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Cryopreservation of zygotic embryos of wild yams (*Dioscorea* spp.) in Korea

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ABSTRACT A simplified technique that cryoprotects zygotic embryos by desiccation was developed for germplasm conservation of wild yam species (*Dioscorea* spp.) in Korea. Comparative studies with three other cryogenic techniques were conducted. The maximum survival of zygotic embryos were achieved at a frequency of 96.6% when embryos were cryopreserved by the desiccation method. For the successful cryopreservation of yam zygotic embryos, those that were excised from immature/mature seeds were dried in the air stream of a laminar flow cabinet for 30 min at room temperature and then directly immersed in liquid nitrogen.

Introduction

The yam belongs to the *Dioscorea* genus which has more than 600 species (Coursey 1967), whereby most of them are distributed in intertropical zones, the humid areas. In East Asia, the genus is widely distributed from the tropics to the subarctic (Prain and Burkill 1936). Yams, which are edible or medicinal tuber crops, are a very important crop in many developing countries. In South Korea, *Dioscorea batatas* Decne. has been cultivated widely to produce edible tubers. Several wild *Dioscorea* species have been used for medicinal drugs. Yam germplasm conservation requires a high labor input for efficient maintenance. Yams, being clonally propagated, are conventionally preserved in the field genebanks as living collections. Field maintenance, however, is expensive and presents a high-risk loss of materials due to biotic and abiotic stress. Therefore, the development of an efficient and reliable protocol for yams is essential for the long-term conservation of its germplasm. The genetic resources of some vegetatively-propagated crop species have been safely cryopreserved and propagated using micropropagation system (Bajas 1983; Fukai et al. 1994; Jaime and Silva 2003; Kim et al. 2004). In yams (*Dioscorea* spp), the cryopreservation using shoot-apices has received wide attention in recent years. The successful cryopreservation of apices, using an encapsulation-dehydration technique, was reported earlier (Mandal et al. 1996; Malaurie et al. 1998). More recently, the use of the same technique, for the cryopreservation of yams, was also reported by another laboratory (Dixit-Shama et al. 2005). Also, there are many previous studies for *in-vitro* micropropagation of yam species (Uduebo 1971; Grewal et al. 1977; Mantell et al. 1978; Ammirato 1984; Sylvia et al. 1995; Shin et al. 2004a; Shin et al. 2004b). The genus *Dioscorea*, however, showed large differences in its response depending on the type of explants, cultivars and concentration levels of plant growth regulators.

In Korea, a wild yam is one of the important crops; this is because of its value as medicinal plants. The yams wide spread in Korea have been called 'San Yak' in oriental medicine because they are able to well withstand against low temperatures as compared to those growing in the tropical regions. For this reason, they have been used for nourishment, extra strength and prevention of diarrhea since ancient times. They have also been known to be beneficial for the lungs and spleen (Toh 1984), for antiphlogistic effects and detoxification, as cough remedy and for discharge of phlegm, urination, neuralgia and rheumatism etc. (HCS 1992). *D. nipponica*, especially have been

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called 'Chun-san-ryong' in oriental medicine, which containing dioscin and diosgenin. They have also been known to be beneficial for discharge of arthritis, lumbago, phlegm, asthenia etc. In the present study, we checked the cryopreservation efficiency of cultivated and wild type yam species.

Cryopreservation of zygotic embryos presents several disadvantages for germplasm conservation. Zygotic embryos were found to be heterozygous in allogamous plants (Hartmann et al. 2002). Zygotic embryos, however, are very small organs from which whole yam plants can easily develop. The development of a long-term preservation method using zygotic embryos is very important for conserving plant germplasm, breeding programs and the floricultural industry (Ishikawa et al. 1997). Most of wild yam species in Korea produced seeds and usually multiplicated by seeding in nature, while the cultivated yams are use usually propagated by planting of vegetative clones. Therefore, in the present study, in order to develop an efficient cryopreservation protocol using zygotic embryos for several yam wild species in Korea, comparative studies with three other cryogenic techniques were conducted.

