Alginate-Encapsulated Technology for the Propagation of the Tropical Forest Trees: *Cedrela odorata* L., *Guazuma crinita* MART., and *Jacaranda mimosaefolia* D. DON.

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Summary

The possibility for encapsulation of shoot-tips or axillary buds in the production of artificial seeds of *Cedrela odorata* L., *Guazuma crinita* MART., and *Jacaranda mimosaefolia* D. DON. was tested. High rate of bud emergence and shoot growth were achieved *in vitro* on substratum containing nutrients but, on substratum containing water only, the bud emergence from beads and later shoot growth were very poor. In an attempt to improve the plant regeneration rate from artificial seeds on substratum without nutrients (to diminish the risk of contamination under non-aseptic conditions), 2 types of beads with a single or double layer were tested. The best result was obtained with double layered beads containing medium at a concentration of 1,000% (w/v) supplemented with 0.5% (w/v) activated charcoal in the inner layer, and at concentration of 100% (w/v) in the outer layer. After 6 weeks of incubation on water substratum solidified with 1% (w/v) agar under *in vitro* conditions, high rates of bud emergence and shoot growth were achieved: 60% and 60% for *C. odorata*, 100% and 80% for *G. crinita*, and, 100% and 100% for *J. mimosaefolia*, respectively. However, when artificial seeds were directly sowed on nutrient-free substratum under non-aseptic conditions, the percentage of beads converted into plants was 6.7%, 3.3%, and 31.7% for *C. odorata, G. crinita* and *J. mimosaefolia,* respectively. But, when artificial seeds of *C. odorata* and *G. crinita* were incubated *in vitro* for about 1 week before sowing on non-sterilized substratum, the plant regeneration rate was increased to 28.6 % and 100 %, respectively.

Key words: artificial seed, alginate-encapsulation, *Cedrela odorata, Guazuma crinita, Jacaranda mimosaefolia,* propagation, tropical tree.

FDC: 165.442; 176.1 *Cedrela odorata;* 176.1 *Jacaranda mimosaefolia.*

Introduction

Since the original theoretical concept of the synthetic seeds (artificial seeds) was proposed by MURASHIGE (1977), artificial seed technology has been investigated in several species in many laboratories around the world. The potential advantages of artificial seed technology in forestry are: (1) rapid propagation of desirable lines, (2) genetic uniformity of plants, (3) easy handling of cultured materials, (4) direct delivery to the field, omitting the transplantating and acclimatization steps, (5) suitability to transporting from one place to another, (6) reduction in space for storage, (7) reduction in costs of vegetatively propagated superior lines, and (8) reduction in the breeding cycle.

Many reviews on somatic embryogeny have emphasized using somatic embryos in the form of synthetic seeds for plant propagation, proposing 4 basic types of synthetic seeds: (1) uncoated, desiccated somatic embryos (GRAY and CONGER, 1985), (2) coated, desiccated embryos (KITTO and JANICK, 1982), (3) encapsulated, hydrated embryos (REDENBAUGH *et al.,* 1984), and (4) hydrated embryos in a fluid-drilling gel (DREW, 1979). A 5th category could be (5) composed of hydrated, uncoated somatic embryos (REDENBAUGH *et al.,* 1991). However, artificial seed technology using somatic embryos has not yet been development except for some crops such as carrot (KITTO and JANICK, 1985a and b), alfalfa (REDENBAUGH *et al.,* 1986, 1987), celery (REDENBAUGH *et al.,* 1986; SAKAMOTO, 1990), and lettuce (SAKAMOTO, 1990). Studies on artificial seed production in forest trees using somatic embryos have been reported in *Eucalyptus citriodora, Santalum album, Pinus lambertiana, Pinus taeda* and *Picea abies*, however with a very low or null percentage of plant regeneration (GUPTA and KREITINGER, 1993). Although somatic embryogenesis will probably be the most appropriate technique for rapid-large scale micropropagation and for artificial seed technology, this technique cannot be used in many plant species in which somatic embryos have not been produced. In such cases, the encapsulation of shoot-tips and/or axillary buds provides an alternative to produce artificial seeds.

This paper shows the possibility of the encapsulation of shoot-tips or axillary buds as a substitute for somatic embryos in the production of artificial seeds of *Cedrela odorata* L., *Guazuma crinita* MART., and *Jacaranda mimosaefolia* D. DON.

Materials and Methods

Construction of artificial seeds

Single layered beads

Shoot-tip and nodal segment (including 1 axillary bud) explants, about 3 mm to 4 mm long, were aseptically excised from *in vitro* plantlets regenerated by the methods described by MARUYAMA *et al.* (1989a and b, 1993, 1996) and ISHII and MARUYAMA (1992) and then immersed into autoclaved culture medium containing 4% (w/v) sodium alginate. Next, the explants mixed with the alginate-medium were picked up by means of tweezers and dropped into sterile solution of 1.4% (w/v) calcium chloride where they remained for 30 min. After that, the calcium chloride solution was decanted and the constructed alginate beads were rinsed three times with autoclaved medium (*Fig. 1*). The standard WPM (LLOYD and MCCOWN, 1980) supplemented with 1 µM BAP (6-benzylaminopurine) and 1 µM KIN (6-furfurylaminopurine) was used for *C. odorata* and *G. crinita*, respectively. The standard B5 medium (GAMBORG *et al.,* 1968) supplemented with 1 µM KIN was used for *J. mimosaefolia*.

