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A protocol for synthetic seed production in *Rosmarinus officinalis*

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Abstract

The synthetic seeds strategy is an effective method for conservation, germplasm exchange, and distribution of *Rosmarinus officinalis*. *R. officinalis* is one of the plants of the Lamiaceae family that originates from the Mediterranean region. According to our knowledge, there is no report on the production of synthetic seeds from somatic embryos obtained from shoots of *R. officinalis*. In this study, a method for synthetic seed production and regeneration was investigated from shoot explants of *R. officinalis*. Shoots of *R. officinalis* were cultured on the MS medium containing various concentrations of NAA and 2,4-D. The best callus induction (41.66%) was obtained on the MS medium fortified with 1 mg/L 2,4-D. The friable embryogenic calli were transferred to a half-strength MS medium with various concentrations of BAP and 2,4-D. Somatic embryos were obtained after 45 days. The highest somatic embryogenesis was obtained on the MS medium containing 1 mg/L 2,4-D and 0.5 mg/L BAP. Somatic embryos at the torpedo stage were encapsulated in sodium alginate and synthetic seeds were obtained. The highest growth ability of synthetic seeds (80.55%) was obtained in the MS medium supplemented with 1.5 mg/L kinetin and 0.5 mg/L BAP. Moreover, the germination percentage of synthetic seeds decreased with increasing storage period and temperature. However, a higher reduction in the synthetic seed germination was recorded at 20±2 °C. The highest germination percentage of the synthetic seeds (85%) was obtained 30 days after storage at 4±1 °C.

***Key words*:** Callus induction, *Rosmarinus officinalis*, Sodium alginate, Somatic embryogenesis, Synthetic seeds.

INTRODUCTION

The synthetic seeds are produced and developed with artificial encapsulation of somatic embryos, shoots or other meristematic tissues (Bapat and Mhatre, 2005; Gantait *et al.*, 2017; Rihan *et al.*, 2017). The synthetic seeds are used for multiplication, preservation, and propagation of plants without real seed (Ikhlaq *et al.*, 2010; Asmah *et al.*, 2011; Siddique and Bukhari, 2018). This technology has the low cost of production and can be used in plant conservation and virus-free germplasm production (Siddique and Bukhari, 2018). The small size of artificial seeds and their easy manipulation facilitate the exchange and distribution of germplasm. It also prevents the bulk transfer of plants and the spread of disease (Bhanuprakash and Umesha, 2015).

Quite a few factors are responsible for the successful production of synthetic seeds, counting the crucial ones such as the level and type of gel required for encapsulation and the extent of exposure of encapsulated seeds to $CaCl_2.H_2O$. Gels such as agar, alginate, carboxy methyl cellulose, carrageenan, gelrite, sodium pectate, etc., were exploited for artificial seed development, of which alginate encapsulation was proved to be appropriate for the same. Several gel

types are exploited for encapsulation; however, sodium alginate established itself to be the most frequently used matrix because of low cost, gelling properties, and its nontoxic nature (Cheruvathur *et al.*, 2013). Mainly, the hardness of the hydrogels is contingent on the number of Na^+ ions in sodium alginate solution, exchanged with Ca^{2+} ions in $CaCl_2.H_2O$ solution, consequently ensuing in the creation of insoluble calcium alginate (Daud *et al.*, 2008). Usage of agar as gel matrix is not preferred because of its inferior nature when compared to alginate relating to long-standing storage. Alginate is the preferred choice since it aids to ameliorate capsule development and additionally, the firmness of alginate beads assures a much improved protection to the encased somatic embryos from mechanical damage (Gantait *et al.*, 2015).

