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EFFECT OF GELLING MATRIX COMPOSITION, STORAGE CONDITIONS AND CAPSULE BREAKAGE ON GERMINATION OF *ROSA INDICA* SYNTHETIC SEEDS

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ABSTRACT

Storage of germplasm using synthetic seed technology is useful to avoid somaclonal variations as well as to cut down the cost and labor of germplasm maintenance under *in vitro* culturing conditions. In the present study, different parameters that affect the viability and regrowth ability of *Rosa indica* germplasm stored as synthetic seeds were optimized. The compact, firm, transparent synthetic seeds showing maximum regeneration rate (100 %) were prepared and stored in three different types of air tight storage vessels (glass jars, polypropylene tubes and plastic bags) at 4 °C and 25 °C for various time durations (0-8 weeks). No significant difference in regeneration frequency of *Rosa indica* synthetic seeds was recorded at different storage conditions. However, the storage temperature significantly influenced the viability and regrowth ability of synthetic seeds. The survival rate (80%) of encapsulated explants was observed up to 8 weeks at 4 °C and declined with elapse of storage duration. Facilitated extrusion of shoot from gel seed and pre-treatment of synthetic seeds with 100 mM $KNO_3$for 5 minutes improved the regeneration frequency while mechanical removal of gel bead resulted in complete loss of explant viability.

Key words: *synthetic seed, viability, Rosa indica, nodal segments.*

INTRODUCTION

Many ornamental plants are rapidly propagated by *in-vitro* culturing techniques. Micropropagation is considered as an efficient method for mass propagation of disease free rose cultivars at commercial scale (Canli and Kazaz, 2009). It gives reliable maintenance of rose germplasm instead of glass-house or field where plants are at risk of disease, pest and environmental stresses (Ali *et al*., 2018). Under *in vitro* culturing conditions, plants are maintained for short to medium term in active or slow growing state (Engelmann and Engels, 2002). The ease of tissue culture has revitalized certain ornamental industries. However, despite of all the advantages, a serious problem encountered during propagation through tissue culture is somaclonal variations that may arise from any types of tissue culture (Neelakandan and Wang, 2012). It has been observed that clonal fidelity is not always the case in tissue culture. It may cause genetic variability in tissue cultured plants through somaclonal variations that is not acceptable where clonal stability is mandatory (Temel *et al*., 2008). Frequent subculturing and the duration of an *in vitro* maintained culture are the leading factors for somaclonal variations (Sun *et al*., 2013). To avoid somaclonal variations, most of the commercial nurseries sample the plants *ex-vitro* periodically or limit the number of subcultures (Skirvin *et al*., 1994).

Synthetic seed technology in which the germplasm is stored under *in vitro* conditions at low temperature (> 0°C) can be exploit to reduce the frequent demand of sub culturing (Mohanty *et al*., 2013). The potential advantages of this technology include the ease of production, handling and transportation (Germana *et al*., 2011), reduced cost and space for germplasm maintenance (Standardi and Piccioni, 1998) with ready availability for propagation and maintaining genetic uniformity of propagated plants (Reddy *et al*., 2012). Synthetic seed technology is particularly useful for propagation of rare hybrids, elite genotypes and varieties whose seeds are rarely available (Mandal *et al*., 2000; Bukhari *et al*., 2014).

Initially, somatic embryogenic propagules were used for synthetic seed preparation (Murashige, 1977). Later on, it has been extended to the use of many non-embryogenic propagules such as shoot tips (Rai *et al*., 2008), nodal segments (Nikhil and Shukla, 2013), micro-plant/cuttings (Adriani*et al*., 2000) and roots (Piatczak and Wysokinska, 2013) as somatic embryogenesis potential is not present in all culturable plants. The use of shoot tips or nodal segments has not only made this technology more easy and cost effective but also has broaden its scope ranging from herb (Siddique and Anis, 2009; Cheruvathur *et al*., 2013), tree (Singh and Sharma, 2006), medicinal plants (Micheli *et al*., 2007), ornamental plants (Haque and Ghosh, 2014) and orchids (Naganand*aet al*., 2011).