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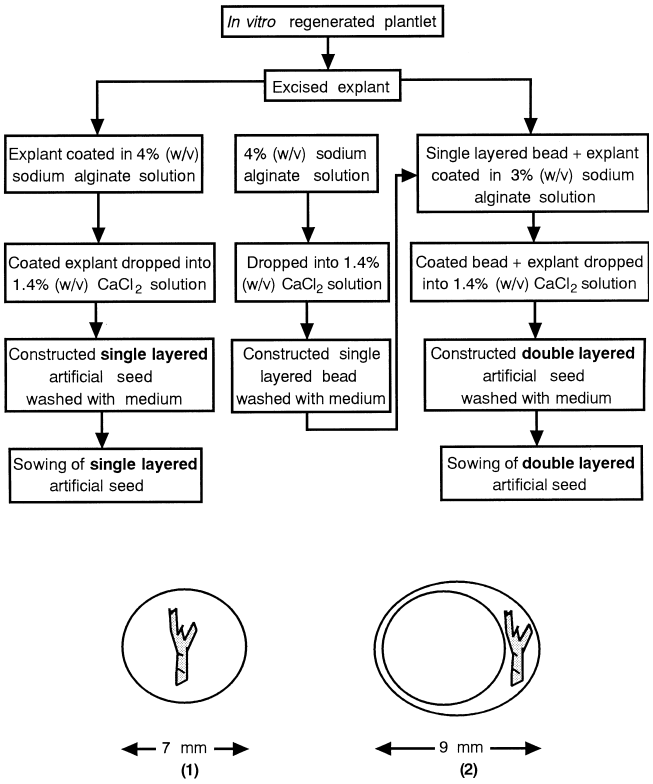

Figure 1. – Construction schemes of artificial seeds: (A) procedures of explants encapsulation, and (B) schematic diagram of single layered (1) and double layered (2) artificial seed.

Double layered beads

Double layered beads were constructed by the method described by KINOSHITA and SAITO (1992). As shown in *figure 1*, shoot-tip explants and calcium alginate single layered beads constructed previously were mixed with a 3% (w/v) sodium alginate medium. Each explant, together with a single layered bead mixed with the gel (sodium alginate medium), was picked up with tweezers and dropped into complexing agent (calcium chloride solution) again. The explant was contained in the outer layer.

Regeneration of plantlets under aseptic conditions

Regeneration of plantlets from encapsulated shoot-tip or nodal segment explants was tested under aseptic conditions. Artificial seeds were cultured under photon flux density of about 65 µmol $m^{-2}s^{-1}$ with a light regime of 16 h daily provided by cool white fluorescent lamps (100 V, 40 W; Toshiba Co. FLR40SW) in a culture room at 25 °C.

Effects of types of encapsulated explants and substrata on the bud emergence, shoot growth, and rooting of artificial seeds

Two types of explants, shoot-tip and nodal segment, were encapsulated into single layered alginate gel beads and cultured *in vitro* on different substrata, namely, water or medium solidified with 1% (w/v) agar (Wako Pure Chem. Ind.) with or without plant growth regulators or sucrose.

Effects of concentrations of medium contained in the gel bead matrix on the bud emergence, shoot growth, and rooting of artificial seeds

Shoot-tip explants were encapsulated into single layered alginate gel beads containing medium at different concentrations (100% to 2,000%) and cultured under aseptic conditions on water substratum solidified with 1% (w/v) agar. Medium at a concentration of 100% represents the standard basal medium. Media at 500%, 1,000%, and 2,000%, represents 5, 10, and 20 times the concentration (w/v) of standard basal medium, respectively, excepting the concentration of calcium components of the media which was added at standard concentration in all treatments.

Effects of types of beads on the bud emergence, shoot growth, and rooting of artificial seeds

Shoot-tip explants encapsulated into 4 types of beads were tested to determine the effect on plant regeneration. Four types of beads were made from combinations of single or double layers, and 2 concentrations of medium (A, B, C, and D) (cf: *Figs. 3* and *5*). They were cultured in ERLENMEYER flasks with water substratum solidified with 1% (w/v) agar under culture room conditions.

Regeneration of plantlets under non-aseptic conditions

Shoot-tip explants encapsulated into different types of beads were placed in plastic pots containing non-sterilized perlite substratum saturated with water, and initially covered with a

Table 1. – Effects of types of encapsulated explants and substrata on the bud emergence, shoot growth and rooting of artificial seeds of *C. odorata, G. crinita,* and *J. mimosaefolia* under aseptic conditions.