The production of synthetic seeds depends on plant species, materials used in coating, their concentrations and conditions that the solutions are maintained (Nhut *et al.*, 2005). In the synthetic seed technique, all kinds of plant explants such as somatic embryos, pollen, axillary bud/nodal segments, shoot or meristem can be applied (Prakash *et al.*, 2018). In some plants due to heterozygosity of seeds, seed size, reduced endosperm, requirement of symbiotic mycorrhizal fungi for seed germination, propagation through seeds is not possible. Therefore, these plants can be propagated by synthetic seed technology (Kinoshita and Saito, 1990). Synthetic seed production has been used in many plants such as *Capparis decidua* (Siddique and Bukhari, 2018), *Plumbago rosea* L. (Prakash *et al.*, 2018), *Solanum nigrum* (Verma *et al.*, 2010), *Vitex negundo* (Ahmad and Anis, 2010), and *Picrorhiza kurroa* (Mishra *et al.*, 2011). Several studies indicated that several compounds such as nutrients, plant growth regulators, anti-pathogens, herbicides, bio-controllers, and bio-fertilizers, are essential to control the growth and facilitate the germination of synthetic seeds (Prewein and Wilhelm, 2003; Kumar *et al.*, 2005; Malabadi and Staden, 2005).

Rosmarinus officinalis (Rosemary) is one of the plants of the Lamiaceae family originated from the Mediterranean region. The height of this perennial and aromatic plant reaches 2 meters. *R. officinalis* is used as an ornamental and medicinal plant and as a natural preservative (Karataş *et al.*, 2020; Lešnik *et al.*, 2021; Nguyen *et al.*, 2021). There are various medicinal compounds in this plant including phenolics, diterpenes, triterpenes such as caffeic acid, carnosic acid, chlorogenic acid, oleanolic acid, rosmarinic acid, ursolic acid, alpha-pinene, camphor, carnosol, eucalyptol, rosmadial, rosmanol, rosmaquinones A and B, secohinokio, and derivatives of eugenol and luteolin (de Oliveira *et al.*, 2019). *R. officinalis* has large, colourful and durable flowers and is the most popular orchid genus in the horticultural industry. *R. officinalis* is a monopodial epiphyte, which is difficult to propagate by vegetative organs (Nieves *et al.*, 2003). According to our knowledge, there is no report on the production of synthetic seeds from somatic embryos obtained from shoots of *R. officinalis*. Therefore, this study aimed to determine the optimum concentrations of plant growth regulators for callus induction, somatic embryogenesis and production of synthetic seeds from *R. officinalis*.

MATERIALS AND METHODS

Callus induction

The experiments were carried out in tissue culture laboratory of the Academic Center for Education, Culture & Research (ACECR) of Ardabil. The shoots of *R. officinalis* were obtained from Agriculture Organization of Ardabil Province and disinfected with 70% (v/v) ethanol for 60 s and 2% (w/v) sodium hypochlorite for 10 min, and rinsed three times with sterile distilled water. After establishment of *in vitro* cultures, shoots were used as explants for callus induction. The explants were cultured on the MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of NAA and 2,4-D (0, 0.5, 1 and 1.5 mg/L) and their reciprocal combinations. The cultures were maintained in a growth chamber at 23±1 °C in the dark and were sub-cultured every 3 weeks.

Somatic embryogenesis

The produced calli were transferred to the MS medium containing 1 mg/L 2,4-D for multiplication and maintained in the growth chamber at 23±1 °C with 16 h light/8 h dark photoperiod and were sub-cultured every 3 weeks. After 45 days, the calli were transferred to the half-strength MS medium supplemented with different concentrations of 2,4-D (0, 0.5, 1 and 1.5 mg/L) and BAP (0, 0.25, 0.5 and 1 mg/L) for somatic embryogenesis.

Synthetic seed production

The synthetic seeds of *R. officinalis* were produced based on Siddique and Bukhari (2018) with a modification. The somatic embryos at torpedo stage were collected and suspended in a matrix of the MS medium containing 4% (w/v) sodium alginate and then dropped into a 100 mM $CaCl_2.2H_2O$ solution by pipetting to accomplish encapsulation. The beads were then shaken in an orbital shaker at 80 rpm for 40 min

for the proper bead formation. The beads containing somatic embryos were washed with sterile double distilled water to remove $CaCl_2.2H_2O$ and treated with 50 mg/L cefotaxime and placed on a sterilized filter paper to remove excess water.

