Korean J Crop Sci 49(4) 354~357(2004)

Cryopreservation of Zygotic Embryos of Herbaceous Peony (*Paeonia lactiflora* Pall.) by Encapsulation-Dehydration

Hyun-Mi Kim*, Jong-Hee Shin, and Jea-Keun Sohn*†**

**Department of Agronomy, Kyungpook National University, Daegu 702-701*
***Institute of Bioresources, Gyeoungbuk Provincial Agricultural Technology Administration, Andong 760-891*

ABSTRACT: A simplified technique which cryoprotects zygotic embryos by encapsulation-dehydration was developed for the germplasm conservation of herbaceous peony (*Paeonia lactiflora* Pall.). The highest survival rate (85%) was obtained from embryos treated by encapsulation-dehydration. The zygotic embryos were precultured on MS medium containing 0.3mg/L GA_3 for 1 day. The precultured embryos were encapsulated in 3% (w/v) alginate beads and immersed for 1 h in MS medium containing 2 M glycerol and 0.5 M sucrose. The encapsulated embryos were dehydrated for 5 h by air drying prior to direct immersion in liquid nitrogen. This encapsulation-dehydration method appears to be a promising technique for germplasm cryopreservation of a herbaceous peony.

Keywards: cryopreservation, encapsulation-dehydration, peony, zygotic embryo

Germplasm preservation plays an important role in the maintenance of biodiversity and avoidance of genetic erosion, but the preservation in the field gene banks requires huge land areas and high cost. More importantly, the genetic resources are threatened by attacks of pests, diseases, and other natural hazards (Engelmann, 1997; Withers & Engelmann, 1998).

For many crops, cryopreservation is currently being employed to overcome the serious limitations encountered by traditional germplasm conservation strategies. Conservation at ultra-temperatures, usually at -196 °C, which is the temperature of liquid nitrogen (LN), allows the long-term and contamination-free storage of plant genetic resources (Helliot *et al.*, 2002). Moreover, the materials are stored in a small volume, which requires only very minimal maintenance. Cryopreservation systems using zygotic embryos have been established in orchid (Ishikawa *et al.*, 1997), barley (Wang & Huang, 2002), and coffee (Dussert *et al*, 2002).

Herbaceous peony is one of the important crops because of its value as medicinal (Choung & Kang, 1994) and ornamental plants. Peony is usually propagated by cloning, and genetic resources of this species are mainly conserved vegetatively in the field gene bank, which is costly and laborious. Cryopreservation using zygotic embryos preserves the genetic resources of peony.

To our knowledge, successful cryopreservation of peony has not yet been established The aim of the present research was to establish a reliable method for the cryopreservation of herbaceous peony using zygotic embryos.

MATERIALS AND METHODS

Plant materials

Mature seeds used for this study were supplied by Institute of Bioresources, Gyeoungbuk Provincial Agricultural Technology Administration. Peony seeds were soaked in 70% ethanol for 1 min, 1% sodium hypochlorite solution for 15 min, and finally washed three times with sterile distilled water. Zygotic embryos excised from sterilized seeds were precultured for 24 h on MS medium containing 0.3 mg/L GA_3.

Desiccation

After preculturing for 1 day, the embryos were placed on sterilized filter paper in 9-cm petri dishes. The embryos were desiccated for 0 h to 3 h under a laminar flow cabinet to determine the optimum desiccation times for cryopreservation. The desiccated embryos were cryopreserved for 1 h and thawed in a water bath at 40 °C for 5 min. The thawing embryos were cultured on MS medium containing 0.3 mg/L GA_3.

Vitrification

The precultured zygotic embryos were transferred to cryotubes (2 ml) and then loaded with a mixture of 2 M glycerol and 0.5 M sucrose. After removing the loading solution, they were dehydrated with PVS2 for 30 min PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide in MS liquid medium with 0.4 M sucrose (pH 5.8). Embryos were immersed in LN for 1 h

†Corresponding author (Phone) +82-53-950-5711 (E-mail) jksohn@knu.ac.kr
<Received August 12, 2004>

and rapidly warmed in a water bath at 40°C for 5 min. The PVS2 solution was drained from the cryotubes and replaced with 1.2 M sucrose in MS liquid medium, pH 5.8; embryos were maintained in that state for 20 min and then placed on MS medium containing 0.3 mg/L GA_3.

