Preservation Methods for Jerusalem Artichoke Cultivars

G.M. Volk¹

U.S. Department of Agriculture, Agricultural Research Service, National Center for Genetic Resources Preservation, 1111 South Mason Street, Fort Collins, CO 80521

K. Richards

Plant Gene Resources of Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK S7N 0X2, Canada

Additional index words. cryopreservation, PVS2, storage, vitrification, short-term storage

Abstract. Preservation methods were evaluated for jerusalem artichokes (*Helianthus tuberosus* L.) to facilitate the back-up of field collections. In vitro plant cultures were established from field-harvested tubers for nine jerusalem artichoke cultivars obtained from the Plant Gene Resources of Canada, Saskatoon Research Centre, Saskatoon, Canada. Most cultivars could be maintained at 5 °C or under 25 °C growth room conditions for at least 6 months. Excised shoot tips from in vitro cultures were cryopreserved using plant vitrification solution 2 (PVS2; 15% w/v ethylene glycol, 15% w/v dimethyl sulfoxide, 30% w/v glycerol, and 13.7% w/v sucrose in half strength media salts) as a cryoprotectant. PVS2 exposures of 15 or 30 min at 0 °C resulted in an average regrowth of 34% across five jerusalem artichoke cultivars after liquid nitrogen exposure. Jerusalem artichokes can be successfully maintained using both reduced temperature and cryopreservation approaches.

Jerusalem artichoke (*Helianthus tuberosus* L.) is a cold-hardy North American wild relative of the sunflower, *Helianthus annuus* L. Jerusalem artichoke plants were spread from Saskatchewan east to Ontario and south to Arkansas and Georgia by Native Americans who cultivated them for edible tubers that can be baked, steamed, or boiled. Raw tubers can be sliced or shredded for salads. The tubers contain high levels of inulin, which has been pursued as a bulking agent for artificial sweeteners (McLaurin et al., 1999). Since jerusalem artichoke plants produce large quantities of biomass with few inputs, this crop has also been considered for silage production (Seiler, 1993). Furthermore, *Helianthus* breeders have interest in jerusalem artichoke germplasm since *Sclerotinia sclerotiorum* (Lib.) resistance has been identified through tissue culture screening (Cassells and Walsh, 1995).

The USDA National Plant Germplasm System maintains seven clonal accessions of *H. tuberosus*; however, 165 *H. tuberosus* (L.) accessions are propagated using tubers at Plant Gene Resources of Canada. These accessions are not currently distributed or listed on the Germplasm Resources Information Network-Canadian version (GRIN-CA) database. Since the tubers must be dug and replanted every 3 years, jerusalem artichoke plants are relatively high maintenance compared to many seed-

propagated species. Labor costs and field space also make maintaining duplicate cultivars at separate locations prohibitively expensive.

This research was performed to identify desirable methods for maintaining back-up collections of clonally propagated *H. tuberosus* in genebanks. Methods for in vitro culture of *H. tuberosus* have been published (Cassells and Walsh, 1995; Devi and Rani, 2002; Roche and Cassells, 1996; Gamborg et al., 1999). Once established as in vitro plants, many temperate crops can be stored for extended lengths of time at low (5 to 15 °C) temperatures (Reed, 1992, 1993). Jerusalem artichoke tubers easily overwinter in Canadian field conditions, but a test for reduced-temperature medium-term storage of *H. tuberosus* in vitro cultures has not been published.

Successful cryopreservation protocols allow for long-term storage of clonally propagated species. While *H. tuberosus* suspension cell cultures have been successfully cryopreserved (Harris et al., 2004; Swan et al., 1999), there are no reports of the cryopreservation of *H. tuberosus* shoot tips. The use of shoot tips limits the risk of somaclonal variation that can occur when plants are maintained as suspension

cell cultures (D'Amato, 1985; Harding, 2004; Scowcroft 1984). Shoot tip cryopreservation protocols have been developed for many temperate plant species, but they have not been shown to be generally applicable. Vitrification solutions such as plant vitrification solution 2 (PVS2) (Sakai et al., 1991) and plant vitrification solution 3 (PVS3) (Nishizawa et al., 1993) reduce ice crystal formation by dehydrating or by altering water properties (Volk and Walters, 2006); however, constituents of the solutions can be cytotoxic (Steponkus et al., 1992). Cultivars within a species differ in their responsiveness to PVS2 and PVS3, though the basis for different responses is not known.