Germination of synthetic seeds under different storage temperatures

Fifty synthetic seeds were kept at 20±2 °C and 50 synthetic seeds were kept at 4±1 °C for the survival test. The synthetic seeds were maintained at these temperatures (20±2 °C and 4±1 °C) for 0, 15, 30, 45, and 60 days. For germination, the synthetic seeds were cultured on the MS medium containing various concentrations of kinetin (0, 0.5, 1 and 1.5 mg/L) and BAP (0, 0.25, 0.5 and 1 mg/L) and maintained in a growth chamber at 25±1 °C with a 16/8h photoperiod.

Experimental designs

In this study, four separate experiments were carried out as a factorial experiment based on a completely randomized design with three replications. Data were analysed using SPSS (SPSS Inc. Version 26.0) software. Mean values were compared by Duncan's multiple range test at a probability level of 0.05.

RESULTS

Callus induction

The callus and produced synthetic seeds are shown in Figure 1. The analysis of variance (ANOVA) indicated that different concentrations of 2,4-D had a significant effect on callus induction, but no significant effects were observed in different concentrations of NAA and its interactions with 2,4-D (Table 1). The MS medium supplemented with various concentrations of 2,4-D produced callus and the mass of obtained callus was closely related to the concentration of 2,4-D. Among the 2,4-D concentrations, the 1 mg/L treatment resulted in the highest callus induction (Figure 2A). The results showed that the highest and lowest callus induction rates were obtained in the MS medium containing 1 mg/L 2,4-D in the absence of NAA (41.66%) and MS medium without 2,4-D and NAA (0.0%), respectively. In this study shoot explants were used for callus induction, however, other explants also can used for callus induction. For instance, Agastian *et al.* (2006) observed that when nodal explants of *Justicia gendarussa* were grown in the MS medium containing 0.1 mg/L BAP and 1 mg/L NAA, compact chlorophyllous calli were produced. 2,4-D either alone or in combination with

