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Embryo Rescue via Artificial Seed Technique and Long-Term Preservation of *Zephyranthes*

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Abstract

Zephyranthes is valued as a native ornamental landscaping plant and a traditional medicinal herb. Due to the low seed viability, this study was carried out to evaluate the potential of seed embryo rescue using the artificial seed technique and long-preservation in *Z. atamasca* and *Z. grandiflora*. Seed embryos were selected for encapsulation with different concentration of sodium alginate (3%, 4%, and 5%) and calcium chloride (either 25, 50, 75, and 100 mM) followed by no encapsulated embryos as a control. The greatest viability of encapsulated embryos was 95% in *Z. grandiflora* and 85% in *Z. atamasca* with the combination of 4% sodium alginate and 100 mM calcium chloride after two weeks at 5˚C. The highest viability with A_{490nm}0.12 and A_{490nm}0.16 were achieved when embryos were cultured in pretreatment medium with 30 g/L sucrose in *Z. grandiflora* and *Z. atamasca*, respectively. The highest viability by TTC assay after cryopreservation was observed with 54% viability for *Z. grandiflora* and 48% viability with *Z. atamasca*, after 2 h of dehydration. Rain lilies embryos were successfully preserved functioned as artificial seed and cryopreservation using encapsulation-dehydration method has been established for both species that can be used for other flower species with some modifications.

Keywords

Rain Lily, Synthetic Seed, Cryopreservation, Encapsulation, Ornamental Landscape, *Zephyranthes atamasc*, *Zephyranthes grandiflora*

1. Introduction

The genus *Zephyranthes* Herb. consist of plants from the family Amaryllidaceae, which comprise approximately 65 species with neotropical distribution [1] [2].

These flowering bulbs are commonly known as rain lily, fairy lily, rain flower, and zephyrlily [3]. These small bulbs earned the name "rain lily" because they often flower within a few days after rainfall. Several species are valued in native landscapes for the small, solitary flowers that vary in color [4]. For example, *Zephyranthes atamasca* (L.) Herb. has white flowers and *Zephyranthes grandiflora* (L.) with pink flowers (**Figure 1**).

Zephyranthes species have been used as medicinal herbs [5] [6] [7] and have been used in various pharmacological activities including antineoplastic, antidiabetic, and anti-HIV [5] [8]. Furthermore, the medicinal and ornamental properties of *Z. grandiflora* have increased market demand for it at both the domestic and international level. Hence, this species is decreasing alarmingly from the wild due to unrestricted collection from natural flora [9]. *Zephyranthes* is usually propagated by dividing bulbs or by seeds. The vegetative propagation method is very slow and can take two years or more for a bulb to flower. Even bulbs micropropagation has some issues with contamination in stage I cultures and genotype differences (somaclonal variations) in tissue culture media [10]. Seed viability diminishes quickly after just a few days of ripening and harvest [11] and keeping seeds in cold storage may prolong viability but is very limited to short term storage only [10]. To overcome this problem, there are two ways of *in-vitro* delivery and preservation through encapsulation, 1) medium term preservation and delivery method using artificial seed and 2) long term preservation with cryopreservation technique.

Artificial seed (synthetic seed) has the ability to pause the growth of the vegetative propagules at the certain stages of the embryo life. Synthetic seeds containing propagules (plants reproducible organs) such as nodes, shoots, callus, embryos, somatic embryos, and protocorms are contained in a gelatinous matrix of a combination of sodium alginate and calcium chloride [10] [11]. *In-vitro* derived axillary buds and shoot tips are good clonal propagules for storage [11] [12]. Synthetic seeds have important advantages including ease of handling during storage and transportation; a channel for new plants to be delivered directly to the greenhouse or field; and allowing economical mass propagation of elite plants [10] [11] [12].

Cryopreservation involves the storage of live plant cells, tissues or organs at

Figure 1. (a). *Zephyranthes atamasca* and (b). *Zephyranthes grandiflora*.

ultra-low temperature (−196˚C) with low risk of genetic and physiological changes even over long periods of time [13]. Conservation is very important for *Zephyranthes* germplasm, breeding programs, and the floriculture industries. Different techniques of cryopreservation are used to develop cryogenic protocols for example, vitrification [14], and encapsulation-dehydration [10] [11] [14] [15] [16]. Encapsulation-dehydration method is preferred for vegetative propagules because the alginate beads allow greater protection of dried materials from mechanical and oxidative stress during storage and ease handling of small samples during pre and post-cryopreservation procedures [11] [17]. However, in order to establish a cryopreservation protocol various factors such as pretreatment and preculture conditions, water content, and dehydration methods have to be investigated. Therefore, the main objectives of this study were: 1) to utilize the synthetic seed technology as a possible method for embryo rescue and delivery, 2) to establish a method for long-term storage of embryos using the encapsulation dehydration method.

