ARTIFICIAL SEED PRODUCTION OF LOBELIA CHINENSIS LOUR

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ABSTRACT

An efficient protocol was developed for production of the artificial seed of Lobelia chinensis Lour. In vitro nodal segments of L. chinensis were used as inclusion materials for artificial seeds by entrapping them in various combinations of sodium alginate and calcium nitrate. The artificial seed was produced by mixing 3.5% sodium alginate and 50 mM calcium nitrate, supporting the optimal in vitro conversion potential. The number of shoots produced by encapsulated nodal segments was 3.4 while non-encapsulated nodal segments produced 3.5 shoots per explant. Plantlets regenerated from the artificial seeds of nodal segments were hardened, acclimatized and established well in the field, showing similar morphology with donor plants. This encapsulation technology would provide an easy and novel propagation system for L. chinensis.

INTRODUCTION

Lobelia chinensis, a species from family Lobeliaceae, is commonly known as Chinese lobelia herb. L. chinensis is used to reduce inflammation, contract tissues, clear toxins, and can serve as a respiratory stimulant and antifungal herb (Bown, 2002; Liu and Peng, 1994). This plant has been used for removal of fever and tumors

through detoxification and diuresis. It possesses some unique pharmaceutical efficacies for schistosomiasis or liver cirrhosis (Wang, 2001; Tada et al., 1995).

Synthetic seeds or artificial seeds are defined as artificial encapsulation of somatic embryos, shoot buds, cell aggregates, or any propagules that can be used for sowing as seeds. They can convert into plants under in vitro or ex vitro conditions

and retain this potential even after storage (Hussain et al., 2000; Capuano et al., 1998). Recently, there has been an increasing interest in the production and use of synthetic seed due to the benefits of offering an alternative way for maintenance of elite germplasm and producing virus-free, genetically uniform planting material for easy handling, transportation and storage (Maziah et al., 2006; Nyende et al., 2005, 2003, 2002; Saiprasad and Polisetty, 2003; Ganapathi et al., 2001; Patel et al., 2000; Maruyama et al., 1997; Padmaja et al., 1995; Senaratna et al., 1989).

L. chinensis is not commonly found in the wild. It has a low growth rate and is easily infected by some pathogens. Artificial seed production ais currently considered an effective, alternative method that could scale up the micropropagation rapidly and economically (Wang et al., 2007). This encapsulation technology and technique can serve as a low-cost and high-volume propagation system (Saiprasad and Polisetty, 2003).

To date, there is no report on the production of artificial seeds using in vitro nodal segments of L. chinensis for clonal propagation. The major objectives of this research were a) to investigate the optimal combination of sodium alginate and calcium nitrate for artificial seed production, b) to examine the effect of encapsulation on shoot regeneration, and c) to investigate the survival potential of plantlets produced from artificial seeds in the field.

MATERIALS AND METHODS

Source of plant materials

Nodal segments of in vitro grown L. chinensis were used as inclusion materials for artificial seed production.

Culture conditions

The pH of the medium was adjusted to 5.7-5.8 using 0.01 M NaOH or 0.01 M HCl before autoclaving at 121°C under 1.2 kg cm-2 for fifteen minutes Gelrite 0.25% (w/v) was included as a gelling agent. All cultures were incubated at 25±2°C under a sixteen-hour photoperiod provided by cool-white fluorescent lamps and eight hours of darkness.

Effect of concentrations of sodium alginate and reaction times on physical appearance and germination of artificial seed

Experiments were carried out to determine the effect of sodium alginate concentrations and reaction times on artificial seed production. Gel complexation was done by mixing the nodal segments of L. chinensis measuring 0.3-0.4 cm with 2.0, 2.5, 3.0, 3.5 and 4.0% (w/v) sodium alginate, dropping these into 50.0 mM calcium chloride solution and agitating in orbital shakers for different time intervals, 0, 15 and 30 minutes to obtain uniform beads. The artificial seeds embedded nodal segments and were collected using a sterilized tea strainer and rinsed two to three times in sterile water to remove traces of calcium chloride. The shape, hardness and color of alginate beads were recorded. The artificial seeds were cultured on Murashige and Skoog medium and maintained under culture conditions in order to retrieve complete plantlets. Thirty replicates were used for each treatment and the experiment was repeated three times. The percentage of germination of artificial seeds was recorded.