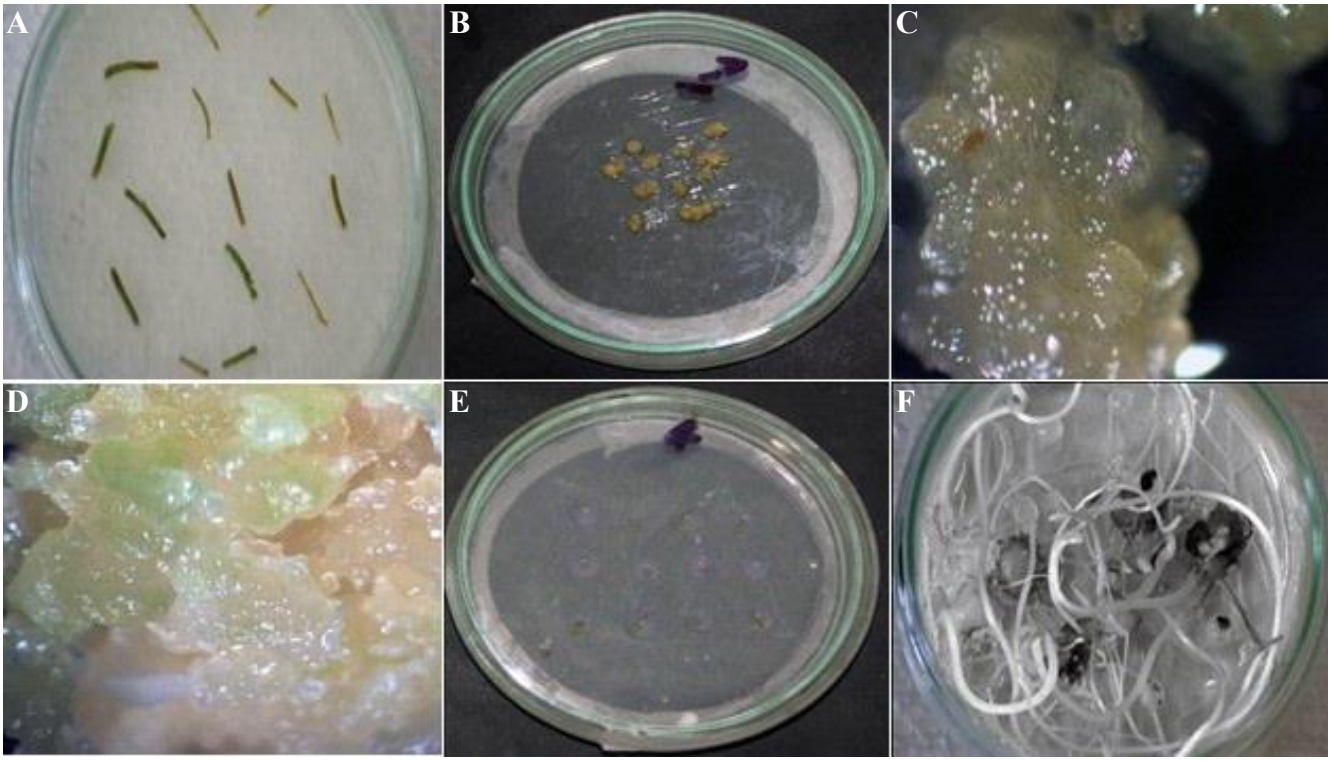

Figure 1. A: Culture of shoot explants of *Rosmarinus officinalis* on MS medium, **B:** the formation of callus on MS medium containing 1.0 mg/L 2,4-D , 0.5 mg/L NAA, **C and D:** embryogenic callus and somatic embryogenesis in MS medium supplemented with 1.0 mg/L 2,4-D with 0.5 mg/L BAP, **E:** synthetic seeds encapsulated by sodium alginate and **F:** germinated synthetic seeds roots in MS medium containing 1.5 mg/L kinetin, and 0.5 mg/L BAP.

a cytokinin is routinely used for callus induction from various explants of *Rhinacanthus nasutus* (L.) Kurz (shoots, cotyledon and epicotyl) (Cheruvathur *et al.*, 2013), *Tridax procumbens* (leaf, shoot tip and internodes) (Wani *et al.*, 2010) and *Withania somnifera* (leaf) (Singh *et al.*, 2011). Krishna Kumar and Thomas (2012) reported that optimum embryogenic calli (75%) were induced on the MS medium supplemented with 2 mg/L 2,4-D. Callus is the least exploited in terms of synthetic seed production in medicinal plants, based on its intricate use during encapsulation and subsequent regeneration. The undifferentiated nature of callus and the requirement of successful differentiation potential presumably restrict its acceptance as an explant for synthetic seed production (Gantait *et al.*, 2015). Kim and Park (2002) and Zych *et al.* (2005) were the only researchers who successfully encapsulated calli of *Allium sativum* and *Rhodiola kirilowii*, respectively, and attained as high as 95–100% regeneration. However, Tabassum *et al.* (2010) employed cell suspension derived from friable callus and ensured 57% regeneration of synthetic seeds from *Cucumis sativus*.