2. Material and Methods

2.1. Seed Collection

Seed pods of *Z. atamasca and Z. grandiflora* (each 10 pods) collected after 10 days of flowering when pods were closed but have begun to turn yellowish in color and kept in the paper bags for 1 day. The research was conducted at the research greenhouse facility at Oklahoma State University, Stillwater campus (1301N. Western, Stillwater, OK; 36˚08'09.9"N 97˚05'10.9"W). Afterwards, embryos encapsulation with different concentration of sodium alginate (3%, 4% and 5%) and calcium chloride (either 25, 50, 75 and 100 mM) followed by no encapsulation as a control. *Z. atamasca* had an average seed size of 0.4 - 0.5 mm and 0.7 - 0.8 mm for *Z. grandiflora*.

2.2. Preparation of the Encapsulation Matrix

For embryo encapsulation, sodium alginate was used with calcium chloride dehydrate for complexation. Two solutions were prepared as follows: 1) sodium alginate 3%, 4% and 5% (w/v) prepared by mixing 5.00, 6.66 and 8.33 g of sodium salt of alginic acid (PhytoTechnology Laboratories, Lenexa, KS, USA) with 250 ml of 1/2 strength liquid Murashige and Skoog (MS) medium free of calcium, growth regulators, and iron followed by autoclaving for 20 minutes to ensure complete sterilization. 2) $CaCl_2{\cdot}2H_2O$ at 25, 50, 75 and 100 mM prepared by mixing 1.47, 2.94, 4.41 and 5.88 g $CaCl_2{\cdot}2H_2O$ in 400 ml of 1/2 strength liquid MS medium free of calcium, growth regulators, and iron.

2.3. Preparation of Artificial Seeds

With the slippery surface of the rain lily seeds, there is no possibility for encapsulation of the seed coat. Hence, the embryos were separated from the seed coat and blot dried, mixed with the sodium alginate solutions (3%, 4% and 5%), and

dropped one by one into $CaCl_2 \cdot 2H_2O$ solution (25, 50, 75 and 100 mM) using a sterile 10 ml disposable pipette cap with one end cut using scalpel and surgical blades. The drops, each containing a single embryo, were left in the $CaCl_2 \cdot 2H_2O$ solution to polymerize for 30 minutes on a gyratory shaker (75 rpm). The resulting beads (4 - 5 mm in diameter) were recovered by decanting the $CaCl_2 \cdot 2H_2O$ solution and washing with sterilized de-ionized water three times. Beads were placed in a Petri dish (5 beads in each Petri dish) with moist Whatman 41 filter paper in a laminar air-flow for 30 minutes, sealed with parafilm, and stored in the dark at 5˚C for two weeks prior to data collection on percent survival. The treatment, which gave the highest survival, was selected for the next experiment. The greatest encapsulating agents were determined by checking the embryo viability using Triphenyl Tetrazolium Chloride (TTC) (Sigma-Aldrich, St. Louis, USA) where high TTC value indicated high viability.

2.4. Viability Test by TTC Staining

In order to evaluate the viability of embryos, TTC solution (0.18 M) was used. In a TTC assay, cell survival is estimated by the amount of formazan produced from the reduction of TTC due to the action of dehydrogenases in living cells or tissue [11] [16] [18]. The absorbance [A] (pink color) at A_{490nm} was measured using a Genesys 10 UV spectrophotometer (Thermo Spectronic, Rochester, NY, USA). The living plant cells should show red color because dehydrogenase enzyme in living plant cells reduces the colorless 2,3,5-Triphenyltetrazolium chloride to triphenylformazan. The results achieved based on wavelength were then converted to a percentage using the control embryo with 100% viability.

2.5. Pretreatment

Embryos, ranging in size between 0.4 - 0.5 mm and 0.7 - 0.8 mm in diameter were selected, from *Z. atamasca* and *Z. grandiflora*. Two factors, namely sucrose concentration and duration of exposure in media, were assessed individually in this study.

