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Re-induction of Embryogenic Tissue from the Cryopreserved Somatic Embryo in Japanese Larch (*Larix leptolepis* Gordon)

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Abstract : The study aimed to develop a cryopreservation method for long-term storage using mature somatic embryo of Japanese larch. In this study, desiccation treatments significantly affected re-induction rates of embryogenic tissue (ET) from dried somatic embryos. In the effect of different dehydration temperature and duration on the re-initiation ET, the highest frequency was shown when somatic embryos were dehydrated at 25°C for 2 (45.5%) or 1 day (43.3%), respectively. In addition, low temperatures [4°C, 2 days (44.2%) or 3 days (43.5%)] were marked higher ET initiation. After that, the initiation value was declined with dehydration duration. For comparison of different relative humidity on re-induction frequency of ET, the best re-induction (43.5%) was obtained from somatic embryos pre-dried at $(NH_4)_2SO_4$ (RH 79%). Both Na_2HPO_4 (RH 97%) and Na_2CO_3 (RH 88%) treatments were showed the similar rate, 34.6, 34.2%, respectively. However the lowest rate (19.6%) was observed in distilled water (RH 100%). In comparison of the various storage temperatures and duration of the dried somatic embryos, the highest frequency (66.9%) of re-initiation was obtained when somatic embryos were cryopreserved for one day. However, the frequency was gradually decreased as the time length of storage increased regardless of types of storage. None of ET re-initiated when stored at 4°C for 1, 2 and 84 days.

Key words **:** *dehydration, liquid nitrogen, re-induction, solutes*

Introduction

Somatic embryogenesis allows large-scale clonal propagation of elite genotypes and provides through cryopreservation, the opportunity to preserve genotype tissues during the period necessary for field tests. However, initiation of somatic embryogenesis from conifer tree species is at present, with few exceptions, only possible from juvenile material with unknown genotype. For this reason, it is essential to develop techniques for long-time conservation of embryogenic cultures during field testing. At present, cryopreservation is an important tool for safely maintaining living tissues or cells in a genetically stable state for prolonged periods of time. Cryopreservation reduces labor and supply costs, decrease chances for contamination from frequent subculture, limits somaclonal variation result from long-term *in vitro* culture. In addition, cryopreservation can also enhance embryogenic capacity by eliminating highly vacuolated non-embryogenic cells (Kartha *et al.*, 1988). Thus cryopreservation is an especially attractive tool for forest tree embryogenic cultures. Among forest species, cryopreservation protocols have been successfully applies to embryogenic cultures of conifer-*Picea glauca* (Moench) Voss (Kartha *et al.*, 1988), *Picea mariana* (Touchell *et al.*, 2002) and *Pinus patula* (Ford *et al.*, 2000) or hardwood species – *Liriodendron tulipifera* (Wagner *et al.*, 2001) and *Aesculus hippocastanum* (Jekkel *et al.*, 1998), respectively. In larch species, using slow cooling procedures, successful cryopreservation, with high recovery and regeneration frequency, has been demonstrated for hybrid larch (Klimaszewska *et al.*, 1992; Lelu *et al.*, 1995) or Japanese larch (Kim *et al.*, 1999). This technique, however, has some drawbacks. The method requires pretreatment with toxic cryoprotectants, such as dimethyl sulfoxide (DMSO). For coniferous species, cryopreservation is routinely achieved through the use of slow cooling procedures (Häggman, 2000), which requires expensive equipment, which are quite costly, to carefully manipulate cooling rate (Percy *et al.*, 2000). In addition, there is always the possibility of severe tissue damage due to ice crystal formation. For this reason, it is necessary to develop a technique not requires pretreatment using DMSO or sorbitol which causing DNA variation after retrieve from cryopreservation. On the contrary, dehydration (desiccation) has also been evaluated as a potential method for the preservation of somatic embryos as shown for conifer