Somatic embryogenesis

The embryogenic calli were developed from the shoot explants on the MS medium supplemented with 2,4-D and BAP (Table 1). The ANOVA showed that different concentrations of 2,4-D and BAP and their interactions had a significant effect on the somatic embryogenesis (Table 1). Among different concentrations of 2,4-D and BAP the highest and lowest percentages of embryogenesis were obtained by applying 0.5 mg/L 2,4-D with 1 mg/L BAP (75.84%) and without plant growth regulators, respectively (Figure 2B). Somatic embryos are reportedly derived either from single cells or from single cells within a proembryonic mass. Somatic embryogenesis is generally promoted by auxins, either alone or in combination with cytokinins (Krishna Kumar and Thomas, 2012). Similarly, addition of cytokinin in the medium has been reported to improve somatic embryogenesis in other plant species (Baskaran *et al.*, 2015). Somatic embryo has been effectively utilized for synthetic seed production in several medicinal plant species, such as *Musa* spp. (Ganapathi *et al.*, 2001), *Rotula aquatic* (Chithra *et al.*, 2005), *Pinus patula* (Malabadi and Staden, 2005), *Vitis vinifera* (Das *et al.*, 2010), *Dalbergia sissoo* (Singh and Chand, 2010), *Catharanthus roseus* (Maqsood *et al.*, 2012), and *Rhinacanthus nasutus* (Cheruvathur *et al.*, 2013). It was evident that there were two major hurdles to overcome in the way of producing synthetic seeds using somatic embryos. In the procedure involving

Table 1. Analysis of variance of the effect of 2,4-D, NAA, BAP and kinetin on measured indices in *R. officinalis* tissue culture.

Source of variation	df	Mean of square					
		Callus induction		Percentage of indirect somatic embryogenesis		Days of germination	Percentage of germination
2,4-D	3	2.78**	2,4-D	3	4.03^{ns}	Kinetin	3	326.00**	7.33**
NAA	3	0.74^{ns}	BAP	3	8.64**	BAP	3	6.00**	7.89**
2,4-D×NAA	9	0.45^{ns}	2,4-D×BAP	9	9.23**	Kinetin×BAP	9	8.67**	8.12**
Error	32	0.36	Error	32	9.70	Error	32	0.55	8.55
Coeffcient of variation (%)		9.60	Coeffcient of variation (%)		14.50	Coeffcient of variation (%)		5.93	10.65

^{ns}, *, **: Non-significant and significant at probability levels of 5% and 1%, respectively.

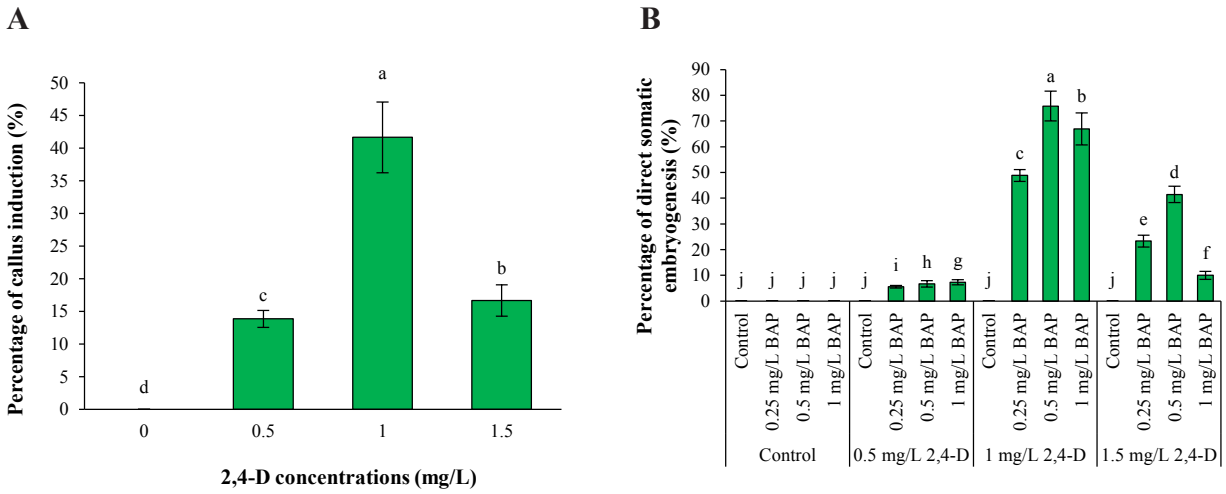

Figure 2. A: Effect of different concentrations of 2,4-D on callus induction and **B:** effect of different concentrations of 2,4-D and BAP on direct somatic embryogenesis. Means followed by different letters in each column are significantly different at P≤0.05.

