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IN VITRO REGENERATION PROTOCOL FOR ARTIFICIAL SEED PRODUCTION IN AN IMPORTANT MEDICINAL PLANT *MENTHA ARVENSIS* L

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Abstract

Context: The application of encapsulated shoot tips and nodal segments may contribute to the protection of rare and threatened medicinal plants. Although the artificial seed technique has been reported for more than two decades, for medicinal plants this method has not been developed sufficiently. The main limitations in conventional propagation of some species with medicinal value are: reduced endosperm, low germination rate and seedless varieties. The above mentioned reasons indicate the need for the production of artificial seeds as a technique which combines the advantages of clonal multiplication with those of seed propagation and storage.

Objectives: The objective of the present investigation was to standardize artificial seed production technology taking shoot tip and nodal explants in *Mentha arvensis and* its *in vitro* regeneration

Materials and Methods: Sodium alginate beads were produced by encapsulation of shoot tip and nodal segments of the plant *M. arvensis*. MS medium was used as basal medium with agar and sodium alginate was used as gelling agent accompanied by $CaCl_2$ solution.

Results: Different concentrations and combinations of BAP, Kin and NAA were used in alginate bead in MS basal medium. Among the different concentrations of phytohormone, highest 80% of shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA from nodal segments of *M. arvensis*. Highest average number of shoot 9.87 ± 0.58 formation was obtained in the same medium but highest length of shoot 6.27 ± 0.29 cm was found in the medium having 1.0 mg/l BAP + 0.5 mg/l NAA.

Conclusion: The present investigation clearly established and demonstrated the method of obtaining the artificial seed production in *M. arvensis* supported by different hormone concentrations

Key words: *Mentha arvensis*, sodium alginate, artificial seed and regeneration.

Introduction

Currently used broader definition of artificial seed is 'an artificially encapsulated somatic embryo, shoot or any other meristematic tissue which can develop into a plant under *in vitro* or *in vivo* conditions' (Bapat and Mhatre 2005). Artificial seed production is a potential technique for plant multiplication and preservation, especially as it has been considered to be promising for propagation of no-seed producing plants, transgenic plants and other plants that need to keep superior traits by means of asexual propagation (Saiprasad 2001). Its effect varied with different species, coating materials, maintained solutions and its concentration and condition (Nhut *et al*. 2005). Kamada (1985) presented a general concept of plant artificial seed, in which all kinds of plant explants with germination ability can be used for artificial seed production. Artificial seed production is an outstanding technique used to propagate and preserve plants and has been applied on many plants (Slade *et al*. 1989, Fukai *et al*. 1994, Stephen and Jayabalan 2000, Ipekci and Gozukirmizi 2003, Halmagyi *et al*. 2004, Nhut *et al*. 2005, Wang and Qi 2010).

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Artificial seed would provide an easy and novel propagation system for the elite as well as difficult to root species (Bapat *et al*. 1987, Ballester *et al.* 1997). Also, encapsulation of propagules that were produced *in vitro* could reduce the cost of micropropagation of plantlets for commercialization and final delivery (Chu 1995, Nieves *et al*. 2003). This technology may be of value in breeding programs and allows the propagation of many elite genotypes in a short time (Nieves *et al*. 1998). In some of the horticultural crops, seeds propagation is not successful due to: a. heterozygosity of seeds particularly in cross pollinated crops, b. minute seed size (orchids), c. presence of reduced endosperm, d. some seeds require mycorrhizal fungi association for germination (orchids), e. no seeds are formed. These crop species can be propagated by vegetative means like artificial seeds.