2.6. Effect of Sucrose Concentration on Embryos Prior to Encapsulation in Pretreatment Media

Before performing the encapsulation procedure, embryos were immersed in 1/2 strength liquid MS medium supplemented with 0, 20, 30, 40 and 50 g/L sucrose for 3 days.

2.7. Effect of Duration of Exposure in Pretreatment Media on Embryos

Once the optimum concentration of sucrose was determined, the pretreatment duration was evaluated; embryos were pretreated at different intervals (0, 1, 2, 3, 4 and 5 days) with the goal of conditioning them to withstand freezing stress. The optimum sucrose concentration and pretreatment duration were used for

the subsequent optimization experiments. The optimum pretreatment conditions (sucrose concentration and exposure duration) were determined by checking viability of protocorms using TTC.

2.8. Desiccation

After pretreatment conditions, beads were rapidly surface-dried on sterile filter paper to remove any remaining liquid from the pretreatment medium and were submitted to an additional physical dehydration by evaporation at room temperature (23˚C). Dehydration occurred under a laminar flow hood at 1, 2, 3, 4, 5 and 6 h. In this regard, standard desiccation curve was obtained using empty beads, which have been pretreated under optimal conditions (0.75 M sucrose after 3 days) to determine standard desiccation conditions.

2.9. Freezing and Thawing

The desiccated beads were transferred to 1.8 mL polypropylene sterile cryotubes (Sigma-Aldrich, St. Louis, USA) with five encapsulated embryos in each cryotube and directly immersed into liquid nitrogen for 1 h in the dark. Cryotubes were then warmed rapidly in a water bath followed by stirring at 38˚C ± 2˚C for 2 minutes with water levels covering cryovials in the cryocanes.

2.10. Viability Test by TTC Staining

After freezing and thawing the viability of embryos dehydrated in different dehydration time were examined with TTC followed by re-growth ability after two weeks.

2.11. Statistical Analysis

All experiments were carried out in factorial on a completely randomized design (CRD) and each treatment was replicated six times with 15 seeds for each replication. The SAS software was used for analysis of variance (ANOVA). Treatment means were compared by using Duncan's Multiple Range Test (DMRT) ($P \leq 0.05$) [19].

3. Results

Different concentrations of sodium alginate (3%, 4%, and 5% (w/v)) and calcium chloride (25, 50, 75, and 100 mM) were used to determine the best combination mixture for embryos in retaining the viability and production of synthetic seed with good shape. The greatest viability of encapsulated seeds using the TTC achieved was 95% in *Z. grandiflora* and 85% in *Z. atamasca* with the combination of 4% sodium alginate with 100 mM calcium chloride after two weeks at 5˚C (**Figure 2**).

Embryos with no-capsule (control) showed only 28% and 31% viability, compared to using the least concentration of gelling agents (3% sodium alginate and 50 mM calcium chloride); with 55% and 58% viability increase in viability were