Materials and methods

Plant materials for cryopreservation by encapsulation-dehydration in three yam genotype

The stock plants of two cultivars, 'Ma1' (short cylinder tubers), 'Db037' (round tubers), and one wild type (Slender tubers) of *Dioscorea batatas* were grown at the field of the Institute of Bioresources, Gyeongbuk Provincial Agricultural Technology Administration. Vine cuttings, with several axillary buds, were collected in late July, and then prepared by removing the leaves. The explants were surface sterilized by dipping the tissue in 70%(v/v) of ethanol for 30 sec and then by immersing them in 2%(v/v) of NaOCl solution for 15 min. The explants were then thoroughly washed 3 times with sterile, distilled water. Shoot-apices excised from the axillary buds were cultured in a MS medium (Murashige and Skoog 1962) with 0.5 mg/L of BA (N_6+benzyladenine) and 0.5 mg/L of kinetin. For the test of cryopreservation efficiency of their yam cultivars, shoot-apices, excised from *in-vitro* grown plantlets in each cultivar, were used for experimental materials.

Cryopreservation methods using shoot-apices by encapsulation-dehydration

Excised shoot-apices from *in-vitro* grown plantlets were precultured on a medium with 0.3 M of sucrose for 16 h. Shoot-apices were suspended in a calcium free MS inorganic medium supplemented with 3% (w/v) Na-alginate and 0.4 M of sucrose. The mixture including the apices was encapsulated with a sterile pipette into 0.1 M of $CaCl_2$ solution containing 0.4 M of sucrose at room temperature for 1 h, in order to form beads (about 4 mm in diameter). Each bead contains one shoot-apex. In order to increase dehydration tolerance before air drying, these encapsulated apices were treated in a MS medium supplemented with 0.5M of sucrose and 2 M of glycerol for 1 h at 100 rpm on a rotary shaker at 26±1℃. They were subjected to air drying for 4 h in petri-dishes. Twenty dried beads were placed in a 2 mL polypropylene sterile cryo-tube and frozen in LN where they were kept for at least 1 h. The cryo-tubes, which had been immersed in LN, rapidly thawed in a water-bath at 40℃ for 3 min. The cryopreserved beads were then placed in petri-dishes on a MS medium with 0.2 mg/L of kinetin and 0.2 mg/L of BA.

Cryopreservation methods for zygotic embryos of wild yam

Materials: This study used immature or mature seeds collected from a mature mother plant of *D. batatas* (wild type) and *D. nipponica* in the field of the Institute. Seeds formed by open-pollination of two wild yam genotype were harvested at the field of the Institute in late August (35DAF; 35 days after flowering) and mid September (50DAF), surface sterilized in 70% (v/v) ethanol for 1 min and 1% (v/v) sodium hypochlorite solution for 15 min followed by four rinses in sterile distilled water. Seed coat and endosperm were removed from the seeds.

Desiccation technique: Zygotic embryos excised from mature and immature seeds of *D. batatas* (wild type, slender tuber) and *D. nipponica* were placed in open, empty petri-dishes, 30 embryos to a 9 cm diameter dish, and desiccated in the air stream of a laminar flow cabinet for 0 and 30 min. For each desiccation amount of time, half of the desiccated embryos

were then transferred to a MS medium with 0.3 mg/L of GA_3 (gibberelic acid), and the other half were placed in 2 mL cryo-vials (30 embryos to a vial), which were immediately plunged into LN. The embryos of the control group that did not receive any desiccation treatment were similarly dealt with, half were cryopreserved (LN+) and the other half were left uncooled (LN-).

Preculture technique: Harvested zygotic embryos were cultured on MS medium containing 0.3 mg/L of GA_3 for a day. Half of the precultured embryos were placed in 2 mL cryo-vials (30 apices to a vial), which were immediately plunged into LN.

