CATRINA (2007), 2 (2): 189 - 192
© 2007 BY THE EGYPTIAN SOCIETY FOR ENVIRONMENTAL SCIENCES
PROCEEDINGS OF THE SECOND INTERNATIONAL CONFERENCE ON THE ROLE OF GENETICS AND BIOTECHNOLOGY IN CONSERVATION OF NATURAL RESOURCES, ISMAILIA, EGYPT, JULY 9-10, 2007

In vitro Formation of Synthetic Seed from Microshoots of *Begonia* x *hiemalis* Fotch.

Asmah Awal[1], Rosna M. Taha[2] and Nor A. Hasbullah[2]
[1]MARA University of Technology, Negeri Sembilan Branch, 72000 Kuala Pilah, Negeri Sembilan, Malaysia.
[2]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

ABSTRACT

Artificial seed or synthetic seed formation of *Begonia* x *hiemalis* Fotch var. *Schwabenland Red* had been induced from leaf explants *in vitro*. The explants were cultured on (Murashige and Skoog (MS) medium supplemented with combinations of 1.0 mg/l Benzylamino purine (BAP) and 1.0 mg/l Naphthalene acetic acid (NAA). After 8 weeks, regenerated microshoots were excised from leaf cultures and microshoots successfully encapsulated in 3% sodium alginate solution together with MS salt solution and polymerized within 1.0M $CaCl_2.2H_2O$ solution for 30 minutes. The beads produced were with firm coats, round beads and uniform size and in good shape for handling. The results demonstrate that the optimum germination and survival rate of encapsulated matrix was in MS medium containing 3% sodium alginate solution supplemented with 3% sucrose, 1.0 mg/l BAP in combination with 1.0 mg/l NAA. The viability of the encapsulated micro shoots after storage period at 4 °C was also determined. High germination rate (100%) was achieved after 1-3 months storage whereas low germination rate (7-53%) was obtained after 4-6 months storage. The seeds were also successfully germinated in three different germination media including MS basal, garden soil and vermiculite.

Keywords: *Begonia* x *hiemalis* Fotch., encapsulated microshoots, synthetic seeds, tissue culture.

INTRODUCTION

Synthetic seed technology has benefited the mass production of plant propagation system *in vitro*. The production of synthetic seeds in tissue culture system could overcome breeding problems in seedless plants. In order to obtain the exact form of synthetic seed, specific applications might be necessary. Such encapsulation provides protection to the microshoots and allows an advisable handling similar to the natural seeds.

Begonias are normally grown as ornamental plants especially as decorative houseplants and for landscaping. Begonias are unique for their sheer beauty and variety of leaves. Other than great horticultural value, Begonias also has medicinal values. It is estimated that there are about 10,000 Begonias hybrids and cultivars worldwide. One of the most popular hybrids of Begonias is *Begonia* x *hiemalis* Fotch., a temperate plant that is commercially used as flower potting plant and propagated by cuttings due to the unavailability of the seeds.

Generally, synthetic seeds production can be induced using somatic embryos, microshoots, protocorm-like bodies, shoot buds as the encapsulated propagule and most of the studies have been carried out using somatic embryos. To date, few studies have used microshoots or multiple shoots for the production of artificial seeds as reported in pineapple (Soneji *et al*, 2002). In Begonia, although somatic embryogenesis can be directly induced (Castillo and Smith, 1997) but organogenesis process have been largely favoured using explants such as leaf discs (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985), inflorescences (Pierik and Tetteroo, 1987), peduncles, petioles (Ringe and Nitsch, 1968; Cassells and Morrish, 1985) and tubers (Samyn *et al*., 1984). Thus, synthetic seeds production obtained from organogenesis process could also overcome regeneration problem in Begonia.