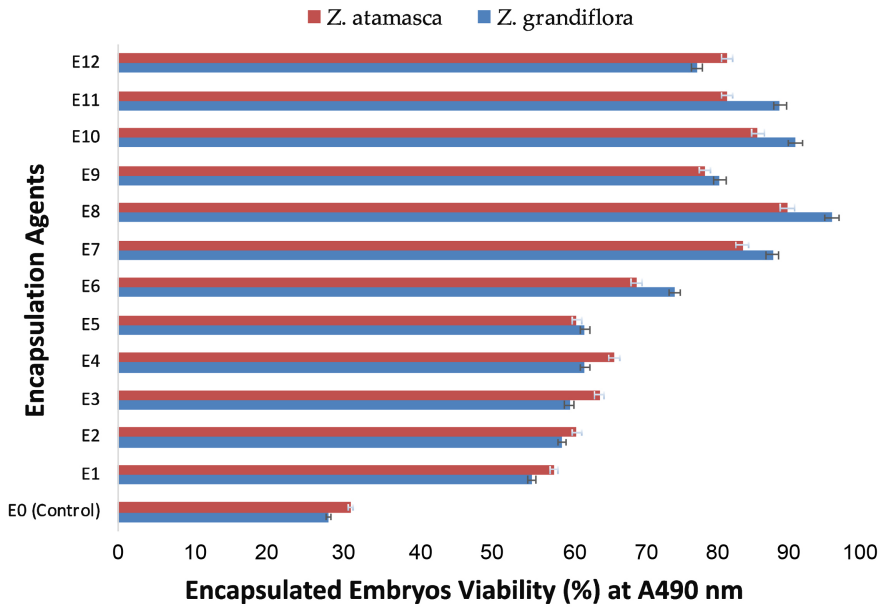

Figure 2. Effect of sodium alginate and calcium chloride concentration on viability of encapsulated embryo at 5˚C after two weeks for *Zephyranthes grandiflora and Zephyranthes atamasca* (n = 6). Bars represent standard error differences of means (±SEM). E0 = Control, E1 = 3% sodium alginate + 25 mM $CaCl_2{\cdot}2H_2O$ ($CaCl_2$), E2 = 3% sodium alginate + 50 mM $CaCl_2$, E3 = 3% sodium alginate + 75 mM $CaCl_2$, E4 = 3% sodium alginate + 100 mM $CaCl_2$, E5 = 4% sodium alginate + 25 mM $CaCl_2$, E6 = 4% sodium alginate + 50 mM $CaCl_2$, E7 = 4% sodium alginate + 75 mM $CaCl_2$, E8 = 4% sodium alginate + 100 mM $CaCl_2$, E9 = 5% sodium alginate + 25 mM $CaCl_2$, E10 = 5% sodium alginate + 50 mM $CaCl_2$, E11 = 5% sodium alginate + 75 mM $CaCl_2$, E12 = 5% sodium alginate + 100 mM $CaCl_2$.

observed after two weeks at 5˚C in *Z. grandiflora* and *Z. atamasca*, respectively (Figure 2). Viability increased with using calcium chloride from 25 mM to 75 mM in all sodium alginate concentrations but was negatively affected when continued to increase until 100 mM. The lowest viability of synthetic seeds obtained in this study was 55% for the combination of 3% sodium alginate with 50 mM $CaCl_2{\cdot}2H_2O$ in *Z. grandiflora* (Figure 2).

The differences in concentrations profoundly affected size, shape, and elasticity of the bead. Encapsulated embryos with 3% sodium alginate with both 25 and 50 mM $CaCl_2{\cdot}2H_2O$ and 4% sodium alginate combined with 25 mM $CaCl_2{\cdot}2H_2O$ were not uniform in size and shape, non-rigid, and not suitable for handling in both species (Figure 3, Figure 4(a), Figure 4(b), Figure 4(e)). Beads, which were formed using 4% sodium alginate solution and dropped in 100 mM $CaCl_2{\cdot}2H_2O$ solution followed by 5% sodium alginate solution and dropped in 50 mM $CaCl_2{\cdot}2H_2O$, were uniform in size, diametric, rigid, clear, and suitable for handling as compared to beads formed by other combinations (Figure 3, Figure 4(h), Figure 4(j)). Also, beads formed using 5% sodium alginate solution and dipped in 75 and 100 mM $CaCl_2{\cdot}2H_2O$ solution were uniform in size, not very clear, diametric, solid, and very rigid (Figure 3, Figure 4(k), Figure 4(l)). Beads formed in 3% sodium alginate with 75 mM and 4% sodium alginate with 50 and