Rose is one of the most important ornamental plants which are generally propagated by conventional vegetative propagation methods (cutting and grafting) (Horn, 1992). Micro propagation is extensively used for mass propagation of rose (Chavan *et al*., 2007). However, frequent sub culturing during *in vitro* propagation can adversely affects the clonal stability of rose cultivars. The aim of the present study was to investigate and optimize the factors affecting the *in vitro* conservation and regeneration frequency of synthetic seeds of rose stored at low temperature using synthetic seed technology.

MATERIALS AND METHODS

All the experiments were performed in plant tissue culture laboratory at Institute of Biochemistry and Biotechnology, University of the Punjab. The*invitro* grown cultures of *Rosa indica* in aforementioned laboratory were used as source plant for nodal segments to be conserved. All the reagents used in the present study were purchased from Sigma Aldrich (USA).

Plant material: Nodal segments from *in vitro* grown cultures of *Rosa indica* were used as explant for synthetic seed preparation. *In vitro* cultures were maintained on MS medium (Murashige and Skoog, 1962) fortified with 0.6 $mg^{-}l$ of BAP(6-Benzyl aminopurine) under 16/8 hrs photoperiod provided by 2000 lux light.

Encapsulation of explant for synthetic seed production: For encapsulation, nodal segments of size 3 mm were excised from *in vitro* culture of rose and dipped in sterile solution of sodium alginate (as gel matrix) of various concentrations (1-4%) prepared in full strength MS salts lacking sucrose and phytohormones. The explants submerged in alginate solution were agitated for 30 minutes. For seed formation, each nodal segment with sufficient volume of alginate sodium was sucked through the pippete tip modified by cutting to have diameter of 7 mm. Each drop having explant was released in $CaCl_2$ solution (50-200mM) for complexation and kept in it for different time durations (5-20min). After defined time of period, the synthetic seeds were removed from $CaCl_2$ solution and washed with sterile distilled water to remove all the traces of $CaCl_2$.

Storage of synthetic seeds: For appropriate storage of synthetic seeds, three storage parameters: storage vessel, storage temperature and storage time were evaluated. Synthetic seeds were stored in three different air tight storage vessels (plastic bags, polypropylene tubes, and glass jars) at ambient temperature i.e. 25°C as well as at 4°C for different time intervals (0-10 weeks). After allocated storage time, the synthetic seeds were sterilized with 0.5%NaOCl, rinsed with water and used for inoculation in the germination medium (MS medium supplemented with 0.6 $mg^{-}l$ of BAP).

Pre-treatment of synthetic seeds: In order to check the observed delay in regeneration caused by gelling matrix (alginate covering), synthetic seeds were pre-treated with sterile 200mM KNO_3 for different time durations (5-30 min). This treatment facilitates the germination of encapsulated explant (Ali *et al*., 2012**)** by self-breakage of gelling matrix.

In parallel, the germination was also facilitated by mechanical removal of gelling matrix of synthetic seeds with the help of a sterile scalpel under aseptic conditions prior to inoculation in germination medium.

Effect of synthetic seed composition on germination: To evaluate the effect of synthetic seed composition on germination rate, synthetic seeds (7mm size) of three different compositions (3% Alginate in MS salts, 3% Alginate in MS salts + 3% sucrose and 3% Alginate in MS salts + 3% sucrose + 0.6 mg/l BAP) were prepared and allowed to germinate after defined period of storage on germination medium under 16/8 hrs photoperiod provided by 2000 lux light.

Experimental design and statistical analysis: The experiment was designed with optimization of nine factors including: i) optimization of alginate concentration, ii) $CaCl_2$ concentration, iii) exposure time to $CaCl_2$, iv) storage vessel, v) storage temperature, vi) storage time, vii) pre-treatment with KNO_3, viii) mechanical removal of alginate gel and ix) synthetic seed composition. Each experiment was consisted on 20 replicate tubes (one synthetic seed per tube). Each experiment was performed in triplicate. The mean standard error and one way ANOVA was performed by SPSS software. The significant difference between the means was assessed by Duncan's multiple range test at $p \leq 0.05$.