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species: dried somatic embryos of *Picea abies* stored for 1 year at -20°C successfully regenerated to plantlets (Attree *et al.*, 1995) or reinduction of embryogenic tissue derived from dried and cryopreserved somatic embryos of *P. mariana* (Bomal and Tremblay, 2000). For herb species, dehydration to low moisture content is a means to achieve long-term storage of dried somatic embryos has been reported for alfalfa (Senaratna *et al.*, 1990), carrot (Lecouteux *et al.*, 1992). For alfalfa, somatic embryos have been dried to 8-15% moisture content and stored for one year at room temperature without loss of vigor (Senaratna *et al.*, 1990). Particularly, dehydration of somatic embryos has been shown highly beneficial treatment for enhancing frequency of germination and plantlet regeneration in black spruce (Bomal and Tremblay, 1999, 2000) and hybrid larch (Lelu *et al.*, 1995).

The present study aimed to develop a cryopreservation method for long-term storage using dried somatic embryo in stead of embryogenic suspension in Japanese larch. In this study, we compared some treatments (kinds of solute, temperature & storage duration) to obtain the highest frequency of re-induction of embryogenic tissue from somatic embryos which preserved in low-temperature including liquid nitrogen. Our data also suggested that mature somatic embryos may provide an alternative source of germplasm for maintaining elite lines in cryopreservation.

Materials and Method

1. Plant material

ET of Japanese larch were initiated from immature embryos and maintained as described by Kim *et al.* (1999). Embryogenic cultures were maintained on LMB medium, ½LM (Litvay *et al.*, 1985) medium composed of half-strength macro-, and micro- salts, full strength vitamins (0.56 μM myo-inositol, 4.06 μM Nicotinic acid, 0.49 μM Pyridoxine HCl and 0.30 μM Thiamine HCl), 10.3 mM L-glutamine, and 58.4 mM sucrose (Sigma, Grade I). The plant growth regulators, 9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 4.4 μM N6-benzylaminopurine (BAP), solidified with 0.4% (w/v) gellan gum (Phytagel, Sigma). The pH of media was adjusted to 5.7 with 1 *N* HCl and 1 *N* NaOH prior to autoclaving at 121°C for 15 min. L-glutamine (Sigma) was sterilized by filtration, added to cooled medium (45-50°C) after autoclaving. Cultures were maintained and matured in the dark at 24±1°C. Subculturing every 2 weeks required to maintain the ET in a healthy proliferative state.

2. Maturation of somatic embryos

Maturation of somatic embryos was carried out with one embryogenic line. The plating technique was as described by Klimaszewska and Smith (1997) with slight modification. The procedure that ET were weighed and dispersed in liquid ½LM medium without growth regulators. After the ET suspensions were homogenized with pipette, 3 mL of the liquid medium containing 90 mg FW (30 mg/mL) of dispersed tissue were poured over a filter paper (Whatman #2, Ø5.5 cm) placed in a Büchner funnel. After draining the medium by low pressure pulse vacuum, the filter paper with ET on it was placed on maturation media with 0.2 M maltose (Sigma), 60 μM Abscisic acid (ABA, Sigma), and 0.8% gellan gum and cultured in darkness for 8 weeks without subculture to fresh medium.

3. Dehydration with different relative humidities

Somatic embryos desiccation, with 6 desiccation treatments, was performed by placing them to the multi-well dish (Falcon) in the dark at 23°C. Relative humidities of 100% (RH100), 97% (RH 97), 88% (RH 88), 79% (RH 79), 63% (RH 63) and 43% (RH 43) were generated using saturated salt solutions of distilled water, Na_2HPO_4,, Na_2CO_3, $(NH_4)_2SO_4$, NH_4NO_3, K_2CO_3, respectively (Rockland, 1960).