Encapsulation-dehydration technique: Excised embryos from a mature seed of *D. batatas* (wild type) were precultured on a medium with 0.3 mg/L of GA_3 for a day. The precultured embryos were then suspended in a calcium free MS inorganic medium supplemented with 3% (w/v) Na-alginate and 0.4M of sucrose. The mixture including the apices was encapsulated with a sterile pipette into 0.1M of $CaCl_2$ solution containing 0.4M of sucrose at room temperature for 1 h, in order to form beads (about 4 mm in diameter). Each bead contains one embryo. In order to increase dehydration tolerance before air drying, these encapsulated embryos were treated in a MS medium supplemented with 0.5 M of sucrose and 2M of glycerol for 1 h at 100 rpm on a rotary shaker at 26±1℃. They were subjected to air drying for 4 h in petri-dishes. Twenty dried beads were placed in a 2 mL polypropylene sterile cryo-tube and frozen in LN where they were kept for at least 1 h. The remaining beads were placed in petri-dishes on a MS medium with 0.3 mg/L of GA_3.

Thawing and post-thaw regrowth

The cryo-tubes, which had been immersed in LN, rapidly thawed in a water-bath at 40℃ for 3 min. Cryopreserved zygotic embryos were post-cultured on a MS medium containing 0.3 mg/L of GA_3. The embryos were maintained in the above conditions for stock culturing.

Measurement

The developmental parameters, such as the post thaw regrowth of cryopreserved embryos, were recorded for all experiments. The survival frequencys of the embryos were determined by regrowth on a solidified MS medium containing 0.3 mg/L of GA_3 at 25±1℃ under continuous light (2,500 lux) for 4 weeks. The germination frequency was evaluated as the ration of the embryos with epicotyl elongation to the total number of cultured embryos. Experiments were done in a completely randomized design with three replicates per treatment. Data were statistically analyzed using the one-way ANOVA followed by Tukey's multiple comparison tests to determine inter-group differences, A level of P=0.05 was accepted as being statistically significant.

Results

Genotypic difference in response to encapsulation-dehydration technique using shoot-apices

The survival frequencies of cryopreserved apices ranged between 0 and 40% for three genotypes, 'Ma1', 'Db037' and wild type of *D. batatas* (Figure 1). There was a clear genotypic difference in response to one cryogenic technique. With *D. batatas* cv. 'Db037', the encapsulation-dehydration method

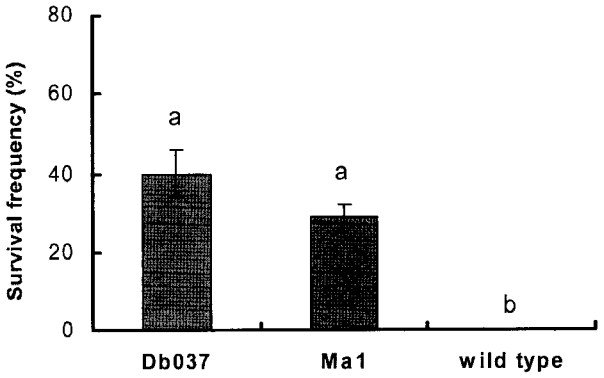

Figure 1. Survival (%) of apices of the three yam genotypes (*D. batatas*) cryopreserved at -196℃ by encapsulation-dehydration. In each treatment, 120 apices were tested with three replicates. Data represent mean ± SD (*P=0.05). Encapsulated apices precultured with 0.3 M of sucrose were placed on sterilized filter papers in 9 cm petri-dishes and dehydrated by air drying in a laminar flow cabinet for 4 h at room temperature and then directly immersed in LN for 1 h. Shoot-apices immersed in LN were thawed in 40℃ water-bath for 3 min. Cryopreserved apices were post-cultured on a solidified MS medium containing 30 g/L of sucrose, 0.2 mg/L of BA and 0.2 mg/L of kinetin, under light conditions for survival assessment.

could produce a high survival frequency from thawed apices. The survival of 'Mal' decreased to 29%, and that of wild type in Korea decreased to nil. But there was no statistically significant difference between *D. batatas* cv 'DB037' and 'Mal' at a level of P=0.05.