RESULTS AND DISCUSSION

To maintain the cultures under *in vitro* conditions while avoiding the frequent sub culturing that result in somaclonal variations,synthetic seeds of rose cultivar were prepared by encapsulating non-embryogenic propagules (nodal segments) of *in vitro* culture in gelling matrix of alginate with $CaCl_2$ as complexation agent. Various parameters of synthetic seed production and storage were optimized as under:

Alginate encapsulation: In order to get the synthetic seeds of well-defined shape and texture, 3 % sodium alginate was used as gelling matrix along with 100mM $CaCl_2$ as complexation agent were used. It was observed that concentration of sodium alginate and $CaCl_2$ along with exposure time to $CaCl_2$ significantly affected the shape, texture, firmness and appearance of synthetic seeds. These factors have synergistic effect on the overall morphology of alginate bead. The uniform, transparent

and firm beads were obtained by using 3% sodium alginate after exposure of 15 minutes to 100mM $CaCl_2$. The hardness of gel beads is mainly based on the exchange of Na^+ ions of alginate with Ca^+ ions resulting in insoluble Ca-alginate (Nikhil and Shuklay, 2013). As the exposure time to $CaCl_2$was increased, thickness of insoluble Ca^+ alginate wall was also increased until a point came where maximal ion exchange has been taken place. Further exposure to $CaCl_2$was impractical with reference to the bead hardness. In our study, that point was attained after 15 min exposure of 100mM $CaCl_2$ for gel beads with 3% sodium alginate (Figure 1). There are several reports in which 3% sodium alginate with 100mM $CaCl_2$ had been used but exposure time to $CaCl_2$ is different ranging from 15-45 minutes (Baskaran *et al*., 2015; Mohanty *et al*., 2013; Ahmad and Anis, 2012; Rai *et al*., 2008; Ray and Bhattacharya, 2008; Naik and Chand, 2006). The report of Sakhanokho *et al*., (2013) supports our observation that after a particular exposure time to $CaCl_2$, there is no further increase in the hardness of bead even though the exposure time is further increased.

Effect of storage conditions on synthetic seed germination: The proper storage of synthetic seed is important to maintain the viability (green color, without necrosis and yellowish appearance) and the regrowth ability of encapsulated explant (Standardi, 2012). To optimize the storage conditions three parameters: i) storage vessels, ii) storage temperature and iii) storage time were evaluated. It was observed that there was no significant difference in rate of synthetic seed germination whether stored in air tight plastic bags, polypropylene tubes or in glass jars (Figure 2a). In several reports, the nature of storage vessels has not been mentioned while in some reports storage of synthetic seeds has been mentioned in glass jars (Ikhlaq *et al*., 2010), petri plates (Sakhanokho *et al*., 2013) and plastic cuvettes (Micheli *et al*., 2007). However, the effect of storage vessels on viability or germination frequency of synthetic seed has not been evaluated yet. In our study, it was established that for storage of synthetic seed glass vessels, plastic bags and polypropylene tubes can equally be used for the storage without affecting the germination and viability of synthetic seeds. The key point is that the storage vessel should be airtight to prevent shrinkage and dehydration of synthetic seed.

The viability and regrowth ability of synthetic seeds was significantly affected by storage temperature and storage duration. The viability and regrowth ability of synthetic seeds declined rapidly after the storage of 1-2 weeks at 25°C.The encapsulated explant became brown in color and showed necrosis after storage of 2 weeks. It might be possible that under storage at 25°C (an ambient temperature for the growth of *in vitro* cultures), encapsulated explant came under stress due to unavailability of sufficient nutrients in alginate gel matrix (i.e. lack of sucrose and phytohormones). It resulted in release of phenolic compounds which gave brownish appearance to synthetic seed and finally necrosis and loss of viability of encapsulated explant. Whereas the synthetic seeds stored at low temperature (above freezing point, 4°C) sustained viability and regrowth ability over the long period of time (8 weeks) (Figure 2b). It might be possible that low temperature induced a visual quiescent state in encapsulated explants that favored the viability of explant in the gel bead (Ikhlaq *et al*., 2010). The result of our study corroborates with the findings of Ikhlaq *et al*. (2010) and Sakhanokho *et al*. (2013).