4. Different low-temperature storage and cryopreservation

Following on from the above, the use of dried and stored on low temperature (4, -20 and -80°C) or liquid nitrogen (LN, -196°C) somatic embryos of Japanese larch as explants for reinduction of ET was tested. Five to six dried somatic embryos were transferred to sterile 1.2 mL polypropylene cryogenic vials (Corning). For low-temperature storage, the vials including somatic embryos were kept at 4, -20 and -80°C in refrigerator and freezer, respectively. Cryopreservation was also carried out without cryoprotectant, by immersing the vials in LN. Somatic embryos were stored at low temperature or LN for 7-84 days.

5. Thawing from cryopreservation

For fast thawing, cryovials were put into warm water (40°C) directly, and leave them on water for 4-5 min.

6. Rehydration

Thawed somatic embryos were immediately transferred to Petri dish (Srerillin, 87×15 mm) containing a filter paper (Whatman #2, Ø5.5 cm) soaked with some sterile distilled water and rehydrated for overnight.

7. Re-induction ET from low temperature stored or cryopreserved somatic embryos

In order to re-induce ET from the rehydrated embryos, the embryos were cultured onto initiation medium includ-

ing ½LM salts, vitamines, 2.0 mg/L 2,4-D, 1.0 mg/L BA, 20 g/L sucrose, 1000 mg/L L-glutamine and 0.4% Phytagel. Reinduction frequency was recorded after 8 weeks. The experimental design was completely randomized, with 20 embryos per replicate (Petri dish) (n = 5).

Results

1. Effect of different dehydration temperature and duration on the re-induction of ET

Two dehydration temperature (25 or 4°C) and duration (1, 2, 3, 4, 5 and 9 days) used significantly affected the production of ET from dried and cryopreserved somatic embryos. The highest reinduction rate was shown in the dehydrated somatic embryos were placed at 25°C for 2 (45.5%) (Figure 2). The lowest one (22.9%) was observed when somatic embryos were dried in treatment with 4°C for 9 days (Figure 2).

2. Effect of different relative humidities on re-induction rates of ET

Desiccation treatments significantly affected re-induction rates of ET from dried somatic embryos. The best re-induction frequency (43.5%) was obtained from cryopreserved embryos pre-dried at $(NH_4)_2SO_4$ (RH 79%). Both Na_2HPO_4 (RH 97%) and Na_2CO_3 (RH 88%) treatments were showed the similar rate, 34.6, 34.2% (Figure 3), respectively, however the lowest value (19.6%) was obtained in distilled water (RH 100%). When the somatic embryos were dehydrated at 63 (NH_4NO_3), 43% RH, the frequency was shown as 30, 27.8%, respectively, which did better than 100% RH.

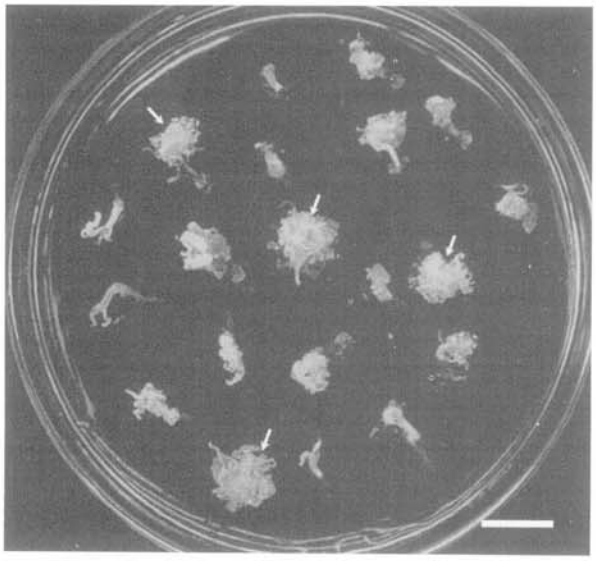

Figure 1. ET (arrows) derived from the cryopreserved somatic embryos. Photograph was taken 4 weeks after thawing (scale bar=1.5 cm).