The main objective of the present study was to develop a procedure for the encapsulation of in vitro-derived microshoots of *Begonia* x *hiemalis* Fotch., which do not form seeds, in alginate for the production of synthetic seeds, as a new propagation method. Five different factors were identified in this study including concentration of sodium alginate, concentration and duration of exposure to $CaCl_2.2H_2O$ solution, presence or absence of hormone in the encapsulation solvent, different types of sowing media and cold storage periods of the artificial seeds.

MATERIALS AND METHODS

Preparation of culture media and microshoots

The explants sources consisting of small pieces of leaves derived from *in vitro* plantlets were established on MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 2.5 g/l gelrite added with 1.0 mg/l BAP in combination with 1.0 mg/l NAA. The pH of the medium employed in the experiment was adjusted to 5.8 sterilization process at 121°C for 21 minutes. Cultures were kept in culture room at 25 ± 1°C, under 16-h light photoperiod of light intensity (1000 lux). Microshoots (approx. 3 mm in length) were excised from cultures after 8 weeks in culture (Fig. 1B). The microshoots were carefully isolated and were blot dried on filter paper. After being encapsulated, these microshoots will be used as artificial seeds.

Formation of beads

The ideal procedure for encapsulation of synthetic seeds were identified by studying the effect of various factors on bead formation which include: (1) concentration of sodium alginate, (2) concentration and duration of exposure to $CaCl_2.2H_2O$ solution,

* *Corresponding author: asmah@nsembilan.uitm.edu.my.*

(3) presence or absence of hormone in the encapsulation solvent, (4) different types of sowing media and (5) storage period of the artificial seeds. For the production of synthetic seed in *Begonia* x *hiemalis* Fotch., microshoots were mixed in the encapsulating matrix which consisted of 2-5% solutions of sodium alginate (Sigma), mixed up in MS basal medium solution (pH 5.8) added with 30mg/l sucrose and 1.0mg/l BAP in combination with 1.0mg/l NAA. Apart from that, the microshoots were also encapsulated with sodium alginate solution devoid of MS basal salts and plant growth regulator. Subsequently, by using sterile micropipette, the microshoots were drawn up with some encapsulation matrix and dropped into the matrix solution ($CaCl_2.2H_2O$ solution). Different concentrations of $CaCl_2.2H2O$ solution (0.1 - 1.0M) were also identified. The seeds were left to harden for a certain period (10-30 min) for complexation. Then, the seeds were washed in MS standard liquid medium avoiding from sticking together and retrieved using nylon mesh. The resulting capsules or beads consisted of one propagule/ bead.

Germination of beads and storage

The beads were germinated on various germination media and substrates, for germination evaluation. Germination media including MS basal media devoid of sucrose (control), MS basal media supplemented with 3% sucrose, garden soil, vermiculite and also sphagnum peat. All the germinating substrates were prepared in the jam jar and autoclaved prior to use. The cold-storage of the beads were also done in the incubator at 4°C from 1-6 months prior to germination process. All the samples were germinated and incubated under the culture room conditions at 25 ± 1°C, under 16-h light photoperiod of light intensity (1000lux). The germination days of the synthetic seeds were recorded manually and germination rate were recorded after 6 weeks of germination.

RESULTS

Effect of alginate matrix on beads formation

Microshoots were successfully encapsulated in 3% sodium alginate. The alginate solution prepared in MS salt solution together with 3% sucrose was left to harden for another 30 min in 1.0M $CaCl_2.2H_2O$, which produced beads with firm coats, round beads and uniform size and in good shape for handling (Fig. 1C). The effect of various concentrations of sodium alginate (2-6%) and calcium chloride (0.25-1.25M) for bead formation from encapsulated microshoots is presented in Table 1. Lower concentrations of sodium alginate (1-2%) formed fragile beads with no definite shapes. At higher concentrations (more than 4%) the beads were isodiametric in shape but too hard. Observations were made after 30 min in $CaCl_2.2H_2O$ solution for hardening process. However, it was found that encapsulated microshoots showed different degree of successes based on ideal beads produced. Further experiments were carried out in order to determine the optimal complexation of encapsulation matrix. In the present study, it was observed that MS medium containing 3% sodium alginate solution supplemented with 3% sucrose, 1.0 mg/l BAP in combination with 1.0 mg/l NAA maintained in 1.0M $CaCl_2.2H_2O$ solution for encapsulation was the optimal concentration to produce artificial seeds from encapsulated microshoots. The beads started to germinate after 7 days in culture with 90.48% germination rate and developed into plantlets 10 days later (Fig. 1D).