It was also observed that the frequency of synthetic seed regeneration declined while time required for regeneration was increased with elapse in storage duration at 4°C (Figure 2c). It was worth noting that drastic decline was observed in regeneration frequency (approximately to zero) of synthetic seeds after storage of 8 weeks at 4°C (Figure2c). The most desirable feature of encapsulated vegetative propagules is to retain their viability after storage for a reasonable period of time (Micheli *et al*., 2007; Rai *et al*., 2008). The decrease in germination frequency over the period of time is in accordance with the previous reports on the other species. However, complete loss of viability and/ or maximum storage duration is different in different reports like: 6 weeks for Olive cv. Moraiolo and *Centauriumerythraea* at 4°C (Ikhlaq *et al*., 2010; Piatczak and Wysokinska, 2013), 40 days for *Rosa hybrid* 'King's Ransom'at 4°C (Jayasree and Devi, 1997), 60 days for pomegranate at 4°C (Naik and Chand, 2006), 45 days for guava at 4°C (Rai *et al*., 2008), 14 weeks for *Rauvolfia serpentine* at 4°C (Ray and Bhattacharya, 2008),4 weeks at 22°C and 8 weeks at 4 °Cin*Begonia* microshoots (Sakhanokho*et al*., 2013), 8 weeks at 4°C for *Decalepishamiltonii* and *Rutagraveolens* (Sharma and Shahzad, 2012; Ahmad *et al*., 2012) and 8 weeks at 4°Cfor *Cassia angustifolia* (Parveen and Shahzad, 2014). Indeed, storage at low temperature (4°C) lessens the rate of explant morbidity over long period of storage in comparison to the storage at 25°C. Even at low temperature storage, the explants are under stress of inhibited respiration impeded by alginate covering (Redenbaugh *et al*., 1991). Upto certain period of storage period, plant survive under stress but as the stress overwhelmed, the explants loss their viability and regeneration ability completely. This duration of plant resistance for survival against stress during low temperature storage (4°C) is different among different plants (as mentioned above). In case of *Rosa indica* synthetic seeds, this duration of resistance last upto eight weeks as regeneration frequency dropped drastically from 80% to 0% after 8 weeks of storage at 4°C. Hence, the storage of germplasm under any condition ultimately affects its viability.

Effect of self-breakage and mechanical breakage of gel bead on germination: Encapsulation procedures are considered efficient in maintaining the viability of encapsulated explant. The synthetic seeds prepared using 3 % sodium alginate with 15 min exposure to 100mM $CaCl_2$ were firm enough to be handled and transported easily without mechanical breakage of seeds. However, remarkable delay or very poor response of germination has been reported when synthetic seeds are used directly for germination (Kumar *et al*., 2005). The coating material cause the hindrance in emergence of shoot from gel bead (Kurusulee *et al*., 1992).The stored synthetic seeds of *Rosa indica* that were not subjected to the any pre-treatment or breakage procedure showed delay in regeneration (60 days) and low germination frequency (10%) as well Table 1. To avoid the delay in regeneration caused by alginate gel bead covering, the synthetic seeds were treated with KNO_3 for self-breakage of gel bead as well as the alginate covering was removed mechanically with the help of scalpel. Synthetic seeds pretreated with 200mM KNO_3 for 5 min showed (80%) frequency of regeneration even after the storage of 8 weeks at4 °C (Figure 2d; Figure 3). Time of exposure to KNO_3 was very important as further increase in exposure time (> 5min) resulted in complete loss of gel bead structure and loss of regeneration ability (Table 1). In contrast, Kumar *et al*., (2005) reported the exposure of 60 min and 40 min with 200mM and 100mM KNO_3 respectively. Sharma *et al*., (2014) reported that treatment of synthetic seeds with 100mM KNO_3 for 30 minutes prior to incubation in germination medium significantly improved conversion frequency. It was observed that treatment of KNO_3 made the synthetic seeds soft and porous and made the developing shoots easy to extrude from the alginate bead. In fact, KNO_3 pre-treatment caused the exchange of K^+ ions with Ca^+ ions of Ca-alginate bead and made the bead soft (Onishi *et al*., 1994).