Effect of concentrations of calcium chloride and reaction times on physical appearance and germination of artificial seed

Experiments were carried out to determine the effect of calcium chloride concentrations and reaction times on artificial seed production. Gel complexation was done by mixing the nodal segments of L. chinensis measuring 0.3-0.4 cm with 3.5 % (w/v) sodium alginate, dropping them into 25.0, 50.0, 75.0 and 100.0 mM calcium chloride solution and agitating in orbital shakers for different time intervals, 0, 15 and 30 minutes, to obtain uniform beads. The artificial seeds embedded nodal segments and were collected using a sterilized tea strainer and rinsed two to three times in sterile water to remove traces of calcium chloride.

Effect of encapsulation on shoot regeneration

Encapsulated and non-encapsulated nodal segments were cultured in solid Murashige and Skoog medium to compare the effect of encapsulation on shoot regeneration. Ten replicates were used for each treatment and the experiment was repeated three times. The percentage germination and number of shoots formed from both encapsulated and non-encapsulated nodal segments were recorded.

Acclimatization and planting plantlets in the field

Acclimatization was carried out to harden in vitro plantlets before transferring to the field. Uniform rooted lobelia plantlets were selected from the glass vessels and transferred to a growth chamber with air temperature of 25±2 °C and a photoperiod of sixteen hours. Inside the growth chamber, each plantlet was immediately transplanted into individual commercial plastic pots containing a steam-sterilized mixture of sand: soil (1:1, v/v). After transplanting, the plantlets were subjected to an acclimatization treatment of seven days of high humidity. By day eight, the vigorously grew and uniformed size lobelia plantlets were transferred to the glasshouse. All pots were fertilized twice a week with 100 mL of Murashige and Skoog nutrient solution. Total survival rate of plantlets were determined on day thirty after transferr to the glasshouse.

Statistical analysis

Analysis was performed using Statistical Analysis System (SAS) (SAS Institute Inc., Cary, NC, 1985). Statistical differences were tested by Duncan's multiple range test for the means. The significance level was established at $p<0.05$.

RESULTS AND DISCUSSION

Effect of concentrations of sodium alginate and reaction times on physical appearance and germination of artificial seed

The shape of artificial seeds was round for 2.0 to 3.5% sodium alginate and oval for 4.0% sodium alginate regardless of reaction times. The artificial seeds without reaction were soft for all the concentrations of sodium alginate tested. The seeds were soft at 2.0 and 2.5% sodium alginate and fifteen-minute reaction; the seeds became elastic from 3.0 to 4.0% sodium alginate. For reaction time of thirty minutes, the seeds were elastic at 2.0 and 2.5% sodium alginate but became firm from 3.0 to 4.0% sodium alginate. The artificial seeds without reaction were transparent for all the concentrations of sodium alginate tested. The color of artificial seeds was white for all the concentration of sodium alginate with reaction time of fifteen and thirty minutes (Table 1, Figure 1).

Table 1. Effect of reaction times and concentrations of sodium alginate on the physical appearance of artificial seeds

Reaction Times (min)	Characteristic of artificial seeds	Concentrations of sodium alginate (%)			
		2.0	2.5	3.0	4.0
0	Shape	round	round	round	oval
	Hardness	soft	soft	soft	soft
	Color	transparent	transparent	transparent	transparent
15	Shape	round	round	round	oval
	Hardness	soft	soft	elastic	elastic
	Color	white	white	white	white
30	Shape	round	round	round	oval
	Hardness	elastic	elastic	firm	firm
	Color	white	white	white	white

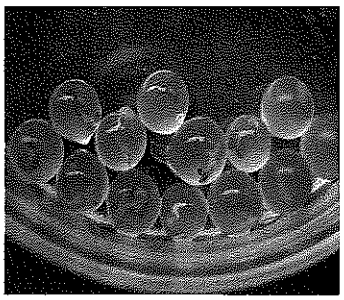

Figure 1. Artificial seeds of L. chinensis

The alginate beads differed morphologically with respect to texture, shape, diameter and transparency, with different combinations of sodium alginate and calcium chloride (Naik and Chand, 2006). Sodium alginate is a copolymer composed of D-mannuronic acid and L-glucuronic acid units which are capable of forming hydrogels in the presence of divalent cations such as Ca2+. The large pore size and rigid structure of these hydrogels are useful for the encapsulation of live cells from plants, because they allow the exchange of substances to and from the surrounding medium. The concentration of polymer, degree of viscosity of the alginate used, concentration of calcium chloride and curing time are important parameters determining the permeability, resistance and hardness of the resulting alginate beads and the subsequent success of the encapsulation method (Block, 2003).