Cryopreservation using zygotic embryos of wild yam species

The survival of cryopreserved zygotic embryos was dependent on the dehydration methods. The maximum survival frequency (96.6%) could be achieved when the embryos were cryopreserved by a desiccation method. The encapsulation-dehydration method, however, had poor results, with a low survival frequency (below 62%) on the regrowth from control (LN-) and cryopreserved (LN+) embryos (Figure 2).

Embryo maturation (Figure 3) and desiccation (Figure 4) affected the survival of cryopreserved zygotic embryos. Desiccation (air drying for 30 min) also markedly affected the germination of zygotic embryos, especially immature embryos of the seeds matured for 35 DAF (Figure 5).

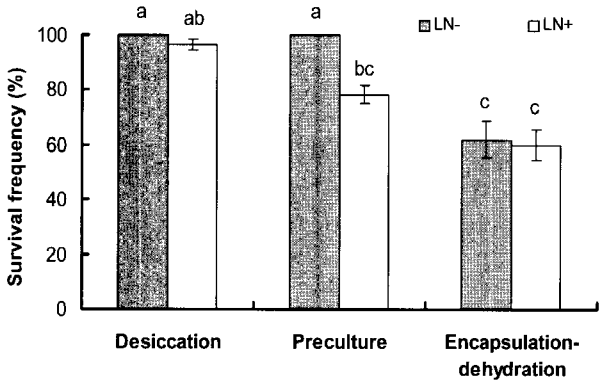

Figure 2. Survival (%) of control (LN-) and cryopreserved (LN+) zygotic embryos (*D. batatas* wild type) in three different techniques. In each treatment, 120 embryos were tested with three replicates. Data represent mean ± SD (*P=0.05). Desiccation techniques: Embryos excised from seeds were dried in the air stream of a laminar flow cabinet for 30 min. Preculture techniques: Zygotic embryos precultured on MS medium containing 0.3 mg/L of GA_3 for a day. Encapsulation-dehydration techniques: Encapsulated embryos were dehydrated by air-drying in a laminar flow cabinet at room temperature for 4 h.

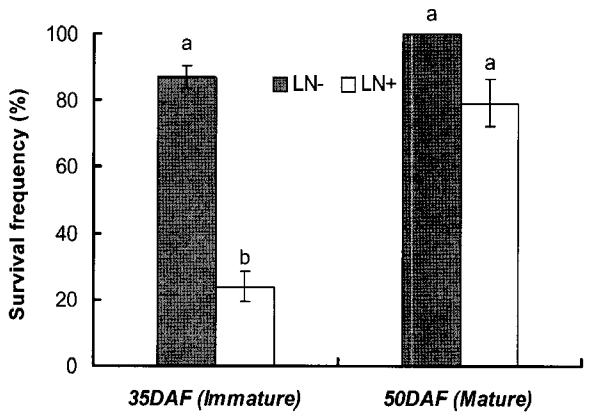

Figure 3. Survival frequency (%) of mature and immature zygotic embryos of *D. batatas* (wild type) cryopreserved at -196℃. In each treatment, 150 embryos were tested with three replicates. Data represent mean ± SD (*P=0.05). Excised embryos were directly immersed in LN. Zygotic embryos immersed in LN were thawed in 40℃ water-bath for 3 min. Embryos were dissected from the seed matured for 35 DAF and 50 DAF.