The results of mechanical removal of gel bead covering were quite surprising. This strategy was opted to avoid the delay in extrusion of shoots. But unexpectedly, the explants failed to germinate and became brown/die just in a week. While, treatment of synthetic seeds with KNO_3 did not affect the viability and regrowth ability of encapsulated explants unless excessive exposure dissolve the alginate gel bead structure. It was inferred from the observations that encapsulation is necessary for the storage as well as to maintain the viability and regrowth of explant. Mechanical removal of gel bead is not recommended as it not only makes the process laborious and time consuming, but also increases the chance of contamination and significantly affects the viability/regrowth ability of explant. Whereas avoid excessive exposure of synthetic seeds with KNO_3 to prevent loss of gel bead structure.

Effect of composition of alginate gel matrix: Gelling matrix of synthetic seeds supplemented with different ingredients such as inorganic nutrient, carbon source and plant growth regulators served as the artificial endosperm and helps in the germination of encapsulated explant (Sharma and Shahzad, 2012). The composition of gelling matrix is among one of the important factors that significantly affects the germination frequency of encapsulated plant material (Sharma *et al*., 2014). In our study, among the different compositions of the gelling matrix tested for *Rosa indica*, the maximum regeneration rate(100%)was recorded with 3% alginate in MS salts devoid of sucrose and plant growth regulators (Figure 2e).An improved germination of explant has been reported with the inclusion of plant growth regulators in gelling matrix (Sundararaj *et al*., 2010). But on the other side, high rate of synthetic seed germination has also been reported in hormone free gel matrix (Winkelmann *et al*., 2004). For effective growth and conversion of encapsulated explant, the definite ingredients of the gel matrix are species specific (Sharma *et al*., 2012). In case of *Rosa indica* reduction in nutrient strength of (lack of sucrose and hormone) in gelling matrix along with low temperature storage has prompted effect on synthetic seed germination after defined period of storage. The findings of Das *et al*., (2011) also strengthen our result.

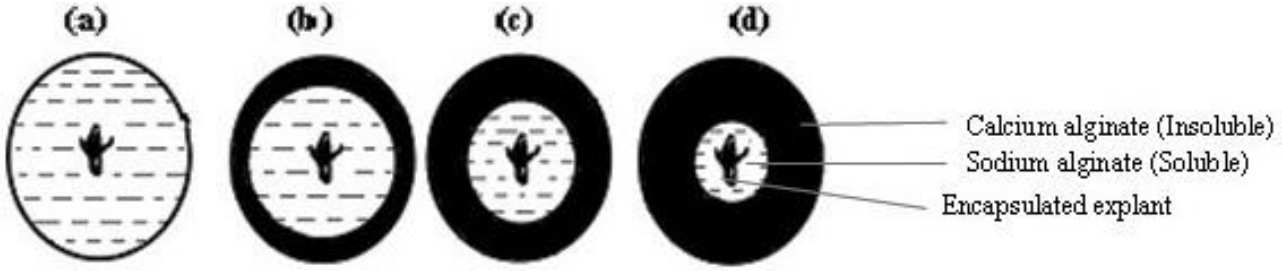

Figure 1.Effect of increase in exposure time of 3% sodium alginate gel matrix to 100mM $CaCl_2$. (a) 2 minutes; (b)5 minutes; (c) 10 minutes; (d) 15 minutes.