Encapsulation-dehydration

The precultured embryos were suspended in a 3% sodium alginate solution and immediately dropped into 100 ml of a 100 mM $CaCl_2$ solution containing 0.6 M sucrose to allow the formation of sodium alginate beads (about 5 mm in diameter) After keeping the embryos in the solution for 30 min, each encapsulated embryo was immersed in MS medium containing 2 M glycerol and 0 5 M sucrose. Then they were placed on sterilized filter paper in 9-cm petri dishes and dehydrated for 0 h to 7 h by placing the dishes under a laminar flow cabinet to reduce water content. The dehydrated beads were transferred to a cryotube, immersed in LN for 1 h, and thawed in a water bath at 40 for 5 min. Cryopreserved embryos were cultured on MS medium containing 0.3 mg/L GA_3.

Survival rate

Survival rates were determined by regrowth on solidified MS medium containing 0.3 mg/L GA_3 at 25°C under continuous light. The development of cryopreserved embryos was investigated at 4 weeks after they were on MS medium containing 0.3 mg/L GA_3. The survival rates were evaluated as the ratio of the germinated embryos to the total number of cultured embryos.

RESULTS

An initial experiment to optimize the desiccation times showed that the survival rate of cryopreserved zygotic embryos changed with desiccation times ranging from 0 h to 3 h. The survival rate of non-desiccated embryos was 14%, but that of the embryos desiccated for 1 h by air drying in laminar flow cabinet was 69% (Fig. 1)

The survival rate of encapsulated embryos after cryopreservation greatly changed with dehydration periods ranging from 0 h to 7 h, as shown in Fig. 2. The highest survival rate (66.7%) was obtained from embryos desiccated for 5 h by air drying.

In order to determine the most suitable cryogenic procedure, we cryopreserved zygotic embryos using three different pretreatment methods: desiccation, vitrification, and encapsulation-dehydration. The maximum survival rate (85%) was achieved from the cryopreserved embryos by

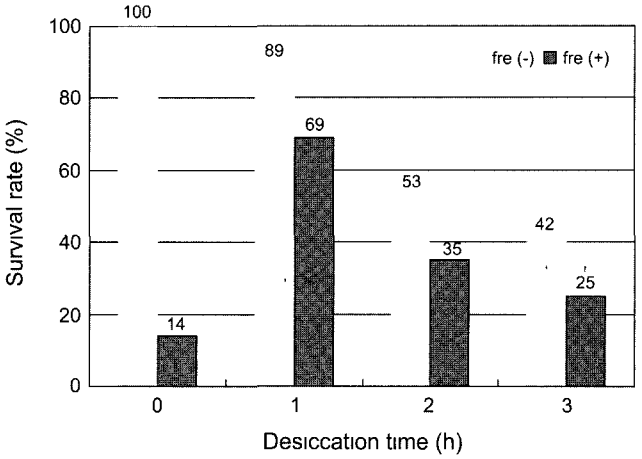

Fig. 1. Changes in survival rate of cryopreserved zygotic embryos by desiccation times. fre (−) : non-cryopreserved embryos, fre (+) : cryopreserved embryos.

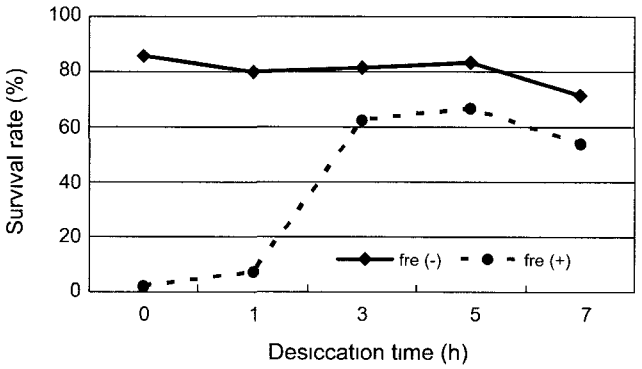

Fig. 2. Effect of dehydration on survival of encapsulated embryos fre (−) non-cryopreserved embryos, fre (+) : cryopreserved embryos

encapsulation-dehydration, followed by desiccation (71%), and then vitrification (65%) (Fig. 3).