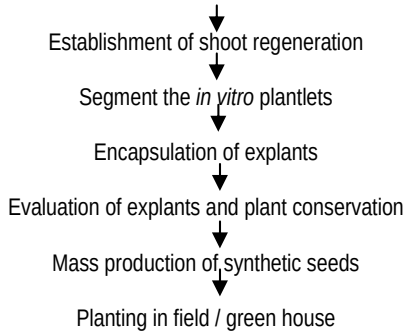
This technology also has been employed for germplasm storage and exchange purposes as reported by Danso and Lloyd (2003). Several researchers suggest that to control growth and facilitate the germination of somatic embryogenesis, the synthetic endosperm can simulate an endosperm of sexual origin, containing one or several compounds such as: nutrients, growth regulators, anti-pathogens, herbicides, biocontrollers and bio-fertilizers, among others, with the aim of ensuring the conversion of the plant and its development in the field (Castillo *et al*. 1998, Kumar *et al*. 2004, Malabadi and Staden 2005).

Mentha arvensis, known as common mint, is a herb with green foliage and white flowers like many other members of this genus, is often used as a domestic herbal remedy, being valued especially for its antiseptic properties and its beneficial effect on the digestion. Leaves are used as a flavouring in salads or cooked foods. An herb tea is made from the fresh or dried leaves. An essential oil from the plant is used as flavouring in sweets and beverages. The leaves contain about 0.2% essential oil. It is a common herb and widely cultivated. The plant was used to produce artificial seed production taking shoot tip and nodal segment as the explants.

Materials and Methods

Shoot tip and nodal segments of *M. arvensis* were used as explants in this investigation for artificial seed production. Shoot tips and nodal segments 3-5 mm long were aseptically excised from *in vitro* cultured plants regenerated by the method described by Maruyama (1996).

Procedure for production of artificial seeds:



Sodium alginate beads were produced by encapsulation according to the method of Kinoshita and Satio (1990). 200 ml ½ strength MS medium was prepared. Sucrose (6.0 gm) was first added to 150 ml of ½ strength MS and then different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg./1) of BAP, Kn, NAA and IAA were added. It was then filled up to 200 ml. Then 0.8 gm of sodium alginate was added to 20 ml of this solution in 50 ml beaker. With a small piece of glass rod efforts were made to mix the alginate in solution, Alginate was partially dissolved and it was then kept aside. During autoclaving alginate was completely

dissolved. $CaCl_2$ (0.7 gm) was dissolved separately in 50 ml of ½ strength MS keeping behind another 130 ml of ½ strength MS to be used during washing the encapsulated beads.

The nodal segments with active buds and shoot tips from *in vitro* grown plants dipped into the beaker containing alginate solution. The dipping explants were taken by a forceps and placed to the beaker of $CaCl_2$ and after 30 minutes each explant became a hardball encoated by alginate.

Results

In vitro grown explants of *M. arvensis* plant were used as the source for providing necessary explants. Nodes and shoot tips were used as explants for artificial seed production. Different concentrations of auxins and cytokinins were used alone or in combinations to investigate the initiation of shoot and its subsequent regeneration. Encapsulated artificial seeds were cultured on to MS agar gelled media supplemented with different concentrations of two cytokinins (BAP and Kn) and one auxins (NAA). It was observed that IBA, 2,4-D alone or in combinations with cytokinin failed to initiate any shoot regeneration. Therefore, IBA and 2,4-D were not used in these experiments. Data on days of germination, percentage of germination, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. The results are presented according to types of explants used under separate heads:

Encapsulated shoot tip explants:

In this present investigation shoot tips of *M. arvensis* were used for artificial seed and their growth performances were evaluated by the application of different hormones. Four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn used alone and in combinations. On the otherhand, four concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) of NAA were treated with two concentrations(1.0 and 2.0 mg/l) of BAP in MS medium for the purpose of multiple shoot induction from encapsulated shoot tip explants of *M. arvensis*. Data were taken after 6 weeks of inoculation and days of germination, percentage of germination, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 1. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot germination started within 6-12 days and it ranged from 20.00-65.00%. Artificial seed with shoot tip exhibited highest percentage (65.00%) of multiple shoot formation observed in MS medium containing 2.0 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 0.2 mg/l NAA. Highest mean number of shoots was 6.20 ± 0.23 in media having 1.0 mg/l BAP + 0.5 mg/l NAA followed by 4.60 ± 0.11 in 1.0 mg/l BAP + 1.0 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 42 days of culture. Highest average length was recorded 6.43 ± 0.08 cm in 1.0 mg/l BAP + 0.2mg/l NAA and the lowest average length was 3.73 ± 0.17 cm in 0.5 mg/l BAP. Experimental results revealed that, 1.0 mg/l of BAP alone and combination of 1.0 mg/l BAP + 0.5 mg/l Kn, 1.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l BAP + 0.5 mg/l NAA were found as most effective media concentrations for multiple shoot induction from artificial seeds in *M. arvensis*.