When the artificial seeds of L. chinensis were inoculated onto solid Murashige and Skoog medium, all of them regenerated and produced plantlets (Figure 2 and 3). Likewise, the encapsulated shoot tips of banana regenerated in vitro on different substrates. Use of White medium resulted in 100% conversion of the encapsulated shoot tips into plantlets (Ganapathi et al., 1992). It was reported that about 96% of cultures of Plumbago zerylanica germinated on media containing Murashige and Skoog + 0.1 mg/L indole-3-butyric acid within a week (Rout et al., 2000) while artificial seeds of Gerbera jamesonii exhibited a germination rate of 96.6% (Rahmad, 1999). However, when the artificial seeds of mulberry variety–S54 (Morus indica) were cultured on Linsmaier and Skoog basal medium supplemented with 6-benzylaminopurine (2.0 mg/L) and 2,3,5-triiodobenzoic acid (1.0 mg/L), they produced shoots after twenty-one days exhibiting only 48.2±0.60% in vitro conversion response (Kavyashree et al., 2006). The artificial seeds of L. chinensis started to root on the second day and produced shoots on the third day. Similarly, Nallammai (1997) reported that artificial seeds of Anubia nana started to germinate after three to four days.

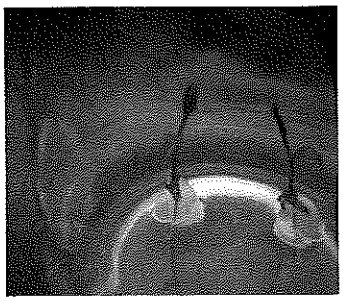

Figure 2. Germination of artificial seeds

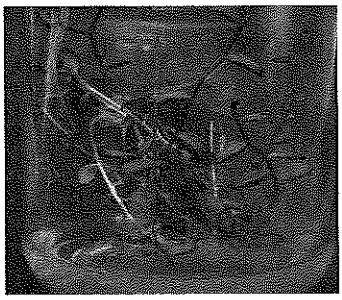

Figure 3. Plantlets produced from artificial seeds after 8 weeks of cultivation

All the concentrations used in this study coated the nodal segments of L. chinensis very well. It was observed that 2.0% and 2.5% alginate beads achieved 100%

germination in the shortest time. All of them germinated within five days and produced 100% plantlets within ten to thirteen days. However, low concentrations of sodium alginate solutions were not suitable because the resultant artificial seeds were too soft to handle. They could be broken easily and were very difficult to transfer to the vessel. This result was in agreement with the finding of Singh et al. (2006) and Nallammai (1997). Production of axillary bud artificial seeds was influenced by the concentrations of the gel matrix and complexing agent (Kavyashree et al., 2006; Pattnaik and Chand, 2000). West et al. (2006) observed that the low concentrations of high viscosity sodium alginate had lower viscosity and coated the nodal segments of Hibiscus moscheutos poorly. Repunte et al. (1995) reported that calcium-alginate beads could not be formed in the case less than 1.5% alginate. At lower concentrations, one to two percent, sodium alginate became unsuitable for encapsulation perhaps because of a reduction in its gelling ability following exposure to a high temperature during autoclaving (Larkin et al., 1988).