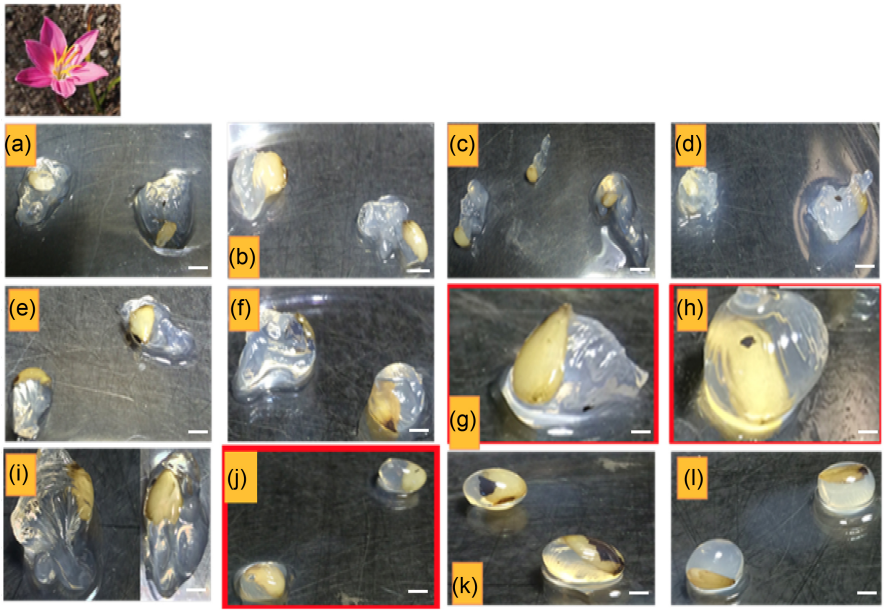

Figure 3. Embryos encapsulated in (a), (b), (c), (d) 3% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$; (e), (f), (g), (h) 4% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$; (i), (j), (k), (l) 5% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$ in *Z. grandiflora* (a), (b), (j), (k), (l), bar 21 mm; (c), (d), (e), bar 26 mm; (f), bar 20 mm; (g), (i), bar 16 mm; (h), bar 13 mm. Red color denotes the highest viability achieved from the gelling agents.

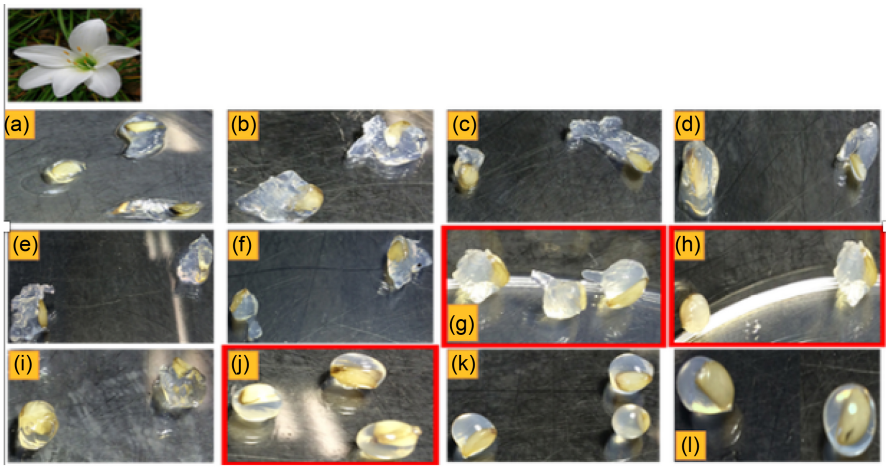

Figure 4. Embryos encapsulated in (a), (b), (c), (d) 3% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$; (e), (f), (g), (h) 4% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$; (i), (j), (k), (l) 5% sodium alginate + 25, 50, 75, and 100 mM $CaCl_2$ in *Z. atamasca* (a), (f), bar 27 mm; (b), (g), (i), (j), (k), (l) bar 26 mm; (f), bar 20 mm; (c), (d), (e) bar 16 mm. Red color denotes the highest viability achieved from the gelling agents.

75 mM $CaCl_2 \cdot 2H_2O$ were not very uniform in size, solid with a long tail, which is not desired for handling and storage (**Figure 3**, **Figure 4(c)**, **Figure 4(f)**, **Figure 4(g)**). Sodium alginate at 3% and 5% in combination with 100 and 25 mM $CaCl_2 \cdot 2H_2O$ were not very clear and rigid, which is undesirable for storage (**Figure 3**, **Figure 4(d)**, **Figure 4(i)**).

The highest viability with A_{490nm}0.12 and A_{490nm}0.16 were achieved when em-

bryos were cultured in pretreatment medium with 30 g·l sucrose in *Z. grandiflora* and *Z. atamasca*, respectively (Figure 5). Viability decreased as the sucrose concentration increased beyond 30 g·l with A_{490nm}0.06 and A_{490nm}0.07 at 50 g·l in *Z. grandiflora* and *Z. atamasca*, respectively (Figure 5) probably because of limited capacity of embryos to absorb the sucrose in high-saturated media.

Pretreatment on medium supplemented with sucrose increased the viability of embryos to A_{490nm}0.07 and A_{490nm}0.06 in *Z. grandiflora* and *Z. atamasca*, respectively as compared with non-pretreated embryos used as control (A_{490nm}0.02) for both species.

Embryo viability increased as the pretreatment duration was extended to 2 days. After 2 days of pretreatment, the viability started to decline to A_{490nm}0.15 and A_{490nm}0.24 after 5 days for *Z. grandiflora* and *Z. atamasca*, respectively (Figure 6).

