Chapter 9

Plant Regeneration of *Agave cupreata* by Somatic Embryogenesis in a Temporary Immersion System with Silver Nanoparticles

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Abstract

Somatic embryogenesis in *Agave* genus has been induced; however, it is desirable to increase the rate of growth to get a more efficient propagation system. In this chapter, we present in detailed a protocol for somatic embryogenesis in *Agave cupreata* and the use of silver nanoparticles in a temporary immersion system. This is an efficient method that can be used commercially to improve the production and germination of somatic embryos.

Key words *Agave cupreata*, Temporary immersion system, Somatic embryogenesis, Silver nanoparticles

1 Introduction

Throughout the world, climate change and the extension of markets for some agave products, such as fructans, inulin, biofuels, tequila, mezcal and fermented sap, etc., have caused a growing interest in these plants in the national and global context. This demand has been accompanied by the overexploitation of wild populations, illegal looting of plants, and the destruction of their habitat [1]. Naturally, these plants reproduce sexually and/or asexually; the first requires 7–35 years to reach maturity and the second is limited in some species [2]. An alternative to conventional propagation in agave is the use of tissue cultures and plant cells, to easily obtain new seedlings in a short time and on a large scale [3]. Agave seedlings obtained by somatic embryogenesis can improve their quality with the use of temporary immersion system (TIS) [4]. Recently, the use of TIS has allowed to reduce production costs and increase the multiplication rate gates in these plants

Marco A. Ramírez-Mosqueda and Carlos A. Cruz-Cruz (eds.), *Micropropagation Methods in Temporary Immersion Systems*, Methods in Molecular Biology, vol. 2759, https://doi.org/10.1007/978-1-0716-3654-1_9,

[5]. However, contamination is a serious problem in TIS during the micropropagation process [6]. The presence of endophytic contaminants causes high losses in the in vitro culture of plants [7]. In A*gave*, *Bacillus licheniformis* has been found as an endophytic organism, which affects in vitro propagation procedure [8]. An environmental friendly strategy for the control of microorganisms is the green synthesis of silver nanoparticles using *Agave* leaves [9, 10], which organs have been reported to have antimicrobial activity due to their saponin content [11, 12].

2 Materials

2.1 Plant Material

1. *Agave cupreata* seeds.

2.2 Reagents

1. Seed sterilization solution: 20 mL anti-benzyl surgical soap and two drops Tween 20.
2. Ethanol: 70% (v/v) solution in water.
3. Sodium hypochlorite: 1% (v/v) solution in water.
4. Silver nanoparticles (AgNP) biosynthesized from *Agave cupreata* with a particle size of 2.1 ± 0.8 nm.

2.3 Instrumentation

1. 50 mL sterile Falcon tubes.
2. Scalpel and forceps.
3. Sterile plastic Petri dishes (100 mm × 15 mm).

2.4 Equipment

1. Magnetic stirrer.
2. Fridge.
3. Stereo microscope.
4. Twin Bottle-type temporary immersion system (*see* Fig. 1).

2.5 Media

The culture media commonly used are based on modifications of the MS medium [13]. Supplements such as growth regulators, gelling agents, and other additives are specifically indicated for each stage in the protocol (Table 1).

1. Embryogenic callus induction medium (ECIM): modified MS medium supplemented with 5 mg/L 2,4-D, 3 mg/L BA, and 60 g/L sucrose. Adjust the pH to 5.7 with 0.1 N NaOH or 0.1 N HCl. Add 8.0 g/L of agar and then autoclave at 121 °C for 20 min. Pour 20 mL of culture medium into Petri dishes (100 mm × 15 mm).
2. Somatic embryo maturation and expression medium (SEMEM): modified MS medium supplemented with 100 mg/L putrescine and 30 g/L sucrose. Adjust the pH to 5.7 with 0.1 N NaOH or 0.1 N HCl. Add 8.0 g/L of agar and

