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Original Article

Encapsulation of Axillary Buds of *Gmelina arborea* Roxb. and *Peronema canescens* Jack.

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1. Introduction

Synthetic seeds or artificial seeds made by encapsulating somatic embryos, shoot buds, or any other meristematic tissue help minimize the cost of micro-propagated plantlets for commercialization (MANOJ *et al*., 2009). A synthetic seed is a functional mimic seed that has the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that can retain this potential even after storage (CAPUANO *et al*., 1998; ARA *et al*., 2000). Synthetic seeds have multiple advantages for propagation by organogenesis, including ease of handling, potential long-term storage, higher scale-up, and low production costs (REDENBAUGH *et al*., 1987).

A synthetic seed is surrounded by a protective coating, which provides necessary protection during storage, handling, and mechanical planting. The coating may also contain nutrients to provide the energy required for germination, which is normally supplied by the endosperm. Plant growth regulators may also be included to aid development during germination (GUPTA and KREITINGER, 1993). The synthetic seed technique is mostly used for mass propagation of somatic embryos (ONISHI *et al*., 1994). However, micro-propagated buds offer an interesting alternative (BAPAT *et al*., 1987; KINOSHITA and SAITO, 1990; PICCIONI and STANDARDI, 1995).

The first synthetic seed was reported by KITTO and JANICK (1985), who successfully germinated carrot somatic embryos coated in a polyoxyethylene water disc. Synthetic seed research has since been extended to forest tree species, including *Santalum album* (BAPAT and RAO, 1988), *Betula platyphylla* var. *japonica* (KINOSHITA and SAITO, 1990), *Picea glauca* (ATTREE *et al*., 1994), *Paraserianthes falcataria* (ISHII *et al*., 1994), *Betula pendula* (PICCIONI and STANDARDI, 1995), *Quercus robur* (PREWEIN and WILHELM, 2003), and *Dalbergia sissoo* (CHANDA and SHING, 2004). These studies described the effect of medium, growth regulators, and sucrose on plantlet regeneration from the encapsulated tissue using systematic screening.

Gmelina arborea and *Peronema canescens* (both Verbenaceae) are suitable tree species for industrial plantations in some tropical areas because of their fast growth and multiple uses. *In vitro* clonal propagation of these species has been successfully developed for the rapid circulation of selected plus trees (SUKARTININGSIH *et al*., 1999; SUKARTININGSIH, 1999). However, a large space is required for mass propagation of plantlets, and frequent subculturing is necessary to maintain cultures. Therefore, the *in vitro* propagation system must be improved. Many tropical

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trees require comparatively high temperatures for survival (BAPAT and RAO, 1988), although sandalwood (*S. album*) somatic embryos encapsulated in sodium alginate gel have been successfully preserved at 4 ° C. Thus, we may be able to apply the synthetic seed system to *G. arborea* and *P. canescens* not only for mass propagation but also for *in vitro* preservation.

Here, we describe an effective encapsulation method for axillary buds of these two species to regenerate complete plantlets and the preservation of the synthetic seeds under various temperatures.

2. Materials and Methods

1. Encapsulation of axillary buds and plant regeneration from synthetic seeds

Encapsulation was conducted based on methods describe by KINOSHITA and SAITO (1990) with some modification. *G. arborea* and *P. canescens* plantlets, originally established *in vitro* by the methods described by Sukartiningsih *et al*. (1999) and Sukartiningsih (1999), were used as materials after a 6-week subculture. Axillary buds with 3-mm-long stem nodal regions were excised from the plantlets and then immersed in sodium alginate (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) solution. The solution used here was MS medium (MURASHIGE and SKOOG, 1962) supplemented with plant growth regulators (0.22 mg/l 6-benzylaminopurine, 0.02 mg/l α-naphthylacetic acid, and 1.00 mg/l 3-indolebuthylic acid, which we refer to as PGRs hereafter), different concentrations of sucrose (3, 6, 12, 24, or 36%), and 4% (w/v) sodium alginate. The explants were picked from the sodium alginate solution with a pair of forceps and dropped into a solution of $CaCl_2 \cdot 2H_2O$ (14 g/l) where they were kept for 30 minutes. The coated explants (or synthetic seeds hereafter) were then picked up with forceps and washed three times with sterile deionized water. All solutions used here were previously autoclaved at 121° C at 2 atm for 20 minutes. The procedures were performed under aseptic conditions at a clean bench.