Medium-term cold storage and long-term cryopreservation approaches are complementary. Cultivars introduced into tissue culture can be maintained in vitro until resources for cryopreserving shoot tips for long-term storage are available. This research was performed to identify a method by which clonally propagated *H. tuberosus* accessions could be backed up within genebanks.

Materials and Methods

Plant materials. In mid-August, tubers were harvested from nine *H. tuberosus* cultivars grown in the fields at the Plant Gene Resources of Canada, Saskatoon Research Centre, Saskatchewan, Canada (Table 1). One-year-old field plants had not begun flowering and had small (<1-cm-diameter) tubers. Tubers were sent to the National Center for Genetic Resources Preservation, Ft. Collins, Colo. and stored at 4 °C for up to 30 d until introduction into tissue culture.

Tubers and rhizomes have many buds that provide shoot tips for introduction into tissue culture. Segments (5 mm) of field-grown tubers or rhizomes were surface-sterilized with either 70% isopropanol for 2 min, 70% isopropanol for 5 min, 95% ethanol for 2 min, or 70% ethanol for 5 min followed by a 20% commercial bleach (1.8% sodium hypochlorite) + 0.0001% Tween 20 (Sigma, St. Louis, Mo.) for 20 min. After three 10-min sterile water rinses, 20 shoot tips were excised under sterile conditions and plated onto one of two media. Growth medium 1 contained MS salts and vitamins, 0.1 mg·L⁻¹ gibberellic acid and 15 g·L⁻¹ sucrose (Cassells and Walsh, 1995) and growth medium 2 contained MS salts (Murashige and Skoog, 1962), MS-G, 1 mg·L⁻¹ thiamine HCl, 1 mg·L⁻¹ indole acetic acid, 20 g·L⁻¹ sucrose, and 9 g·L⁻¹ agar.

Table 1. Jerusalem artichoke accession information. All varieties are maintained at the Plant Gene Resources of Canada, Saskatoon Research Centre, Saskatoon, Canada.

Accession no.	Alternative Canadian no.	Name	Origin	Date of acquisition
JA 14	NC10-16	HM-3	Manitoba, Canada	1970
JA 37	NC10-40	Comber	Manitoba, Canada	1974
JA 38	NC10-41	B.C. #1	Native American collection, Canada	1976
JA 40	NC10-43	USDA-P1	Unknown	1976
JA 41	NC10-44	Sunchoke-Fiesda's	California	1976
JA 64	NC10-79	W-3X Branching 7611	Canada	1979
JA 92	NC10-114	Industrie	Russia	1984
JA 103	NC10-130	#1	Ontario, Canada	1988
JA 172	SR	SR	Unknown	Unknown

Received for publication 30 Sept. 2005. Accepted for publication 3 Nov. 2005. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Technical assistance was provided by Kate Rotindo, Jackie Harris, and Dallas Kessler.

¹To whom reprint requests should be addressed; e-mail gvolk@lamar.colostate.edu.

Nine jerusalem artichoke cultivars were introduced into tissue culture by surface sterilizing tuber segments with 70% isopropanol for 2 min, 1.8% sodium hypochlorite for 20 min, and then rinsing three times with sterile water. Shoot tips were excised from the tuber segments and cultured on medium 1 for in vitro plant establishment. Established shoot tip cultures were maintained in a growth room at 25 °C and 16-h days ($32 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Cold storage of in vitro plants. One-month cultures of two- to three-node sections of nine *H. tuberosus* cultivars (Table 1) were placed into incubators maintained at 5, 10, or 15 °C or were kept under the growth room conditions described above. Fluorescent bulbs provided $35 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination in the incubators for 8 h each day. Each of the 20 single-plant tubes of each cultivar at each temperature was evaluated at 3- and 6-month intervals. Cultures were determined to be healthy (green culture without visual evidence of damage), recoverable (cultures grew after transferring them to 25 °C and fresh medium), or dead (cultures didn't grow after transfer to 25 °C).