Encapsulated nodal explants:

Nodal explants of *M. arvensis* were used for artificial seed and their growth performance was also evaluated by the application of different hormones. Four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn used alone and in combinations. Four concentrations (0.1, 0.2, 0.5 and 1.0 mg/l)of NAA were applied with two concentrations (1.0 and 2.0 mg/l) of BAP in MS medium for the purpose of multiple shoot induction from encapsulated nodal explants of *M. arvensis*. Data were taken after 6 weeks of inoculation and days of germination, percentage of germination, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 2. Morphogenic responses of the explants were

found to vary with hormonal formulations present in the culture media. Shoot germination started within 6-12 days and the germination ranged from 25.00-80.00%. In artificial seeds encapsulated with nodal segment, highest 80.00% multiple shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA followed by 75% in MS medium containing 1.0 mg/l BAP + 0.5 mg/l NAA. Highest mean number of shoots was 9.87 ± 0.58 in media having 2.0 mg/l BAP + 0.2 mg/l NAA followed by 7.87 ± 0.33 in 1.0 mg/l BAP + 0.2 mg/l NAA. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 42 days of culture. Highest average length was recorded 6.27 ± 0.29 cm in 1.0 mg/l BAP + 0.5 mg/l NAA. Experimental results revealed that, 1.0 mg/l BAP alone and combination of 2.0 mg/l BAP + 0.2 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l BAP + 0.2 mg/l NAA were found most effective concentrations for multiple shoot induction in *M. arvensis* when nodal explants were used as artificial seeds.

Table 1. Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation in shoot tip explants of *M. arvensis.* Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Days of germination	Percentage of germination	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.5	8-12	20	1.33 ± 0.06	3.73 ± 0.17
1.0	8-12	50	2.20 ± 0.20	4.27 ± 0.17
2.0	8-12	40	4.20 ± 0.30	4.93 ± 0.29
3.0	8-12	30	2.60 ± 0.23	4.47 ± 0.24
BAP + Kn				
1.0 + 0.2	7-10	45	2.60 ± 0.23	5.40 ± 0.11
1.0 + 0.5	7-10	60	2.93 ± 0.06	5.80 ± 0.23
1.0 + 1.0	7-10	52	4.60 ± 0.11	5.53 ± 0.24
1.0 + 2.0	7-10	46	3.93 ± 0.13	5.20 ± 0.11
2.0 + 0.2	7-10	44	2.80 ± 0.11	5.13 ± 0.06
2.0 + 0.5	7-10	65	4.33 ± 0.17	6.13 ± 0.29
2.0 + 1.0	7-10	54	3.60 ± 0.23	5.33 ± 0.06
2.0 + 2.0	7-10	40	2.87 ± 0.17	4.73 ± 0.17
BAP + NAA				
1.0 + 0.1	6-8	50	2.60 ± 0.11	5.33 ± 0.06
1.0 + 0.2	6-8	65	4.27 ± 0.29	6.43 ± 0.08
1.0 + 0.5	6-8	55	6.20 ± 0.23	6.20 ± 0.11
1.0 + 1.0	6-8	45	4.53 ± 0.17	5.67 ± 0.17
2.0 + 0.1	6-8	52	2.87 ± 0.17	4.00 ± 0.11
2.0 + 0.2	6-8	60	4.20 ± 0.11	5.33 ± 0.13
2.0 + 0.5	6-8	55	4.53 ± 0.06	4.53 ± 0.17
2.0 + 1.0	6-8	50	2.67 ± 0.17	4.40 ± 0.11

* Values are the mean of three replicates with 5 explants.