Five types of 1% (w/v) agar medium, which contained only agar, 3% sucrose, PGRs, basal components of MS medium without sucrose, and MS medium containing PGRs and 3% sucrose were prepared for the synthetic seed shoot elongation bed. Ten synthetic seeds were laid aseptically in 40 mm ϕ x 120 mm culture tubes containing about 25 ml of agar medium and kept in an incubator at 25° C under fluorescent light at 5,000 lux with a 16 h photoperiod. The number of shoot-sprouting synthetic seeds was counted, and the shoot elongation and root formation trend was recorded after 6 weeks of culture. Twenty synthetic seeds (two culture tubes) were prepared for each treatment combination.

Elongated shoots with or without roots were subsequently transplanted onto sterilized vermiculite in an "Agripot" (polycarbonate pot 80 mm ϕ x 115 mm, Asahi Glass Co., Tokyo, Japan) and cultured at 25° C under fluorescent light at 5,000 lux with a 16 h photoperiod for 1 month.

2. Preservation of synthetic seeds

Synthetic seeds coated with the calcium alginate gel containing MS medium, PGRs, and 6% sucrose were packed in a Petri dish, covered with aluminum foil, and stored at different temperatures (4, 10, 15, 20, or 25° C) and different durations (1, 2, 3, or 4 weeks) in incubators under a dark condition. They were subsequently cultured in about 25 ml of MS 1% agar medium containing 3% sucrose and PGRs in 40 mmϕ x 120 mm culture tubes at 25° C under fluorescent light at 5,000 lux with a 16 h photoperiod for 6 weeks. Twenty synthetic seeds were used for each treatment combination.

3. Results

1. Encapsulation of axillary buds and plant regeneration from synthetic seeds

The encapsulation procedure resulted in round-shaped capsules of calcium alginate gel covering the explants perfectly. Capsule size depended on explant size. Table 1 lists the proportions of synthetic *G. arborea* and *P. canescens* seeds from which axillary shoot sprouting were observed.

Gmelina arborea: The first shoot sprouts from the axillary buds of encapsulated explants were observed on MS medium containing PGRs and 3% sucrose after 5 days of culture. All shoots sprouted on this medium after 6 weeks of culture irrespective of the sucrose concentration in the encapsulation medium. High (80 − 100%) shoot sprouting was also achieved on the simple agar medium (Fig. 1, left). The proportion of shoot sprouting was lowest on the shoot elongation medium containing 3% sucrose only. All shoots sprouted on specific combinations of the encapsulation medium. Although callus formation at the base of shoot-sprouted explants was prominent, some explants developed roots within 6 weeks of culture in several combinations of encapsulation medium and the shoot elongation bed. Shoot elongation from synthetic seeds encapsulated with higher sucrose concentration (24 and 36%) medium deteriorated in comparison

Table 1. The proportion of shoot sprouting and root formation from synthetic seeds of *Gmelina arborea* and *Peronema canescens* after 6 weeks of culture[1)].

(%)

Species	Shoot elongation medium[2)]	Sucrose in encapsulation medium[3)] (w/v %)				
		3	6	12	24	36
G. arborea	Agar only	100	100	100	80	100
	3% sucrose	50	0	20	0	0
	PGRs[4)]	0	0	75	100	50
	MS[5)]	100	20	0	0	0
	MS[5)] with 3% sucrose and PGRs[4)]	100	100	100	100	100
P. canescens	Agar only	40	55	50	100	75
	3% sucrose	20	25	40	45	35
	PGRs[4)]	100	100	100	100	100
	MS[5)]	0	25	30	10	10
	MS[5)] with 3% sucrose and PGRs[4)]	40	20	50	15	20

Note: 1) Twenty synthetic seeds were used for each treatment combination, 2) 1% (w/v) agar solution, 3) MS medium (MURASHIGE and SKOOG, 1962) containing PGRs and 4% (w/v) sodium alginate, 4) 0.22 mg/l BAP, 0.02 mg/l NAA, and 1.00 mg/l IBA, 5) Basic components of MS medium.

with lower concentrations (3, 6, and 12%) (personal observation). When elongated shoots were transplanted to vermiculite, they grew into rooted plants within 1 month, irrespective of root development on the shoot elongation medium (Fig. 2, left).