Cryopreservation. An initial cryopreservation experiment was conducted using JA-40 shoot tips excised directly from surface sterilized tubers and rhizomes. Under sterile conditions, shoot tips were treated with 2 M glycerol + 0.6 M sucrose and half strength MS salts for 20 min at 22 °C and then immersed in PVS2 (15% w/v ethylene glycol, 15% w/v dimethyl sulfoxide, 30% w/v glycerol, and 13.7% w/v sucrose in half strength MS salts; Sakai et al., 1991) for 10, 20, or 30 min at 0 °C. Single shoot tips were placed within droplets of PVS2 on 4 × 2-cm aluminum-foil strips. Foil strips were plunged directly into liquid nitrogen (LN) for at least 1 h before thawing by immersing the foil strips with shoot tips into a petri dish containing 1.2 M sucrose and half strength MS salts for 20 min at 22 °C and then returned to medium 1 and growth room conditions for recovery.

Subsequent cryopreservation experiments used shoot tips excised from in vitro materials derived from JA-14, JA-41, JA-64, JA-92, and JA-103. Single-node in vitro stem sections were subcultured onto solidified MS medium without hormones for 3 d. Axillary shoot tips 1 mm long were harvested from the nodal sections and placed into 0.3 M sucrose and half strength MS liquid medium in the dark at 22 °C for 24 h. Shoot tips were exposed to 2 M glycerol + 0.6 M sucrose and half strength MS salts for 20 min at 22 °C and then treated with PVS2 for 15 or 30 min at 0 °C. As described above, shoot tips were plunged into LN on foil strips, thawed, and diluted into 1.2 M sucrose and half strength MS salts for 20 min at 22 °C before plating onto medium 1.

For PVS3 experiments, shoot tips of JA-14 and JA-92, two cultivars that grow quickly under tissue culture conditions, were subjected to a protocol similar to one that has been successful for garlic shoot tips (Makowska et al., 1999). In vitro shoot tips were treated with 2 M glycerol + 0.6 M sucrose and half strength MS salts for 20 min at 22 °C, followed by PVS3 (50% w/v sucrose, 50% w/v glycerol;

Nishizawa et al., 1993) for 120 min at 22 °C, and placed into 1.2-mL cryo-vials (Corning Costar Corporation, Cambridge, Mass., USA) containing 0.5 mL PVS3. Cryo-vials were plunged into LN for at least 1 h and then thawed in a 40 °C water bath for 2 min. PVS3 was removed and 1.2 M sucrose containing half strength MS salts was added to the shoot tips in the vial for 10 min at 22 °C. Shoot tips were plated in petri dishes containing medium 1. Plates with shoot tips were kept in the dark at 22 °C for 1 week and then exposed to light.

Bacterial contamination was not evident within the *H. tuberosus* tissue cultures before explant excision. However, after excision and treatment with either PVS2 or PVS3, many of the JA-14 shoot tips displayed a low level of contamination. This slow-growing bacterial growth did not appear to affect the ability of the explants to regenerate after cryoprotectant exposure. Vigorous young JA-14 shoots that emerged outgrew the bacteria and could be transferred to the same medium, supplemented with 2 mL $\cdot$ L⁻¹ plant preservative mixture (PPM) (Plant Cell Technology, Inc., Washington, D.C.), which limited the bacterial proliferation.

Each treatment included a minimum of 20 shoot tips and each cryopreservation experiment was performed at least twice. Viability and growth were assessed after 6 weeks of culture. Plants exhibiting regrowth formed shoots without callus proliferation. Statistical analyses were performed using the JMP software package (SAS Institute Inc., Cary, N.C.).

Results and Discussion

Sterilization protocols and a preferred growth medium were determined for jerusalem artichoke shoot tips excised from soil-harvested rhizomes or tubers. Surface sterilization procedures that were performed with 70% isopropanol for 2 min or 95% ethanol for 2 min followed by a 20 min bleach exposure resulted in low levels of fungal and bacterial contamination (0% to 30%). Noncontaminated green shoots could be subcultured in vitro without further contamination. Growth medium 1 (see methods) promoted primary shoot elongation in explants excised from tubers. In contrast, medium 2 promoted callus formation, and therefore its use was discontinued. Our data

Table 2. Percent of healthy jerusalem artichoke cultures after 3- and 6-month storage intervals in incubators at 5, 10, or 15 °C or growth room conditions (25 °C).