Table 2. Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation in nodal explants of *M. arvensis*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Days of germination	Percentage of germination	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.5	7-10	42	2.33 ± 0.06	4.13 ± 0.17
1.0	7-10	64	5.67 ± 0.29	4.40 ± 0.11
2.0	7-10	56	4.20 ± 0.11	5.07 ± 0.17
3.0	7-10	25	2.33 ± 0.06	4.60 ± 0.11
BAP + Kn				
1.0 + 0.2	7-10	50	4.00 ± 0.11	5.40 ± 0.11
1.0 + 0.5	7-10	65	5.87 ± 0.17	5.67 ± 0.06
1.0 + 1.0	7-10	45	3.40 ± 0.11	5.73 ± 0.06
1.0 + 2.0	7-10	30	2.13 ± 0.17	4.80 ± 0.11
2.0 + 0.2	7-10	55	4.47 ± 0.17	4.93 ± 0.17
2.0 + 0.5	7-10	72	6.47 ± 0.13	6.07 ± 0.17
2.0 + 1.0	7-10	52	4.40 ± 0.11	5.53 ± 0.24
2.0 + 2.0	7-10	36	3.60 ± 0.11	4.60 ± 0.11
BAP + NAA				
1.0 + 0.1	5-8	50	6.00 ± 0.11	5.40 ± 0.11
1.0 + 0.2	5-8	72	7.87 ± 0.33	5.93 ± 0.06
1.0 + 0.5	5-8	75	7.60 ± 0.30	6.27 ± 0.29
1.0 + 1.0	5-8	62	4.47 ± 0.06	5.20 ± 0.30
2.0 + 0.1	5-8	54	4.33 ± 0.29	4.27 ± 0.17
2.0 + 0.2	5-8	80	9.87 ± 0.58	5.27 ± 0.24
2.0 + 0.5	5-8	66	5.47 ± 0.17	4.87 ± 0.17
2.0 + 1.0	5-8	55	3.53 ± 0.17	4.33 ± 0.17

* Values are the mean of three replicates with 5 explants.

