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Encapsulation-dehydration based cryopreservation of *Rauwolfia* shoot tips and their regeneration potential in vitro

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ABSTRACT

The glory of R.serpentine has faced its alarming stage due to over exploitation of natural resource by the pharmaceutical industries. Hence a conservation approach is necessary to keep them alive in nature. Cryopreservation of synthetic seed based approach of in vitro conservation is most convenient and safe method for long term germplasm conservation. For this reason, an in vitro germplasm conservation method through encapsulation dehydration followed by cryopreservation has been developed for this plant using the shoot tip as explants. The optimal dose of sucrose during preculture of encapsulated seeds and the dehydration duration using laminar airflow has been tested during experiment. During dehydration, moisture content was found least after 7 hours of dehydration (6.78±0.93). After cryopreservation, maximum responses of seeds were recorded for pre-treatment at 0.3 M sucrose followed by 9 hours dehydration and 0.5 M sucrose followed by 7 hours dehydration respectively. Addition of RAPD based approach suggested there was no variation between the mother plant and cryopreserved plant after regeneration.

Keywords*:* Cryopreservation, encapsulation dehydration, R. serpentine, synthetic seed

Rauwolfia serpentine is a well-known medicinal plant for the production of several pharmaceutically important products. In *Ayurveda*, the root of this plant is known as "*Sarpagandha*", "*Nakuli*", and popularly uses for the treatment of the mental disorders (Bhat *et al.*,2008). Among the secondary metabolites of this plant, *reserpine* is popular due to its ability to lower down the hypertension (Vida, 1953; Susila *et al.*,2013). In addition to secondary metabolites, vitamins such as ascorbic acid, riboflavin, thiamine, and niacin are also available in this plant (Ahmed, 2015). Unfortunately this plant has been categorised as endangered plant species by IUCN (International Union for the Conservation of Nature) (Deshmukh *et al.*,2012) due to the overexploitation of natural resources to meet the commercial demand of the pharmaceutical industries (Nayar and Sastry, 1987; Sharma and Chandel, 1992). Hence large scale plantation is essential to keep them alive in nature but problem is lack of saplings. Seed-based propagation of *R. serpentine* does not effective because of the low germination rate (Baksha *et al*., 2007) while the traditional vegetative propagation methods are time-consuming, laborious and environment dependent. These facts clearly demands an urgent need to find out an alternative way of regeneration and conservation of this endangered plant species to keep balance in agro-biodiversity of nature. *In vitro* conservation is widely applicable, cost effective and permits the conservation within short space due to small size of sample (Engelmann, 2011).Alginate-encapsulation is commonly used for both production of synthetic seeds and *in vitro* germplasm conservation (Hasan and Takagi, 1995; Dansoand Ford-Lloyd, 2003). Again, the conservation of plant materials under extremely low temperature (- 196°C) in liquid nitrogen (known as cryopreservation) has been proved as an economically feasible alternative method for long duration storage in a small space with no or little rate of contamination (Benelli *et al.*,2013; Bettoni *et al.*,2016, and Engelmann, 2004). In compare to other methods, this method is easy to handle, simple and inexpensive, and uses only sucrose as crypotectants (Martinez *et al.*,1999; Maneerattanarungroj *et al.*,2007). Here, the encapsulated explants are precultured in liquid medium with a high sucrose concentration and partially desiccated before freezing (Gonzalez-Arnao and Engelmann, 2006). Hence developing an efficient protocol for cryopreservation through the encapsulation-dehydration method and maximizing the regeneration potential without any genetic modification, sucrose concentration as osmoprotectant and dehydration duration needs to be critically standardized. Till date reports related to conservation of *Rauvolfia* species through synthetic seed based approach followed by plant regeneration are very less; short time conservation at 4ÚC have been reported by Ray and Bhattacharya (2008) for *Rauvolfia serpentine;* Faisal *et al.*(2006) for *Rauvolfia tetraphylla*

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Table 1: Regeneration potential at different sucrose concentration at varied dehydration duration