2. Re-induction ET from low temperature stored or cryopreserved somatic embryos

In comparison of the various storage temperature & duration with dried somatic embryos, the highest frequency (57.5%) of reinduction was obtained when somatic embryos were cryopreserved into nitrogen for one day (Figure 1, 4). However, the re-initiation value was gradually decreased as the storage duration increased. None of ET re-initiated when stored at 4°C for 84 days. The declination in re-induction frequency as increased storage time may be due to desiccation conditions, which generally provide different drying rates. Cryopreserved embryos first turned brown, then began to recover 2-3 weeks after thawing. Some embryos started to produce ET on the surface of explants after 4weeks of recovering LN, some of them produced abundantly ET as shown in Figure 1.

Discussion

The present study demonstrates that mature dried (dehydrated) somatic embryos are amenable to store for long-term that as shown in *Picea mariana* and *P. glauca* (Bomal and Tremblay, 2000). The results provide evidence that pre-dehydrated embryos can tolerate immersion in liquid nitrogen without need for cryoprotectants, and can be used directly to induce new ET. Several protective measures were applied to somatic embryos (desiccation, chemical protectors, hardening by culture at low temperatures, encapsulation in alginate beads). Among them, somatic embryos were easily cryopreserved by means of the simplest technique.

Re-induction frequency from dried somatic embryos is influenced by method of desiccation (duration or temperature) or temperature kinds of storage (Bomal and Tremblay, 2000). In addition, the cryopreservation of somatic embryos is influenced by the genotype and physiological state of the donor plant, the size and development of the embryo, its water content, the method of freezing, and the thawing temperature. Reduction of water content to critical level seems to be a necessary step for successful cryopreservation of somatic embryos of plant species (Janeiro *et al.*, 1996; Bomal and Tremblay, 2000). In the present experiment, the method of desiccation was shown to play a key factor in the re-induction from cryopreserved somatic embryos in this species. During desiccation, drying conditions must be controlled to ensure high frequency of stored somatic embryos. Data from the re-induction showed that the highest values were obtained when embryos were desiccated at 79% RH and 2 days at 25°C (Figure 2). However, Bomal and Tremblay (2000) showed the best results were obtained when embryos were desiccated at

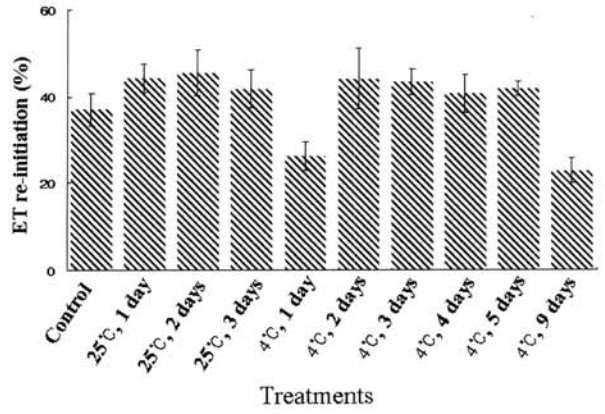

Figure 2. Effect of dehydration of different temperature and duration on the re-induction frequency of ET in *L. leptolepis*. (Bars: Standard deviation)

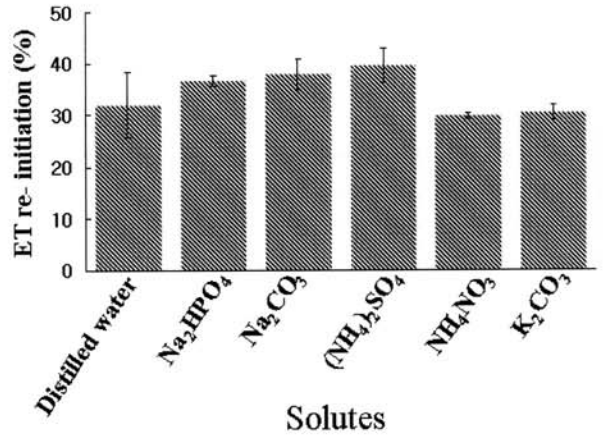

Figure 3. Effect of dehydration of different relative humidity on re-induction frequency of ET in *L. leptolepis*. (Bars: Standard deviation)

97% RH before cryopreservation in *Picea* species. This means that re-induction rate after cryopreservation of somatic embryos was influenced greatly by genotype and physiological state, size and stage of development, water content and thawing temperature shown by Florin *et al.* (1993) or Bomal and Tremblay (2000).