DISCUSSION

Dussert *et al* (2002) achieved maximum survival (96%) of cryopreserved zygotic embryos of coffee after 0.5 h desiccation. However, no embryos survived if they were excessively desiccated. These results are consistent with those of the present research and indicate that optimal desiccation is the key factor for successful cryopreservation of zygotic embryos. For most plant-cell and organ cultures, injury during freezing is associated with intracellular ice formation in highly vacuolated cells; therefore, survival of cryopreservation could be increased by reducing the water content of the cell (Wang *et al.*, 2000). The optimal water content for survival varies among plant species because of their differences in dehydration tolerance (Withers & Engelmann, 1998).

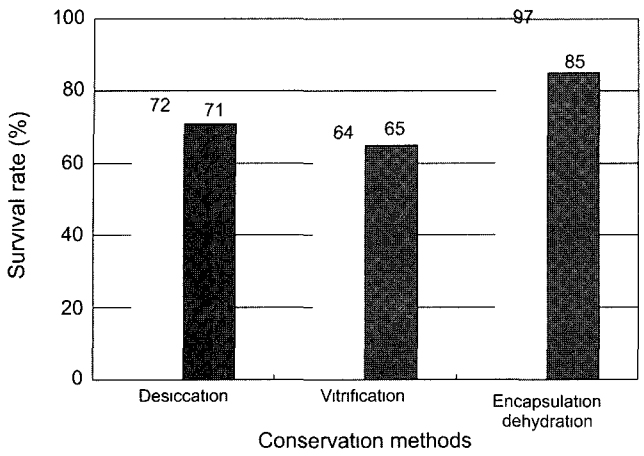

Fig. 3. Effect of desiccation, vitrification and encapsulation-dehydration on survival of cryopreserved embryos in herbaceous peony. fre (–) . non-cryopreserved embryos, fre (+) cryopreserved embryos. Desiccation: Embryos were desiccated for 1 h by air drying Vitrification: Embryos were dehydrated with PVS2 for 30 min prior to a plunge into LN Encapsulation-dehydration· Embryos were encapsulated with 3% sodium alginate solution and dehydrated by exposure to a sterile air flow before being frozen in LN

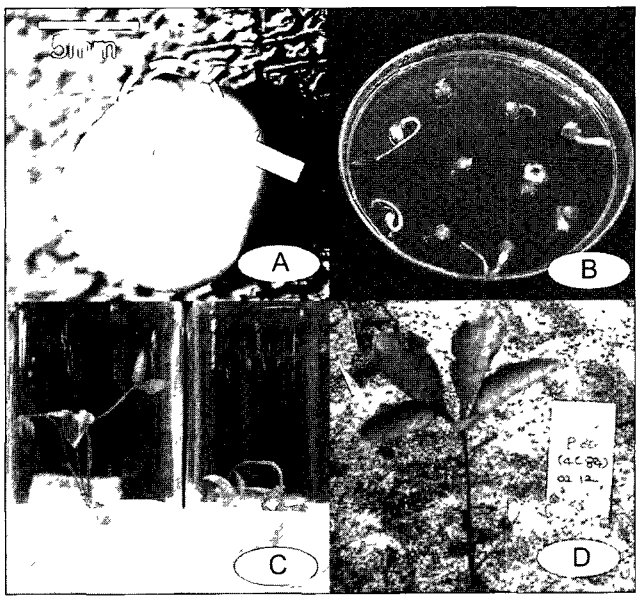

Fig. 4. Plant regeneration from cryopreserved zygotic embryos of herbaceous peony. A . Zygotic embryo in seed of herbaceous peony, B Germination from the encapsulated embryos after cryopreservation, C . Plants recovered from cryopreserved embryos at 50 days after culture, D Plant transplanted in pot

Therefore, it is, important to emphasize that, in order to achieve the highest survival of cryopreserved embryos, the optimum desiccation condition should be carefully determined before any application (Wang *et al.*, 2000)

