Microshoots encapsulation and plant conversion of *Musa sp*. cv. 'Grand Naine'

Encapsulamento de microbrotos e conversão em plantas de *Musa sp*. cultivar 'Grand Naine'

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ABSTRACT

Synthetic seed technologies are useful tools for the field delivery of in vitro derived plantlets. In the present study, different encapsulation procedures and their efficacy in the plantlet regeneration using microshoots of banana cv. 'Grand Naine' were evaluated. Two encapsulation systems were evaluated: i) single encapsulation in beads or droplet hardening method; and ii) double layer or hollow beads. The use of different compounds to enhance the capsule conservation and the conversion to plantlets was also evaluated. The conversion capacity was assessed in vitro on water-agar culture medium or in ex vitro conditions with Gerbox® boxes. The single encapsulation system showed 80% conversion. The capsules with MS saline formulation treated with 100mM KNO_3 showed 76% conversion. Capsules with 1.5g L^{-1} activated charcoal, and 0.5g L^{-1} benomyl sucrose-free capsules showed 75% conversion. The encapsulated and non-encapsulated microshoots exhibited 100% germination in response to MS culture medium, and polyethylene glycol after 10 days of storage at 4°C. Sucrose-free capsules showed significantly higher germination (83.3%) than those sucrose-enriched capsules (56.7%). The ex vitro conversion of encapsulated microshoots was 20% in the Gerbox™. These results indicate the feasibility using synthetic seeds in the large-scale micropropagation of banana cv. 'Grand Naine'.

Key words: *artificial seeds, banana,* in vitro *propagation, pharmaceutical capsules.*

RESUMO

As tecnologias de semente sintética são ferramentas promissoras para a micropropagação de plantas. O objetivo do presente trabalho foi desenvolver um protocolo de encapsulamento e armazenamento de unidades encapsuláveis a partir de microbrotos de bananeira da cultivar 'Grand Naine'. Foram avaliados dois sistemas de encapsulamento: i) formação de cápsula pelo método de camada simples e ii) camada dupla. Foi avaliada também a adição de diferentes adjuvantes na matriz de alginato para promover a conservação da cápsula e favorecer a conversão em plantas. Para a conversão em planta foram avaliadas as condições in vitro sobre o meio de cultura composto de agar-água e as condições ex vitro em caixas Gerbox®. O melhor sistema de encapsulamento para os microbrotos da bananeira da cultivar 'Grand Naine' foi o de camada simples, com 80% de conversão. O emprego da formulação salina MS na forma de endosperma artificial e a descomplexação com 100mM KNO_3 favoreceram a conversão de microbrotos (76%). A adição de carvão ativado (1,5g L^{-1}) e de benomyl (0.5g L^{-1}), na ausência de sacarose na matriz de alginato, resultou em 75% de conversão, além de reduzir a oxidação e a contaminação dos microbrotos. Microbrotos encapsulados e não-encapsulados revelaram 100% de germinação em resposta ao meio de cultura MS e PEG após 10 dias de estocagem a 4°C. Cápsulas sem a adição de sacarose resultaram em 83,3% de conversão, valor superior ao valor obtido com a adição de sacarose (56,7%). A conversão de microbrotos encapsulados foi de 20% nas condições ex vitro. Os resultados indicam a viabilidade de uso destas tecnologias para a micropropagação em larga escala da cultivar de banana 'Grand Naine'.

Palavras-chave: *banana, semente sintética, propagação* in vitro, *cápsula farmacêutica.*

INTRODUCTION

Synthetic seeds consist of artificially encapsulated somatic propagules (embryos, shoots or other *in vitro*-derived propagule) capable of conversion

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into plantlets *in vitro* or *ex vitro* conditions (AITKEN-CHRISTIE et al., 1995). Encapsulated technology using *in vitro*-grown propagules offers an efficient means for mass clonal propagation and conservation of elite or endangered plant species (MALLÓN et al., 2007). The main advantages using synthetic seed techniques are associated with the elimination of the final micropropagation stages (i.e. rooting and acclimatization) allowing direct delivery as well as the development of cost-effective propagation systems (SINGH et al., 2006). Nevertheless, to reach such objective, the encapsulation technique must be firstly improved regarding its composition and conditions for short term storage.