The present study shows that somatic embryos of Japanese larch can be cryopreserved for germplasm storage and subsequently can be used as explants to re-induce ET. The 79% RH and 2 days at 25°C dehydration treatment used for Japanese larch greatly improved the reinduction frequency of dehydrated and cryopreserved somatic embryos.

We have shown that cryopreservation of dried somatic embryos is one of candidate methods for log-term embryo storage of Japanese larch. This method is rapid, effective, and does not require the use of expensive and possibly phyto-toxic cryoprotectant such as DMSO (Bomal and Tremblay, 2000). DMSO in 2-10% solutions was involved in generating genetic and epigenetic variations

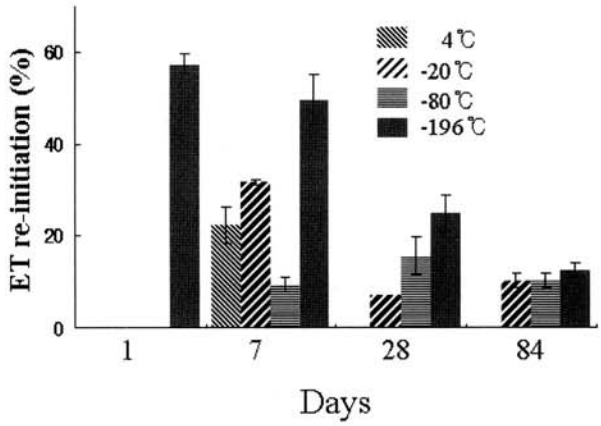

Figure 4. Comparison of storage types on re-induction of ET in *L. leptolepis*. (Bars: Standard deviation)

(Finkle *et al.*, 1985).

An alternative to ESM is the cryopreservation of mature somatic embryos, which has been suggested by Touchell *et al.* (2002) as a potential strategy for conserving conifer germplasm. In previous reports, spruce and western red pine somatic embryos have survived LN treatments if dried to remove freezable water (Percy *et al.*, 2000). In addition, for Japanese larch, we have demonstrated that nature somatic embryos can survive LN exposure with appropriate desiccation treatments. Our system with dried somatic embryos, as compared ET, could be applicable for conifer genetic transformation.

In conclusion, cryopreservation of Japanese larch somatic embryos using dehydration has been achieved for the first time. Further experiments should be accomplished to elucidate comparison of efficiencies in terms of somatic embryos production after reinduction, plant regeneration and genetic variation between parent lines and sublines in this species. The responsiveness of maturation and plantlet regeneration capacities from sublines should be show to that of the parent line.

Literature Cited

1. Attree, S.M., Pomeroy, M.K. and Fowke, L.C. 1995. Development of white spruce (*Picea glauca* (Moench) Voss) somatic embryos during culture with abscisic acid and osmoticum, and their tolerance to drying and frozen storage. J. Exp. Bot. 46: 433-439.
2. Bomal, C. and Tremblay, F.M. 1999. Effect of desiccation to low moisture content on germination synchronization of root emergence, and plantlet regeneration of black spruce somatic embryos. Plant cell Tiss. Org. Cult. 56: 193-200.
3. Bomal, C. and Tremblay, F.M. 2000. Dried cryopreserved somatic embryos of two *Picea* species provide suitable material for direct plantlet regeneration and

germplasm storage. Ann. Bot. 86: 177-183.