Substratum	*Cedrela odorata*						*Guazuma crinita*						*Jacaranda mimosaefolia*					
	Shoot-tip			Nodal segment			Shoot-tip			Nodal segment			Shoot-tip			Nodal segment		
	E	G	R	E	G	R	E	G	R	E	G	R	E	G	R	E	G	R
W	0	0	0	20	20	0	0	0	0	0	0	0	30	0	0	0	0	0
W+H	0	0	0	20	0	0	0	0	0	0	0	0	40	0	0	0	0	0
W+S	0	0	0	0	0	0	10	0	80	0	0	0	100	100	40	60	20	0
W+S+H	0	0	0	0	0	0	0	0	60	10	0	0	100	100	0	50	20	0
M	0	0	0	40	40	0	0	0	0	0	0	0	20	20	0	0	0	0
M+H	20	0	0	40	20	0	0	0	0	0	0	0	40	20	0	0	0	0
M+S	90	90	60	100	100	20	100	90	60	90	90	30	100	100	10	80	40	0
M+S+H	100	100	40	100	100	0	100	100	70	100	100	40	100	100	0	100	100	0

Data were calculated from 10 artificial seeds for each treatment after 45 days of culturing.
Shoot-tip and nodal segment explants about 3 mm to 4 mm long were encapsulated by immersion into medium containing 2% (w/v) sucrose, 1 µM BAP or KIN and 4% (w/v) sodium alginate, and then dropped into the same medium with 1.4% (w/v) calcium chloride.
E: Percentage of bud emergence, G: percentage of shoot growth, R: percentage of rooting.
W: Water, H: growth regulator (1 µM BAP for *C. odorata*, 1µM KIN for *G. crinita* and *J. mimosaefolia*), S: 2% (w/v) sucrose, M: sucrose and growth regulator-free medium (WPM for *C. odorata* and *G. crinita*, B5 for *J. mimosaefolia*).
All substrata were solidified with 1% (w/v) agar.

Petri dish and sealed with Parafilm (American Can Co.) to prevent drying. Plastic pots with artificial seeds were kept in a growth cabinet at 25 °C to 30 °C under a 16 h photoperiod with a photon flux density of about 35 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (100 V, 20 W; Toshiba Co. FL20SW). They were irrigated with water until the evident appearance of roots from grown shoots, and then fertilized with 0.1% (v/v) Hyponex 5-10-5 plant-food solution (The Hyponex Co., Inc.).

As in the experiments under aseptic conditions, when the expanded leaves extruded out from the beads, the growth stage of artificial seeds was regarded as bud emergence. When the shoot stem extruded out from the beads, it was regarded as the start of shoot growth.

Results and Discussion

Regeneration of plantlets under aseptic conditions

Effects of types of encapsulated explants and substrata on the bud emergence, shoot growth, and rooting of artificial seeds

Table 1 shows the bud emergence, shoot growth, and rooting rates of 2 types of encapsulated explants on different substrata. When encapsulated explants of both shoot-tip and nodal segment were cultured on a medium substratum supplemented with sucrose, high percentages of bud emergence and shoot growth were obtained in the three species. However, the percentages of rooting ranged from 0 % to 70 %. The best percentage of rooting was observed in *G. crinita* with shoot-tip explants, and the worst was in both shoot-tip and nodal segment explants of *J. mimosaefolia*. Rooting of encapsulated shoot-tips was much better than that of encapsulated nodal segment explants. Addition of auxins, 0.49 µM IBA (indole-3-butyric acid) only, or in combination with 0.054 µM NAA (α-naphthaleneacetic acid) into the gel bead matrix and/or into the substratum was not beneficial because it barely increased the rooting rate in *J. mimosaefolia* and *C. odorata*. It decreased the bud emergence and shoot growth rate and increased the percentage of browning, and in *G. crinita*, it promoted callus formation only (data not presented). This result may be attributable to the inappropriate timing of the auxin action, because it is not essential for bud emergence and shoot growth but possibly necessary for rooting, although in an appropriate stage.

When the encapsulated explants were cultured on water substratum, the percentages of bud emergence, shoot growth, and rooting, except for *J. mimosaefolia* explants, were null or very low. This result suggests that, (1) some of the nutrients in beads such as sucrose and plant growth regulators should have diffused into water substratum, leaving insufficient amounts of components for good growth, and (2) the requirements of the medium and/or sucrose for bud emergence, shoot growth, or rooting are different in each species. Thus, although the components reduced in the gel bead matrix were not sufficient to promote bud emergence and then shoot growth in encapsulated explants of *C. odorata* and *G. crinita*, they were sufficient in encapsulated explants of *J. mimosaefolia*, but with less impact on nodal segments. Moreover, these results suggest that both medium and sucrose are necessary for bud emergence, shoot growth, and rooting of *C. odorata*, and for bud emergence and shoot growth of *G. crinita*, and that sucrose is the essential material to promote good bud emergence and shoot growth of *J. mimosaefolia,* and for rooting of *G. crinita.*

Effects of concentrations of medium contained in the gel bead matrix on the bud emergence, shoot growth, and rooting of artificial seeds

In an attempt to improve the plant regeneration rate from artificial seeds on substratum without nutrients, the concentration of medium contained in the gel bead matrix was increased to 5, 10, and 20 times. Results shown in *table 2* indicate that the artificial seeds of the 3 species showed increased bud emergence and shoot growth rates proportionately to the concentration of medium contained in the gel bead matrix. However, improvement of rooting rate was obtained only in artificial seeds of *G. crinita*. Although the diffusion of nutrients from beads to substratum was very fast (*Fig. 2*), some of the nutrients which remained in the beads constructed with medium and sucrose at a high concentration (2,000%) were able to induce bud emergence and shoot growth in the three species in ranges of 50% to 80% and 30% to 50% respectively, and a rooting rate of 80% in *G. crinita* was attained. Similar results were obtained in artificial seeds of Japanese white birch (*Betula platyphylla* var. *japonica*) (KINOSHITA and SAITO, 1990). It was reported that there was an improvement in the plant regeneration rate using encapsulated axillary buds and constructing beads with a large quantity of sucrose (39%) cultured on agar substratum containing only distilled water. In contrast to these results, BAPAT *et al.* (1987) have shown that axillary bud of *in vitro* propagated mulberry (*Morus indica* L.)

