PLANTLETS FROM ENCAPSULATED SHOOT BUDS OF *CATALPA OVATA* G. DON

HALINA WYSOKIŃSKA, KATARZYNA LISOWSKA,
KATARZYNA FLORYANOWICZ-CZEKALSKA

Department of Biology and Pharmaceutical Botany, Medical University
Muszyńskiego 1, 90-151 Łódź, Poland

(Received: September 21, 2001. Accepted: April 18, 2002)

ABSTRACT

Shoot buds isolated from in vitro shoot cultures of *Catalpa ovata* G. Don were encapsulated using 3% sodium alginate with sucrose (3%) and 50 mM calcium chloride. The morphogenic response of encapsulated buds was affected by such factors, like composition of the media and the presence of growth regulators. The highest frequency of plantlet germination from encapsulated buds (70% within 4 weeks) was obtained on Woody Plant medium (WP) (Lloyd and McCown 1980) containing indole-3-butyric acid (IBA) (1 mg/l). The process was substantially inhibited by cold-storage (4°C) of encapsulated buds. In this case, the frequency response ranged from 3% to 22% dependent on storage period (28 or 42 days) and the presence of the paraffin coat covering the alginate capsules. The plantlets developed from both unstored and stored encapsulated buds of *C. ovata* were transplanted to soil and grew in pots to phenotypically normal plants.

Key words: *Catalpa ovata*, encapsulation, cold storage, paraffin coat.

INTRODUCTION

Catalpa ovata G. Don (Bignoniaceae) is a woody plant distributed in East Asia. *Catalpa* trees are cultivated in many countries for their ornamental and medicinal importance. Their medicinal value includes treatment for asthma and cough, as well as diuretic and wound healing properties (Koriyone and Kimura 1962).

Catalpa is mainly propagated by seeds and rarely by root cuttings (Hryniewicz-Sudnik et al. 1987). The plants produce viable seeds when they are at least thirty years old; the seeds remain viable usually for a year (Bärtels 1982). To overcome these problems micropropagation of *C. ovata* using shoot tips and nodal segments have been established (Lisowska and Wysokińska 2000).

Synthetic seed technology (a concept first put forward by Murashige in 1978) has recently received considerable attention as a potentially cost-effective clonal propagation system. The technology could be also used for storage and transfer of sterile germplasm. Plants from synthetic seeds in vegetable and ornamental crops, conifers, cereals and other value plants, which are difficult to propagate by other methods, have been reported (Redenbaugh et al. 1991; Redenbaugh 1993). In most cases synthetic seeds are made by encapsulating somatic embryos. However, this technique is not appropriate for use with *C. ovata* because somatic embryogenesis has not yet been achieved for the plant. Therefore, there is a need of using non-embryogenic, vegetative parts of plants, like shoot tips and axillary buds for enca-

psulation. Production of whole plantlets from encapsulated shoot buds has been previously reported with various frequency for several herbaceous (Mathur et al. 1989; Ganapathi et al. 1992; Sharma et al. 1994), as well as woody plant species (Bapat et al. 1987; Maruyama et al. 1997; Pattnaik and Chand 2000; Piccioni and Standardi 1995). In this work we present the encapsulation of shoot buds of *C. ovata* and the formation of plantlets from the alginated buds on different nutrient media. In addition, the effect of storage period (28 or 42 days at 4°C), and the presence of paraffin coating over the alginate surface on development of plantlets from encapsulated buds of *C. ovata* was also investigated.

MATERIALS AND METHODS

Plant material

Shoot buds (0.3-0.5 cm long) of *Catalpa ovata* excised from seedlings were transferred to Schenk and Hildebrandt (1972) agar medium (SH) supplemented with indole-3-acetic acid (IAA) (0.1 mg/l) and 6-benzyladenine (BA) (1 mg/l) (Lisowska and Wysokińska 2000). From callus tissue, which formed at the basis of explants, adventitious shoots were regenerated. The 4-week old buds (0.3 cm long) excised from the shoots were used as the experimental material. The cultures were maintained at 26°C under continuous fluorescent light (40 $\mu\text{m} \times \text{m}^{-2} \times \text{s}^{-1}$).

Fig. 1. Shoot development from encapsulated buds of *Catalpa ovata* on various agar media: Murashige and Skoog (MS), Schenk and Hildebrandt (SH) and Woody Plant (WP) without growth regulators.