4. Finkle, B.J., Zavala, M.E. and Ulrich, J.M. 1985. Cryoprotective compounds in the viable freezing of plant tissues. In: Kartha KK, ed. Cryopreservation of plant cells and organs. New York; CRC Press, 75-113.
5. Florin, B., Tessereau, H., Lecouteux, C., Didier, C. and Petiard, V. 1993. Long-term preservation of somatic embryos. In: Redenbaugh K, ed. Synseeds. Applications of synthetic seeds to crop improvement. London: CRC Press, 133-161.
6. Ford, C.S., Jones, N.B. and van Staden, J. 2000. Cryopreservation and plant regeneration from somatic embryos of *Pinus patula*. Plant Cell Rep. 19: 610-615.
7. Häggman, H.M., Aronen, T.S. and Ryynanen, L.A. 2000. Cryopreservation of embryogenic cultures of conifers. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 6, Kluwer, Dordrecht, pp707-728.
8. Janeiro, L.V., Vieitez, A.M. and Ballester, A. 1996. Cryopreservation of somatic embryos and embryonic axes of *Camellia japonica* L. Plant Cell Rep. 15: 699-703.
9. Jekkel, Zs., Gyulai, G., Kiss, J., Kiss, E. and Heszky, L.E. 1998. Cryopreservation of horse-chestnut (*Aesculus hippocastanum* L.) somatic embryos using three different freezing methods. Plant cell Tiss. Org. Cult. 52: 193-197.
10. Kartha, K.K., Fowke, L.C., Leung, N.L., Caswell, K.L. and Hakman, I. 1988. Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). J. Plant Physiol. 132: 529-539.
11. Kim, Y.W., Kim, J.C., Youn, Y., Noh, E.R. and Son, S.H. 1999. Cryopreservation of embryogenic tissue and plant regeneration in *Larix leptolepis*. Kor. J. Plant Tiss. Cult. 26: 229-233.
12. Kim, Y.W., Youn, Y., Noh, E.R. and Kim, J.C. 1999. Somatic embryogenesis and plant regeneration from immature zygotic embryos of Japanese larch (*Larix leptolepis*). Plant Cell, Tiss. Org. Cult. 55: 95-101.
13. Klimaszewska, K. and Smith, D.R. Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol. Plant. 100: 949-957; 1997.
14. Klimaszewska, K., Ward, C. and Cheliak, W.M. 1992. Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix* × *eurolepis*) and black spruce (*Picea mariana*). J. Exp. Bot. 43: 73-79.
15. Lecouteux, C., Tessereau, H., Florin, B., Courtois, D. and Petiard, V. 1992. Preservation of carrot (*Daucus carota* L.) somatic embryos by dehydration after pretreatment with sucrose. C.R. Acad. Sci. Ser. III. 314: 423-428.
16. Lelu, M.A., Klimaszewska, K., Pflaum, G. and Bastien, C. 1995. Effect of maturation on desiccation tolerance in hybrid larch (*Larix* × *Leptoeurapaea* Dengler) somatic embryos. In Vitro Cell. Dev. Biol. 31: 15-20.
17. Litvay, J.D., Verma, D.C. and Johnson, M.A. Influence of a loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Darcus carota* L.). Plant Cell Rep. 4: 325-328; 1985.
18. Percy, R.E., Klimaszewska, K. and Cyr, D.R. Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can. J. For. Res 30: 1867-1876; 2000.
19. Rockland, L.B. Saturated salt solutions for static control of relative humidity between 5 and 40. Anal. Chem. 32: 1375-1376; 1960.
20. Senaratna, T., McKersie, B.D. and Bowley, S.R. Artificial seeds alfalfa (*Mediacago sativa* L.). Introduction of desiccation tolerance in somatic embryos. In Vitro Cell. Dev. Biol. 26: 85-89; 1990.
21. Touchell, D.H., Chiang, V.L. and Tsai, C.J. Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. Plant Cell Rep. 21: 118-124; 2002.
12. Wagner, A.V., Christopher, P.H., Paul, M.M. and Dale, R.S., Scott, A.M. Cryopreservation of yellow-poplar and sweetgum embryogenic cultures. New For. 21: 283-292; 2001.

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