Peronema canescens: Up to 100% shoot sprouting was observed on the shoot elongation medium containing PGRs alone. Comparatively low proportions of shoot sprouting were observed on the other combinations of shoot elongation medium and the encapsulation medium except for the combinations of simple agar medium and encapsulation medium with 24 or 36% sucrose (Fig. 1, right). Some synthetic seed-sprouted shoots developed roots in several combinations of encapsulation medium and the shoot elongation medium until 6 weeks of culture. Shoot elongation was promoted with an increase in the sucrose concentration (personal

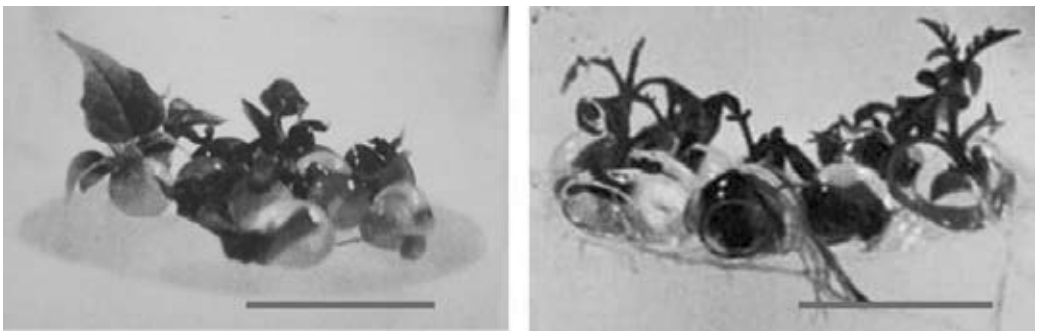

Fig. 1. Sprouted shoots from synthetic seeds of *Gmelina arborea* (left) and *Peronema canescens* (right). After 6 weeks of culture. Bar = 10 mm.
Left: *G. arborea* encapsulated in medium containing 6% sucrose and cultured on simple agar medium.
Right: *P. canescens* encapsulated in medium containing 36% sucrose and cultured on simple agar medium.

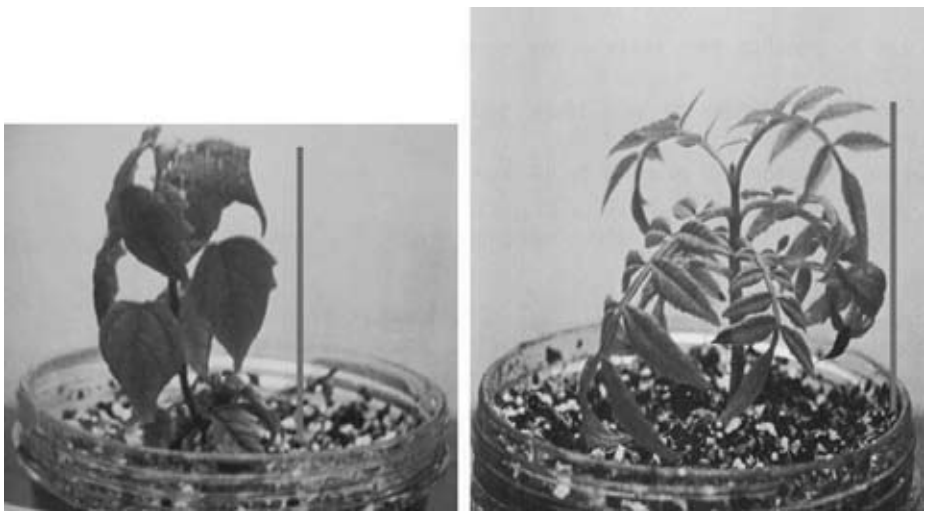

Fig. 2. Regenerated plants derived from synthetic seeds of *Gmelina arborea* (left) and *Peronema canescens* (right). One month after being transplanted to vermiculite. Bar = 50 mm.

observation). When elongated shoots were transplanted to vermiculite, they grew into rooted plants within 1 month irrespective of root development on the shoot elongation medium (Fig. 2, right).

2. Synthetic seed preservation

The proportion of *G. arborea* and *P. canescens* synthetic seeds from which axillary shoot sprouting was observed after preservation is shown in Table 2.

Gmelina arborea: Most of the synthetic seeds sprouted shoots after 6 weeks of subsequent culture irrespective of the durations or temperatures of the preservation, except for those kept at 4° C. All synthetic seeds kept at 4° C turned white during preservation.

