FOLIA
HORTICULTURAE
Ann. 20/1, 2008, 61-71

DOI: 10.2478/fhort-2013-0106

Employment of encapsulation-dehydration method for liquid nitrogen cryopreservation of ornamental plant explants propagated *in vitro*

Bożena Pawłowska

Department of Ornamental Plants
Faculty of Horticulture, Agricultural University in Kraków
29 Listopada 54, 31-425 Kraków, Poland
e-mail: ropawlow@cyf-kr.edu.pl

Key words: cold storage, rose, snowdrops, golden polypody, *in vitro* cultures

ABSTRACT

In the present studies, an attempt has been made to develop a method of liquid nitrogen preservation of plant explants propagated *in vitro* in the laboratory of the Department of Ornamental Plants, of Agricultural University in Kraków: shoot apical and axillary meristems of *Rosa* 'New Dawn', somatic embryos of snowdrops *Galanthus nivalis* L. and *G. elwesii* Hook, and gametophyte of *Phlebodium aureum* (L.) J. Sm. (golden polypody). After encapsulation of plant material, it was dehydrated by quick method (capsules were placed in liquid media containing 0.75 M sucrose for 18 h) or by gradual method (capsules were transferred to liquid solutions of media with increasing sucrose concentrations from 0.3 M to 1 M for consecutive 7 days). Moreover, some explants for cryopreservation were treated with the medium containing elevated sucrose level (0.25 M) for 8 weeks.

The obtained results indicate that survival rate of plant tissue after freezing in liquid nitrogen depended on plant material: apical meristems of roses regenerated callus but axillary meristems did not survive the freezing, gametophyte of golden polypody regenerated multiplying gametophytes, somatic embryos of snowdrops, although did not change appearance (did not blacken), did not regenerate. Cutting capsules after freezing did not influence regeneration of the cryopreserved plant explants.

It was shown that employment of slow dehydration method for preparation of plant material for cryopreservation allowed to obtain a viable callus tissue of *Rosa* 'New Dawn' and propagating *Phlebodium aureum* gametophytes. Rose axillary buds and golden polypody gametophytes prepared for cryopreservation by fast dehydration method did not survive freezing.

INTRODUCTION

Cryopreservation was introduced into horticulture in 1970s and new techniques were implemented at the beginning of 1990s. It is now used worldwide for protection of different biological materials in gene banks, selection of unique specimens and protection of morfogenetic potential of *in vitro* cultures or plant material capable of production of specific secondary metabolites. Application of cryopreservation in *in vitro* cultures reduces somaclonal variability and danger of material loss due to human error. Cell divisions and metabolic processes are restrained at ultralow temperature of liquid nitrogen (-196°C), thus, plant material can be preserved unchanged without loss of characteristic properties for unlimited time. Initially, very diverse specimens from *in vitro* cultures were preserved in liquid nitrogen (LN). In recent years, a tendency of combining different methods and simplifying procedures dominates in order to adjust methods to routine preservation of numerous specimens (Wang et al. 2000, Engelmann and Engels 2002, Wang et al. 2002, Wang and Laamanen 2005, Halmagyi and Pinker 2006).

Liquid nitrogen preservation techniques for vegetatively propagated plants are known but have not been commonly used for plant material preservation in gene banks. Progress in studies of plant material preservation depends on development and practical verification of cryopreservation protocols in laboratories of different research centers worldwide. So far, about a hundred species of vascular plants and many alga species have been cryopreserved in liquid nitrogen (Reed et al. 1998, Benson 1999, Reed et al. 2001, Harding et al. 2005).

In Poland, cryopreservation is used in a few research centers, e.g. in the Botanical Garden CZRB PAN in Powsin and Department of Potato Genetics IHAR in Młochow. For its values, intense development and focusing much interest

worldwide, it should be improved and implemented in Poland, as well (Mikuła and Rybczyński 2006).

The aim of the present studies was to develop a procedure of liquid nitrogen cryopreservation (using encapsulation-dehydration method) of plant explants propagated *in vitro*: apical and axillary meristems of roses, somatic embryos of snowdrops and prothalia of ferns.

MATERIAL AND METHODS

Plant material for the study were as follows: prothalia of *Phlebodium aureum* (L.) J. Sm., somatic embryos of snowdrops *Galanthus nivalis* L. and *G. elwesii* Hook, and meristems of the *Rosa* 'New Dawn', originated from plant collection cultivated in an *in vitro* laboratory of the Department of Ornamental Plants of Agriculture University in Kraków.