Accession	3 months at storage temp (°C)				6 months at storage temp (°C)			
	5	10	15	25	5	10	15	25
JA-14	88	0	0	100	20	0	0	50
JA-37	79	63	88	100	63	33	38	100
JA-38	100	67	62	100	50	52	64	100
JA-40	100	0	7	100	36	0	0	0
JA-41	83	39	61	100	32	39	26	53
JA-64	100	87	97	100	87	10	33	100
JA-92	100	82	79	100	48	76	27	91
JA-103	97	81	88	100	63	81	97	100
JA-172	93	55	70	100	67	55	60	82
Average ± SE	93 ± 3	53 ± 11	61 ± 12	100 ± 0	52 ± 7	38 ± 10	38 ± 10	75 ± 12

Fig. 1. Percent (± standard error) of healthy + recoverable in vitro jerusalem artichoke plants after 3 months (black bars) or 6 months (gray bars) of storage at 5, 10, 15, or 25 °C. Data for nine cultivars are combined.

confirm that jerusalem artichoke plants can be easily introduced into culture (Gamburg et al., 1999; Gautheret, 1969; Minocha and Halperin, 1974).

Reduced temperature storage. In vitro jerusalem artichoke plants were placed at 5, 10, or 15 °C and compared with plants that remained under growth room conditions for 6 months. This time interval was sufficient to identify trends in survival at the reduced temperatures. Survival was highest for plants kept for 3 months at 5 °C or under 25 °C growth room conditions, but decreased at intermedi-

ate temperatures (Table 2). After 6 months, the 5 °C storage conditions had 52% healthy cultures overall, with at least 20% healthy cultures for each of the cultivars (Table 2). These healthy cultures could be successfully kept under cold-storage conditions for at least another 3 months. The percentage of recoverable cultures at each temperature for 3- and 6-month storage intervals was also recorded. These recoverable cultures were alive, but they needed to be returned to the growth room for regrowth. Most of these recoverable cultures would not survive another 3 months at the re-

duced temperature conditions. Culture survival was greater than 50% at all storage conditions when the percentage of recoverable samples was summed with the percentage of those that are healthy (Fig. 1).

Cryopreservation results. Initially, 1 mm³ shoot tips of JA-40 were excised from buds of rhizomes or tubers, surface sterilized, and directly employed in cryopreservation protocols. This approach is comparable to that used for garlic cryopreservation, where shoot tips are excised directly from surface sterilized cloves. Shoot tips from tubers grew in 80%, 56%, and 50% of the cultures with 10, 20 and 30 min PVS2 exposures at 0 °C. Shoot tips from rhizomes grew in 0%, 23%, and 55% of the cultures with 10, 20, and 30 min PVS2 exposures at 0 °C. None of the shoot tips from either the tubers or the rhizomes exhibited shoot growth after LN exposure.

Jerusalem artichoke shoot tips could be cryopreserved when they were excised from in vitro cultures and treated with PVS2. Shoot tips not treated with PVS2 had regrowth of 90 to 100%. There were no statistical differences in regrowth between the 15 and 30 min PVS2 exposures among JA-14, JA-41, JA-64, JA-92 and JA-103 cultivars (Fig. 2). The average regrowth of LN-treated shoot tips was 34% when explants were treated with PVS2 for 15 or 30 min at 0 °C (Fig. 2). PVS2 exposure may cause damage to shoot tips. This toxic effect of PVS2, particularly at 25 °C, has been reported (De Carlo et al., 2000; Sakai et al., 2000; Tanaka et al., 2004; Thammasiri 1999; Volk et al., 2004); however, the specific biological and chemical nature of this damage has not been documented. Cultivars that stored well at 5 °C were not necessarily more amenable to cryopreservation treatments.