In the case of banana (*Musa sp*), the use of synthetic seed techniques may improve the quality of the plantlets, and decreasing the production costs. This technique was successfully employed in the cvs. 'Basrai' by GANAPATHI et al. (1992), 'Nanicão' by MATSUMOTO et al. (1995) and 'Rasthali' by GANAPATHI et al. (2001) and resulting in high conversion rates (100%, 75% and 66%, respectively). However, in all these cases only *in vitro* conversion was tested on sucrose and/or plant growth regulators-enriched culture medium.

The general methodologies for the production of synthetic seeds using sodium alginate were described by REDENBAUGH et al. (1993), following by the development of the technique using pharmaceutical capsules (DUPUIS et al., 1994). Later, it was proposed the double layer or hollow beads techniques based upon production of capsules with a gelled external layer surrounding a liquid internal layer (PATEL et al., 2000). Thus, the first step towards the development of an efficient and reliable protocol for the production of synthetic seeds is the correct choice of the encapsulation methodology, associated with the composition of the matrix.

The synthetic seed technology application in commercial scale is still hampered by the low efficiency of the protocols and logistic behind it. For instance, the conversion step should be already accomplished in *ex vitro* conditions or very simple *in vitro* conditions, making the logistic process easier without requiring specialized facilities for their conversion. To the best of our knowledge, up to now in the systems developed for banana synthetic seeds only *in vitro* conversion was evaluated. The direct transference of the synthetic seeds to *ex vitro* conditions was already successfully described in bromeliads (RECH FILHO, 2004). Therefore, an optimized system using synthetic seeds should include the best conversion rate of these, feasibility to short-term storage regarding logistic and *ex vitro* conversion or on simplified conditions.

The objective of this study was to evaluate different encapsulation systems, short-term storability and incorporation of co-adjuvant (fungicide and activated charcoal), as well as the regeneration capacity of synthetic seeds containing microshoots of banana cv. 'Grand Naine'.

MATERIAL AND METHODS

Apical buds (3mm) of banana cv. 'Grand Naine' were excised and inoculated in 200mL glass flasks containing 30mL of culture medium with the following composition: MS saline formulation (MURASHIGE & SKOOG, 1962) supplemented with 6-benzylaminopurine (BAP) (4.4µM), sucrose (30g L^{-1}) and gelled with agar Merse® (7.5g L^{-1}). The pH of the culture medium was adjusted to 5.8 prior adding the gelling agent and autoclaved at 1.5kgf cm^{-2} at 121°C for 15min. After 30 days, the cultures were transferred to 300mL glass flasks containing 20mL of MS liquid culture medium supplemented with 44.4µM of BAP and subcultured at six week intervals. At the end of the fourth subculture, microshoots here defined as clusters of unipolar axis 6-8mm in length were obtained and subjected to a fast dehydration (5min) in flow chamber over sterile paper filter, being subsequently used for the encapsulation procedures. The sodium alginate, the starch and the pharmaceutical capsules were sterilized under UV light during 30min. The MS saline formulation incorporated into the matrix gel had the pH adjusted to 5.8, then autoclaved at 1.5kgf cm^{-2} 121°C for 15min before ionotropic gelation.

Microshoots of 6-8mm were used as explant source for obtaining synthetic seeds. Different encapsulation systems were evaluated: i) single encapsulation in beads or droplet hardening method (REDENBAUGH et al., 1993) consisting of MS salts plus 1% (w/v) sodium alginate which was dropped into 100mM CaCl$_2$ solution for 20min for hardening; ii) double layer or hollow beads, adapted from PATEL et al. (2000), with the carboxymethylcelulose replaced by 5% starch in a CaCl$_2$ 100mM solution for 20min. The resulting capsule was then immersed in a solution of 1% sodium alginate, then drooped in a CaCl$_2$ solution and hardened for 20min; and, iii) pharmaceutical capsules (DUPUIS et al., 1994) filled with liquid MS salts and the microshoots. Thereafter synthetic seeds for procedure 1 and 2 were subjected to 100mM KNO$_3$ for 20min. In the procedure 2 (hollow beads) the carboxymethylcelulose was replaced by 5% starch for being readily available, biocompatible, cost-effective

and presenting adequate viscosity. The conversion percentage was recorded after 4 weeks in 300mL glass flasks containing 40mL of MS culture medium gelled with agar Merse® (7.5g L^{-1}).

