Chapter 30

From Somatic Embryo to Synthetic Seed in *Citrus* spp. Through the Encapsulation Technology

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Abstract

In vitro propagation by somatic embryogenesis represents an efficient alternative method to produce high-quality and healthy plants in *Citrus* species. The regenerated somatic embryos need protection from mechanical damages during manipulation and transport, as well as nutritive support for their evolution in plantlets after sowing. The encapsulation technology allows to obtain synthetic seeds by covering somatic embryos with a gel of calcium alginate enriched by nutrients. This chapter describes the procedure for producing synthetic seeds containing somatic embryos from different *Citrus* genotypes.

Key words Artificial seed, Calcium alginate matrix, Plant tissue culture, Somatic embryogenesis, Synseed

1 Introduction

The increasing world's demand for new and promising *Citrus* genotypes requires effective and innovative technologies for high-quality plant production. Consequently, research is looking for an innovative procedure able to join the advantages of micropropagation (high productive efficiency, sanitary plant conditions, and reduced space requirements) with the technologic characteristics of the zygotic seed, as handling, storability, and transportability [1], actually represented by the synthetic seed technology. The original concept of synthetic seed (artificial seed or synseed) was applied to desiccated or hydrated somatic embryos (SEs) and did not involve the encapsulation [2, 3]. Later Murashige [4] gave the first definition of synthetic seed as "an encapsulated single SE inside a covering matrix."

The large use of sodium alginate as encapsulating agent is due to its moderate viscosity, low spin ability of solution, low toxicity, quick gellation, low cost, and biocompatibility characteristics [5–7]. The encapsulation technology was proposed to safeguard the SEs from mechanical damages during handling in the nursery and

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transportation in the farms, as well as to provide nutrients (*artificial endosperm*) during their evolution in plantlets under in vivo or in vitro conditions (*conversion*). In fact, SEs are structurally similar to gamic or zygotic embryos, but lack nutritive and tegument structures [5]. Nevertheless, the first experiments on the encapsulation were conducted employing SEs, as their bipolar nature, able to convert in plantlets in a single step, made them suitable for synthetic seed production [2, 8]. SEs develop from somatic cells, and this regenerative pathway allows the clonal propagation. Their use as encapsulated explants for synthetic seed preparation is however limited because of the involved difficulties, due to asynchronism during SEs formation and development, somaclonal variation, recurrent embryogenesis [8], and embryo dormancy [9]. Moreover in vitro SE production requires expensive manual labor, even though they could be obtained by bioreactors [10]. Therefore, different propagules were tested to produce synthetic seeds. New perspectives emerged with the use of non-embryogenic unipolar plant propagules. In fact, the most recent concept involves every meristematic tissues (in vitro or in vivo derived), as long as able to convert in a whole plantlet after encapsulation and possible storage [5, 7, 8, 11–14]. However, the abovementioned limitations of SEs for synthetic seeds production seem to be infrequent in *Citrus* spp., and several studies are focused on the application of the encapsulation technology to citrus [7, 15–21].

2 Materials

2.1 Plant Material

Since some researchers found that the SE size affects the conversion in different plant species [22–25], we carried out preliminary experiments using different sized SEs of *Citrus* genotypes for encapsulation (unpublished data). The results indicated that the largest SEs (5–6 mm) showed the highest values in terms of *viability* (green appearance of explants, with no necrosis or yellowing), *regrowth* (increasing in size of the explants with consequent breakage of the involucre and extrusion of at least one visible shoot or root after the sowing), and conversion [16, 17]. Nevertheless their encapsulation involves the formation of an irregular alginate layer around the propagule, reducing the protective and nutritive functions. So, in our experiments, we used only medium-sized SEs (3–4 mm) discarding the larger and the smaller ones (Fig. 1), hence limiting the negative effects of asynchronism (*see* **Note 1**) and recurrent embryogenesis (*see* **Note 2**).

Usually our experiments were carried out using hydrated SEs of *Citrus reticulata* Blanco cv Mandarino Tardivo di Ciaculli, *Citrus limonimedica* Lushington, and *Citrus clementina* Hort. ex Tan. cvs Nules and Monreal, obtained according the procedures described