In particular, vitrification is the most widely used technique. It enables the cryopreservation of the shoot tips, meristems, and embryogenic tissues of many plants. Using this technique, high concentrations of dimethylsulfoxide, glycerol, and ethylene glycol are neccessary to achieve sufficient dehydration of materials. For example, a representative vitrification solution, PVS2, contains the above chemicals at 15% (w/v), 30% (w/v), and 15 (w/v), respectively (Sakai *et al.*, 1990). These chemicals must be washed out or considerably diluted after rewarming the cryopreserved materials to eliminate their toxic effect (Hirata *et al.*, 2002). On the other hand, the encapsulation-dehydration technique is more suitable for the cryopreservation of embryos in comparison with the vitrification technique; the washing step after rewarming can be eliminated because no toxic chemicals are used. The main advantages of the encapsulation-dehydration protocol are an easier manipulation of organs by alginate embedding, storage of large number of delicate explant (shoot tips, embryos, meristems, callus, and pollen), direct protection of embryos during dehydration, higher regrowth of cryopreserved embryos, and an efficient cryopreservation of different varieties (Paul *et al.*, 2000).

In conclusion, desiccation for 1h under a laminar flow cabinet enhanced survival of cryopreserved zygotic embryos. Survival of embryos after cryopreservation by encapsulation-dehydration (85%) was greater than by desiccation (71%) and vitrification (65%). Thus, the encapsulation-dehydration appears to be a potentially valuable cryogenic protocol for cryopreservation of peony germplasm.

ACKNOWLEDGEMENTS

This Study was Supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

REFERENCES

Choung, M G and K H Kang 1994 Extraction Methods and HPLC Analysis Conditions of Paeoniflorin in Peony, *Paeonia lactiflora* Pall. Korean J. Crop. Sci 36 542-547.

Dussert, S , N Chabrillange, F Engelmann, F Anthony, N Vasquez, and S Hamon. 2002. Cryopreservation of *Coffea* (Coffee) In· Towill, L. E , and Y. P S Bajaj (eds) Biotechnology in Agriculture and Forestry 50 Cryopreservation of Plant Germplasm (pp 220-233), Springer, Berlin.

Engelmann, F. 1997 In vitro conservation methods In Callow, J. A , Ford-Lloyd & Newbury, H J (eds) Biotechnology and Plant Genetic Resources (pp 119-161) CAB International, Oxford.

Helliot, B , B. Panis, Y. Poumay, R. Swennen, P Lepoivre, and E Frison 2002 Cryopreservation for the elimination of cucum-

ber mosaic and banana streak viruses from banana (*Musa* spp.). Plant Cell Rep 20 : 1117-1122.

Hirata, K., M. Mukai., S Goda., M Ishio-Kinugasa, K Yoshida , A Sakai, and K Miyamoto 2002 Cryopreservation of hairy root cultures of *Vinca minor* (L) by encapsulation-dehydration Biotechnology Letters 24 371-376

Ishikawa, K., K Harata, M Mii, A. Sakai, K Yoshimatsu, and K. Shimomura. 1997 Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification Plant Cell Rep. 16 : 754-757.

Paul, H , G. Daigny, and B S Sangwan-Norreel 2000 Cryopreservation of apple (*Malus domestica* Borkh) shoot tips following encapsulation-dehydration or encapsulation-vitrification Plant Cell Rep. 19 · 768-774

Sakai, A , S. Kobayashi, and I Oiyama 1990 Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb var *brasiliensis* Tanaka) by vitrification Plant cell Rep 18 : 749-753

Wang, J H and C N. Huang 2002. Cryopreservation of Hordeum (Barley) In Towill, L E and Y P. S Bjai (eds) Biotechnology in Agricuture and Forestry 50 Cryopreservation of Plant Germplasm (119-135), Springer, Berlin

Wang, Q , E Tanne, A. Arav, and R Gafny. 2000. Cryopreservation of *in vitro*-grown shoot tips of grapevine by encapsulation-dehydration Plant Cell Tiss. Org Cult 63 41-46

Withers, L A. and F. Engelmann. 1998 *In vitro* conservation of plant genetic resources. In: Altman A (ed) Agricultural Biotechnology (pp. 57-88), Marcel Dekker, New York