The collected prothalia of *P. aureum* (L.) J. Sm. (diameter approximately 1 mm) developed from spores on the medium containing (as the basic composition) 50% of mineral components according to Murashige and Skoog (1962) (MS), pH 5.3 under 16-h photoperiod, irradiance PPFD (photosynthetic photon flux density) of 20 μmol m^{-2} s^{-1}, at a temperature of 23/25°C (night/day), and 80% relative humidity of air.

Somatic embryos of snowdrops *Galanthus nivalis* and *G. elwesii* for cryopreservation (mean height 2.5mm) were obtained from embryogenic callus initiated on MS medium supplemented with 10 μM auxin (Picloram) and 1 μM cytokinin (BA). Differentiation and maturation of embryos were carried out on MS medium containing 10 μM BA and 1 μM NAA (8 weeks), and then 5 μM BA and 0.1 μM NAA. All propagation stages proceeded in the dark at 20°C.

In addition, apical and axillary buds (from leaf axils) of about 0.4 mm in diameter were collected from park rose 'New Dawn' propagated on the mineral medium according to Quoirin et al. (1977) (QL) supplemented with 5 μM BA, 0.3 μM GA_3 and 0.5 μM NAA, and sucrose concentration 0.06 or 0.25 M. Some plants were cultivated on the medium with the higher sucrose concentration for 8 weeks. Cultures were cultivated under 16-hour photoperiod, with irradiance PPFD of 30 μmol m^{-2} s^{-1}, at a temperature of 23/25°C (night/day) and 80% relative humidity.

Plant material for encapsulation-dehydration was collected in a sterile laminar flow hood and prepared according to two different schemes as follows:

I. 'Fast dehydration method'

- Encapsulation of explants in capsules made of 3% sodium alginate in

a. 100% QL/MS medium (depends on species);

b. distilled water or

c. distilled water containing 0.5 g dm^{-3} of activated charcoal

and 0.75 M sucrose;

- Solidification of capsules in 0.1 M $CaCl_2$ solution and rinsing thrice with sterile distilled water;

- Placing of capsules in liquid QL medium (0.75 M sucrose) on a laboratory shaker (100 rpm) in the dark for 18 hours;

- Drying of capsules in a sterile laminar flow hood for 4 hours (desiccation).

II. 'Gradual dehydration method'

- Encapsulation of explants in capsules made of 3% sodium alginate in QL medium (rose), MS medium (snowdrops), 50% MS medium (golden polypody) with 0.75 M sucrose;

- Solidification of capsules in 0.1 M $CaCl_2$ solution and rinsing thrice with sterile distilled water;

- Transferring of capsules into liquid solutions of QL medium (rose), MS medium (snowdrops) and 50% MS medium (golden polypody) with increasing concentrations of sucrose for 7 consecutive days (day 1 – 0,3 M; day 3 – 0,5 M; day 5 – 0,75 M; day 7 – 1 M) for tissue dehydration;

- Desiccation in air in a sterile laminar flow hood for 5 hours.

Capsules containing plant material were placed in special 5 ml cryo-vials (Sigma), 10 specimens in each, and then in bottles containing liquid nitrogen (LN, -196°C).

Samples of all species were thawed according to the same procedure. Immediately after removal of cryo-vials from liquid nitrogen, they were transferred to a water bath at 35°C for 2 minutes. Then the samples were placed on regenerative media. Rose samples were regenerated on 50% QL medium, supplemented with 0.06 M of sucrose and growth regulators: 5 μM BA, 0.3 μM GA_3 and 0.5 μM NAA, pH 5.6. Rose callus was regenerated on QL medium supplemented with cytokinins: kinetin, zeatin, 2iP or thidiazuron, at concentrations of 0.1, 1 and 5 μM. Cultures were maintained under 16-hour photoperiod, irradiance PPFD of 30 μmol m^{-2} s^{-1}, at a temperature of 23/25°C (night/day), 80% relative humidity.

Snowdrops were placed on the medium containing 50% of mineral salts according to MS, 30 g dm^{-3} of sucrose and 5 μM BA and 0.1 μM NAA (pH 5.8). Further stages of regeneration were carried out on MS medium containing 1 μM BA and 0.1 μM IAA, and on the medium enriched in 1 μM IAA and 0.1 μM BA. Cultures conditions were the same as before freezing: darkness and temperature of 20°C.