Regrowth of beads

The optimum seedling rate of synthetic seeds under sterile conditions was 90.48% (Table 2). To evaluate the germination of beads is to observe the increase in size of explants, with breakage of the capsule and extrusion of the shoot or of a leaf bud. The explants were considered alive if they remained green, with no necrosis or yellowing and continued to enlarge after encapsulation.

Table (1): Effect of different concentrations of sodium alginate and calcium chloride on beads formation. Observations were taken after 30 min of complexation process in $CaCl_2.2H_2O$ solution.

$CaCl_2.2H_2O$ (M)	Sodium alginate concentration (%)				
	2.0	3.0	4.0	5.0	6.0
0.25	+	+ + +	+ + +	+ + +	+ + +
0.50	+	+ + +	+ + +	+ + +	+ + +
0.75	+ +	+ + +	+ + +	+ + +	+ + + +
1.00	+ +	+ + + +	+ + + +	+ + + +	+ + + +
1.25	+ +	+ + + +	+ + + +	+ + + +	+ + + +

\+ Very fragile bead with no definite shape
+ + Fragile beads with no definite shape
+ + + Soft, solid and uniform shape
+ + + + Optimal, firm, uniform and round shape

Table (2): Growth response of microshoots of Begonia encapsulated in different capsule matrix after being transplanted into MS media for 10 and 30 days. Unencapsulated microshoots were used as controls.

Capsule matrix	Germination rate (%) (after 10 days)	Survival rate (%) (after 30 days)
Control	80.00 ± 0.08	80.00 ± 0.08
Ca-free MS + dist. water	80.95 ± 0.06	30.00 ± 1.58
Ca-free MS + 3% sucrose	90.48 ± 1.08	64.00 ± 1.20
Ca-free MS + 3% sucrose + 1mg/l BAP + 1mg/l NAA	90.48 ± 1.08	83.33 ± 0.06

Effect of sowing media on germination

Table 3 demonstrated that most of the beads managed to survive after 8 weeks in culture on MS basal medium (100%) and sterile garden soil (83.33%). The survival rate of seedlings reached 80.0% after they were transferred to pots containing garden soil.