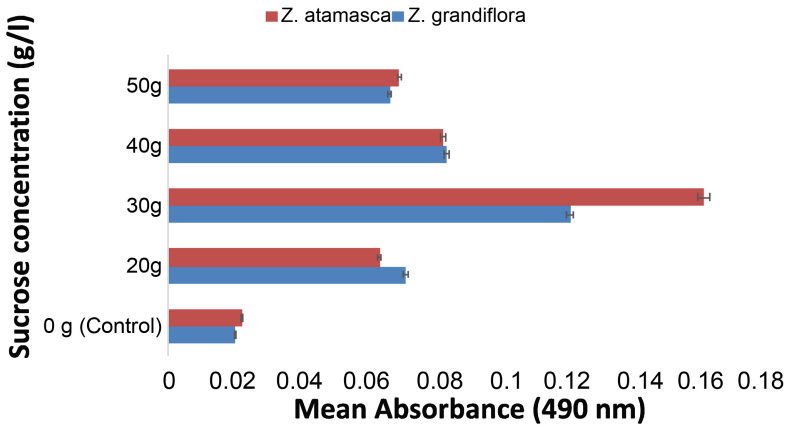

Figure 5. Absorbance following TTC reduction by embryo pretreated with different sucrose concentration in *Zephyranthes grandiflora and Zephyranthes atamasca* (n = 6). Bars represent standard error differences of means (±SEM).

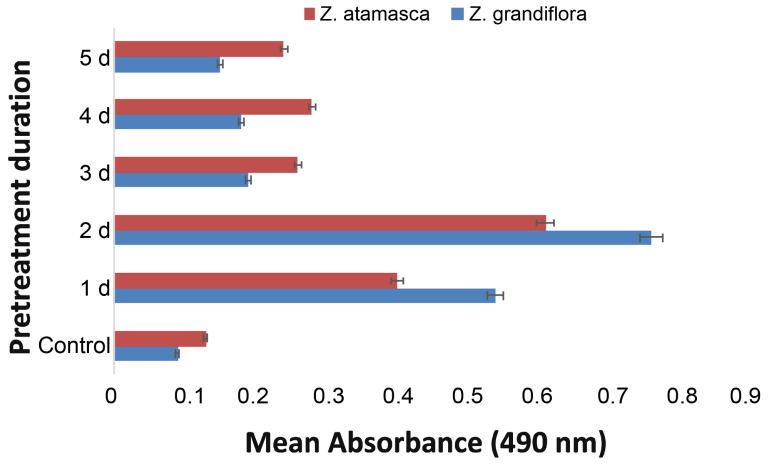

Figure 6. Absorbance following TTC reduction by embryo of *Zephyranthes grandiflora* and *Zephyranthes atamasca* in 1/2 MS medium supplemented with 30 g sucrose at different days of culture (n = 6). Bars represent standard error differences of means (±SEM).

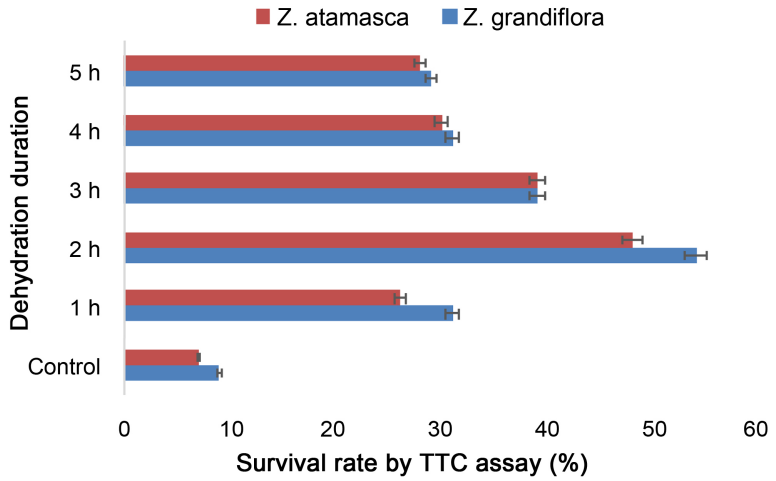

Figure 7. Changes of the water content of encapsulated embryo during dehydration by air-drying in a laminar flow chamber for 0 to 5 hours in *Z. grandiflora* and *Zephyranthes atamasca* (n = 6). Bars represent standard error differences of means (±SEM).