Table 2. – Effects of concentrations of medium contained in bead gel matrix on the bud emergence, shoot growth and rooting of artificial seeds of *C. odorata, G. crinita,* and *J. mimosaefolia* under aseptic conditions.

Concentration of medium (%)	*Cedrela odorata*			*Guazuma crinita*			*Jacaranda mimosaefolia*		
	E	G	R	E	G	R	E	G	R
100	0	0	0	0	0	0	30	0	0
500	20	0	10	30	0	30	50	0	0
1,000	30	10	0	60	10	90	60	30	0
2,000	50	30	0	80	50	80	70	50	0

Data were calculated from 10 artificial seeds for each treatment after 45 days of culturing on water substratum solidified with 1% (w/v) agar.
Shoot-tip explants about 3 mm to 4 mm long were encapsulated into bead gel matrix containing each concentration of medium (WPM for *C. odorata* and *G. crinita*, and B5 for *J. mimosaefolia*) and supplemented with 1 µM BAP (*C. odorata*) or 1 µM KIN (*G. crinita* and *J. mimosaefolia*). Concentration of 100% medium represents the standard basal medium. In all treatments, calcium components of the medium were added at a concentration of 100%.
E: Percentage of bud emergence, G: percentage of shoot growth, R: percentage of rooting.

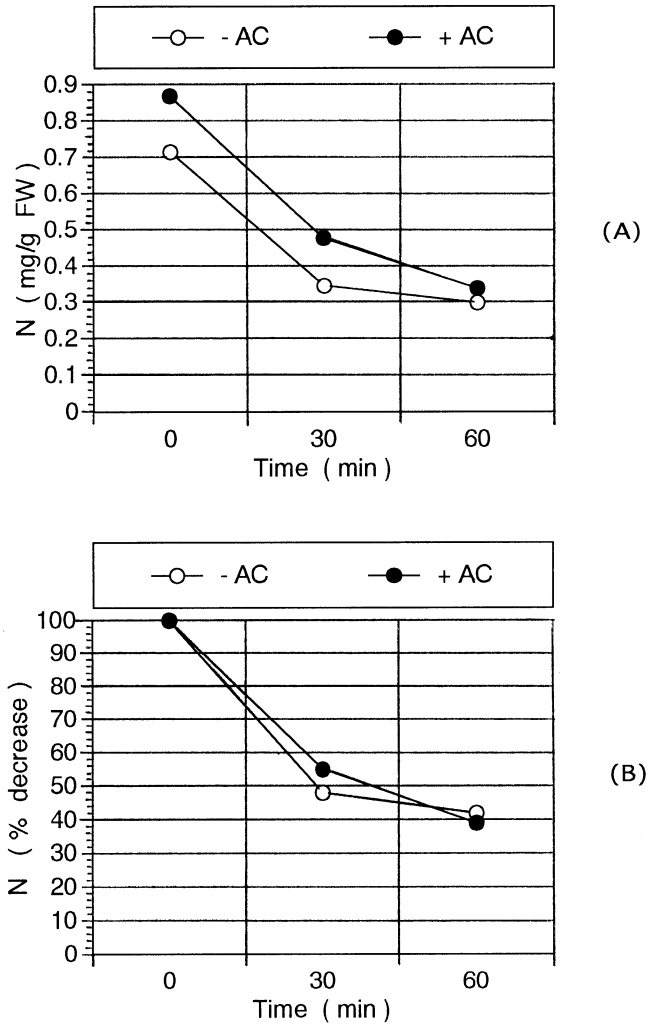

Figure 2. – Change of nitrogen (N) content in gel matrix (with or without AC) of artificial seeds at different times after construction, setting the beads in distilled water and determinated by colorimetric analysis.
(A) Change of nitrogen content calculated in mg/g of fresh weight (FW).
(B) Change of nitrogen content calculated in percentage regarding to the initial content.
AC: 0.5% (w/v) activated charcoal.