Based on the results obtained new experiments were carried out to improve shoot emergence of encapsulated microshoots. Each microshoot was included in alginate solution and then dropped into $CaCl_2$ 100mM. The beads formed by ionotropic gelation were hardened in the same solution for 20min.

The *in vitro* conversion of the capsules was analyzed to evaluate the effects of such components in the shoot emergence of the microshoots. Firstly, it was evaluated the effect of the presence or absence of MS salts (factor A) in the sodium alginate matrix and the presence or absence of a KNO_3 treatment (Factor B). The capsules were immersed in 100mM KNO_3 for 20min as proposed by ONISHI et al. (1994). Such treatment replaces the calcium by potassium in the alginate matrix. Distilled water was used in the control treatment. Secondly, it was evaluated the effect of activated charcoal (0.0 or 1.5g L^{-1}, Factor A), benomyl (0.0; 0.5 or 1.0g L^{-1} – Benlate™, DuPont Co., Factor B), and the MS salts in the alginate matrix as co-adjuvant. Finally, it was evaluated the tolerance of the microshoots to low temperatures in response to different osmoprotectors as follow: 1) MS; 2) MS + DMSO – dimethyl sulfoxide (10% w/v); 3) MS + DMSO 20%; 4) MS + polyethylene glycol (PEG) (FW 8000) 10%, and 5) MS + PEG-8000 20%. The encapsulated and non-encapsulated microshoots were then cold-hardened at 4°C for 10 days in the dark. The encapsulated microshoots were incubated in sterile Petri dishes and sealed with Parafilm®. The non-encapsulated microshoots were placed in Petri dishes containing the culture medium supplemented with the osmoprotector treatments, gelled with agar Merse® (7.5g L^{-1}). After the short-term storage (10 days) the synthetic seeds were subjected to KNO_3 100mM for 20min. The synthetic seeds of the three experiments were then inoculated in flasks (300mL) containing 40mL water-Agar (7.5g L^{-1}- Merse®) to evaluate their shoot emergence percentage and data were recorded after 4 weeks in culture.

The optimized conditions described above were used to evaluate the effect of sucrose (0 and 30g L^{-1}) in the matrix solution was also evaluated and the shoot emergence percentage was measured both in *in vitro* and *ex vitro* conditions. The synthetic seeds were aseptically transferred to *in vitro* conditions for their conversion in flasks (300mL) containing 40mL water gelled with Agar (7.5g L^{-1}- Merse®) and autoclaved for

15 min at 121°C (1.5kgf cm^{-2}.) or in standard Gerbox® boxes with filter paper moistened with distilled water and benomyl 1.0g L^{-1}. All the cultures were maintained in a culture room at 25±2°C under 16h light with an intensity of 50-70μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent (Sylvania) and high pressure sodium vapor lamps (Empalux—VST).