Peronema canescens: All synthetic seeds remained green, but no shoot sprouting was observed at any temperatures or durations of preservation except for those kept at 4° C after 6 weeks of culture. All synthetic seeds kept at 4° C turned white during preservation.

Discussion

1. Encapsulation method and plant regeneration

We obtained perfect plants from synthetic seeds of both *G. arborea* and *P. canescens* in this experiment under aseptic conditions. The basic encapsulation protocol used here to make synthetic seeds was primarily developed for *Betula platyphylla*, a temperate tree (KINOSHITA and SAITO, 1990). Our study reveals that this protocol is also applicable to tropical species.

Although there were interactions between the encapsulation and shoot elongation media, synthetic seeds attained 100% shoot sprouting in any of the shoot elongation media. It seemed that the amount of sucrose in the encapsulation medium did not affect the proportion of shoots that sprouted. PICCIONI and STANDARDI (1995) achieved high regrowth from encapsulated axillary buds of six woody species using encapsulation medium without sucrose. However, they

Table 2. The proportion of shoot elongation from preserved synthetic seeds of *Gmelina arborea* and *Peronema canescens* under different temperatures and various durations after 6 weeks of culture at 25° C.[1)]

(%)

Species	Duration (weeks)	Temperature (°C)				
		4[2)]	10	15	20	25
G. arborea	1	0	80	90	80	100
	2	0	85	90	90	100
	3	0	90	90	100	100
	4	0	85	90	95	90
P. canescens	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0

Note: 1) Twenty synthetic seeds were used for each treatment combination, 2) All the explants preserved at 4° C turned white.

noted the definitive lack of nutrition for further growth. In our case, sucrose concentrations had different effects on *G. arborea* and *P. canescens*. Shoot elongation of the former was promoted at lower sucrose concentration and that of the latter increased at higher sucrose concentrations (personal observation), indicating that the species have different sucrose demands. Based on the proportions of shoot sprouting and shoot elongation, we selected 3-12% and 24-36% as adequate sucrose concentrations in the encapsulation medium for *G. arborea* and *P. canescens*.

The shoot elongation medium had different effects on shoot sprouting of *G. arborea* and *P. canescens*. Whereas high proportions of shoot sprouting from *G. arborea* synthetic seeds were achieved on the simple agar bed and on MS medium containing PGRs and 3% sucrose, these media were not effective for *P. canescens*. The shoot elongation medium with only PGRs promoted remarkable shoot sprouting in *P. canescens*, but its effect was limited in *G. arborea*, indicating that a continuous supply of plant growth regulators is essential for shoot sprouting in *P. canescens* but that MS nutrition and sucrose are not required as much. In contrast, *G. arborea* did not always require plant growth regulators, but some amount of sucrose was essential for shoot sprouting. Then we determined simple agar medium and/or MS agar medium with 3% sucrose and PGRs for *G. arborea*, and agar medium containing PGRs for *P. canescens* as the proper shoot elongation medium.

2. Synthetic seed preservation

All synthetic seeds preserved at 4 °C died. PATTNAIK and CHAND (2000) stored encapsulated buds of six mulberry species (*Morus* spp.) for 90 days at 4° C without losing regenerative ability, and IKHLAQ *et al*. (2010) succeeded in regenerating olive (*Olea europaea*) from synthetic seed made of nodal segments preserved for 60 days at 4° C. There is no available literature on synthetic seed preservation of tropical tree species derived from nodal stem at the relatively low temperature of 4° C. However, synthetic seeds derived from node cutting and shoot tips of cassava (*Manihot esculenta*), a tropical perennial crop, stored at 4° C did not regenerate after 7 days of storage, whereas they regenerated when they were stored at 22° C. These results suggest that temperate species can survive at 4° C, but this temperature is likely beyond the physiological limits of tropical species.

Gmelina arborea was successfully preserved even at 10° C for 4 weeks, and shoot sprouting was achieved, indicating that comparatively long preservation of propagules without any subculture has been realized. NASSAR (2003) succeeded in slow growth storage of *Coffea arbica*, a typical tropical tree species, at 15° C for 12 months. Thus, we are hopeful that we may be able to preserve *G. arborea* synthetic seeds for a longer period at this temperature. In contrast, shoot sprouting failed for *P. canescens* preserved synthetic seeds. The explants survived after 4 weeks of preservation except for those kept at 4° C. According to the results of the shoot sprouting experiment, supplementing the shoot elongation medium with plant growth regulators was effective for *P. canescens*. Thus, it may be possible to regenerate this species from preserved synthetic seeds using plant growth regulators.

