Cryopreservation of sievers wormwood (Artemisia sieversiana Ehrh. Ex Willd.) seeds by vitrification and encapsulation

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ABSTRACT

Artemisia sieversiana is being used for the medicinal purpose, there are not many conservation objects, and the species is registered as a rare plant in Korea. There is also no research on the preservation of A. sieversiana seeds. In this paper, we investigated the preservation of seeds using cryopreservation. The vitality and germination rate of seeds were investigated with pretreatment, vitrification, and encapsulation. The initial germination rate of the seeds was 95%. PVS3 solution treated for 60 min showed the highest vitality, and germination rate. When encapsulation, both vitality and germination rate decreased. Changes in vitality rate during storage period were measured. As a result, the seed-maintained vitality when the vitrification solution used. Encapsulation is unavailable because of too much change. Therefore, seeds can be safely preserved without deterioration of vitality at cryopreservation by using PVS 3 solution and it will be helpful in future studies.

Introduction

The common species, Artemisia sieversiana Ehrh. Ex Willd. is an annual or biennial herbaceous rhizome plant of the Compositae family and grows in sandy clay, moist habitats, and distributes. A. sieversiana is a plant belonging to Artemisia, which is distributed in Korea, Japan and China. The plant grows up to 150 cm long and the stem is thick and straight, and it grows in the field. It has been used for the treatment of outbreak, bronchitis, tonsillitis, dermatitis, mastitis, hepatitis, and urinary inflammation (Park and Chung 2013). The aerial parts of A. sieversiana afforded, in addition to β-sitosterol, stigmasterol and daucosterol, two novel lignans as well as one known and three new guaianolides (Tan et al. 1998). A. sieversiana possesses insecticidal activity against the maize weevil Sitophilus zeamais (Liu et al. 2010).

Germplasm preservation plays an important role in the maintenance of biodiversity and avoidance of genetic erosion. It has been amply stated that germplasm preservation is valuable not only for plant improvement and utilization for food, fiber, and medicinal crops, but also for conservation of rare and endangered species (Iriondo and Hond 2008). Cryopreservation has long been considered an important tool for the long-term storage of plant germplasm (Sakai et al. 1990).

In vitro culture technology is an alternative to seed banking and is a good way to cryopreserve plant somatic cells or plant tissues for long-term storage. Cryopreservation can safely and cost-effectively preserve plant genetic resources (Engelmann 2004). In addition, cryopreservation technology can be applied to simple and broad resource plants, with minimal space and maintenance, and long-term preservation of the desired plant material (Kaviani 2011).

Cryopreservation technology was developed for vitrification (Yamada et al. 1991; Sekizawa et al. 2011) and encapsulation-dehydration technology (Fabre and Dereuddre 1990; Kaviani et al. 2008). This technique allows storage at very low temperatures by removing frozen cell osmosis through exposure to concentrated vitrification solutions (vitrification procedures) or air drying (encapsulation-dehydration procedures) rather than freezing. Simple and reliable cryogenic protocols such as simple freezing, vitrification, and encapsulation-dehydration have been proven and the number of cryopreserved species or cultivars has increased markedly over the last few years. Successful cryopreservation of embryos has been reported in many plants (Tessereau et al. 1994; Ishikawa et al. 1997; Wu et al. 2003; Nadarajan et al. 2006).

Very limited studies have been carried out on cryopreservation of Artemisia and plants. Artemisia annua L. Callus of two lines was treated with three kinds of cryoprotectants, and callus preservation, regeneration
field and plant differentiation were reported (Chenshu et al. 2003). The shoot-tips of Artemisia herba-alba, native to arid and semi-arid climates, were found to have a survival rate of 68% through vitrification and encapsulation (Sharaf et al. 2012).

There have been no cryopreservation studies comparing vitrification and encapsulation methods, respectively. This study was conducted to investigate the optimal cryopreservation conditions for A. sieversiana seed preservation, and to investigate the effect of cryopreservation on plant growth after germination.

Materials and methods

Plant material

Seeds of A. sieversiana Ehrh. ex Willd. were obtained from the Korea National Arboretum, South Korea. The seeds washed with distilled water and stored in a plastic bag with silica gel at 4°C for 6 months. The experiments were carried out at Gyeongsang National University, Jinju in 2017. Before the experiment started, the initial rate of seed germination and moisture content of seed were 95% and 9%, respectively.