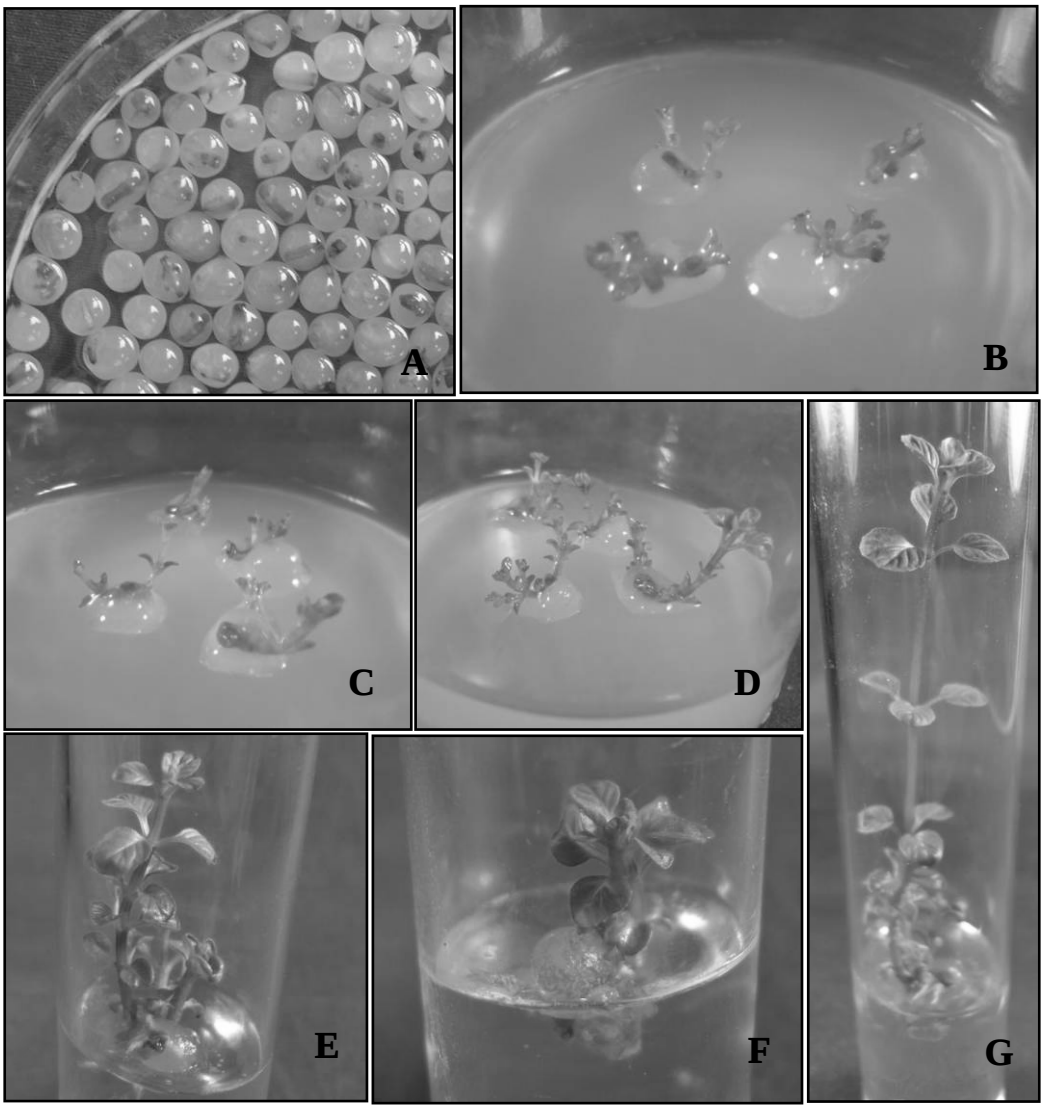


Plate 1. Artificial seeds and different stages of germination

A. Artificial seeds encapsulated by sodium alginate; B. Germinated artificial seeds in 2.0 mg/l BAP+ 0.2 mg/l NAA; C-E. Artificial seed derived plant of nodal explants in 2.0 mg/l BAP + 0.2 mg/l NAA after 2-4 weeks of culture; F. Artificial seed derived plant of shoot tip explants in 2.0 mg/l BAP + 0.2 mg/l NAA after 4 weeks of culture; G. Root induction from germinated artificial seeds.

Survival test of artificial seed in *M. arvensis* under different storage temperatures:

In the present investigation, 50 artificial seeds were kept in storage under growth chamber at 20 ± 2°C, 50 were kept in refrigerator at 4±1°C and 50 were kept in refrigerator at 0°C for survival test. Both shoot tip and nodal encapsulated artificial seeds were used for this survival test. At the end of each storage period,

artificial seeds were immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds were regrown under *in vitro* conditions on nutrient media for shoot development and root induction.

The survival percentage of encapsulated shoot tips and nodal explants decreased significantly with increased storage periods and temperature. However, the reduction in viability was recorded more at 20 ± 2° C in contrast to storage at 4 ± 1° C and O° C. Under the storage of 50 artificial seeds, the mean number of encapsulated shoot tip and nodal explants that survived after 15 days at 20 ± 2°C storage temperatures was 25-45 percent, whereas the survival percentage of encapsulated explants at the storage of 4 ± 1° C was 70-73 percent. After 30 days of storage at 20 ± 2° C, 0-20 percent was found while it was 62-64 percent at 4 ± 1° C. After 45 days of storage at 20 ± 2°C, no survived encapsulated explants was found, while it was 54-55% at 4 ± 1° C. But after 60 days of storage at 20 ± 2°C, no survived encapsulated explants was found, while it was 44-46% at 4 ± 1°C. On the other hand, storage at 0º C temperature no survived encapsulated explants was found. After 60 days and above no survived encapsulated explants were found at 20 ± 2° C. On the other hand, survivability found extended around 44-46 % under storage temperature 4 ± 1°C (Table 3).

Table 3. Effect of preservation on the viability of artificial seed (For each treatment 50 explants were used).