The experiments were carried in a randomized block design, with four repetitions of 10 synthetic seeds. Data of shoot emergence percentage were scored until stabilization or after four weeks in culture. Data were subjected to analysis of variance (ANOVA) and means separation using SNK at 0.05 level using Statgraphics software, version 7.0.
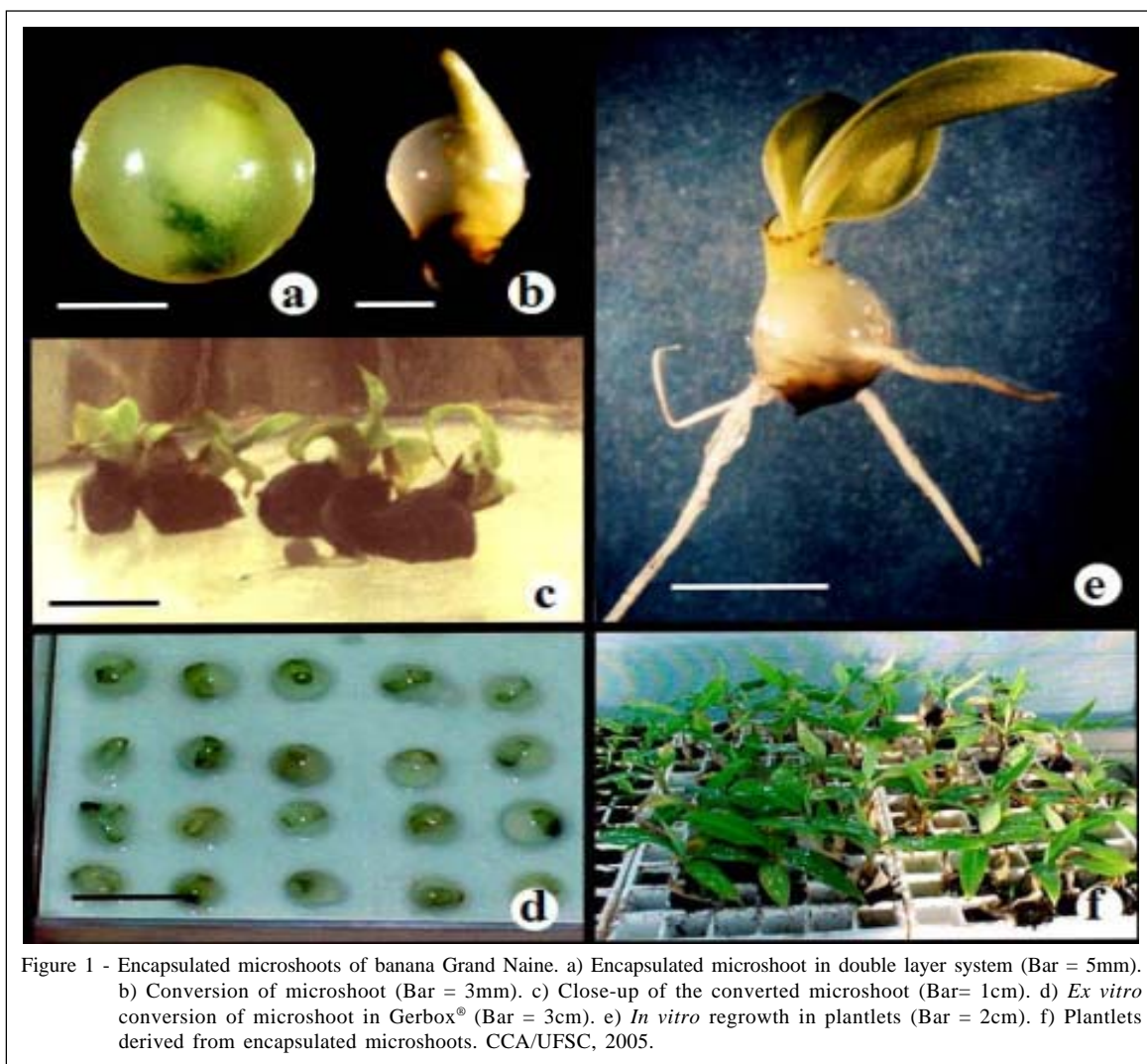
RESULTS

The ionotropic gelation of sodium alginate by Ca^{2+} ions was an efficient procedure for the production of synthetic seeds of banana (Figure 1a). The systems of single layer and double layer showed 80% and 70% conversion, respectively, without statistical differences between these treatments (Table 1). A significant low conversion percentage (15%) was observed using pharmaceutical capsules. Leakage of culture medium was often observed using pharmaceutical gelatin capsules, which in turn drastically reduced the conversion percentage.

The highest (76%) shoot emergence was obtained in the treatment with KNO_3 and MS (Table 2). The conversion occurred after two weeks in culture (Figure 1b). The lowest shoot emergence rate (32%) was observed in the capsules without MS and KNO_3 (Table 2).