The surface of seed was sterilized by immersion in ethanol 70% for 1 min and sodium hypochlorite 1.0% for 1 min. The seeds were rinsed three times with sterile distilled water.

In vitro cultivation of cryoprotectant treated seed

Surface-sterilized seeds were suspended in MS (Murashige and Skoog 1962) liquid medium with different concentration of sucrose for 0, 0.3, 0.5, 0.7 M for 30 min and pH 5.6 for 0–60 min. Vitrified and encapsulated seeds were cultured on solidified MS medium supplemented with 3% sucrose, 0.2% gel-lite, and pH 5.6. Cultures were maintained under a 16/8(light/dark) hours at 25°C.

Vitrification of seed

Some modified methods were applied for vitrification (Hu et al. 2013). Seeds were transferred into 1.8 ml cryotube, and then treated with or without loading solution (LS) with 2 M glycerol and 0.4 M sucrose, pH 5.6 at 25°C for 30 min. Following removal of LS, the seeds were dehydrated with plant vitrification solution 2 (PVS2) or plant vitrification solution 3 (PVS3). The PVS2 consisted of 30% glycerol, 15% ethylene glycol, and 15% DMSO in liquid MS medium with 0.4 M sucrose and pH 5.6 for 0–60 min. The PVS3 consisted of 50% glycerol, 50% ethylene glycol in liquid MS medium with 0.4 M sucrose, and pH 5.6 for 0–60 min. The seeds were suspended into 1 ml of vitrification solution in 1.8 ml cryotube and then directly plunged into liquid nitrogen. After storage for period (0, 1 day, 1 week, 2 week, 1 month), the cryotubes were rapidly warmed in a water bath at 40°C for 1 min. Vitrification solution was removed from cryotube and then 1 ml of liquid MS medium supplemented with 1.2 M sucrose were added to each tube and held for 30 min.

Encapsulation of seed

Encapsulation method has been used with some modifications to the Flachslan et al. 2006. Seeds were suspended in MS medium supplemented with 3% Na-alginate and 0.6 M sucrose for 1 h with slow agitation. Then, seeds were dropped into MS medium containing 100 mM CaCl2 and 0.1 M sucrose for 1 h with slow agitation. Seeds were washed three times with sterile distilled water. Following removal of distilled water, the capsules were supplemented with 0.75 M sucrose in MS medium and pH 5.6 for 0–3 h. Encapsulation seeds were transferred to empty open petri dishes and desiccated in the laminar flow chamber, then directly plunged into liquid nitrogen (LN). After storage for period (0, 1 day, 1 week, 2 week, 1 month), the cryotubes were rapidly warmed in a water bath at 40°C for 1 min.

TTC staining for viability assessment

The viability of the seeds following each treatment was evaluated using the 2,3,5-triphenyltetrazolium chloride (TTC) test (Lee et al. 2015). Seeds were incubated in 1% TTC solution for 1 day at 27 ± 2°C in the dark. The number of embryos stained by TTC was counted, and the percentage of TTC-stained seeds represented the survival rate.

Thawing and plantlet formation

After leaving for 60 min in LN, cryovials were removed and rapidly rewarmed in a 40°C water bath for 1 min. Cryopreservation solutions were removed from cryovials with a sterile disposable transfer pipette under a laminar flow hood. Seeds were rinsed with 1/2 strength MS culture medium +1.2 M sucrose (pH 5.7) for 15 min, transferred to Petri dishes containing 1/2 strength MS medium +0.06 M sucrose (pH 5.7) solidified with 7.0 g/l agar and incubated under controlled environmental conditions (27±2°C; 60 µmol m⁻² s⁻¹; 16 light/8 dark hours). Petri dishes were visually monitored weekly for seed germination. Germination percentage was assessed from week 4 through 12 for the control and different treatments by counting the number of germinated seeds under a microscope. Seed survival was assessed by counting the number of germinated seeds that survived and which continued growing.

Rooting and acclimation

The in vitro cultured plants were taken out of the culture bottle and then the agar was removed from the roots. Roots were washed with distilled water and
transferred to a plastic pot containing sterilized artificial soil for them to acclimate in the greenhouse.