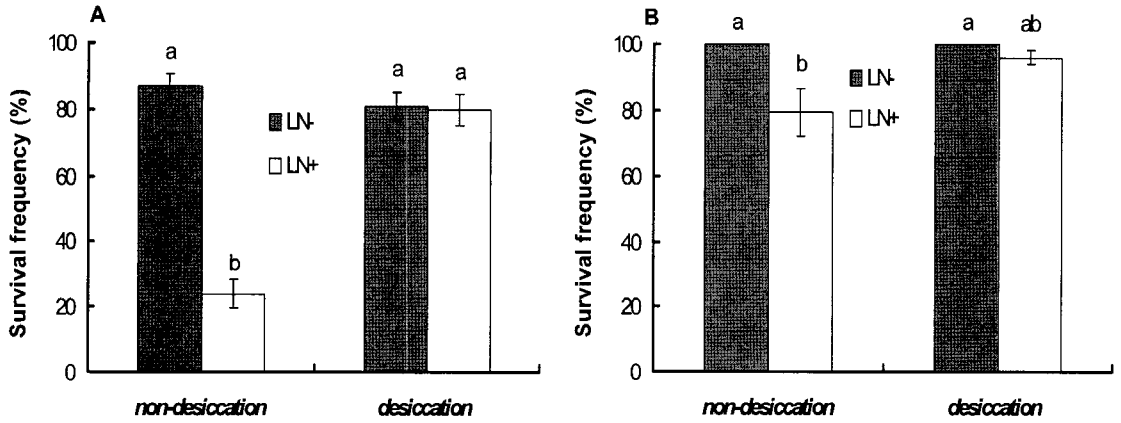

Figure 4. Survival frequency (%) of immature (A) and mature (B) zygotic embryos of *D. batatas* (wild type) cryopreserved at -196℃ by desiccation method. In each treatment, 150 embryos were tested with three replicates. Data represent mean ± SD (*P=0.05). Excised embryos were dried in the air stream of a laminar flow cabinet for 0 and 30 min at room temperature and then directly immersed in LN. Zygotic embryos immersed in LN were thawed in 40℃ water-bath for 3 min. Embryos were dissected from the seed matured for 35 DAF and 50 DAF.

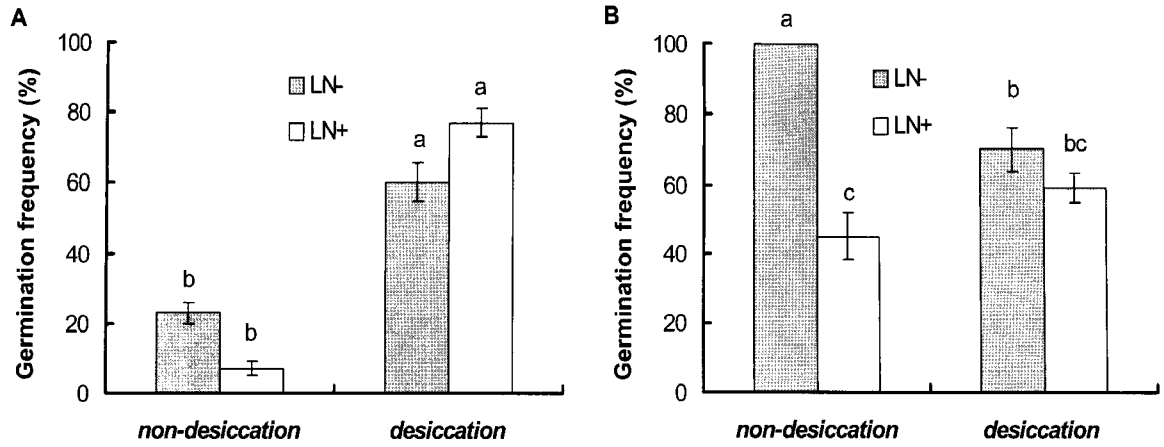

Figure 5. Germination frequency (%) of immature (A) and mature (B) zygotic embryos of *D. batatas* (wild type) cryopreserved at - 196℃ by desiccation method. In each treatment, 150 embryos were tested with three replicates. Data represent mean ± SD (*P=0.05). Excised embryos were dried in the air stream of a laminar flow cabinet for 0 and 30 min at room temperature and then directly immersed in LN. Zygotic embryos immersed in LN were thawed at 40℃ water-bath for 3 min. Embryos were dissected from the seed matured for 35 DAF and 50 DAF.