The incorporation of a fungicide to avoid contaminants is an appropriate strategy to increase shoot emergence or conversion of synthetic seeds. In the present study, *in vitro* microshoot emergence capacity was influenced by the interaction between activated charcoal and the fungicide benomyl. Shoot emergence increased significantly (P≤0.05) to 75.0% in the presence of 1.5g L^{-1} activated charcoal and 0.5g L^{-1} benomyl (Table 3). However, the use of fungicide alone in the matrix (i.e. without charcoal) negatively affected the microshoots conversion (36.2%) to plantlets. Additionally, activated charcoal reduced the browning of microshoots and enhanced the plantlets vigor (Figure 1c).

The encapsulated and non-encapsulated microshoots showed 100% shoot emergence in response to treatments with MS solely or in combination with PEG (10% and 20%), after a short-term storage at 4°C for 10 days. In the treatment with

Figure 1 - Encapsulated microshoots of banana Grand Naine. a) Encapsulated microshoot in double layer system (Bar = 5mm). b) Conversion of microshoot (Bar = 3mm). c) Close-up of the converted microshoot (Bar= 1cm). d) *Ex vitro* conversion of microshoot in Gerbox® (Bar = 3cm). e) *In vitro* regrowth in plantlets (Bar = 2cm). f) Plantlets derived from encapsulated microshoots. CCA/UFSC, 2005.

10% DMSO, the encapsulated and non-encapsulated microshoots showed viability of 90 and 60% respectively. The use of 20% DMSO resulted in 20% viability in encapsulated microshoots. No conversion of the non-encapsulated microshoots was observed and this may be due to DMSO toxicity (data not shown).

Since the presence of sucrose may affect the further growth of the capsules, the presence or absence of sucrose in the capsule was tested. Sucrose-free capsules showed high (P≤0.01) *in vitro* shoot emergence (83.3%) compared to those containing 30g l^{-1} sucrose (56.7%). Sucrose-free synthetic seeds cultivated in Gerbox® boxes (Figure 1d) had 20% conversion. On the other hand, all the capsules containing sucrose got contaminated (data not shown). Sucrose-free capsules did not contaminate and enhanced the quality of the converted plantlets (Figure 1e).

In the present study, the microshoots encapsulated in sucrose-free capsules had a high potential to convert into plantlets on culture medium containing only agar and water (Figure 1c). These

Table 1 - Conversion rate of banana cv. Grand Naine microshoots in response to different encapsulating techniques. CCA/UFSC, 2005.

Technique	Conversion rate (%)[1]
Single layer	80.0a
Double layer	70.0a
Pharmaceutical capsules	15.0b
Mean	55.0
CV (%)	14.8

[1]Treatments followed by the same letter are not different (SNK test at P=0.05).

Table 2 - Conversion rate (%) of banana cv. 'Grand Naine' microshoots in response to MS saline formulation (MURASHIGE & SKOOG, 1962) culture medium-enriched matrix and self-breaking with KNO_3. CCA/UFSC, 2005.

------------Treatments------------		Conversion rate (%)[1]
MS	KNO_3	
Absence	0	32.0b
Absence	100	56.0a
Presence	0	68.0a
Presence	100	76.0a
Mean		58.0
CV (%)		24.8

[1]Treatments followed by the same letter are not different (SNK test at P=0.05).

plantlets were successfully acclimatized, and after 13 weeks 90% survival was observed (Figure 1f). Therefore, different strategies may be used regarding the germination of the encapsulated microshoots (i.e. direct transfer to *ex vitro* conditions or *in vitro* conditions with only water-agar culture medium).

DISCUSSION

Synthetic seeds are useful strategies for the field delivery of *in vitro*-grown propagules, and different systems for the encapsulation have been developed, including the encapsulation with calcium alginate or the use of pharmaceutical capsules. The system of single encapsulation based on sodium alginate beads hardened in $CaCl_2$ was developed by REDENBAUGH et al. (1993), and subsequently it described the system

Table 3 - Conversion rate (%) of banana cv. 'Grand Naine' microshoots in response to activated charcoal and benomyl supplemented to the alginate matrix. CCA/UFSC, 2005.