**Statistical analysis**

The survival rate and germination rate of the seeds were analyzed by Duncan’s multiple test method and significance level was 5%. Statistical analysis was performed using SPSS statistics 23. (IBM, Corp, USA)

**Results**

**Seed viability and germination according to sucrose pretreatment**

Sucrose concentration significantly influenced the seed viability of *A. sieversiana* seeds (Table 1). Seed viability was 64% in non-sucrose treatment, and seed viability was increased in sucrose treatment. The viability of *A. sieversiana* seeds was highest (93.2% at 0.3 M), and 73% and 82% at 0.5 M and 0.7 M treatment, respectively.

The seed germination rate of *A. sieversiana* varied according to sucrose concentration. After 4 weeks of sucrose treatment, the highest germination rate was in 0.3 M, followed by 0.5 M and 0.7 M. However, the germination rate of sucrose untreated seeds was much lower than that of treated seeds.

The seed germination rate in sucrose treatments was different according to germination period. Seed germination started after 1 week of incubation with 0.3 M sucrose treatment. However, seed germination at 0.5 M and 0.7 M sucrose treatment started after 4 weeks of culture.

The treatment time with sucrose did not affect seed viability. Seed viability immediately before sucrose treatment was 64%, but it increased to 90% after 10 and 30 min of treatment. And it was slightly lower at 80% after 60 min respectively.

Sucrose treatment time also affected seed germination rate (Table 2). Seed germination started 1 week after incubation for 30 min and 60 min, but there was no difference in the seed germination rate after that. However, germination was not observed at 10 min treatment.

**Seed viability and germination rate after vitrification**

Vitrification solution affected seed viability (Table 3). Seed viability differed according to treatment time. Compared to the control, the vitality of the seed that treated solution increased. When each solution treated for 30 min, it showed the highest vitality.

The PVS treatment time also affected seed germination rate. The germination rate of treated seeds was significantly higher than those without treatment. Seed germination rate increased slightly with longer PVS solution treatment time. The germination rate increased rapidly after 2 weeks of incubation for 10 min in PVS2, but there was no difference thereafter. After 30 min treatment, germination rate increased from 1 week after culture and gradually increased thereafter. The highest germination rate was at 60 min after incubation, with 85% germination in 2 weeks. The germination rate at 120 min treatment showed low germination rate after 1 week of culture, but high germination rate after 2 weeks stored.

Seed germination rate with PVS3 treatment was higher than that of PVS2 treatment. The highest germination rate was recorded at 60 min. Germination

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**Table 1.** Effects of sucrose concentration on the TTC staining and the germination rate of *A. sieversiana* cryopreserved seeds.

| Sucrose concentration | TTC staining (%) | Germination (%) |
|-----------------------|------------------|-----------------|
| 0 M                   | 64.43 ± 1.96c    | 3.57 ± 1.84b    |
| 0.3 M                 | 93.21 ± 1.02a    | 12.82 ± 1.28a   |
| 0.5 M                 | 73.08 ± 0.84c    | 8.33 ± 2.40c    |
| 0.7 M                 | 81.70 ± 1.40b    | 7.69 ± 2.22b    |

Seed were cryopreserved for 30 min. Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test ($p=0.05$). Germination rates were calculated after 4 weeks of culture.

**Table 2.** Effects of sucrose treatment time on the TTC staining and the germination rate of *A. sieversiana* cryopreserved seeds.

| Sucrose treatment time | TTC staining (%) | Germination (%) |
|-----------------------|------------------|-----------------|
| 0 min                 | 64.43 ± 1.96c    | 3.57 ± 1.84b    |
| 10 min                | 90.00 ± 1.86a    | 12.82 ± 1.28a   |
| 30 min                | 93.21 ± 1.02a    | 80.70 ± 0.88b   |
| 60 min                | 81.70 ± 1.40b    | 10.00 ± 1.92a   |

Seed were cryopreserved for 30 min. Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test ($p=0.05$). Germination rates were calculated after 4 weeks of culture.