The highest viability by TTC assay after cryopreservation was observed with 54% viability for *Z. grandiflora* and 48% viability with *Z. atamasca*, after 2 h of dehydration (**Figure 6** and **Figure 7**). Whereas the control treatment (0 h drying) showed 9% viability for *Z. grandiflora* and 7% viability with *Z. atamasca* viability followed by ~96% viability in non-freezing treatment (**Figure 7**).

4. Discussion

Embryos encapsulated with 4% sodium alginate in 100 mM calcium chloride showed the best combination among gelling agents on survival of embryos after two weeks at 5˚C in both species. By using 3% and 5% sodium alginate in combination with either 100 mM $CaCl_2 \cdot 2H_2O$, viability decreased to 62% and 77% in *Z. grandiflora* and *Z. atamasca*, respectively (**Figure 2** and **Figure 3**). Studies on somatic embryos of eggplant, asparagus and carrot showed that the sodium alginate at 5%, 6%, and 7% was too viscous and the harder beads hindered emergence of shoots and roots [20] [21] [22]. Possible reason might be an unsuitable elasticity of the gel bead and oxygen deficiency within the gel bead [23]. The highest survival of protocorm like bodies with 70% reported from the combination of 4% sodium alginate and 100 mM calcium chloride after two weeks at 5˚C in *Phalaenopsis bellina* (Rchb. f.) Christenson [11]. In another study, bulbs of *Z. grandiflora* showed 90% viability using 4% sodium alginate and 1% sucrose as encapsulating agents [24].

The clear beads were formed with the combination of 4% sodium alginate and 75 mM $CaCl_2 \cdot 2H_2O$ in an epiphytic orchid *Acampe praemorsa* (Roxb) Blatter and McCann [25]. The concentration of 3% sodium alginate was most effective for shoot encapsulation in *Ananas cosmosus* L. Merr [26]. In addition, 3% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ was found to be very effective in encapsulation of micro shoots of *Saintpaulia ionantha* [10]. Also, the combination of

4% sodium alginate and 100 mM calcium chloride showed the greatest survival of protocorm like bodies with 70% in *Phalaenopsis bellina* [11]. In the study on *Z. grandiflora*, the bulbs of *Z. grandiflora* showed 90% viability using 4% sodium alginate and 1% sucrose [24]. A concentration of 4% sodium alginate with 75 mM $CaCl_2 \cdot 2H_2O$ was selected for further encapsulation experiments.

As the embryos are the sensitive material, prior to encapsulation, they can be submitted to a pretreatment medium which aims at conditioning the explants to withstand freezing by using the medium with standard or high sucrose concentration [15] [27] [28]. Four concentrations of sucrose were tested for the pretreatment condition, which ranged from 20 to 50 g·l for three days and embryos cultured on medium without sucrose were used as the control. Sucrose is very important in pretreatment media because it acts as cellular osmolyte, which may protect cells by equalizing the osmotic strength of the cytosol with that of the vacuole and the external medium and may interact with cellular macromolecules such as proteins and membranes [15] [29]. Using sucrose can help in avoiding a problem that may happen in the next stage before liquid nitrogen immersion.

In *Phalaenopsis bellina*, the highest viability of protocorm like bodies was observed after 3 days of pretreatment with 0.75 M [16]. In another study, pretreatment duration with sucrose has to be sufficiently long to obtain survival and the time required is different even among the same species [29]. Researchers test different combinations of factors used in cryopreservation in order to optimize a desired protocol for new species. There are established protocols that have been reported for many plant genera, which can be used as starting points and, in some cases, can be directly applied to plants with little modifications [30]. Viability tests in different dehydration duration (0, 1, 2, 3, 4, and 5 h) and non-freezing samples were performed using TTC after thawing. Triphenyl Tetrazolium Chloride values are frequently employed to determine the viability of cells subjected to various stress factors such as cold, salinity, heat, and to provide a guide for predicting the success of experiments [16] [31] [32] [33]. Artificial seeds of rain lily using the embryos were successfully preserved and functioned as an artificial seed in both *Z. atamasca* and *Z. grandiflora*. In addition, the cryopreservation technique using encapsulation-dehydration method has been established for the rain lilies embryo which can be used for other plants with some modifications.

Data Availability Statement

The dataset is available upon reasonable request to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

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