In conclusion, a fundamental protocol to encapsulate micro-propagated buds of two tropical species was successfully established for the first time. Additionally, although we failed to sprout shoots from preserved *P. canescens*, the possibility of long-term preservation of the synthetic seeds from these two tropical species at relatively low temperatures was shown.

Summary

This study developed a method of synthetic seed formation by encapsulating axillary buds from *in vitro* grown plantlets of two fast-growing tropical tree species, *Gmelina arborea* and *Peronema canescens*, under aseptic conditions. MS medium containing plant growth regulators (0.22 mg/l 6-benzylaminopurine, 0.02 mg/l α-naphthylacetic acid, and 1.00 mg/l 3-indolebuthylic acid) and different concentrations of sucrose (3, 6, 12, 24, and 36%) were used as the encapsulation medium in combination with 4% (w/v) sodium alginate. Nodal segments of plantlets were encapsulated with calcium alginate gel. Synthetic seeds were cultured on five types of agar medium containing different shoot sprouting components. Both *G. arborea* and *P. canescens* were successfully sprouted from axillary buds in any combination of encapsulation medium and shoot elongation medium after 6 weeks of culture. The MS shoot sprouting medium containing plant growth regulators and 3% sucrose appeared to be suitable for sprouting *G. arborea* shoots. Shoot sprouting of *P. canescens* was prominent on medium containing only plant growth regulators, indicating that a continuous supply of plant growth regulators is necessary for *P. canescens* shoot sprouting. Shoot elongation in *G. arborea* was promoted at lower sucrose concentrations in the encapsulation medium, whereas that of *P. canescens* was promoted at higher sucrose concentrations. Plants of these species were established within 1 month by transplanting the elongated shoots into vermiculite. When synthetic seeds were stored at different temperatures (4 10, 15, 20, and 25° C) and for different durations (1, 2, 3, and 4 weeks), they were successfully preserved at all combinations of temperature and duration except for those stored at 4° C. Preserved *G. arborea* synthetic seeds successfully sprouted shoots in a succeeding culture at 25° C, whereas *P. canescens* synthetic seeds did not sprout, although the explants remained green in color.

Keywords: tropical tree, synthetic seed, sodium alginate, *in vitro* preservation, plant regeneration

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Gmelina arborea Roxb. と *Peronema canescens* Jack. の腋芽のカプセル化

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要　旨

二種の熱帯早生樹，*Gmelina arborea* と *Peronema canescens* の培養中の幼植物体の腋芽部分をカプセル化して人工種子を作成する方法を開発した。植物成長調節物質（0.22mg/l 6-benzylaminopurine, 0.02mg/l α-naphthylacetic acid, 1.00mg/l 3-indolebuthylic acid）と異なる濃度のショ糖（3, 6, 12, 24, 36%）を含む MS 培地に 4%（w/v）のアルギン酸ナトリウムを添加したものをカプセル化培地とし，アルギン酸カルシウムで包まれた人工種子を作成した。人工種子をシュートの伸長を期待する 5 種類の培地上で培養したところ，6 週間後には，両種ともカプセル化培地とシュート伸長培地の特定の組み合わせにおいて，シュートの発生と伸長が見られた。*G. arborea* では，MS 培地に成長調節物質とショ糖 3% を添加した培地でのシュートの発生が良好であった。*P. canescens* では成長調節物質のみを添加した培地でのシュートの発生が良好であった。これは，*G. arborea* では成長調節物質は必ずしも必要ではないが，*P. canescens* ではその補給がシュートの発生に必要なことを示している。シュートの伸長は，*G. arborea* ではカプセル化培地中のショ糖濃度が低い方で，*P. canescens* では高い方でそれぞれ良好であった。人工種子を異なる温度と期間で保存したところ，4°C での保存を除いてすべての組み合わせで保存が可能であった。保存した人工種子を 25°C で培養したところ *G. arborea* ではシュートの発生が見られた。一方，*P. canescens* では外植体は緑色を呈していたが，シュートの発生は見られなかった。

キーワード：熱帯樹木・人工種子・アルギン酸ナトリウム・試験管内保存・植物体再生