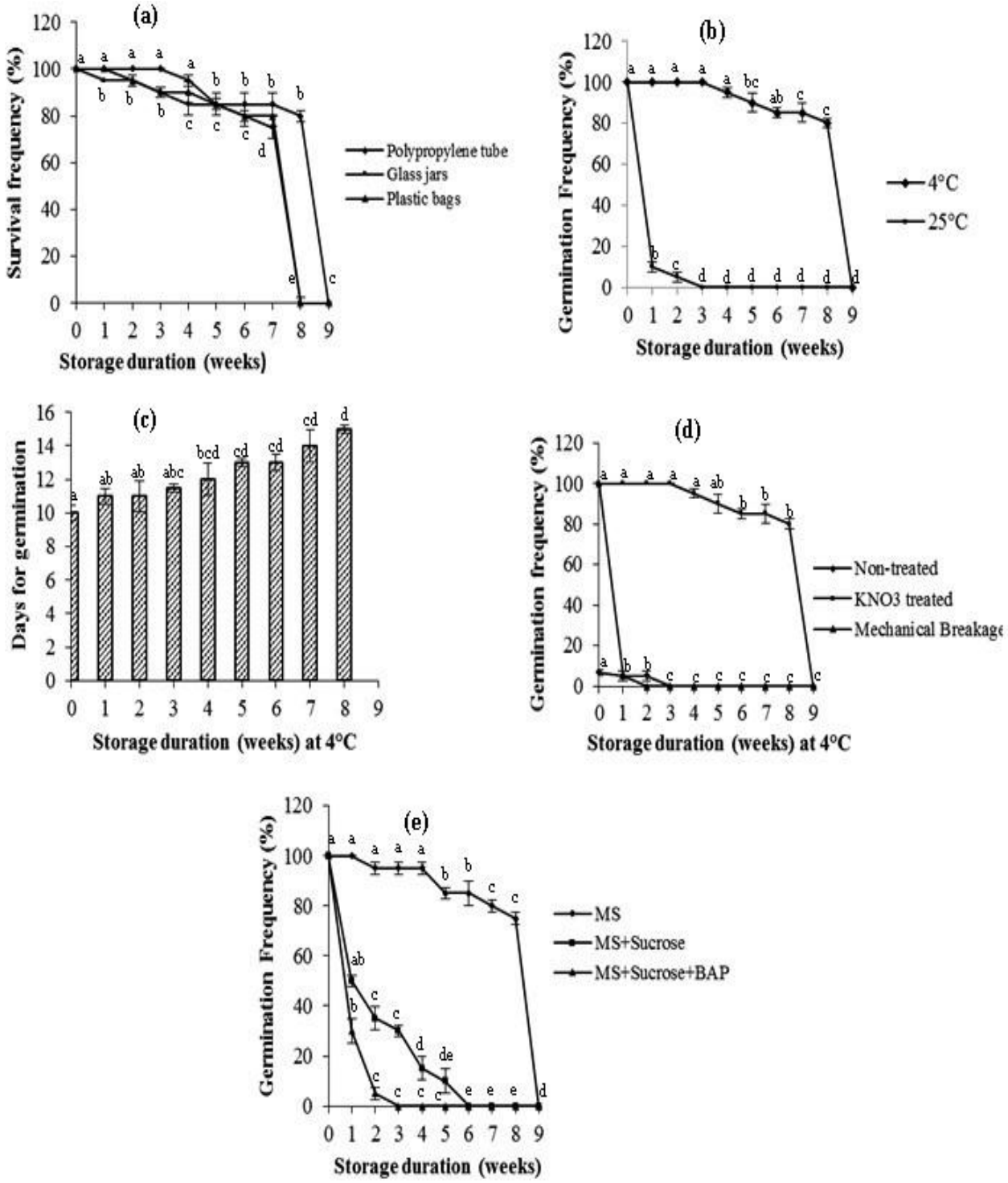

Figure 2. Optimization of different parameters affecting germination frequency of encapsulated nodal segments of *Rosa indica*. *(a)*: Effect of different storage vessels on survival of encapsulated nodal segments; *(b)*: Effect of storage temperature on germination frequency of encapsulated nodal segments; *(c)*: Effect of storage duration on time required for germination; *(d)*: Effect of pre-treatment for facilitated extrusion of shoots from seed gel on germination frequency of synthetic seeds; *(e)*: Effect of synthetic seed composition on germination frequency of encapsulated nodal segments. Mean values having the same letter do not differ significantly ($p \leq 0.05$) according to Duncan's multiple range test.