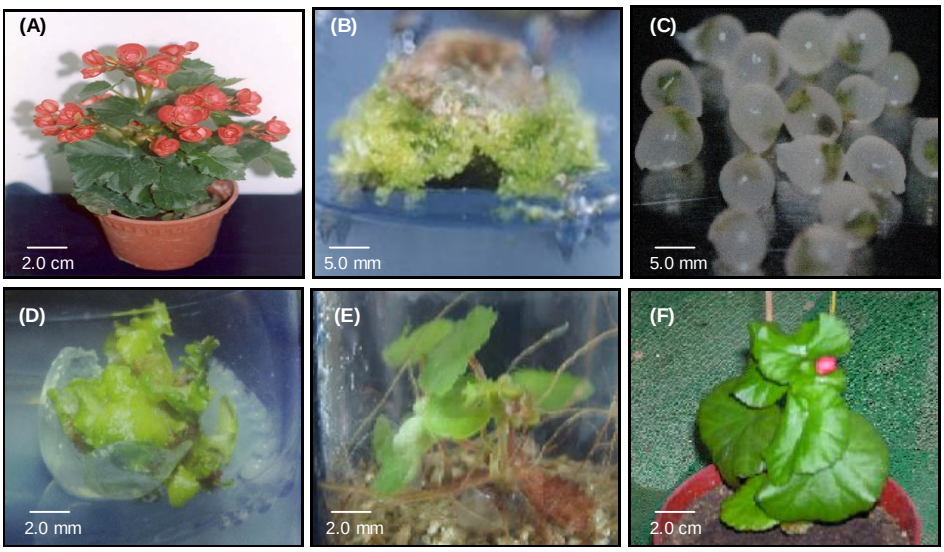

Figure (1): (A) the intact plant of *Begonia* x *hiemalis* Fotch. var. *Schwabenland Red*., **(B)** microshoots derived from leaf explants after 8 weeks in culture, **(C)** microshoots encapsulated in alginate matrix, **(D)** after 7 days, plantlet emerged from artificial seed on germination medium, **(E)** synthetic seeds germinating on vermiculite after 3 months, and **(F)** Plantlet regenerated from artificial seeds produced flower after 6 months transferred into soil.

Storage period effects

Plantlet regeneration from cold-stored synthetic seeds was morphologically similar from regeneration cultures. The results in table 4 showed that 96.67-100% germination rate could be obtained from 0-60 days storage, whereas 90 days storage also showed precocious germination and the seeds germinated at 83.33%. The 120-150 days storage seeds showed 7-54% germination. Most of the artificial seeds developed into normal seedlings. No phenotypic changes were observed from the plantlets and over 90% of the plantlets developed into healthy, field-grown plants with about 5 cm height after 4 months cultured into MS basal medium. After 9 months of acclimatization process, a number of plants produced flower (Fig. 1F).

Table (3): Effect of different sowing media on germination rate of synthetic seeds of Begonia. Unencapsulated microshoots were used as controls.

Sowing medium	Germination rate (%)
Control	80.00 ± 0.08
MS + 3% sucrose	100.00 ± 0.00
Garden soil	83.33 ± 0.06
Vermiculite	56.67 ± 1.20
Sphagnum	36.67 ± 1.18

Table (4): Effect of storage time (days) at 4°C on germination of synthetic seeds on MS basal medium.

Day storage	Germination rate (%)
0	100 ± 0.00
30	96.67 ± 0.03
60	100 ± 0.00
90	83.33 ± 0.06
120	53.33 ± 1.00
150	6.67 ± 0.00
180	0

DISCUSSION

The present study showed that synthetic seed was successfully developed from microshoots *in vitro*. The optimum germination capability of the seeds prior to storage period was also identified. The germination rate of synthetic seed was affected by various factors such as sodium alginate concentrations, different concentrations of $CaCl_2.2H_2O$ solution, different types of sowing substrates and also storage period of the seeds. The highest germination rate of microshoots can be obtained with 3% sodium alginate and maintained in 1.0M $CaCl_2.2H_2O$ for 30 minutes. Previous research showed that different types of encapsulation matrix could be used as coating agents but sodium alginate was as the most popular for encapsulating matrix. Redenbaugh *et al*. (1986) also identified that the production of sodium alginate from different commercial sources may be due to differential purity of alginic acid or the variation in the mannuronic acid: guluronic acid ratio. Alginate was chosen as the encapsulation matrix because of its moderate viscosity, low toxicity, quick gelation and low cost (Onishi *et al*, 1992).

Hardening process of the synthetic seeds carried an important role in the germination rate of the beads. An ideal bead formation was successfully achieved using complexation solution ie. 1.0M $CaCl_2.2H_2O$ solution for 30 minutes. Lisek and Orlikowska (2004) had also identified the optimum bead production of strawberry and raspberry using 3% of sodium alginate and 0.75M

$CaCl_2.2H_2O$. Their finding is similar to the present work except for the complexation solution whereby 1.0M $CaCl_2.2H_2O$ was selected in this study.

Germination and survival rate of the seeds were identified using two different factors including different types of encapsulating matrix and different types of sowing substrates. The four encapsulating matrix tested during the preparative procedures (Table 2) indicated that the presence of hormonal combinations affected the emergence of the microshoots. From the present investigation, it was found that the encapsulated microshoots were successfully sown in MS basal media and sterile garden soil.