PVS3 did not provide adequate protection during cryoexposure. Two jerusalem artichoke accessions were tested, JA-14 and JA-92, and <20% of the explants recovered following at 2 h exposure to PVS3 at 22 °C and rapidly frozen into LN (Fig. 3). Treatment with PVS3 protected shoot tips of other cold-hardy species from damage during LN exposure (Baek et al., 2003; Kim et al., 2004; Makowska et al., 1999). Although *H. tuberosus* is also a cold hardy species, shorter PVS3 solution exposures may improve cryopreservation results using PVS3.

Cryoprotected *H. tuberosus* shoot tips were warmed after 1 h LN exposures. It is generally accepted that surviving LN short-term exposures is indicative of successful regrowth after long term LN storage (Towill, 2002; Yamada et al., 1991). Cryopreserved plant materials have been successfully regenerated after >4 years in storage (Mix-Wagner et al., 2002; Niino et al., 1995).

Jerusalem artichokes can be easily introduced into tissue culture. The nine cultivars we tested can be kept at either 5 or 25 °C for >6 months without subculturing. Using vitrification techniques, we have successfully cryopreserved and regenerated shoot tips of *H. tuberosus*. Field collections can be backed-up with cryopreserved in vitro shoot tips that can be thawed to produce viable plants when necessary.

Fig. 2. Percent ($\pm$ standard error) of jerusalem artichoke shoot tips that grew into healthy plants after exposure to plant vitrification solution 2 and liquid N. Five cultivars (as indicated) were evaluated.

Fig. 3. Percent ($\pm$ standard error) of jerusalem artichoke shoot tips that grew into healthy plants after exposure to plant vitrification solution 3 and liquid N. Two cultivars (as indicated) were evaluated.