Encapsulation procedure

Shoot buds were mixed with 3% sodium alginate (Sigma) solution (prepared in distilled water supplemented with 3% sucrose) and dropped individually into 50 mM complexing solution of calcium chloride in flask placed on a magnetic stirrer. Both alginate gel matrix and the complexing agent were sterilized by autoclaving at 121°C for 20 min. Calcium alginate capsules containing one bud were left in the calcium chloride solution for 30 min. Then they were collected and rinsed three times for 15 min in sterile water to remove traces of calcium chloride. The diameter of capsules ranged between 0.5-0.6 cm (Fig. 3A).

Culture media

The resulting encapsulated shoot buds (Fig. 3A) were placed on 0.7% agar-solidified SH (Schenk and Hildebrandt 1972), WP (Lloyd and McCown 1980) and MS

Fig. 2. Rooting of shoots developing from encapsulated buds of *Catalpa ovata* on various agar media: Murashige and Skoog (MS), Schenk and Hildebrandt (SH) and Woody Plant (WP), supplemented with auxin IAA 0.1 mg/l (hatched bars) or IBA 1 mg/l (white bars).

(Murashige and Skoog 1962) media without growth regulators or containing auxin (0.1 mg/l IAA or 1 mg/l IBA). In some experiments, SH medium supplemented with 0.1 mg/l IAA and 1 mg/l BA was also used for shoot proliferation. For each treatment 20-30 encapsulated buds were tested and all the experiments were conducted at 26°C under fluorescent light ($40 \mu\text{m} \times \text{m}^{-2} \times \text{s}^{-1}$). Data on the percentage of shoot development from encapsulated buds and rooting shoots on various media were recorded after 7, 14, 21 and 28 days.

Storage experiments

In the experiments *C. ovata* buds encapsulated in alginate and covered with paraffin coats over the alginate gel surface were used. The procedure for coating the alginate capsules with paraffin was followed as described by Repunte et al. (1995). The alginate capsules (prepared like described in encapsulation procedure) were dropped into melted paraffin in a water bath kept at 52°C. They were picked up immediately after the formation of paraffin coats, followed by solidification of the coats at room temperature. All the operations were made under sterile conditions. The capsules with and without paraffin coats were placed in petri-dishes. The petri-dishes were sealed with Parafilm and kept in a refrigerator at 4°C in the dark for 28 and 42 days. After

Fig. 3. Encapsulated buds of *Catalpa ovata*: A – in calcium alginate; B – shoot development from encapsulated bud on SH agar medium without growth regulators (8 days); C – capsules with paraffin coats; bar = 1 cm.

the storage period the capsules were transferred to WP agar medium containing IBA (1 mg/l) and were incubated for 30 days. The incubation conditions were the same, as those used for unstored encapsulated buds.

The following data were recorded after 10, 20 and 30 days in culture: the plantlet formation frequency (defined as the percentage of buds developed both shoots and roots with respect to the total number of encapsulated buds); the mean of shoot length (data on average shoot length was calculated from only these encapsulated buds which formed complete plantlets).

Transfer of plantlets to soil

Plantlets having a well developed root system (Fig. 4A) were washed under running tap-water to remove agar from the roots and were transferred into pots (10 cm diameter) in sterilized mixture of soil, sand and peat (4:4:3 v/v). Plantlets were covered with glasses to maintain a high humidity and were kept at 26°C under continuous fluorescent light ($40 \mu\text{M} \times \text{m}^{-2} \times \text{s}^{-1}$). The glasses were opened after 7 days and removed after 14 days.

Fig. 4. Plantlet developed from encapsulated buds: A – after 28 days on WP medium containing IBA (1 mg/l); B – after 28 days in the soil; bar = 1 cm.

RESULTS AND DISCUSSION

Shoot and plantlet development from encapsulated buds

Encapsulation of *C. ovata* shoot buds was carried out in 3% sodium alginate and in the complexing 50 mM of calcium chloride solution (droplet hardening method). In order to protect the shoot buds against dehydration, sucrose (3%) as osmotic agent was incorporated into the alginate gel matrix. Encapsulated buds were placed on three agar-basal media (MS, SH and WP) for “germination”, i.e. emergence of shoots and roots. There were no differences among media tested with respect to shoot development from encapsulated buds. The percentage of buds forming well-developed shoots (about 1 cm long) was 75, 80 and 81.3% on growth regulator-free MS, WP and SH media, respectively, within 4 weeks (Fig. 1). The shoots emerged from buds during the first week of culture (Fig. 3B) and the process was continued up to 28 day, but only 10% of the shoots produced roots. In order to stimulate roots from encapsulated buds the auxin (such as IAA 0.1 mg/l or IBA 1 mg/l) was added to WP, MS or SH agar media. Among the media tested the best response was achieved on WP me-