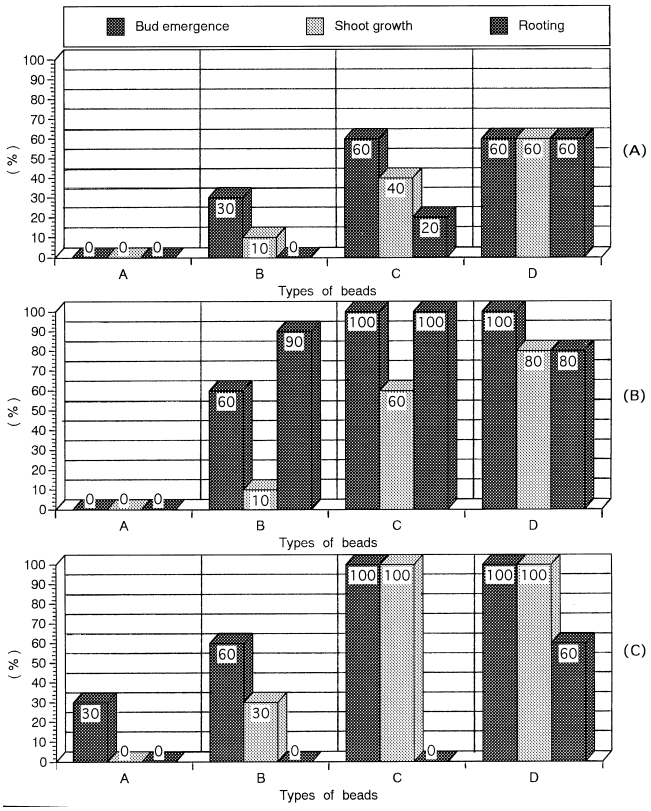

Figure 3. – Effects of types of beads on the bud emergence, shoot growth, and rooting of artificial seeds of *C. odorata* (A), *G. crinita* (B), and *J. mimosaefolia* (C) under aseptic conditions.
Data were calculated from 10 artificial seeds for each treatment after 45 days of culturing on water substratum solidified with 1% (w/v) agar.
A: Single layered beads containing medium at a concentration of 100%.
B: Single layered beads containing medium at a concentration of 1,000%.
C: Double layered beads containing medium at a concentration of 1.000% in the inner layer, and a concentration of 100% in the outer layer.
D: Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer.
In all types of beads calcium components of the medium were added at a concentration of 100%. WPM supplemented with 1 µM BAP and KIN was used for *C. odorata* and *G. crinital* respectively. B5 medium with 1 µM KIN was used for *J. mimosaefolia.*

plantlets, encapsulated in calcium alginate gel matrix containing standard basal MURASHIGE and SKOOG medium, grew well on nutrient-free substratum.

Effects of types of beads on the bud emergence, shoot growth, and rooting of artificial seeds

In order to improve the regeneration rate of artificial seeds on nutrient-free substratum, construction of double layered beads and addition of activated charcoal (AC) into gel matrix were tried. The results after 45 days of culturing shown in *figures 3* and *4*, indicate that double layered beads were much better than single layered beads in stimulating bud emergence, shoot growth, and rooting of artificial seeds of the three species studied. The bud emergence rate and shoot growth rate of the double layered beads were 1.7 to 2.0 and 3.3 to 6.0 times greater, respectively, than those of the single layered beads. Moreover, addition of 0.5 % (w/v) AC into the gel matrix of the inner layer was beneficial because it increased the shoot growth rate in *C. odorata* and *G. crinita*, the rooting rate of *C. odorata* and *J. mimosaefolia*, and even improved the plant regeneration rate of the 3 species. The beneficial effect of AC on plant regeneration from artificial seeds may be attributable to their ability to absorb unwanted exudates, such as 5-hydroxymenthylfurfural (a toxic breakdown product of sucrose formed during autoclaving) and other harmful phenolic oxidation products. LULSDORF *et al.* (1993) reported that the addition of 0.5 % (w/v) AC to the alginate beads matrix significantly enhanced root development and germination of encapsulated somatic embryos of interior spruce (*Picea glauca engelmanii* complex) and black spruce (*Picea mariana* MILL.).

Regeneration of plantlets under non-aseptic conditions

Figure 5 shows the results of plant regeneration from encapsulated shoot-tips under non-aseptic conditions. When single layered artificial seeds were sowed directly into non-sterilized perlite substratum, plant regeneration of *C. odorata* and *G. crinita* was very poor (0% to 6.7% and 0% to 3.3% respectively) but, in contrast, about 30 % of buds from double layered artificial seeds emerged in both species. Moreover, when shoot-tips were encapsulated into double layered beads containing