Sl. No.	Storage period (days)	Survival (%) of encapsulated shoot tip explants Storage temperature			Survival (%) of encapsulated nodal explants Storage temperature		
		Storage at 20 ± 2° C	Storage at 4± 1° C	Storage at 0° C	Storage at 20 ± 2°C	Storage at 4± 1° C	Storage at 0° C
1	07	52	80	-	60	80	-
2	15	25	73	-	45	70	-
3	30	-	62	-	20	64	-
4	45	-	54	-	-	55	-
5	60	-	46	-	-	44	-
6	90	-	-	-	-	-	-

Discussion

In some medicinal plant species seed propagation has not been successful. This is mainly due to heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of seed with mycorrhizal fungi association for germination and also in some seedless varieties plants. Some of these species can be propagated by vegetative means. However, *in vivo* vegetative propagation techniques are time consuming and expensive and the propagules carry the diseases and pest from the mother plant to the seedlings. Development of artificial seed producing technology is currently considered as an effective and efficient alternate method of propagation in several commercially important agronomical and horticultural crops. It has been suggested as a powerful tool for mass propagation of elite plant species with high medicinal value.

A number of encapsulating agents have been tried out of which agar, agarose, alginate, carragenan, gelrite and polyacrylamide are important (Kitto *and* Janick 1985). However, it has been suggested that most suitable encapsulating agent is sodium alginate (Bapat *et al*. 1987) due to its solubility at room temperature and its ability to form completely permeable gel with calcium chloride ($CaCl_2 + 2H_2O$). Our findings revealed that this method provided an efficient mechanism for encapsulating the shoot tip and nodal segment in *M. arvensis*.

Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is available in large quantities, is inert, non toxic, cheap and can be easily handled (Endress 1994, Jaiswal *et al*. 2008). However, studies on *in vitro* germplasm conservation using alginate encapsulation techniques have been reported for only a few species. Embryogenic tissue of *Samtalum album* (Bapat and Rao 1988) and axillary buds of *Morus indica* (Bapat *et al*. 1987) have been encapsulated in alginate beads.

In the present investigation *in vitro* shoot tip and nodal segments of *M. arvensis* were encapsulated in sodium alginate prepared using MS basal medium. Different concentrations and combinations of BAP, Kin and NAA were used in alginate bead. These encapsulated synthetic seeds were cultured on MS medium containing same growth regulators. Among the different concentrations of phytohormone, highest 80% of shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA from nodal segments of *M. arvensis.* Highest average number of shoot 9.87 ± 0.58 formation was observed in the same medium and highest length of shoot 6.27 ± 0.29 cm was found in the media having 1.0 mg/l BAP + 0.5 mg/l NAA. Same phytohormone combination was also found best effective in seed bead of Mulberry (Machii 1992) and in *Withania somnifera* (Siddique 2005).

The influence of storage at 20 ± 2°C, 4ºC and 0ºC temperature on germination rate was also examined. This investigation indicates that artificial seed could be stored 20 ±2º C for 15 days and the germination rate was 25% and after 15 days at 20±2º C no response found of the artificial seeds. But at 4±1ºC for 60 days the germination rate was 45% and after 60 days synthetic seed did not give any response being stored at 4±1 ºC. Ipekci and Gozukirmizi (2003) also observed that the encapsulated embryos of *Paulownia elongate* was survived when the synthetic seeds were stored at 4±1ºC for 60 days and the germination rate was 32.40%. This type of result was also supported the result of Alfalfa seeds (Redenbaugh *et al*. 1987), *Asparagus cooperi* (Ghosh and Sen 1994), *Eucalyptus citrisdora* (Muralidharan and Mascarenhas 1995), *Camellia* (Janeiro *et al*. 1995), Mulberry (Machii 1992, Bapat *et al*. 1987). But no result found at 0°C temperature. Our experimental results clearly demonstrated an efficient method for production of artificial seed of an important medicinal plant which can be used as the means for easy delivery of propagules for this medicinal plant. Bur further research is needed to make investigation for developing the methods for long time preservation of these artificial seeds.

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