Concentrations of Sucrose	Dehydration time (in hour)	Germination % (±SE)		Avg. shoot no. plant^{-1}	
		Control	+LN	Control	+LN
0.3 M	3	100.00±0.00^{a}	26.66±6.66^{abc}	5.00±0.11^{a}	1.06±0.06^{de}
	5	86.66±6.66^{ab}	33.33±6.66^{ab}	4.33±0.13^{b}	1.13±0.06^{cde}
	7	73.33±6.66^{bc}	40.00±0.00^{ab}	3.00±0.11^{c}	1.26±0.06^{bcd}
	9	66.66±6.66^{bcd}	46.66±6.66^{a}	1.80±0.23^{d}	1.46±0.06^{ab}
0.5 M	3	86.66±6.66^{ab}	33.33±6.66^{ab}	2.86±0.17^{c}	1.06±0.06^{de}
	5	73.33±6.66^{bc}	40.00±0.00^{ab}	1.93±0.13^{d}	1.20±0.00^{cde}
	7	66.66±6.66^{bcd}	46.66±6.66^{a}	1.46±0.13^{de}	1.66±0.06^{a}
	9	46.66±6.66^{de}	33.33±6.66^{ab}	1.06±0.06^{ef}	1.13±0.06^{cde}
0.7 M	3	53.33±6.66^{cde}	40.00±11.54^{ab}	1.20±0.11^{e}	1.33±0.17^{bc}
	5	33.33±6.66^{e}	20.00±0.00^{bc}	1.13±0.06^{ef}	1.00±0.00^{e}
	7	33.33±6.66^{e}	13.33±6.66^{cd}	1.06±0.06^{ef}	1.00±0.00^{e}
	9	13.33±6.66^{f}	0.00±0.00^{d}	0.66±0.33^{f}	0.00±0.00^{f}

Table 2: Rate of change of moisture content in *R. serpentine* explants at different dehydration duration.

Dehydration Time (hours)	moisture content (%)
1	75.12±1.26a
2	51.50±1.57b
3	39.66±1.48c
4	31.66±1.03d
5	26.06±1.35e
6	14.36±1.00f
7	6.78±0.93g

L.. Keeping view on this objective, we have determined sucrose concentration as osmoprotectant and dehydration duration required to facilitate the maximum regeneration of encapsulated shoot tips of *R. Serpentine* after cryopreservation. Though long term storage under ultra-low temperature is common approach for germplasm conservation but sometimes it is susceptible to genetic and epigenetic changes in the plant materials. For this reason, we adopted molecular study to check the variation at molecular level in regenerated plants after cryopreservation with the mother plant.

MATERIALS AND METHODS

Preparation of synthetic seed from pre-existing shoot tips of *R. serpentine*

Shoot-tips of *R. Serpentine*were pre-cultured on autoclaved solid hormone-free MS (Murashige and Skoog, 1962) media supplemented with 0.3 M sucrose under aseptic conditions of laminar air flow (manufacturer) and kept for 72 hours. Prior to synthetic seed preparation, the pre-cultured shoot-tips were transferred to a modified liquid hormone free-MS medium containing sodium alginate (3%). To prepare the beads, the shoot-tips were collected individually from sodium alginate containing solution and then transferred into liquid hormone-MS medium containing 75 mm calcium chloride to form beads of about 5 mm in diameter and allowed to polymerize for 30 min with continuous stirring under the laminar air flow. After 30 minutes of polymerization, beads were washed with sterile double distilled water and subjected to next phase of experiment *i.e*. dehydration after pre-treatment of various concentration of sucrose.

Pre-treatment of sucrose and encapsulation dehydration

The encapsulated shoot tips were shifted to modified MS media supplemented with various concentrations of sucrose (0.3, 0.5 and 0.7 M)]. After 24 hours of incubation on rotary shaker (100 rpm) at 25°C, the encapsulated shoot-tips were transferred on sterile filter paper in uncovered petridishes for various time durations (3, 5, 7, or 9 hours) under laminar airflow cabinet at room temperature. After each dehydration period, 5 beads were weighed. MC % was determined by using the following equation:

MC% = {(Beads fresh weight - Beads dry weight)/ Beads fresh weight} X 100

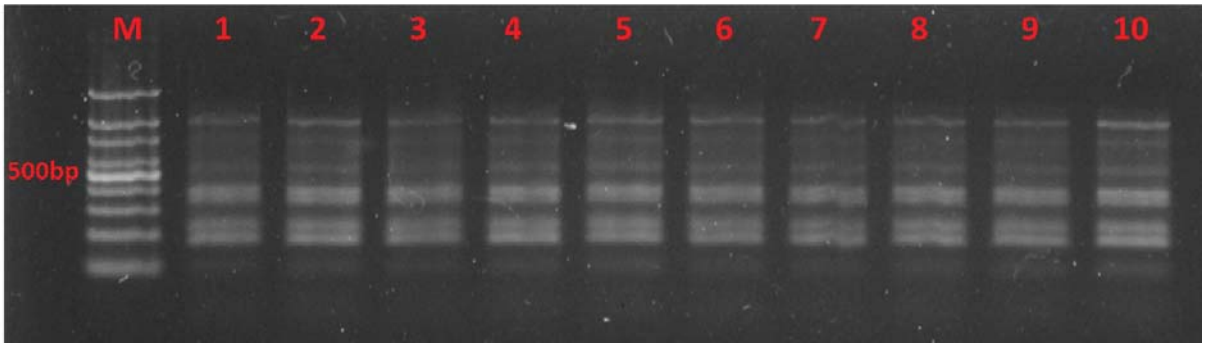

Fig. 1: RAPD analysis of regenerated plantlets with mother plant culture with OPE-4

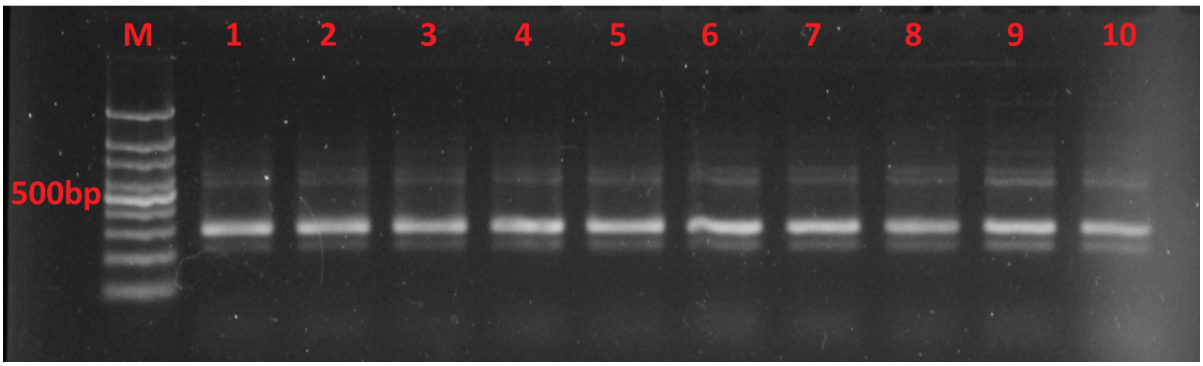

Fig. 2: RAPD analysis of regenerated plantlets with mother plant culture with OPE-6

Liquid Nitrogen (LN) treatment and cryopreservation

After each dehydration period, the dehydrated beads were transferred to pre sterilized cryovials (total 40 beads for 8 cryovials) prior to cryopreservation (liquid nitrogen treatment) for 1 hour. Another 40 beads without LN (control treatment) and were cultured directly to the hormone-free MS medium and incubated for 21 days.

Regeneration of plant from cryopreserved seeds

Prior to plant regeneration the cryopreserved cryovials were subjected to rapid thawing for short time (max. 2–3 minutes) at 37 °C in water bath. *In vitro* shoot regeneration was carried by inoculating the cryopreserved synthetic seed in hormone-free MS medium after proper thawing. The standard method of *in vitro* culture was followed. Data on survival percentage and shoot numbers were measured after 21 days of inoculation in the hormone-free MS medium.