utilization of Somatic embryo for synthetic seed production, the major impediment is a metachronus and delayed development of the embryonic terminal (Gantait *et al.*, 2015). Even though the encapsulation phase was achieved fruitfully, yet the regeneration of encapsulated somatic embryo was not successful in comparison to the other explants. The regeneration was around 50% in majority of the medicinal plants taken into account. It is significant to mention, there was only 26% regeneration in case of Quercus robur synthetic seeds cultured on the P24 medium fortified with 0.9 µM BA and 0.1 µM IBA (Prewein and Wilhelm, 2003). There was only one instance where synthetic seeds developed from Somatic embryo resulted in 100% regeneration in the MS medium containing 2 µM BA plus 0.5 µM IBA (Cheruvathur *et al.*, 2013). Interestingly, both of these extreme results were achieved in comparable media formulations which explain the species specificity of Somatic embryo in terms of its regeneration potency. Hence, to improve plantlet conversion from synthetic seeds, an adept embryogenic system is indispensable (Gantait *et al.*, 2015).

Germination of synthetic seeds under different concentrations of kinetin and BAP

The MS medium supplemented with various concentrations of kinetin and BAP were used for synthetic seed germination. The ANOVA presented significant differences between different levels of kinetin, BAP, and their interactions (Table 1). Among different concentrations of kinetin, the highest number of days to germination (19 days) and percentage of germination (72.25%) were obtained from the kinetin free medium and the medium containing 1.5 mg/L kinetin. In the case of different levels of BAP, the control had the highest days to germination (13.5 days) and 1 mg/L had the highest percentage of germination (43.07%). Thus, the best condition for germination of synthetic seeds was 1.5 mg/L of kinetin and 0.5 mg/L BAP (80.55%) after four days of cultivation. Furthermore, the medium containing 1.5 mg/L of kinetin in combination with 0.5 mg/L of BAP presented the lowest numbers of days to germination. The highest germination days (20 days) were obtained in the MS medium without plant growth regulators and the MS medium containing 0.25 mg/L BAP (Figure 3). In this study we used 4% w/v sodium alginate and 100 mM $CaCl_2.2H_2O$ for synthetic seed production. Several aspects come into play in moderating the environmental elements of temperature and humidity in order to safeguard the biological substances, and additionally offering a sizeable nutrient pool. Evidently, it can be said that matrix materials play a crucial role in the evaluation of the definitive sustainability of synthetic seeds. Over the times, the conception of matrix materials has evolved into a moderately refined interaction that centers on the transformability of synthetic seeds. It was evident from the researches showed that the majority of the optimum results in terms of nicely formed spherical beads were obtained with 100 mM $CaCl_2.H_2O$ and 3% sodium alginate, for example, in *Vitex negundo* (Ahmad and Anis, 2010), *Simmondsia chinensis* (Kumar *et al.*, 2010), *Cucumis sativus* (Tabassum *et al.*, 2010), *Stevia rebaudiana* (Ali *et al.*, 2012), *Ruta graveolens* (Ahmad *et al.*, 2012), *Ceropegia bulbosa* (Dhir and Shekhawat, 2013), *Rauvolfia tetraphylla* (Faisal *et al.*, 2013), *Begonia semperflorens* (Sakhanokho *et al.*, 2013), *Ochradenus baccatus* (Al-Qurainy *et al.*, 2014), *Anethum graveolens* (Dhir *et al.*, 2014), and *Balanites aegyptiaca* (Varshney

