and Le Grice (1998).

In the present study, when cells were frozen to a temperature of -6 °C, the damage became severe and plasma membranes observed were to be ruptured. There is ample evidence in the literature, most from physiological observations that plasma membranes are one of the major targets of freezing injury (Palta *et al.* 1982). Freezing injury may result in specific alterations in membrane transport properties or breakdown of membrane semipermeabilty and membrane rupture (Sukumaran and Weiser 1972, Levitt 1980). In the present study, at -10 °C the damage became so severe that in some cells organelles could no longer be recognised. Many vesicles also appeared in the cytoplasm in some cells. These vesicles did not appear to be discharged into the vacuole, probably due to the damaged state of the tonoplasts and the prevention of normal cellular activities. Moreover, the cytoplasm became fragmented and again no fragments were found in the vacuole. Ristic and Ashworth (1993) have already observed fragmented protoplasm with indistinguishable plasma membranes and damaged cell ultrastructure without any evidence of intracellular ice in the tissue 2 - 3 cm thick, current years growth of flowering dogwood (*Cornus florida* L.) subjected to -5 °C and lower temperatures. Shi *et al.* (1998) have reported changes in mechanical property of potato samples during freezing. After freezing, as the temperature was lowered, more ice was formed and the material became more brittle, indicating the severity of damage.

Fig. 3. Cells subjected to -6 °C: *a* - a general view of distorted cells showing severe freezing injury (*bar* = 2 μm); *b* - a part of cytoplasm surrounded by plasma membrane and cell wall; a damaged nucleus (N) can also be seen (*bar* = 2 μm); *c* - a part of a cell wall and plasma membrane; the plasma membrane (*arrow*) appears to be discontinuous (*bar* = 0.5 μm).

Fig. 4. Cells subjected to -10 °C: *a* - a general view of freeze-damaged cell, showing plasmolysis (*arrow*) (*bar* = 2 μm); *b* - a severely-injured cell, showing only fragments of cytoplasm remaining (*bar* = 2 μm); *c* - a part of severely-injured cell with the cytoplasm showing a flocculated appearance, no organelles can be recognised (*bar* = 2 μm).

References

Anjum, M.A.: Cytology of potato callus cells in relation to their frost hardiness. - Biol. Plant. **44**: 325-331, 2001.

Anjum, M.A., Amjad, M.: Effect of exogenously-applied proline on the growth and frost tolerance of tissue cultures of *Solanum spp.* - J. anim. Plant Sci. **10**: 27-30, 2000.

Fuller, M.P., Le Grice, P.: A chamber for the stimulation of radiation freezing of plants. - Ann. appl. Biol. **133**: 111-121, 1998.

Lee, S.P., Zhu, B., Chen, T.H.H., Li, P.H.: Induction of freezing tolerance in potato (*Solanum commersonii*) suspension-cultured cells. - Physiol. Plant. **84**: 41-48, 1992.

Levitt, J.: Response of Plants to Environmental Stress. Vol. 1. 2nd Ed. - Academic Press, London 1980.

Li, P.H., Fennell, A.: Potato frost hardiness. - In: Li, P.H. (ed.): Potato Physiology. Pp. 457-479. Academic Press, London 1985.

Lindsey, K., Jones, M.G.K.: Plant Biotechnology in Agriculture. - Open University Press, Milton Keynes 1989.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Palta, J.P., Jensen, K.G., Li, P.H.: Cell membrane alterations following a slow freeze-thaw cycle: ion leakage, injury and recovery. - In: Li, P.H., Sakai, A. (ed.): Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications. Vol. 2. Pp. 221-242. Academic Press, New York 1982.

Rajashekar, C., Li, P.H., Carter, J.V.: Frost injury and heterogeneous ice nucleation in leaves of tuber-bearing *Solanum* species. - Plant Physiol. **71**: 749-755, 1983.

Ristic, Z., Ashworth, E.N.: Ultrastructural evidence that intracellular ice formation and possibly cavitation are the sources of freezing injury in supercooling wood tissue of *Cornus florida* L. - Plant Physiol. **103**: 753-761, 1993.

Shi, X., Datta, A.K., Throop, J.A.: Mechanical property changes during freezing of a biomaterial. - Trans. ASAE **41**: 1407-1414, 1998.

Sukumaran, N.P., Weiser, C.J.: Freezing injury in potato leaves. - Plant Physiol. **50**: 564-567, 1972.

Tiedemann, J., Hohenberg, H., Kollmann, R.: Higher pressure freezing of plant cells cultured in cellulose microcapillaries. - J. Microscopy **189**: 163-171, 1998.

Van Swaaij, A.C., Talsma, K., Krijgsheld, H., Jacobsen, E., Feenstra, W.J.: Frost tolerance in cell cultures of potato. - Physiol. Plant. **69**: 602-608, 1987.

Wang, H.-C., Mi, R.-Q., Wang, Y.-Q., Li, J.-S.: Freezing response of plasma membrane. - In: Li, P.H., Sakai, A. (ed.): Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications. Vol. 2. Pp. 285-295. Academic Press, New York 1982.