In this study, a high concentration of sodium alginate (4%) was not preferred because the artificial seeds produced were too hard and slow down the germination process. The resultant artificial seeds took seven days to achieve 100% germination. Artificial seeds produced by 4.0% sodium alginate and 30 min of reaction time took the longest time, seventeen days, to produce 100% plantlets. Naik and Chand (2006) and Singh et al. (2006) also found that at higher concentrations, the beads were isodiametric but were hard enough to cause considerable delay in sprouting and suggested that this differential response may be due to a synergistic effect of alginate and calcium concentration. Nallammai (1997) reported that the artificial seeds of Anubia nana made of 4.0% sodium alginate only produced root after fifteen days of inoculation. Both the higher and lower concentrations had encapsulation limitations related to the viscosity of the matrix medium. Although there were no differences in shoot or root number or growth, there were differences in handling of the matrix medium (West et al., 2006).

It was observed that polymerization using 3.0 and 3.5% sodium alginate (with reaction duration) were more suitable because the artificial seeds produced were easily germinated and were elastic and firm enough to be handled. The artificial seeds achieved 100% germination between six to seven days. They produced whole plantlet within thirteen days. The middle concentration was determined to be the best because it coated the nodal segments very well and was the easiest to drop into the calcium chloride solution. The resulting encapsulation beads held the nodal segments in place and still provided enough resistance to external mechani-

cal pressure for ease of handling (West et al., 2006). Likewise, the shoot tips of Plumbago zerylanica were encapsulated in 3.5% sodium alginate. About 96% of them germinated within a week. The developing shoots and roots emerged through the alginate matrix and grew into plantlets in two weeks (Rout et al., 2000). Shoot buds of Pogonatherum paniceum (Wang et al., 2007) and shoot tips of banana (Ganapathi et al., 1992) were encapsulated in 3% sodium alginate using different gel matrices. The same finding occurred in the artificial seeds production of Morus indica (Kavyashree et al., 2006), Arnebia euchroma (Manjkhola et al., 2005) and Pinellia ternate (Xue et al., 2004).

In this study, 3-4 mm long nodal segments of L. chinensis were used as plant material for artificial seed production. Ganapathi et al. (1992) reported that 4 mm shoot tips of banana were encapsulated in gel matrices. West et al. (2006) reported larger segments were more difficult to encapsulate because the internode ends tended to protrude out of the encapsulation beads, thus drying out the nodal segments. Furthermore, the smaller-sized nodal segments were easier to obtain from proliferating axillary shoot cultures and encapsulating.

Effect of concentrations of calcium chloride and reaction times on physical appearance and germination of artificial seed

When in vitro nodal segments of L. chinensis were mixed into 3.5% sodium alginate solution and reacted with 25.0, 50.0, 75.0 and 100.0 mM calcium chloride at different reaction times (0, 15, 30 min), artificial seeds with different physical appearances were produced. All of these artificial seeds regenerated and produced shoots when they were inoculated onto hormone-free solid Murashige and Skoog medium.

Encapsulation using 3.5% sodium alginate and 25.0 mM calcium chloride produced artificial seeds that were soft and transparent for all the reaction time tested (Table 2). They were round in shape except for 0 min reaction time where the seeds were broken. They germinated within six days and produced whole plantlet within twelve days. However, they were too soft for handling and transferring. The artificial seeds formed by mixing 3.5% sodium alginate and 50.0 mM calcium chloride were soft at 0 min reaction time, but the hardness of the alginate beads increased with the increasing reaction time. They were elastic at fifteen minutes and firm at thirty minutes. They were all round in shape and the color changed from transparent at 0 min to white at fifteen and thirty minutes reaction times. They germinated

within seven days and formed plantlets within thirteen days. It was noticed that artificial seeds made of 3.5% sodium alginate and 50.0 mM calcium chloride with thirty-minute reaction time stimulated the faster germination and conversion to whole plantlets. Polymerization by reaction of 3.5% sodium alginate and 75.0 mM calcium chloride produced artificial seeds that were all round in shape. They were soft at 0 minutes reaction time, elastic at fifteen minutes and firm at thirty minutes reaction time. Their color changed from transparent at 0 minutes to white at fifteen and thirty minutes reaction times. The artificial seeds germinated within nine days and produced whole plant within eighteen days. The artificial seeds produced using 3.5% sodium alginate and 100.0 mM calcium chloride were soft and transparent at 0 minutes reaction time. However, they became firm and white at fifteen and thirty minute reaction times. They were round in shape regardless of reaction time. They germinated within ten days and produced plantlets within twenty-two days. The results showed that soft-type artificial seeds were not suitable because they could not be handled easily though they germinated faster. In contrast, hard-type alginate beads were not preferred because they slow down the germination process. Pratap (1992) stated that increasing the concentrations of sodium alginate and calcium chloride as well as the reaction times reduced the germination ability of the artificial seeds.