Genetic fidelity testing of regenerated plantlets after cryopreservation

Genomic DNA was isolated from leaves of regenerated plantlets through using Cetyltrimethyl ammonium bromide (CTAB) method and quantitative, qualitative analyses of genomic DNA were checked by spectrometric and electrophoretic methods respectively. 10 different RAPD markers were employed to test the genetic uniformity of regenerated plants of *R. serpentine*. The polymerase chain reaction was carried out the amplification reaction was carried out in a thermal cycler (Applied Biosystem, Veriti®, USA). The PCR reaction mixture of 25 ìL was composed of 25 ng genomic DNA, 1 ìL PCR buffer, 2.0 mM $MgCl_2$, 200 mMdNTPs, 1 mM primer (RAPD), and 1 U Taq DNA polymerase (all from Xcelris Labs Ltd. except genomic DNA). The amplification conditions involved a preliminary denaturation at 94 °C for 5 min and subsequently 35 cycles of 94 °C for 1 min, 1 min annealing at 35 °C, and 2 min extension at 72 °C. A final extension was carried out at 72 °C for 7 min. After completion of cycles, amplified products were allowed to cool down at a holding temperature of 4°C. PCR products were then separated on 1% agarose gel electrophoresis by using TAE (Tris Acetic acid EDTA) buffer. The size of amplicons was estimated using a 1 Kb DNA ladder (manufacturer). The gels were photographed using a gel documentation system (manufacturer) and only clear and scorable DNA bands were considered for the analysis.

Data analysis

Statistical analysis of recorded data was performed using SPSS v16.0 for Windows (SPSS Inc., USA) with a 5% probability level of statistical significance.

RESULTS AND DISCUSSION

Effect of pretreatment of sucrose and dehydration duration on cryopreserved plants regeneration

In this experiment we have used the *in vitro* grown shoot tip as explants for synthetic seed conservation at ultra low temperature because of its genetic stability as well as ability to grow (Engelmann, 2000; Matsumoto *et al.*,2001). *R. serpentine* synthetic seeds were pretreated with various concentrations of sucrose as osmoprotectant *viz.* 0.3M, 0.5M and 0.7M (Table 1) prior to dehydration under laminar air flow. The optimal dose of both treatments *i.e.* preculture of encapsulated seeds at sucrose as well as air dehydration using laminar airflow has been tested during cryopreservation of these encapsulated synthetic seeds. The seeds pretreated with 0.3M sucrose followed by 3 hours of dehydration in absence of liquid nitrogen (control) have shown 100% germination as well as regenerated high number of shoots/plant (5.00±0.11) in media without any visible damage due to the desiccation process. With respect to this control, cryopreservation reduced germination rate as well as avg. shoot number plant^{-1} upto 3.75 and 4.71fold *i.e*. 26.66±6.66% and 1.06±0.06 respectively (Table 1).Though there was a negative impact of liquid nitrogen on plant response after cryopreservation but we found it could be adjustable using pretreatment of high concentration of sucrose to the synthetic seeds.In compare to 3.75 fold reduction in germination response, pretreatment of high concentration of sucrose *i.e*. 0.5 M and 0.7 M followed by dehydration for 3 hours prior to cryopreservation resulted reduction in the germination response upto 2.6 and 1.33 fold with respect to the respective control (Table 1). With the increase of dehydration time, both the germination percentage and shoot number were gradually reduced irrespective of the concentration of sucrose in non-cryopreserved seeds as well as cryopreserved seed. Increasing the dehydration time for both desiccated and cryopreserved seeds pretreated with high concentration of sucrose (0.7 M) to 9 hours yielded very poor response *i.e*. only 0.66±0.33 avg. no. shoots plant^{-1} with a growing frequency of 13.33% in dehydrated seeds, pertaining to extreme loss of cellular water while no response was recorded for cryopreserved seed. A clear trend in response of synthetic seeds after cryopreservation pointed out significance of both of pretreatment of sucrose and dehydration duration on survivality of plant. Negative impact of cryopreservation may be arises either from intracellular ice-crystal formation or lethal concentrations of solutes (Huebinger *et al.*,2016). Use of cryoprotectants in cryopreservation reduces the scope of ice crystal hence enhances the survaivility. In our experiment use sucrose as cryoprotectants for this purpose. Prior to cryopreservation, pretreatment of high concentration sucrose acted as osmoprotectants as well as enhanced the intracellular solutute concentration (Dument *et al.*,1993; Shatnawi and Johnson, 2004). From our experiment we found pretreatment of higher concentration of sucrose followed by short duration dehydration as well as low concentration of sucrose followed by long duration dehydration to synthetic seed enhanced the survivality of plant sample after cryopreservation. Hence the higher response of cryopreserved plant sample is a cumulative effect of both of sucrose concentration and dehydration duration. Though high levels of sucrose were toxic to the explants and resulted in a decrease in survival.