dium followed by SH and MS (Fig. 2). Since IBA (1 mg/l) gave higher response (70% of rooted shoots) as compared to those rooted on the medium supplemented with IAA (57%), subsequent experiments on stored-encapsulated buds were then restricted on WP medium with addition of IBA (1 mg/l). On the basis of these studies we drew the conclusion that the presence of auxin in the medium is necessary to develop balanced root and shoot system; only in the presence of the phytohormone regeneration of complete plantlets from encapsulated buds in one-step procedure could be achieved. Under these conditions, however, only single shoot emerged from the encapsulated bud. Our earlier observations (Lisowska and Wysokińska 2000) had shown that multiple shoot formation could be effectively induced from shoot tips of *C. ovata* on SH or WP agar media supplemented with BA alone or in combination with IAA. Encapsulated buds of *C. ovata* placed on SH medium supplemented with IAA (0.1 mg/l) and BA (1 mg/l) produced 2-3 shoots per encapsulated bud within 4 weeks of culture on the medium. Average shoot length was 0.75 cm and small callus was produced at the shoot base. These shoots were rooted on SH medium with no growth regulators or containing only auxin (IBA 1 mg/l) with the frequency 22% and 70%, respectively.

Encapsulated buds of *C. ovata* usually formed shoots and roots between 14 and 21 days of culture on media tested (MS, SH or WP) supplemented with auxin (Fig. 2). During the next week mainly shoot elongation was observed. After 4 weeks average shoot length ranged from 0.8 cm (SH medium with IBA 1 mg/l) (Fig. 4A) to 2.8 cm (WP medium with IAA 0.1 mg/l).

Low temperature storage

The frequency of plantlet formation from encapsulated shoot buds of *C. ovata* stored at low (4°C) temperature was also investigated. After 28 or 42 days encapsulated buds were transferred to WP medium supplemented with 1 mg/l IBA. It was found that 46% of encapsulated buds developed shoots on the medium following 28-days of storage. However, 50% of them were classified as "short" (i.e. small buds mostly less than 0.5 cm in length) and did not form roots within 30-days culture. Only 23% of encapsulated buds stored for 28 days were capable of developing into plantlets (Table 1). The percentage was 3-fold lower than that obtained with unstored-encapsulated buds of *C. ovata* cultured on the same medium (Fig. 2). Increasing pe-

riod of storage resulted in further decrease in the ability of encapsulated buds to plantlet development. Only 4% of encapsulated buds stored for 42 days and then placed on WP basal medium with IBA (1 mg/l) formed complete plantlets (Table 1). It has been also demonstrated by other authors that storage at low temperature significantly reduced the viability and plant recovery frequencies of alginate-encapsulated embryos of many plant species, such as *Camellia japonica* (Janeiro et al. 1997), *Asparagus cooperi* baker (Ghosh and Sen 1994) and *Santalum album* (Bapat and Rao 1988). Pattnaik and Chand (2000), working with encapsulated shoot buds of six different *Morus* species, noted that 8-22% of them developed shoots following storage at 4°C for 60 or 90 days. However, there is no information about the root development from these shoots. Low frequency of plantlet formation of long-term stored encapsulated buds or somatic embryos may be attributed to: (1) lack of the sufficient nutrients to sustain growth; (2) inhibition of respiration in plant tissue due to anaerobic environmental inside alginate bead; (3) rapid desiccation of the alginate beads. According to Fujii et al. (1987) using a hydrophobic thin coat over an alginate gel surface can overcome the last of above mentioned problems. The coat can also act as a shield against microbial and chemical contamination (Repunte et al. 1995). As it has been previously described (Repunte et al. 1996), among the three examined coating materials (Elvax 4260, polyorganosiloxane and paraffin), paraffin was the best for the maintenance of a stable environment during storage of alginate beads. Repunte et al. (1995) reported that coating with paraffin was effective for the long-term storage of alginate capsules containing cell aggregates from horseradish hairy root culture. We studied the effect of paraffin coats on the plantlet development from encapsulated buds of *C. ovata* (Fig. 3C). It was found that 21% of buds stored for 28 days developed into plantlets when the paraffin-coated capsules were used. The value was almost identical as that obtained with capsules kept without the coats (23%) (Table 1). When the encapsulated buds covered with paraffin coats were stored at 4°C for 42 days, their capacity to plantlet development was reduced to 11%; nevertheless it was 3 times higher than that obtained with encapsulated buds without the paraffin coating (Table 1).