Cultures of golden polypody were maintained on 50% MS at pH 5.3. Cultures were placed in a phytotrone under 16-hour photoperiod, with irradiance PPFD of 20 μmol m^{-2} s^{-1}, at a temperature of 23/25°C (night/day) and 80% relative humidity.

Five weeks after cryopreservation half of capsules were cut. The explants were taken off and placed directly on the solid media.

Encapsulated explants which were not exposed to the temperature of -196°C, served as a control.

All experiments were performed in 5 replicates, 15-20 explants in each; 700 capsules in total were prepared. Data significance was evaluated using a combined two-factor analysis for independent data. The level of significance was set to $\alpha = 0.05$. Data were analyzed by Duncan test.

RESULTS AND DISCUSSION

Encapsulation-dehydration method allows for cryopreservation of both differentiated and undifferentiated plant material (Engelman 2004). In the present studies prothalia of *Phlebodium aureum* (L.) J. Sm., somatic embryos of snowdrops (*Galanthus nivalis* L. and *G. elwesii* Hook) and shoot apical and axillary meristems of *Rosa* 'New Dawn' were used. Regeneration of explants encapsulated and cryopreserved is presented in Table 1.

Table 1. Regeneration of explants from the selected ornamental plant species after cryopreservation by encapsulation-dehydration method

Preparation of capsules	Treatment after freezing	Gametophyte of *Phlebodium aureum*	Somatic embryos of *Galanthus*		Buds of *Rosa* 'New Dawn'	
			G. nivalis	*G. elwesii*	apical	axillary
Control – capsules not frozen		+*	+	+	+	+
I. Fast dehydration	Cut	not tested	Lack of data	Lack of data	+	–
	uncut	–	Lack of data	Lack of data	+	–
II. Gradual dehydration	Cut	not tested	–	–	+	–
	uncut	+	–	–	+	–

*+ explants regenerate
– xplants do not regenerate

Capsules, prepared using MS medium (golden polypody, snowdrops) or QL medium (rose), and subjected to fast or gradual dehydration (procedure I or II) and then to desiccation in air in a sterile laminar flow hood, were of good quality. Capsules with centrally located plant explants were selected for freezing (about 10% were rejected). At this stage, blackening of golden polypody prothalia and, partly, rose explants was noted, whereas of white color somatic embryos of snowdrops remained unchanged.

Capsules prepared using distilled water and distilled water supplemented with 0.5 g dm^{-3} of activated charcoal were successfully hardened in $CaCl_2$, but

disintegrated during rinsing with 0.75 M sucrose solution (90% of capsules). Probably capsule shell integrity depends not only on the presence of Ca kations in calcium chloride but also on macro- and microelements of MS or QL media.

Phlebodium aureum (L.) J. Sm. prothalia subjected to cryopreservation blackened, independently of the preparation method, but 2% of capsules prepared by gradual dehydration regenerated after removing them from liquid nitrogen and placing on medium containing 50% MS, pH 5.3. About 45% of control capsules (that were not frozen) regenerated on this medium and explants developed prothalia (Table 1).

Somatic embryos of snowdrops *Galanthus nivalis* L. and *G. elwesii* Hook, prepared by the fast dehydratation method for freezing got infected after removing them from liquid nitrogen and placing on the MS solid medium.

Somatic embryos of both snowdrop species subjected to gradual dehydration (procedure II) after removing from liquid nitrogen looked exactly like unfrozen embryos. They were white, with no blackened tissue. They were placed on the medium containing 50% MS, 3% sucrose, 5 μM BA and 0.1 μM NAA, pH 5.8. In the next two subcultures (lasting 5 weeks each), frozen and unfrozen embryos did not commence regeneration, therefore, they were taken out of capsules, cut into halves and placed on the auxin-containing (1 μM IAA and 0.1 μM BA) or cytokinin-containing (1 μM BA and 0.1 μM IAA) regenerative medium. During cultivations control explants developed axillary buds on bulb scales (Table 1). Literature reports on preservation of somatic embryos in liquid nitrogen are scarce, but indicate that improved tolerance to freezing and dehydration is achieved by using exogenous ABA and elevated sugar contents (Fang et al. 2004).