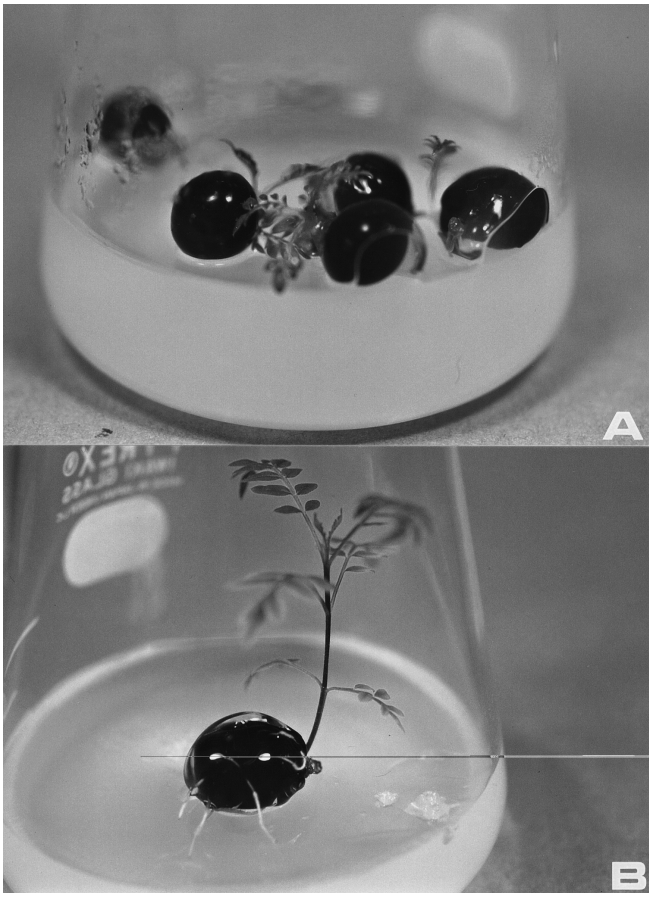

Figure 4. – Bud emergence (A) and plant regeneration (B) of artificial seeds of *J. mimosaefolia* under a septic conditions. Double layered beads containing medium at aconcentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer, were placed on water substratum solidified with 1% (w/v) agar.

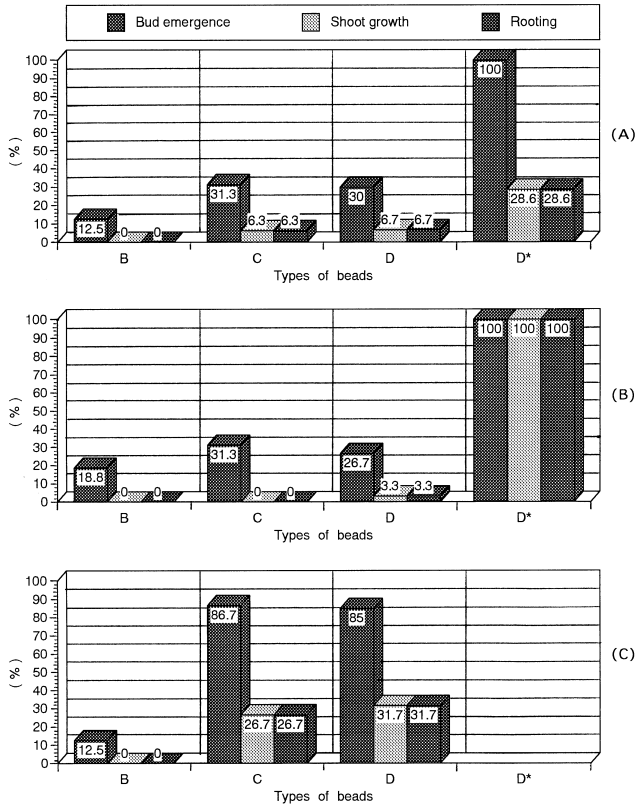

Figure 5. – Effects of types of beads on the bud emergence, shoot growth and rooting of artificial seeds of *C. odorata* (A), *G. crinita* (B), and *J. mimosaefolia* (C) under non-aseptic conditions.
Data were taken from 14 to 60 artificial seeds for each treatment 75 days after sowing on non-sterilized perlite substratum.
B: Single layered beads containing medium at a concentration of 1,000%.
C: Double layered beads containing medium at a concentration of 1,000% in the inner layer, and a concentration of 100% in the outer layer.
D: Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer.
D*: D beads pre-cultured *in vitro* on WPM for about 1 week before sowing on non-aseptic conditions.

0.5% (w/v) AC in the inner layer, and then incubated *in vitro* on WPM for about 1 week before sowing in non-aseptic conditions, the plant regeneration rate of *C. odorata* and *G. crinita* attained was 28.6% and 100%, respectively (*Figs. 6* and *7*). These results suggest that the period of incubation of the artificial seeds on substratum with nutrients promoted swift initiation of the growth process and, when they were transferred into pots containing non-sterilized perlite substratum, a high plant regeneration rate under non-aseptic conditions. Rapid development of leaves for photosynthesis activity is very important for plant regeneration under non-aseptic photoautotropic conditions. Emerged shoots that grew fast, rooted easily and showed a high resistance to microbial contamination, resulting in healthy plants. In contrast, shoots that did not grow fast were very susceptible to microbial contamination and subsequent deterioration. RAO and BAPAT (1993) reported that the addition of a fungicide to the alginate matrix prevents the beads from attack of microorganisms and allows growth in a non-aseptic environment. However, in this study, although the addition of fungicide (benomyl) and antibiotic-antimycotic solution (penicillin, streptomycin and amphotericin) at different concentrations into the gel bead matrix were tested, the plant regeneration rates were not enhanced (data not presented).

Artificial seeds of *J. mimosaefolia* sowed directly into non-sterilized perlite substratum showed a high percentage of bud emergence (about 85%) and a plant regeneration rate of about 30% (*Fig. 8*). This result was much better than that of both *C. odorata* and *G. crinita* artificial seeds. Pre-culture *in vitro* treatment before sowing under non-aseptic conditions was not carried out in this species. Just like *C. odorata* and *G. crinita*, in *J. mimosaefolia* shoot-tips encapsulated into single layered beads containing medium at a high concentration (1,000%), falled to regenerate plants under non-aseptic conditions.