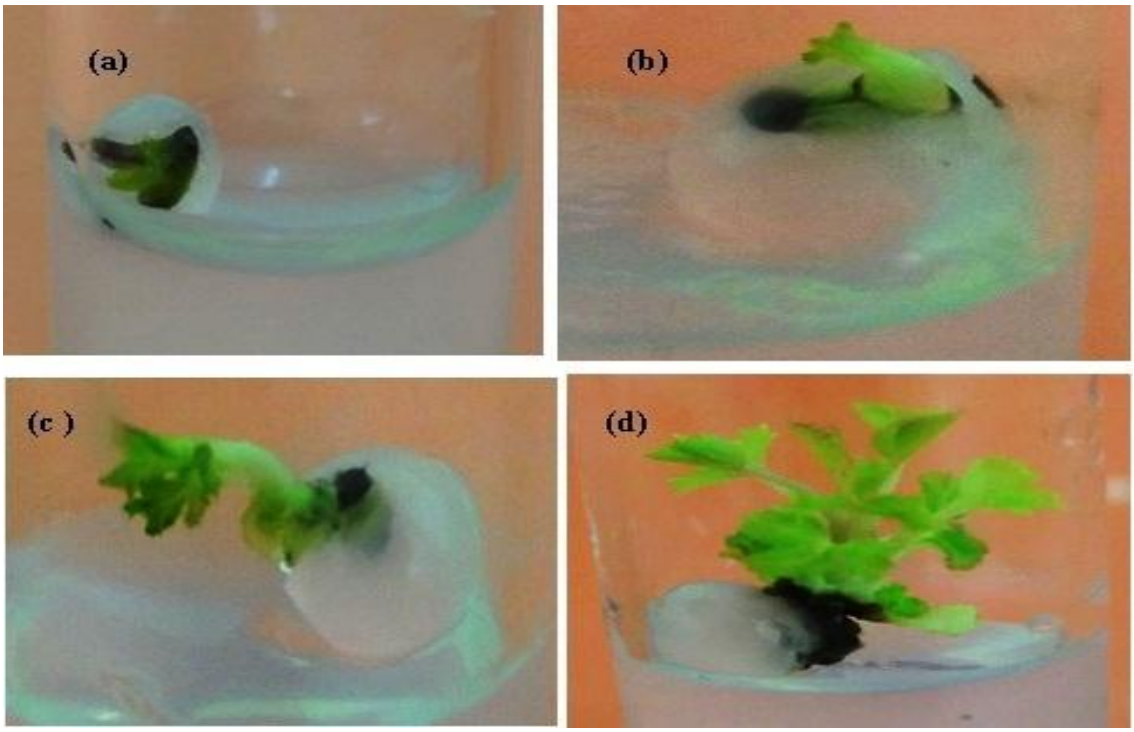

Figure 3. Different stages of synthetic seed germination prepared using 3 % sodium alginate in MS medium with 100mM $CaCl_2$ after storage of 8 weeks at 4 °C pre-treated with 200mM KNO_3 for 5 minutes before inoculation in germination medium. *(a)*: synthetic seed; *(b)*: extrusion of shoot after 13 days; *(c)*: after 24 days, *(d)*: after 30 days.

Table1.Effect of KNO_3 pretreatment on germination frequency of *Rosa indica* synthetic seeds.

Exposure time (minutes)	Alginate gel bead structure	Germination frequency after 8 weeks of storage (%)
0	Firm	10 ± 2.35^{b}
5	Soft, porous	80 ± 4.71^{a}
10	Structure loss (gel dissolved)	0 ± 0.00^{c}
15	Structure loss (gel dissolved)	0 ± 0.00^{c}
20	Structure loss (gel dissolved)	0 ± 0.00^{c}
25	Structure loss (gel dissolved)	0 ± 0.00^{c}
30	Structure loss (gel dissolved)	0 ± 0.00^{c}

Values are mean of three independent experiments. Means having the same letter do notdiffer significantly at $p\leq0.05$ according to Duncan's Multiple Range Test.

Conclusion: By the use of the procedure established in the present study, germplasm of *Rosa indica* can be maintained under *in vitro* conditions for 8 weeks at 4 °C without the need of subculturing. This practice reduces the burden in term of cost, labor and loss of clonal stability.

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