From among diverse plant material, shoot apical meristems are currently the most often chosen for cryopreservation (Talagi 2000, Lambardi et al. 2002). Our studies conducted in the Department of Ornamental Plants demonstrated the ability of shoot apical meristems of *Rosa* 'New Dawn' to survive cryopreservation. The applied encapsulation-dehydration method allowed to obtain alive, growing callus tissue from explants of *Rosa* 'New Dawn' after cryopreservation. Control explants, encapsulated but not frozen at ultralow temperature (-196°C) formed also buds. Differentiation of tissues is associated with morphological cell variability and different degree of their hydration, which usually limits good preservation of all explant cells. The used dehydration method can cause to cell damage resulting from excessive plasmolysis or osmotic shock, what leads to the regeneration in post-freezing culture *via* indirect pathway, i.e. *via* callus. The same was observed in the present work and in the study on *Cosmos atrosanquineus* cultures (Wilkinson et al. 2003).

Apical buds of rose were shown to react well to cryopreservation, since 69.8% of them regenerated callus while encapsulated axillary buds did not commence regeneration (Table 2). Similar proportion (69.4%) of control explants prepared

from axillary buds of *Rosa* 'New Dawn' regenerated callus. Only 33.1% of apical buds without freezing started to regenerate.

Table 2. Influence of explant type of *Rosa* 'New Dawn' on percent of callus-regenerating explants after cryopreservation (encapsulation-dehydration, method II)

Explant type	Percent of callus-regenerating explants	
	Non- frozen plant material (Control)	Frozen plant material
Apical buds	33.1 b	69.8 c
Axillary buds	69.4 c	0 a

*means with the same letter do not differ significantly at p = 0.05

Apical meristems of *Rosa* 'New Dawn' frozen and then cultured on regenerative medium containing 50% QL supplemented with 0.06 M concentration of sucrose and growth regulators: 5 μM BA, 0.3 μM GA_3 and 0.5 μM NAA, pH 5.6, regenerated compact green callus after 9-11 weeks from placing capsules on the medium. Significantly more explants regenerated when slow dehydration was applied (procedure II) in comparison with quick dehydration method (76 and 26%, respectively) (Table 3) and the former value was similar to encapsulated non-frozen explants. Similarly in raspberry cultures (Wang and Laamanen 2005), survival of apical meristems during dehydration was high when capsules were transferred to solutions with gradually increasing sucrose concentration (from 0.3 to 1 M) in comparison with a direct preculture; for instance, at 0.5 M sucrose shoot apical meristems died. Results of studies on black currant (Pluta 2005) allowed the authors to obtain regenerating plants from meristems prepared by quick dehydration method.

Table 3. Influence of encapsulation-dehydration method on percent of regenerating apical buds of *Rosa* 'New Dawn' 5 and 12 weeks after thawing

	Percent of regenerating explants		
	Control	Method I Fast dehydration	Method II Gradual dehydration
After 5 weeks	78 c*	0 a	0 a
After 12 weeks	89 c	26 b	76 c

* see Table 2

The present studies did not show any effect of mechanical cutting of capsules after freezing on percent of regenerating rose explants (Fig. 1). A large proportion of rose buds either from undamaged or cut capsules regenerated after removal from

liquid nitrogen (80% and 60%, respectively), and the difference between them was not statistically significant.

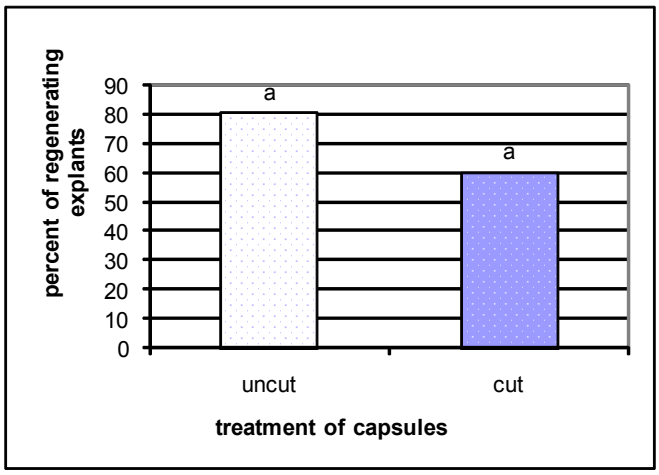

Fig. 1. Effect of post-thawing treatment of capsules (uncut, cut) on percent of regenerating *Rosa* 'New Dawn' explants

When the influence of plant material pretreatment was studied, buds were collected from plants cultured on the sucrose-enriched medium (0.25 M) and on standard sucrose-containing medium (0.06 M). Tip buds of roses from high sucrose medium regenerated callus better than those from standard sucrose medium (Fig. 2).