BAPAT *et al.* (1987) reported the *in vitro* propagation of *Morus indica* by encapsulating axillary buds. MACHII (1992) reported *in vitro* growth of encapsulated adventitious buds in *Morus alba*. KINOSHITA and SAITO (1990) reported the propagation of *Betula platyphylla* var. *japonica* by encapsulated axillary buds. RAO and BAPAT (1992) reported the *in vitro* germination of encapsulated somatic embryos in sandalwood (*Santalum album* L.). MURALIDHARAN and MASCARENHAS (1995) reported the germination of encapsulated embryos of *Eucalyptus citriodora* on aseptic medium. These authors and others (JAIN *et al.*, 1995a and b) also reported propagation by encapsulated techniques under aseptic conditions. However, information about plant regeneration from artificial seeds of woody plants under non-aseptic conditions was not available (BAPAT and RAO,

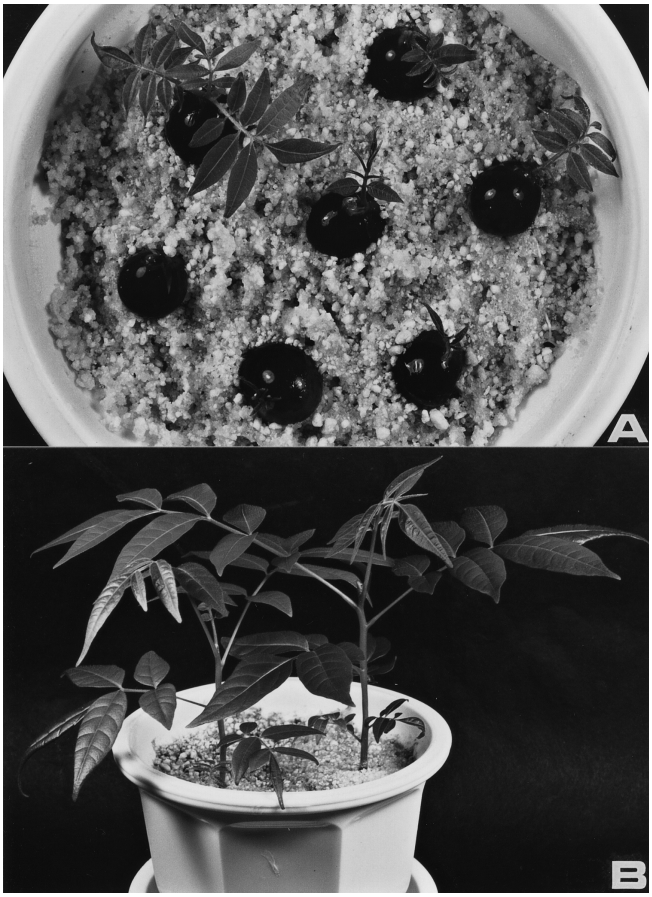

Figure 6. – Bud emergence (A) and plant regeneration (B) of artificial seeds of *C. odorata* under non-aseptic conditions. Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer, were sowed on non-sterilized perlite substratum.

1988), except for reports of recent success in plant regeneration of alginate-encapsulated axillary buds of *Betula platyphylla* var. *japonica* on non-sterilized perlite substratum (KINOSHITA and SAITO, 1992) and plant regeneration of mulberry (*Morus indica* L.) axillary buds encapsulated with 50 mg/l of Carbendazim in a non-aseptic environment (RAO and BAPAT, 1993). The protocols for artificial seed production in *C. odorata*, *G. crinita* and *J. mimosaefolia* are summarized in *table 3*.

Forestry provides one of the most valuable opportunities for artificial seed technology because of the need for vegetative propogation and the lack of other cost-effective methods for propagation of planting stocks. Moreover, the use of artificial seeds of selected plus lines of valuable tree species as substitutes of biological seeds can solve the problems regarding seed supply and seed storage, and has the potential to reduce the tree breeding cycle 5 to 20 years by eliminating the need for seed production orchards (REDENBAUGH *et al.,* 1991). In addition, artificial seeds require less space for storage and are suitable for easy handling and economic transportation.

Conclusions

Artificial seeds of *C. odorata, G. crinita,* and *J. mimosaefolia* grew well when were cultured *in vitro*. However, when they were sowed directly in a non-sterilized substratum, the plant regeneration rate decreased considerably. Regeneration of plants under non-aseptic conditions is the major hurdle for artificial seed technology. Development of double layered beads containing medium at a high concentration in the inner layer, and *in vitro* incubation of artificial seeds before sowing, were found to be beneficial to enhance the plant regeneration rate under non-aseptic conditions. However, further studies including development of suitable coating for artificial seeds that permit easy plant conversion under non-aseptic conditions are required.