**Table 3.** Effects of PVS on the TTC staining and germination rate of cryopreserved *A. sieversiana* in LN.

| Treatment period | TTC staining (%) | Germination (%) |
|-----------------|------------------|-----------------|
| 0 min           | 63.40 ± 1.12d    | 32.00 ± 0.73g   |
| 10 min          | 91.53 ± 1.11bc   | 73.81 ± 1.50e   |
| 30 min          | 91.95 ± 1.91bc   | 88.08 ± 2.39f   |
| 60 min          | 94.63 ± 2.03ab   | 98.08 ± 2.39f   |
| 120 min         | 97.15 ± 1.49a    | 98.08 ± 2.39f   |

Seed were cryopreserved for 30 min. Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test ($p=0.05$). Germination rates were calculated after 4 weeks of culture.
occurred after one week in all treatments, but there was no difference in germination rate.

Seed viability and germination rate of cryopreserved seeds in liquid nitrogen after vitrification

The viability of the seeds was also significantly different according to the liquid nitrogen storage time (Table 4). Seed viability was lower in the treatment than in the control. The viability of the seeds decreased as the cryopreservation time increased.

The germination rate of seeds cryopreserved in liquid nitrogen did not vary according to liquid nitrogen storage time. Seeds that cryopreserved in liquid nitrogen germinated in 1 week of incubation. Seed germination showed little difference due to increasing liquid nitrogen storage time.

Seed viability and germination rate according to encapsulation

When encapsulated seeds were treated with sucrose solution, seed viability was also affected (Table 5). The vitality of the encapsulated seeds decreased with the treatment time. However, there were no trends over time.

The germination rate of seeds encapsulated in liquid nitrogen did not differ according to incubation period. 1 week treatment showed highest germination rate but the control has highest viability with no germination.

The preservation of encapsulated seeds in liquid nitrogen also affected seed viability (Table 6). The vitality of the encapsulated seeds was not observed in liquid nitrogen due to storage time.

When encapsulated seeds were stored in liquid nitrogen, the seeds germination rate was also affected. There was no difference in germination rate of the encapsulated seeds after 3 days of culture. The germination rate was higher than that of 30 min treatment on the 1 h and 3-day treatments.

Seeds stored in liquid nitrogen successfully grew after germination (Figure 1). Germinated plants rooted, and multiple shoots were induced.

Growth of seedling stored in LN

The fresh weight of the germinated plants showed differences growth rate between the plants stored in LN and those not stored in LN (control). The seedlings treated with PVS2 and PVS3 also showed differences in growth (Figure 2). The fresh weight of A. sieversiana seedlings was highest in the control. As the treatment time increased, the actual growth of seedlings decreased. Seedlings treated in PVS3 for 10 min had favorable growth, while PVS2 treated seedlings showed low growth.

Comparison of seedling growth between vitrification and encapsulation

Seedlings obtained from vitrified and encapsulated seeds showed very different growth (Figure 3). The growth of young seedlings was better than that of vitrified seedlings. The fresh weight of seedlings was highest at 0 min after cryoprotectant was added by vitrification with the liquid nitrogen not treated. This is much higher than for other treatments. On the other hand, the encapsulation method showed the lowest value at 0 min, and the 3 days treatment time was the best, and it was judged that the liquid nitrogen treatment time did not have a great influence on the live weight.

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**Table 4.** TTC staining and germination according to different periods of liquid nitrogen storage after vitrification.

| LN storage time | Control | 1 day | 1 week | 2 weeks | 1 month |
|-----------------|---------|-------|--------|---------|---------|
| TTC staining (%) | 95.08 ± 2.81a | 91.14 ± 3.35ab | 84.68 ± 2.32ab | 91.06 ± 1.41bc | 81.87 ± 2.28bc |
| Germination (%) | 89.89 ± 2.37ab | 84.44 ± 3.72ab | 84.32 ± 4.75ab | 80.24 ± 2.03ab | 73.09 ± 4.13ab |

Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test (p = 0.05). Germination rates were calculated after 4 weeks of culture.

**Table 5.** TTC staining and germination according to different sucrose treatment time in encapsulation.

| Sucrose treatment | 0 min | 30 min | 1 h | 2 h | 3 h |
|-------------------|-------|--------|-----|-----|-----|
| TTC staining (%)  | 77.60 ± 1.39a | 52.33 ± 1.45b | 20.73 ± 0.37e | 59.20 ± 1.44b | 41.73 ± 1.04d |
| Germination (%)   | 1 – | 45.84 ± 1.74b | 12.22 ± 3.91c | 55.56 ± 1.86c | – |

Seed were cryopreserved for 30 min. Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test (p = 0.05). Germination rates were calculated after 4 weeks of culture.