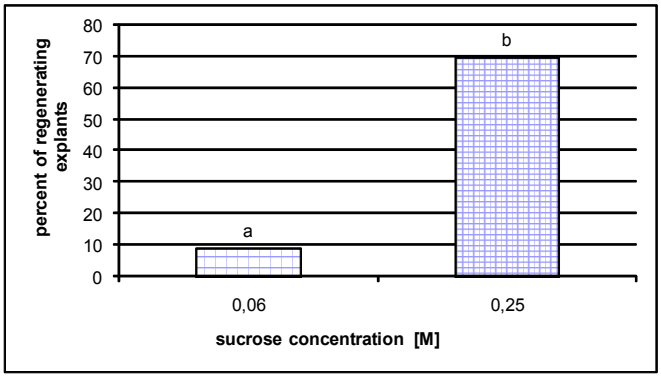

Fig. 2. Effect of sucrose concentration in the medium before freezing on percent of regenerating tip buds of *Rosa* 'New Dawn'

The obtained calluses of *Rosa* 'New Dawn' were regenerated by placing them on media containing different concentrations of cytokinins: kinetin, zeatin, 2iP or thidiazuron (0.1, 1, 5 μM). They did not regenerate axillary buds in the study period.

CONCLUSIONS

- The use of slow dehydration method for preparation of capsules with plant material for cryopreservation allowed to obtain, after removal from liquid nitrogen, alive callus tissue of *Rosa* 'New Dawn' and *Phlebodium aureum* gametophytes. Axillary buds of rose and gametophytes of golden polypody prepared for cryopreservation by quick dehydration method did not survive freezing.
- Cutting of capsules after freezing did not influence regeneration of plant explants after cryopreservation.
- The obtained results of studies on cryopreservation conducted in the Department of Ornamental Plants demonstrated that survival rate of plant tissue after freezing in liquid nitrogen depended on plant material: apical meristems of roses regenerated callus but axillary meristems did not survive the freezing, gametophytes of golden polypody regenerated by multiplying gametophyte, whereas somatic embryos of snowdrops did not regenerate although they did not change their appearance (they did not blacken).

REFERENCES

BENSON E.E., 1999. Plant Conservation Biotechnology. London: Taylor and Francis Ltd., UK.

ENGELMANN F., ENGELS J.M.M., 2002. Managing Plant Genetic Diversity. Engels J.M.M., Ramanatha Rao V., Brown A.H.D., Jackson M.T. (eds), Wallingford and Rome, CAB International and IPGRI: 89-104.

ENGELMAN F., 2004. Plant cryopreservation: progress and prospects. In vitro Cell. Dev. Biol.-Plant 40: 427-433.

FANG J.Y., WETTEN A., HADLEY P., 2004. Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. Plant Sci. 166: 669-675.

HALMAGYI A., PINKER I., 2006. Plant regeneration from *Rosa* shoot tips cryopreserved by a combined droplet verification method. Plant Cell Tiss. Org. Cult. 84: 145-153.

HARDING K., JOHNSON J., BENSON E., 2005. Contributing to a Sustainable Future. Bennet I.J., Bunn E., Clarke H., McComb J.A. (eds). Proceedings of the Australia Branch of the IAPTC&B, Perth, Western Australia, 21-24th September: 205-211.

LAMBARDI M., LYNCH P.T., BENELLI C., MEHRA A., SIDDIKA A., 2002. Towards the cryopreservation of olive germplasm. Adv. Hort. Sci. 16: 165-174.

MIKUŁA A., RYBCZYŃSKI J.J., 2006. Krioprezerwacja narzędziem długoterminowego przechowywania komórek, tkanek i organów pochodzących z kultur in vitro. Biotechnol. 4(75): 145-163.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

PLUTA S., 2005. Możliwości wykorzystania dwóch metod krioprezerwacji do przechowywania merystemów porzeczki czarnej w ciekłym azocie. Michalik B., Żurawicz E. (eds): Zmienność genetyczna i jej wykorzystanie w hodowli roślin ogrodniczych: 351-356.

QUOIRIN M., LEPOIVRE P., BOXUS P., 1977. Un premier bilan de 10 annees de recherches sur les cultures de meristemes st la multiplication in vitro de fruitiers ligneus. In: C.R.Rech. 1976-1977 et repports de Synthese, Stat. des Cult. Fruit. Et Maraich. Glemblous: 93-117.