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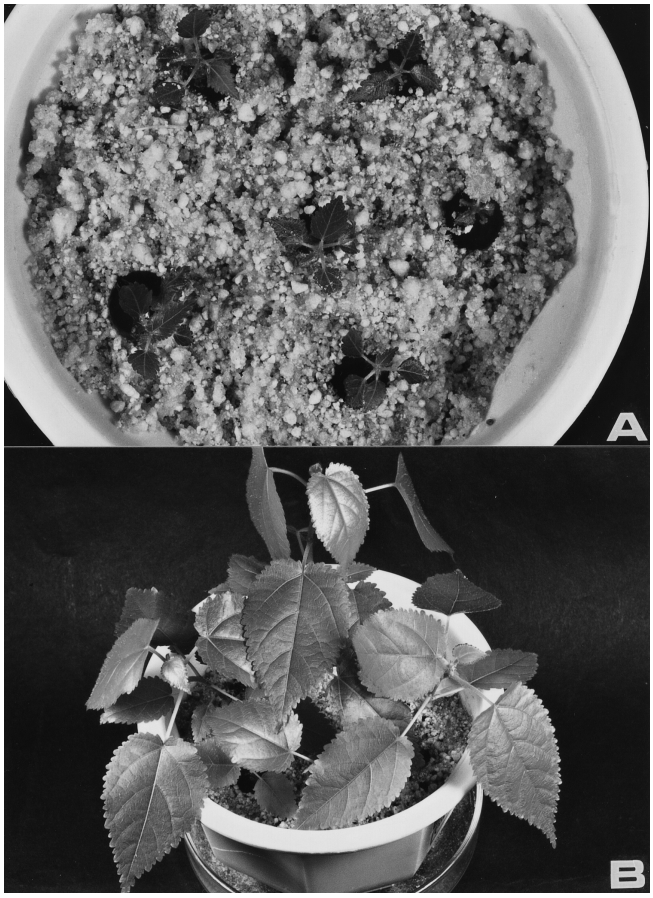

Figure 7. – Bud emergence (A) and plant regeneration (B) of artificial seeds of *G. crinita* under non-aseptic conditions. Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer, were sowed on non-sterilized perlite substratum.

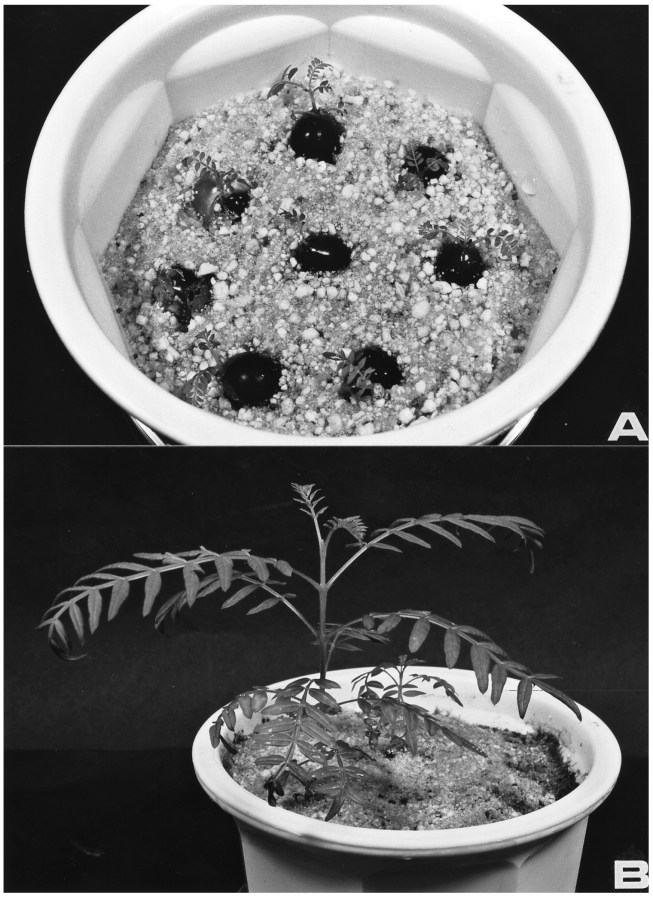

Figure 8. – Bud emergence (A) and plant regeneration (B) of artificial seeds of *J. mimosaefolia* under non-aseptic conditions. Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and a concentration of 100% in the outer layer, were sowed on non-sterilized perlite substratum.

Table 3. – Appropriate protocols for artificial seed production in *C. odorata*, *G. crinita*, and *J. mimosaefolia.*

Regeneration conditions	*C. odorata*	*G. crinita*	*J. mimosaefolia*
Under aseptic conditions			
Encapsulated explant	Shoot-tip (3-4 mm)	Shoot-tip (3-4 mm)	Shoot-tip (3-4 mm)
Substratum	WPM	WPM	B5
Types of beads	Single layered beads [1)]	Single layered beads	Single layered beads
Bud emergence rate	100%	100%	100%
Shoot growth rate	100%	100%	100%
Under non-aseptic conditions			
Encapsulated explant	Shoot-tip (3-4 mm)	Shoot-tip (3-4 mm)	Shoot-tip (3-4 mm)
Substratum	Perlite	Perlite	Perlite
Types of beads	Double layered beads [2)]	Double layered beads	Double layered beads
Bud emergence rate	100%	100%	85%
Shoot growth rate	28.6%	100%	31.7%

[1)] Single layered beads containing medium at a concentration of 100%.
[2)] Double layered beads containing medium at a concentration of 1,000% supplemented with 0.5% (w/v) activated charcoal in the inner layer, and at a concentration of 100% in the outer layer. Shoot-tip was contained in the outer layer.
Artificial seeds of *C. odorata* and *G. crinita* were incubated *in vitro* for about 1 week before sowing in non-aseptic conditions.

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