Role of moisture content in desiccated and cryopreserved seeds

Moisture content of cell is essential for survivality but have a negative impact when cells are subjected to cryopreservation. During experiment gradual decrease in total moisture content in synthetic seeds of *R. serpentine* was observed as we increased the dehydration time (Table 2). The cells retain enough water during the first 3 hours of dehydration (approx. 75.12 to 39.66%) and critical loss of cellular moisture was observed after 7 hoursof dehydration and beyond where the moisture content drastically drops below 6.78%. Strikingly cryopreservation of synthetic seeds pretreated with 0.5 M sucrose followed by 7 hours of dehydration produced significantly higher number of shoots (1.66±0.06) and shoot growths (46.66±6.66) in compare to other treatment. In our experiment pretreatment of high concentration of sucrose followed by long time dehydration reduced the plant response after cryopreservation. A similar trend was also observed under the desiccated condition without liquid nitrogen. This finding may be pointed out the relationship between sucrose concentration, dehydration duration and moisture content. Minimum response of cryopreserved encapsulated shoot tips than desiccated seeds were recorded after first 3 hours of dehydration which pointed out the negative impact of intracellular moisture content on sample during cryopreservation. As moisture level decreased there was comparatively better response recorded for the cryopreserved encapsulated seeds. Similarly pretreatment of high concentration of sucrose

followed by short time dehydration provided similar pattern of response. Hence moisture content, dehydration duration and treatment of sucrose has played a vital role on cryopreservation of plant sample.

Genetic uniformity assessment

Testing the genetic uniformity of cultured plantlets post cryopreservation is imperative to the success of the procedure since storage in sub-zero temperatures followed by slow growth can risk causing genetic variation. To assess the genetic uniformity RAPD markers are most commonly used for their ability to screen the whole genome.Random Amplified Polymorphic DNA (RAPD) is the simple and cost-effective molecular marker system in nature (Babu *et al.*,2014). In addition this marker system is easy to handle, requires less amount of DNA and don't need any prior knowledge about genomic DNA sequence (Esselman, *et al.*,1999). Due to these advantages, RAPD based approach have been reported for checking genetic fidelity of several plant species after *in vitro* storage *i.e*. *Cineraria maritime* (Srivastava *et al.*,2009); *Picrorhiza kurrooa* (Mishra *et al.*,2010)*; Rauvolfia tetraphylla* (Alatar and Faisal, 2012); *Pistacia vera* L. (Akdemir *et al.*,2013); *Elaeis guineensis* Jacq (Gantait *et al.*,2014) *etc*.This is probably the first report on RAPD based approach has been adopted to check the genetic fidelity of plant regenerate after cryopreservation for *R. serpentine*. 10 RAPD markers have been employed (OPE-04 and OPE-06 in Fig 1 and 2 respectively)for the assessment of uniformity among the regenerated synthetic seedlings. A total of 110 scorable and reproducible bands were observed (an average of 11.0 bands per primer).This study justified the authencity of genetic similarity between the regenerated plants with the mother plant.

This is the probably first report on cryopreservation of *R. serpentine* based on encapsulation dehydration using the shoot tip as explants. The suitable dose of sucrose as well as dehydration duration has been standardized for this purpose. We found the response of cryopreserved plant sample was depending on both of sucrose treatment and dehydration duration. Synthetic seed pretreated of high concentration of sucrose followed by short time dehydration as well as pretreated with low concentration sucrose followed by long time dehydration can sustain the effect of ultra low temperature. Response of cryopreserved plant samples are depends on moisture content hence samples with low moisture content will be much more effective for the cryopreservation due to less scope of intracellular freezing at ultra low temperature.

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