Table 2. Effect of reaction times and concentrations of calcium chloride on the physical appearance of artificial seeds

Reaction times (min)	Characteristic of artificial seeds	Concentrations of calcium chloride (mM)			
		25.0	50.0	75.0	100.0
0	Shape	broken	round	Round	round
	Hardness	soft	soft	Soft	soft
	Color	transparent	transparent	transparent	transparent
15	Shape	round	round	Round	round
	Hardness	soft	elastic	elastic	firm
	Color	transparent	white	white	white
30	Shape	round	round	round	round
	Hardness	soft	firm	firm	firm
	Color	transparent	white	white	white

Different plant species required different concentrations of the gel matrix and complexing agent. Artificial seeds of shoot tips of Ceratopetalum gummiferum

(Shatnawi and Johnson, 2004) and nodal segments of Punica granatum (Naik and Chand, 2006) were produced by mixing 3% sodium alginate solution and 100 mM calcium chloride for thirty minutes. Nodal segments of Hibiscus moscheutos were coated with 2.75% high-viscosity sodium alginate and 50 μM calcium chloride solution for thirty minutes (Preece and West, 2006). In addition, somatic embryos of Rotula aquatica (Chithra et al., 2005) and Daucus carota (Liu et al., 1992) were encapsulated by 3% alginic acid and 50 mM calcium chloride for thirty minutes and twenty to forty minutes, respectively. It was reported that the best gel composition of encapsulation of Withania somnifera (Singh

et al., 2006) and Dendrobium 'Sonia' (Saiprasad and Polisetty, 2003) were achieved using 3% sodium alginate and 75mM CaCl2.

Effect of encapsulation on shoot regeneration

When encapsulated and non-encapsulated nodal segments were inoculated in Murashige and Skoog medium, all of them germinated and produced green and normal plantlets. This phenomenon indicated that trimming and encapsulation had no effect on viability of nodal segments and that they still maintained their regenerative characteristics. This agreed with Nyende and colleagues' (2003) finding.

Encapsulated nodal segments produced 3.4 shoots per explant while non-encapsulated nodal segments formed 3.5 shoots. Data analysis showed that the differences between the means were not significant. Similarly, encapsulation did not affect the survival of shoot tips of Ceratopetalum gummiferum, since 100% regrowth was obtained for both encapsulated and non-encapsulated control shoot tips (Shatnawi and Johnson, 2004). It was reported that 81% encapsulated axenic nodal segments of Punica granatum germinated in Murashige and Skoog

medium with the best shoot development achieved two to three shoots/nodal segment in forty days, whereas 89% non-encapsulated axenic nodal segments of Punica granatum sprouted in Murashige and Skoog (Naik and Chand, 2006).

Encapsulation could affect the regeneration of moss gametophytes, as in Bryum rubens (Burch 2003). It was reported that frequency of plantlet development of Armoracia rusticana from encapsulated propagules was only 10% at fifteen days culture and 58% at forty-five days. On the other hand, non-encapsulated plantlets developed into healthy plants at higher frequency, 92% at thirty days. These results indicated that encapsulation resulted in a decrease in plantlet development frequency (Nakashimada et al., 1995). However, in Splachnum ampullaceum neither protonematal diameter nor number of buds was affected (Mallón et al., 2007).

Acclimatization and plantin
g plantlets in the field

After acclimatization, plantlets transferred to glasshouse exhibited a 100% survival rate. They grew well and showed no difference in morphological characters with their parent plants. They developed new leaves and flowered normally when they achieved maturity.

CONCLUSION

The artificial seeds of L. chinensis using in vitro nodal segments were produced by a combination of 3.5% (w/v) sodium alginate and 50.0 mM calcium chloride solution. The alginate beads showed 100% germination in Murashige and Skoog medium. This study suggested that the production of artificial seed would serve as an alternative propagation system for L. chinensis. In conclusion, a simple and efficient protocol has been developed for artificial seed production of L. chinensis.

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