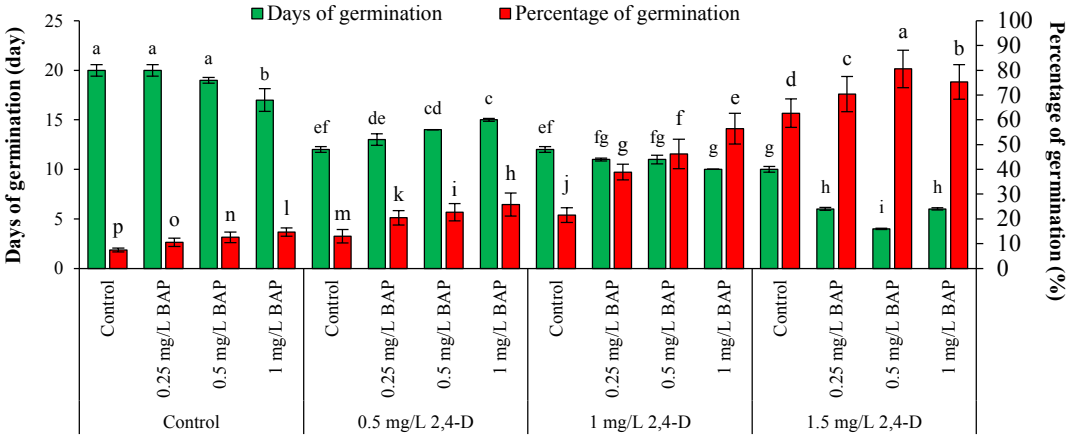

Figure 3. Effect of different concentrations and combinations of kinetin and BAP on days of germination and percentage of germination of synthetic seeds of *R. officinalis*. Means followed by different letters in each column are significantly different at P≤0.05.

and Anis, 2014), and the regeneration of synthetic seeds was around or more than 90% in majority of the instances.

Germination of synthetic seeds under different storage temperatures

Induction, somatic embryogenesis, and produced synthetic seeds are illustrated in Figure 3. The germination percentage of encapsulated somatic embryos decreased significantly with increasing the storage periods and temperatures. However, a more reduction in germination was recorded at 20±2 °C compared to the one recorded at 4±1 °C. The mean number of synthetic seeds that germinated after 15 days at 20±2 °C storage temperature was 40%, whereas the germination percentage was 65% at 4±1 °C. After 30 days of storage at 20±2 °C, 35% of synthetic seeds germinated, while it was 85% at 4±1 °C. No germinated synthetic seeds were observed after 45 and 60 days of storage at 20±2 °C, while it was 70% and 50% at 4±1 °C, respectively (Table 2). Similar results were also reported for other plant species such as *C. ternatea* (Krishna Kumar and Thomas, 2012). Poor viability of stored synthetic seeds may be related to both oxygen deficiency in the gel bead and rapid drying (Redenbaugh *et al.*, 1991). Baskaran *et al.* (2015) reported that germination frequency of synthetic seeds was 51.6% after 50 days of storage at 4 °C.

Table 2. Effect of different temperature and storage intervals on the germination of synthetic seeds of *R. officinalis*.

Storage interval	Mean germination percentage	
	Cold storage (4±1 °C)	Room storage (20±2 °C)
S0 (0 day)	60^{d}	50^{a}
S1 (15 day)	65^{c}	40^{b}
S2 (30 day)	85^{a}	35^{c}
S3 (45 day)	70^{b}	0^{d}
S4 (60 day)	50^{e}	0^{d}

Means followed by different letters in each column are significantly different at P≤0.05.

CONCLUSION

This is the first study on callus induction, somatic embryogenesis, and synthetic seed production in *R. officinalis*. The present investigation demonstrated a method to obtain the artificial seed production in *R. officinalis* using different plant growth regulator. Among the 2,4-D concentrations used for callus induction 1 mg/L was the best concentration. The highest percentage of embryogenesis was observed by using 1 mg/L 2,4-D and 0.5 mg/L BAP. The suitable concentration of kinetin and BAP for germination of synthetic seeds was 1.5 mg/L and 0.5 mg/L, respectively. Also, the highest germination percentage of the synthetic seeds was obtained 30 days after maintaining at 4±1 °C.

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