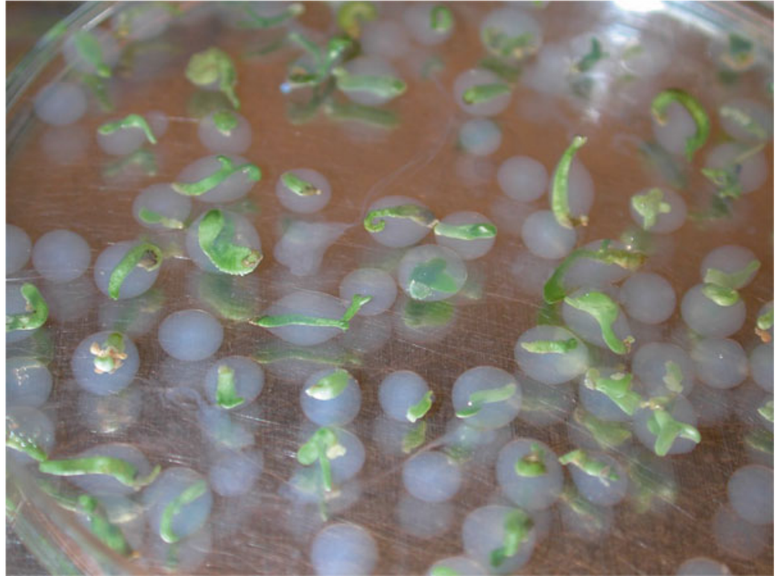

Fig. 1 Synthetic seeds obtained from different sized SEs of *Citrus*

by Germanà and co-workers [26–30]. The synthetic seeds of these genotypes were sown and maintained in aseptic conditions. In addition, synthetic seeds of *Citrus reticulata* Blanco cv Mandarino Tardivo di Ciaculli were sown also in non sterile conditions.

2.2 Encapsulation Solutions

1. Tissue culture facilities: Graduate cylinders, pipettes, lab pipettor, glass beakers, magnetic stirrer, spin bar, analytical balance, lab spoons, weighing boats, pH meter, NaOH and HCl solution (0.1 N), 100 mL screw capped Pyrex glass jars, autoclave, horizontal flow cabinet, forceps, scalpels, blades, and electric incinerator.

2a. Aseptic conditions: Distilled water, half strength MS basal medium [31], 0.25 g/L malt extract, 0.25 g/L ascorbic acid, 1 mg/L gibberellic acid, (GA_3), 0.02 mg/L a-naphthalene acetic acid (NAA), and 68 g/L sucrose (artificial endosperm).

b. Nonsterile conditions: Artificial endosperm and 100 mg/L Thiophanate-methyl TM® (Pestanal, Riedel-de-Haen).

3. Alginate sodium salt, medium viscosity (2.5 % w/v).
4. Calcium chloride anhydrous (1.1 % w/v).

2.3 Sowing Media and Culture Conditions

1. Tissue culture facilities: Graduate cylinders, pipettes, lab pipettor, glass beakers, magnetic stirrer, spin bar, analytical balance, lab spoons, weighing boats, pH meter, NaOH and HCl solution (0.1 N), Magenta® jars (7 × 7 × 7 cm), autoclave, horizontal flow cabinet, forceps, scalpels, blades, electric

incinerator, and growth chamber (temperature of 21 ± 2 °C, photosynthetic photon flux density of 40 μmol/m²/s, and photoperiod 16 h).

2a. Aseptic conditions: Distilled water, full strength MS basal medium [31], 0.5 g/L malt extract, 0.5 g/L ascorbic acid, 68 g/L sucrose and 7 g/L agar, and filter paper bridges.

2b. Nonsterile conditions: Filter paper bridges, perlite, soil (Compo-Cactea®), or Jiffy-7 Pellets (J7).

3 Methods

Three solutions are required to encapsulate SEs: *coating*, *complexing*, and *rinsing* solutions (Fig. 2). The common component is represented by the artificial endosperm (*see* Subheading 2.2) added of 2.5 g/L sodium alginate (coating matrix) and 1.1 g/L calcium chloride (complexing solution). The rinsing solution is composed only by the artificial endosperm. All solutions and media are adjusted to pH 5.5 and autoclaved at 115 °C for 20 min just after their transferring into the containers. During the autoclaving cycle, the sodium alginate is completely dissolved forming a dense dark yellow solution. The artificial endosperm of the synthetic seeds sown in nonsterile conditions is enriched by Thiophanate-methyl TM® (*see* **Note 3**).

Fig. 2 Coating, complexing, and rinsing solutions employed for encapsulation of *Citrus* SEs (*from left to right*)

3.1 Encapsulation

1. Single SEs are immersed in alginate solution for a few seconds (*see* **Note 4**).
2. The alginate-coated SEs are then dropped into the complexing solution for 25–30 min (*see* **Note 5**).
3. The encapsulated SEs are washed 2–3 times in the rinsing solution for 10–15 min in order to remove the toxic residual ions of chloride and sodium (*see* **Note 6**). The whole procedure is carried out in aseptic conditions under a horizontal flow cabinet.

3.2 Sowing and Evaluation

1a. Aseptic conditions: After washing, the synthetic seeds are aseptically transferred into closed Magenta® jars, containing sterilized agar sowing medium or filter paper bridge, moistened with 10 mL of artificial endosperm (*see* **Note** 7).

b. Non-sterile conditions: After washing the synthetic seeds are aseptically transferred into Magenta® jars containing sterilized filter paper bridge, perlite, soil (Compo-Cactea®), or "Jiffy-7 Pellets" (J7) moistened with appropriate amount of artificial endosperm (*see* **Note** 7).