REED B.M., DUMET D., DENOMA J.M., BENSON E.E., 2001. Validation of cryopreservation protocols for plant germplasm conservation: A pilot study using Ribes L. Biodiversity and Conservation 10(6): 939-949.

REED B.M., DENOMA J.M., LUO J., CHANG Y., TOWILL L., 1998. Cryopreservation and long term storage of pear germplasm. In Vitro Cell. Dev. Biol. – Plant 34: 256-260.

TALAGI H., 2000. Cryopreservation of tropical plant germplasm. Engelmann F., Takagi H. (eds). Current Research Progress and Apliction, IPGRI Rome, Italy: 178-193.

WANG Q.C., TANNE E., ARAV A., GAFNY R., 2000. Cryopreservation of in vitro-grown shoot tips of grapevine by encapsulation-dehydration. Plant Cell Tiss. Org. Cult. 63: 41-46.

WANG Q.C., BATUMAN O., LI P., BAR JOSEPH M., GAFNY R., 2002. Cryopreservation of in vitro-grown shoot tips of 'Troyer' citrange (*Poncirus trifoliata* Raf. × *Citrus sinensis* (L.) Osbeck.) by encapsulation-dehydration. Plant Cell Rep. 20: 901-906.

WANG Q.C., LAAMANEN J., 2005. Cryopreservation of in vitro-grown shoot tips of raspberry (*Rubus idaeus* L.) by encapsulation-vitrification and encapsulation-dehydratation. Plant Cell Rep. 24: 280-288.

WILKINSON T., WETTEN A., PRYCHID CH., FAY M.F., 2003. Suitability of cryopreservation fort he long-term storage of rare and endangered plant species: a case history for *Cosmos atrosanquineus*. Ann. Bot. 91: 65-74.

WYKORZYSTANIE METODY KAPSUŁKOWANIA – DEHYDRATACJI DO PRZECHOWYWANIA W CIEKŁYM AZOCIE EKSPLANTATÓW ROŚLIN OZDOBNYCH ROZMNAŻANYCH TECHNIKĄ *IN VITRO*

Streszczenie: W przeprowadzonych badaniach podjęto próbę opracowania sposobu przechowywania w ciekłym azocie metodą kapsułkowania – dehydratacji eksplantatów pochodzących z roślin rozmnażanych techniką *in vitro* w laboratorium Katedry Roślin Ozdobnych Akademii Rolniczej w Krakowie: merystemów wierzchołkowych i bocznych róż *Rosa* 'New Dawn', zarodków somatycznych śnieżyczek *Galanthus nivalis* L. i *G. elwesii* Hook oraz przedrośli paproci *Phlebodium aureum* (L.) J. Sm. Po kapsułkowaniu poddano materiał roślinny dehydratacji metodą szybką (umieszczenie kapsułek w pożywkach płynnych o stężeniu sacharozy 0,75 M na 18 godzin) lub stopniową, (kapsułki przenoszono do płynnych pożywek, o wzrastającym stężeniu sacharozy, od 0,3 M do 1 M, przez kolejne 7 dni). Ponadto część eksplantatów pozyskiwanych do krioprezerwacji hartowano na pożywce z podwyższonym stężeniem sacharozy (0,25 M) przez 8 tygodni.

Uzyskane wyniki wskazują, że przeżywalność tkanki roślinnej po mrożeniu w ciekłym azocie zależy od rodzaju użytego materiału roślinnego: merystemy wierzchołkowe róż regenerowały kalus, a merystemy boczne nie przetrwały mrożenia; gametofity paprotki złocistej regenerowały namnażając gametofit; zarodki somatyczne śnieżyczek, choć nie zmieniły wyglądu (nie czerniały) nie podjęły regeneracji. Natomiast nacinanie kapsułek po mrożeniu nie wpłynęło na regenerację poddanych krioprezerwacji eksplantatów roślinnych.

Wykazano, że zastosowanie powolnej metody dehydratacji podczas przygotowywania kapsułek z materiałem roślinnym do krioprezerwacji pozwola – po wyjęciu z ciekłego azotu – na uzyskanie żywej tkanki kalusowej róży 'New Dawn' i namnażających się gametofitów *Phlebodium aureum*. Pąki boczne róży i gametofity paprotki złocistej przygotowane do krioprezerwacji metodą szybkiej dehydratacji nie przeżywają mrożenia.

Received February13, 2008; accepted June 23, 2008