Storage of microshoots in alginate beads results in 96.67-100% survival after 60 days storage at 4 °C. The synthetic seeds managed to germinate 53-83% with storage period of 90-120 days although it decreases multiplication of Begonia. Lisek and Orlikowska (2004) had also observed 90-100% survival of synthetic seeds of strawberry and raspberry after 90 days (3 months) in storage at 4°C. The results showed that the synthetic seeds stored at 4°C for 3 months retained their ability to germinate and grow into normal plants.

In conclusion, the synthetic seeds production obtained from encapsulation of microshoots can be used as a potential method to solve problems of propagation for *Begonia* x *hiemalis* Fotch. var. *Schwabenland Red* that have no seed. The results obtained showed that encapsulated explants could be handled like true seeds. Furthermore, plantlets produced from synthetic seeds have been successfully transferred to soil for hardening and managed to survive as any other normally field grown plant. The present work suggested that the production of uniform beads with high frequency of germination would be useful for cloning and mass propagation especially for commercial purposes.

ACKNOWLEDGMENTS

The authors would like to thank the University of Malaya for the vote F grant number F F0150/2004A/01 and the Ministry of Science Technology and Environment of Malaysia for the IRPA grant 09-02-03-1018.

REFERENCES

CASSELLS, A. C. AND F. M. MORRISH. 1985. Growth measurements of *Begonia rex* Putz. plants regenerated from leaf cuttings and *in vitro* from leaf, petioles, axenic leaves, re-cycled axenic leaves and callus. Scientia Hortic., **27**: 113-121.

CASTILLO, B. AND M. A. L. SMITH. 1997. Direct somatic embryogenesis from *Begonia gracillis* explants. Plant Cell Reports, **16**: 385-388.

LISEK, A. AND T. ORLIKOWSKA. 2004. *In vitro* storage of strawberry and raspberry in calcium-alginate beads at 4 °C. Plant Cell, Tissue and Organ Culture, **78**: 167-172.

MURASHIGE, T. AND F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. **51**: 473 – 497.

ONISHI, N., T. MASHIKA AND A. OKAMOTO. 1992. Cultural system producing encapsulatable units of synthetic seeds in celery. Acta Horticulturea, **319**: 113-118.

PIERIK, R. L. M. AND F. A. A. TETTEROO. 1987. Vegetative propagation of *Begonia venosa* Skan. *in vitro* from inflorescence explants. Plant Cell, Tissue and Organ Culture, **10**: 135-142.

REDENBAUGH, K., B. D. PAASCH, J. W. NICHOL, M. E. KOSSLER, P. R. VISS AND K. A. WALKER. 1986. Somatic seeds: encapsulation of asexual plant embryos. Biotechnology, **4**: 797-801.

RINGE, F. AND J. P. NITSCH. 1968. Conditions leading to flower formation on excised *Begonia* fragments cultured *in vitro*. Plant & Cell Physiol., **9**: 639-652.

ROEST, S., M. A. E. VAN BERKEL, G. S. BOKELMANN AND C. BROERTJES. 1981. The use an *in vitro* adventitious bud technique for mutation breeding of *Begonia* x *hiemalis*. Euphytica, **30**: 381-388.

SAMYN , G. L., P. C. DEBERGH, AND D. VERMAERKE. 1984. Field performance and phenotypic stability of virus-free tissue-cultured *Begonia* x *tuberhybrida* 'Multiflora'. Scientia Hortic., **24**:185-191.

SONEJI, J. R., P. S. RAO AND M. MHATRE. 2002. Germination of synthetic seeds of pineapple (Ananas comosus L. Merr.). Plant Cell Reports, **20**: 891-894. (visited Jan 4, 2007) In http://www.lib.um.edu.my.

Received July 9, 2007
Accepted November 20, 2007