**Table 6.** TTC staining and germination according to different LN storage time in encapsulation.

| Liquid nitrogen storage time | Control | 1 day | 1 week | 1 month |
|------------------------------|---------|-------|--------|---------|
| TTC staining (%)             | 96.33 ± 1.86a | 54.58 ± 0.96c | 63.36 ± 2.42b | 62.58 ± 1.11c |
| Germination (%)              | 94.44 ± 1.86a | 46.67 ± 2.02c | 61.11 ± 2.53b | 60.42 ± 2.38b |

Values are means of 3 replicates (30 seeds per replicate). Means followed by the same letters are not significantly different by Duncan’s test (p = 0.05). Germination rates were calculated after 4 weeks of culture.
Pre-treatment with cryoprotectants showed an improvement of the viability and germination of *A. sieversiana*. Our results showed that pretreatment of 0.3 M sucrose increased seed viability and germination rates. Also, pretreatment time of sucrose affected seed germination rate. Pretreatment with 0.3 M sucrose for 3 days before vitrification increased the survival rate of immature seeds of 3–4 MAP (Months after pollination) when cooled in liquid nitrogen during the vitrification process (Hirano et al. 2005). However, the difference was not statistically significant as has been previously shown for the mature zygotic embryos of the same species (Ishikawa et al. 1997).

The vitrification solution also affected seed viability and germination rate. The vitrification method involves treating the sample with a high concentration of anti-freeze solution, and PVS2 (Sakai et al. 1990), and PVS3 (Nishizawa et al. 1993), which induces intracellular dehydration and reduces the chance of intracellular ice formation in liquid nitrogen. With the vitrification method, pretreatment with a medium containing a high level of sorbitol and sugar has been reported to be extremely useful in improving the survival of cryopreserved cells and tissues (Uragami et al. 1989; Yamada et al. 1991). PVSs solution composed of different concentrations and combinations of the four main components: dimethyl-sulfoxide, sucrose, glycerol and ethylene glycol. The cryoprotective substances should fulfil several basic parameters, such as cell permeability, viscosity, toxicity and the minimum concentration necessary for vitrification, which eliminates the formation of ice crystals.

The increased efficiency of vitrification methods was achieved by treating plants in the pre-cultivation step before cryopreservation of plant shoot tips in so called LS (Matsumoto et al. 1995; Dumet et al. 2002). Vitrification protocols have been used for the cryopreservation of mature and immature seeds of *Doritaenopsis pulcherrima* (Thammasiri 2000), mature seeds and pollen of Dendrobium hybrids (Vendrame et al. 2008), seeds of *Paphiopedilum tayleri* (Hirano et al. 2009), seeds of *Cymbidium* species (Hirano et al. 2011).

The results of this study showed that the encapsulation method was worse than the vitrification method in seed viability and seed germination. All the treatments decreased seed germination rate and viability. The results showed that the germination percentage of cryopreserved seeds treated with loading solution was
The growth of seedlings that emerged from cryopreserved seeds differed between the two methods (encapsulation and vitrification). Growth of plants resulting from seeds cryopreserved by encapsulation was better than for the untreated seeds, which is like the findings of Jitsopakul et al. (2012).

The growth of seedlings emerged from cryopreserved seeds differed between the two methods (encapsulation and vitrification). Growth of plants resulting from seeds cryopreserved by encapsulation was better than for vitrification method. These results indicate that the cryopreservation method of the seeds is important, but the growth of the plant after germination should also be considered. Cryopreserved seeds are subject to physical, chemical and physiological stress. The growth of plants that emerged from cryopreserved seeds was lower than that of the control, which seems to be due to cryoinjury. In addition, the difference of further growth of plants obtained from the cryopreservation method is considered due to physico-chemical stress.

We have succeeded in cryopreservation of mature seeds of *A. sieversiana*, a useful resource plant. The results of this study can be widely used for cryopreservation of other recalcitrant seeds of useful plants as well as elite cultivar of *A. sieversiana*.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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