2a. Aseptic conditions: The Magenta® jars are hermetically closed, and the cultures are transferred into the growth chamber.

b. Nonsterile conditions: The cultures are then transferred into the growth chamber, and the Magenta® jars are not hermetically closed, allowing the gas exchanges and the water evaporation. To prevent the synthetic seeds dehydration, the substrates moisture is periodically monitored and restored with distilled water.

3. After 1 week, fungal or bacterial contamination is monitored.
4. After 45 days, viability, regrowth and conversion (Fig. 3) are evaluated.

4 Notes

1. The asynchronism involves the simultaneous presence of different sized SEs at the end of regenerative cultures. Their encapsulation determines the formation of heterogenous synthetic seeds with different ability and energy of conversion. So, the synchronism is a crucial step in taking advantage of somatic embryogenesis for the commercial production of plants by synthetic seeds.
2. Recurrent or secondary somatic embryogenesis in the production of new SEs from the mature ones.
3. The application of synthetic seeds in the nurseries should imply their conversion in non-sterile conditions using substrates as perlite, sand, paper, or peat. In this case, the protection of the

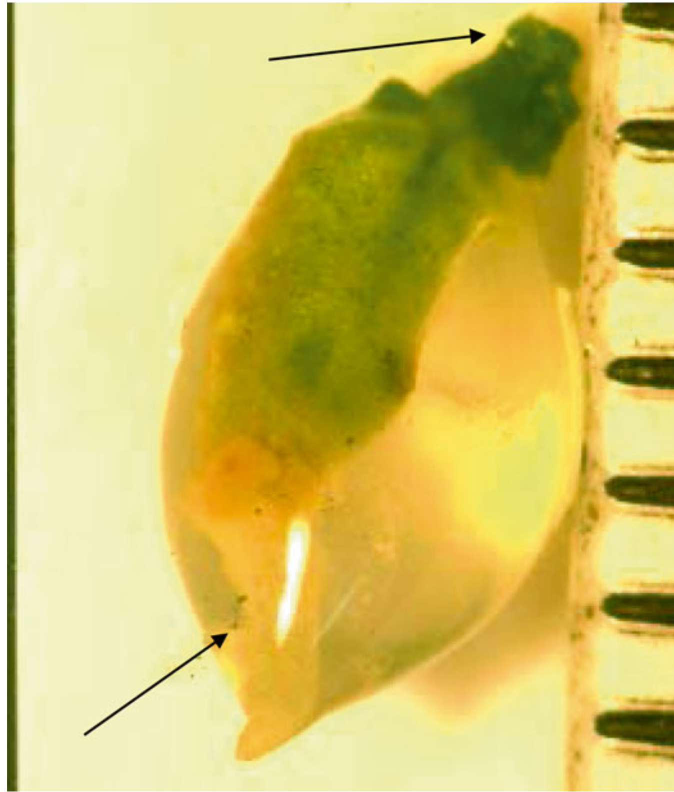

Fig. 3 Extrusion of shoot and root apex (*black arrows*) from the alginate matrix at the beginning of conversion

synthetic seeds from fungal and bacterial contaminations during conversion is essential. The beneficial effect of Thiophanate-methyl fungicide on the *Citrus* synthetic seeds conversion has been showed [17].

4. In substitution to sodium alginate, several substances were tested, like mixture of sodium alginate with gelatin, potassium alginate, polyco 2133, carboxymethyl cellulose, carrageenan, Gelrite, guar gum, sodium pectate, and tragacanth gum [5–7].

5. During the complexation step, ion exchange occurs through the replacement of Na^{+} by Ca^{2+}, forming calcium alginate by ionic cross-linking among the carboxylic acid groups and the polysaccharide molecules and producing a polymeric structure called "egg box" [13, 32, 33]. Hardening of calcium alginate bead is affected by the concentration of sodium alginate and calcium chloride, as well as the complexing time. Usually, at higher consistence corresponds good protection during transport and manipulation but higher difficulty of explants in breaking the alginate coat [8].

6. Automation systems have been proposed, as somatic embryogenesis and encapsulation are expensive techniques due to the high manual labor requirement. The use of bioreactors for temporary immersion system has shown to be effective for the production of *Citrus deliciosa* SEs [34]. Concerning automation, several devices are available for the encapsulation of SEs or other in vitro-derived vegetative propagules, using systems based on concentric tube nozzle, multiple wire loops, rotating disks, perforated plates, or precision dripping [10, 35, 36].
7. Before sowing, the synthetic seeds can be stored at 4–6 °C in darkness, using closed sterile dishes or vials containing some drops of artificial endosperm solution to avoid dehydration.

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