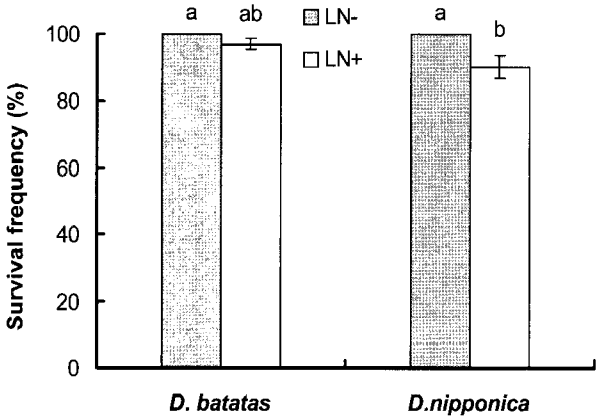

Figure 6. Survival frequency (%) of zygotic embryos of two wild yam species cryopreserved at - 196℃ by desiccation method. In each treatment, 90 embryos were tested with three replicates. Data represent mean ± SD (*P=0.05). Embryos that were excised from the seeds matured for 50 DAF were dried in the air stream of a laminar flow cabinet for 30 min at room temperature and then directly immersed in LN. Zygotic embryos immersed in LN were thawed in 40℃ water-bath for 3 min.

When the zygotic embryos were desiccated for 30 min, the survival frequencies were above 90% in two wild species of *Dioscorea* (*D. batatas* wild type and *D. nipponica*) (Figure 6, 7).

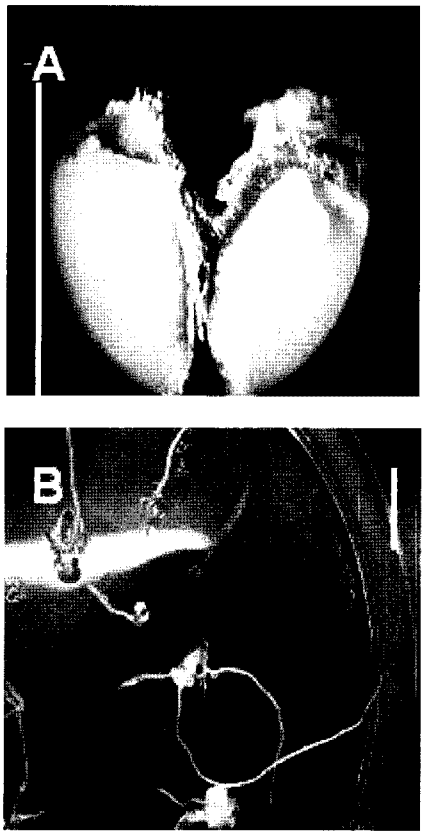

Figure 7. Zygotic embryos(A) of *D. batatas* (right), *D. nipponica* (left) and germinated seedings from cryopreserved zygotic embryos of *D. batatas* (B). Scale bars = 5 mm.

Discussion

The desiccation method was easier to apply than the more complicated cryopreservation method. Desiccation was evaluated as a potential method for the zygotic embryos and embryo axes in many species such as chayote (Abdelnour-Esquivel and Engelmann 2002), coffee (Dussert et al. 2002), tea (Kim et al. 2002), peony (Kim et al. 2004), *Citurs* (Lambardi et al. 2004).

Dussert et al. (2002) achieved maximum survival (96%) of cryopreserved zygotic embryos of coffee after 0.5 h desiccation. However, 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. The main advantages of the encapsulation-dehydration protocol are an easier manipulation of organs by alginate embedding, storage of large number of delicate explants (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 this study, however, the maximal survival of a zygotic embryo (96.6%) could be achieved from the cryopreserved embryos by desiccation, followed by preculture (78.4%) and than encapsulation-dehydration (60%). The results were also different from cryopreservation of zygotic embryos in peony (Kim et al. 2004). For peony germplasm, the encapsulation-dehydration appears to be a potentially valuable cryogenic protocol. In this study, Desiccation also markedly affected the germination of cryopreserved zygotic embryos of immature/ mature embryos of wild yams. In the previous study with Citurs intact seeds (Lambardi et al. 2004), the dehydration/ cryopreservation procedure promotes the germination of zygotic embryos and reduces the number of apomictic seedlings per seed. In conclusion, for the successful cryopreservation of yam zygotic embryos, those that were excised from immature/ mature seeds were dried in the air stream of a laminar flow cabinet for 30 min at room temperature and then directly immersed in LN.

Acknowledgements

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