Studies with encapsulated buds of *C. ovata* indicate that the time required for the emergence of shoots and roots was dependent both on the storage period and the presence of the paraffin coats on the alginate surface. Encapsulated buds with paraffin coats after 28 days of storage initiated shoots and roots within the first ten days of culture, whereas in the absence of coats the process was delayed and started between 20 and 30 day of culture. Similar results were also obtained with encapsulated buds after 42 days of storage, although in this case the response time was longer and encapsulated buds did not show any sign of "germination" during the first ten days of culture even when alginate-paraffin matrix was used (Table 1).

It was found that the presence of paraffin coats did not influence growth of plantlets in terms of shoot elongation. The parameter was slightly affected by cold storage period. After 28-days storage in both types of encapsulated buds (with and without paraffin coats) average shoot length was 2.5 cm within 30 days of culture; those after 42 days of storage developed shoots which grew to a height of 1.8-2 cm within the same period.

TABLE 1. Effect of paraffin coat on plantlet development (%) from encapsulated in calcium alginate buds of *C. ovata* stored at 4°C for 28 (A) and 42 (B) days.

All encapsulated shoot buds after storage period were cultivated on WP agar medium with IBA 1 mg/l, in the light.

For each treatment 26-30 shoot buds were taken.

Values in parentheses present average length of shoots developing from encapsulated buds (cm).

Days of culture	Alginate encapsulated buds (%)			
	without the paraffin coat		with the paraffin coat	
	A	B	A	B
10	0	0	6 (0.7)	0
20	0	0	11 (2.2)	7 (1.4)
30	23 (2.5)	4 (1.8)	21 (2.5)	11 (2.0)

The present report provides a procedure for the using of micropropagated shoot buds of *C. ovata* for encapsulation and the production of synthetic seeds. The selection of appropriate nutrient medium supplemented with growth regulators, such as WP medium containing IBA (1 mg/l), evidently increased (up to 70%) plantlet formation frequency from encapsulated buds. It should also be noted that on the medium the alginate-encapsulated buds of *C. ovata* did not loose the capacity for plantlet development, even after storage at 4°C for 42 days. In this case, however, the capacity of plantlet formation was substantially reduced, in spite of the application of paraffin coatings on the alginate gel surface, which could guarantee a suitable water in the gel for shoot-buds during storage. More work is needed to optimize the nutritional requirements in the culture medium and in the beads for improvement the frequency of plantlet development after storage of encapsulated buds of *C. ovata*. Importantly, *C. ovata* plants from both unstored- and stored-encapsulated buds can be successfully transferred into soil, where they quickly adopted and developed into phenotypically normal plants (Fig. 4B). It opens the opportunity to use synthetic seeds of *C. ovata* containing micropropagated buds for clonal propagation of the important trees.

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ROŚLINY Z KAPSUŁKOWANYCH PĄKÓW *CATALPA OVATA* G. DON

STRESZCZENIE

Pąki przybyszowe, z wyhodowanych aseptycznie, 4-tygodniowych pędów *Catalpa ovata*, kapsułowano z użyciem 3% roztworu alginianu sodu z dodatkiem 3% sacharozy. Substancją utwardzającą był 50 mM roztwór CaCl_2 . Odpowiedź morfogenetyczna kapsułowanych pąków zależna była od składu podłoża i obecności regulatorów wzrostu. Najkorzystniejsze do otrzymywania roślin ze „sztucznych nasion” okazało się podłoże WP zawierające IBA (1 mg/l). W tych warunkach w ciągu 4 tygodni 70% kapsułowanych pąków tworzyło pędy i korzenie. Przechowywanie alginianowych kapsułek w niskiej temperaturze (4°C) zmniejszało ich zdolność do wzrostu i w tych warunkach wynosiła ona od 3 do 22% zależnie od okresu przechowywania (28 lub 42 dni) oraz obecności otoczki parafinowej na powierzchni alginianowych kapsułek. Rośliny wyhodowane ze „sztucznych nasion” były zdane do dalszego rozwoju w doniczkach ze sterylną ziemią.

SŁOWA KLUCZOWE: *Catalpa ovata*, kapsułkowanie pąków, przechowywanie w niskiej temperaturze, otoczka parafinowa.