Treatments		Conversion rate (%)[1]
Benomyl (g L^{-1})	Activated charcoal (g L^{-1})	
0.0	0.0	58.5b
0.5	0.0	52.7bc
1.0	0.0	36.2c
0.0	1.5	52.5bc
0.5	1.5	75.0a
1.0	1.5	56.5b
Mean		55.2
CV (%)		17.4

[1]Treatments followed by the same letter are not different (SNK test at P=0.05).

of hollow beads or double layer based on the ionotropic gellification of the calcium alginate (PATEL et al., 2000). In the present study, no difference was observed between the two encapsulation techniques using calcium alginate (single and double layer). The technique of pharmaceutical capsules resulted in low conversion rate since the microshoots were not able to disrupt the synthetic layer of the gelatin capsule or severe leaking of medium was observed. In contrast, carrot somatic embryos were successfully encapsulated using this technique and resulted in 90% conversion (DUPUIS et al., 1994).

It has been shown that the nutrients to be included in the alginate matrix should be adapted according to propagule type and plant species (NAIK & CHAND, 2006; SINGH et al., 2006). However, the complex composition of endosperm makes difficult its reconstitution and the use of standard culture medium in the capsule may increase the conversion of the propagule to plantlets (MAMIYA & SAKAMOTO, 2000). This is in agreement with our results, where the addition of MS in the alginate matrix together with KNO_3 treatment increased the microshoots conversion, however there were no differences with the treatment without MS but there were with the KNO_3 treatment. These results show that capsule weakening through KNO_3 treatment results in high conversion capacity of the shoots, which is improved by an additional nutrient supply.

Fungicides and antibiotics have beneficial effects against contamination of the synthetic seeds in several species, including banana (GANAPATHI et al., 1992). Similarly, in pineapple the use of antibiotics in the matrix capsule resulted in 86.1% conversion of microshoots (GANGOPADHYAY et al., 2005). In the present work the presence of benomyl (1g L^{-1}) alone negatively affected the viability of the encapsulated microshoots. However, the use of a lower concentration (0.5g L^{-1}) combined with activated charcoal significantly increased the conversion of the microshoots. The incorporation of a fungicide in the capsule to eradicate contaminants without harming the cultures was also an appropriate strategy for the micropropagation of *Hibiscus moscheutos* (WEST & PREECE, 2006).

An interesting strategy for the delivery of synthetic seeds involves their storage (NAIK & CHAND, 2006). In the present study, no dehydration was necessary for the short-term storage (10 days at 4°C) and the microshoots showed 100% shoot emergence in response to the treatments containing MS solely or in combination with PEG (10% and 20%). Treatments employing DMSO actually reduced the shoot emergence.

The contamination of the synthetic seeds was low in the absence of sucrose and the synthetic seeds also showed higher *in vitro* conversion capacity when compared to sucrose-enriched gelling matrix. In contrast, it was showed (MATSUMOTO et al., 1995) that encapsulated banana shoot tips were able to grow only in the presence of sucrose in the culture medium or in the capsules. Probably in the absence of sucrose the microshoots were forced to undergo photosynthesis. It is well known that a regulative feed-back occurs in response to the presence of sugars and the photosynthetic capacity of the plants, as shown in banana (NGUYEN & KOZAI, 2001). The conversion of encapsulated somatic embryos of asparagus showed an inverse correlation among the levels of sucrose in the capsule and the fresh weight of the plantlets (MAMIYA & SAKAMOTO, 2000). In Kiwi, however, the addition of sucrose to the conversion medium enhanced the conversion to plantlets of microcuttings encapsulated in a calcium-sodium alginate solution (ADRIANI et al., 2000).

In summary, the protocol for the production of banana synthetic seeds of microshoots includes the use of alginate capsules containing sucrose-free MS salts, activated charcoal (1.5g L^{-1}) and benomyl (0.5g L^{-1}), and KNO_3 solution (100mM) treatment for 20min for weakening of the capsules. The synthetic seeds showed high survival rate after short-term (10 days) storage, where no special treatments were required. The encapsulation system tested with banana microshoots without any sucrose and plant growth regulators enables direct delivery of the *in vitro* propagules without any *in vitro* rooting step. The main advantage over other technologies developed for banana microshoots refers to the use of sugar-free capsules and the *ex vitro* growth using Gerbox® with moistened paper or the *in vitro* conversion on a very simple culture medium, containing only gelled water. Therefore, this new technology has direct impact on the delivery of the *in vitro* propagules with suitable costs.

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