Literature Cited

- Baek, H.-H., H.-H. Kim, E.-G. Cho, Y.-A. Chae, and F. Engelmann. 2003. Importance of explant size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification. *CryoLetters* 24:381–388.
- Cassells, A.C. and M. Walsh. 1995. Screening for *Sclerotinia* resistance in *Helianthus tuberosus* L. (Jerusalem artichoke) varieties, lines and somaclones, in the field and *in vitro*. *Plant Pathol.* 44:428–437.
- D'Amato, F. 1985. Cytogenetics of plant cell and tissue cultures and their regenerates, p. 73–112. In: B.V. Conger (ed.). *Critical reviews in plant sciences*. CRC Press, Boca Raton, Fla.
- Devi, P. and S. Rani. 2002. *Agrobacterium rhizogenes* induced rooting of *in vitro* regenerated shoots of the hybrid *Helianthus annuus* × *Helianthus tuberosus*. *Scientia Hort.* 93:179–186.
- De Carlo, A., C. Benelli, and M. Lambardi. 2000. Development of a shoot-tip vitrification protocol and comparison with encapsulation-based procedures for plum (*Prunus domestica* L.) cryopreservation. *CryoLetters* 21:215–222.
- Gamburg, K.Z., E.F. Vysotskaya, and L.V. Gamanets. 1999. Microtuber formation in micropropagated Jerusalem artichoke (*Helianthus tuberosus*). *Plant Cell Tissue Organ Cult.* 55:115–118.
- Gautheret, R.J. 1969. Investigations on the roof formation in the tissues of *Helianthus tuberosus* cultured *in vitro*. *Amer. J. Bot.* 56:702–717.
- Harding, K. 2004. Genetic integrity of cryopreserved plant cells: A review. *CryoLetters* 25:3–22.
- Harris, W., P.T. Lynch, A.J. Hargreaves, and P.L.R. Bonner. 2004. Cryopreservation of *Helianthus tuberosus* cell suspension cultures: The effect of preculture treatments on cytoskeletal proteins and transglutaminase activity. *CryoLetters* 25:213–217.
- Kim, H.-H., E.-G. Cho, H.-J. Baek, C.-Y. Kim, E.R.J. Keller, and F. Engelmann. 2004. Cryopreservation of garlic shoot tips by vitrification: Effects of dehydration, rewarming, unloading and regrowth conditions. *CryoLetters* 25:59–70.
- Makowska, Z., J. Keller, and F. Engelmann. 1999. Cryopreservation of apices isolated from garlic (*Allium sativum* L.) bulbils and cloves. *CryoLetters* 20:175–182.
- McLaurin, W.J., Z.C. Somda, and S.J. Kays. 1999. Jerusalem artichoke growth, development, and field storage. I. Numerical assessment of plant part development and dry matter acquisition and allocation. *J. Plant Nut.* 22:1303–1313.
- Minocha, S.C. and W. Halperin. 1974. Hormones and metabolites which control tracheid differentiation, with or without concomitant effects on growth, in cultured tuber tissue of *Helianthus tuberosus* L. *Planta* 116:319–331.
- Mix-Wagner, G., H.M. Schumacher, and R.J. Cross. 2002. Recovery of potato apices after several years of storage in liquid nitrogen. *CryoLetters* 24:33–41.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.
- Niino, T., K. Shirata, and S. Oka. 1995. Viability of mulberry winter buds cryopreserved for 5 years at -135°C . *J. Seric. Sci. Jpn.* 64:370–374.
- Nishizawa, S., A. Sakai, Y. Amano, and T. Matsuzawa. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant-regeneration by vitrification. *Plant Sci.* 91:67–73.
- Reed, B.M. 1992. Cold storage of strawberries *in vitro*: A comparison of three storage systems. *Fruit Var. J.* 46:98–102.
- Reed, B.M. 1993. Improved survival of *in vitro* stored *Rubus* germplasm. *J. Amer. Soc. Hort. Sci.* 118:890–895.
- Roche, T.D. and A.C. Cassells. 1996. Gaseous and media-related factors influencing *in vitro* morphogenesis of Jerusalem artichoke (*Helianthus tuberosus*) 'Nahodka' node cultures. *Acta Hort.* 440:588–593.
- Sakai, A., S. Kobayashi, and I. Oiyama. 1991. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var *brasiliensis* Tanaka) cooled to 196°C . *J. Plant Physiol.* 137:465–470.
- Sakai, A., T. Matsumoto, D. Hirai, and T. Niino. 2000. Newly developed encapsulation-dehydration protocol for plant cryopreservation. *CryoLetters* 21:53–62.
- Scowcroft, W.R. 1984. Genetic variability in tissue culture: Impact on germplasm conservation and utilization. *Tech. Rpt. International Plant Genetic Resources*, Rome, Italy.
- Seiler, G.J. 1993. Forage and tuber yields and digestibility of selected wild and cultivated genotypes of Jerusalem artichoke. *Agron. J.* 85:29–33.
- Steponkus, P.L., R. Langis, and S. Fugikawa. 1992. Cryopreservation of plant tissues by vitrification, p. 1–61. In: P.L. Steponkus (ed.). *Advances in low-temperature biology*. JAI Press Ltd., Greenwich, Conn.
- Swan, T.W., D. O'Hare, R.A. Gill, and P.T. Lynch. 1999. Influence of preculture conditions on the post-thaw recovery of suspension cultures of Jerusalem artichoke (*Helianthus tuberosus* L.). *CryoLetters* 20:325–336.
- Tanaka, D., T. Niino, K. Isuzugawa, T. Hikage, and M. Uemura. 2004. Cryopreservation of shoot apices of *in vitro* grown gentian plants: Comparison of vitrification and encapsulation-vitrification protocols. *CryoLetters* 25:167–176.
- Thammasiri, K. 1999. Cryopreservation of embryonic axes of jackfruit. *CryoLetters* 20:21–28.
- Towill, L.E. 2002. Cryopreservation of *Mentha* (mint), p. 151–163. In: L.E. Towill and Y.P.S. Bajaj (eds.). *Biotechnology in agriculture and forestry*, vol. 50.
- Volk, G.M. and C. Walters. 2005. Plant vitrification solution 2 (PVS2) lowers water content and alters freezing behavior in shoot tips during cryoprotection. *Cryobiology* (in press).
- Volk, G.M., N. Maness, and K. Rotindo. 2004. Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2. *CryoLetters* 25:219–226.
- Yamada, T., A. Sakai, T. Matsumura, and S. Higuchi. 1991. Cryopreservation of apical meristems of white clover (*Trifolium repens* L.). *Plant Sci.* 73:111–116.