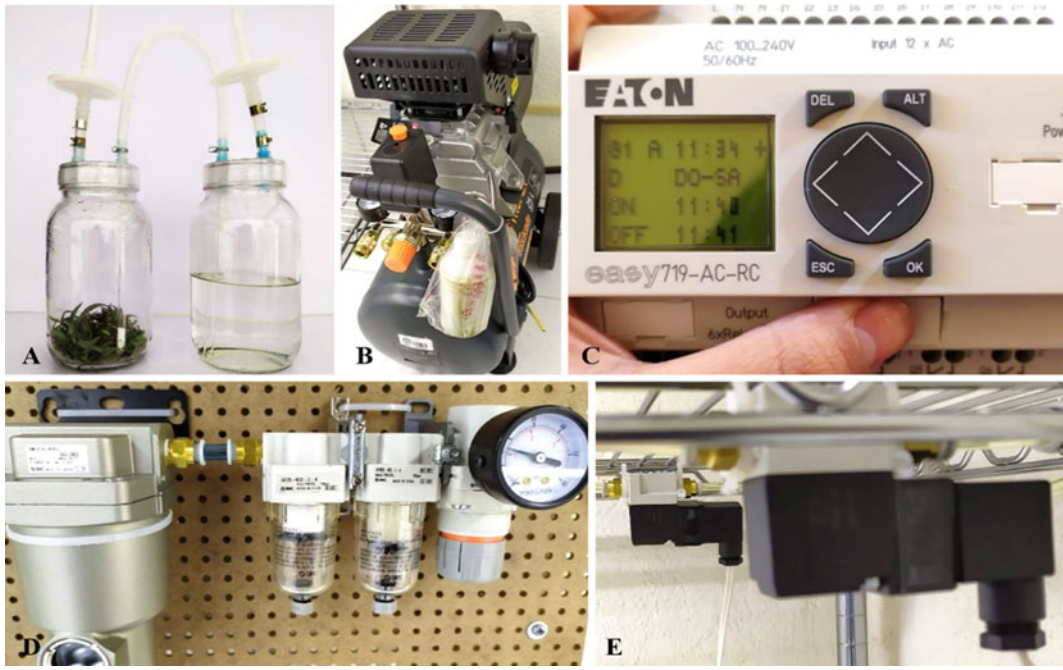

Fig. 1 Parts of a twin vial temporary immersion system (TIS). (**a**) TIS with two twin flasks, one is used as a deposit of the plant material and the other contains medium culture, (**b**) air compressor that allows the flow of air from one container to another, (**c**) programmable logic controller (PLC) for establish the times and frequencies of immersion, (**d**) filters and manometer to purify and regulate the flow of air, (**e**) solenoid valves to control the flow of air between containers

then autoclave at 121 °C for 20 min. Pour 20 mL of culture medium into Petri dishes (100 mm × 15 mm).

3. Somatic embryo germination and growth medium (SEGGM): modified MS liquid medium supplemented with 30 g/L sucrose. Adjust the pH to 5.7 with 0.1 N NaOH or 0.1 N HCl. Autoclave at 121 °C for 20 min.

3 Methods

3.1 Seed Sterilization

1. In the laminar flow hood, transfer seeds to a Falcon tube with the seed sterilization solution, and shake with a magnetic stirrer for 15 min.
2. Discard the solution anti-benzyl surgical soap and Tween 20, and add 70% ethanol solution and shake for 1 min.
3. Discard the 70% ethanol solution, add 1% sodium hypochlorite solution, and shake for 1 min.
4. Discard the 1% sodium hypochlorite solution, and rinse three times with sterile distilled water.
5. Store the seeds in a Falcon tube with sterile distilled water in a refrigerator at 4 °C for 24 h (*see* **Note 1**).

Table 1
Components of the media culture used in each phase of the somatic embryogenesis in *Agave cupreata*, including mineral salts, growth regulators, vitamins, and pH

Component	Embryogenic callus induction medium (mg/L)	Somatic embryo maturation and expression medium (mg/L)	Somatic embryo germination and growth medium (mg/L)
Ammonium nitrate	412.5	825	825
Potassium nitrate	475	950	950
Calcium chloride	110	220	220
Magnesium sulfate	92.5	185	185
Potassium phosphate	42.5	85	85
EDTA disodium salt	9.325	18.65	18.65
Ferrous sulfate	6.95	13.9	13.9
Manganese sulfate	5.575	11.15	11.15
Zinc sulfate	2.15	4.3	4.3
Boric acid	1.55	3.1	3.1
Potassium iodide	0.2075	0.415	0.415
Sodium molybdate	0.0625	0.125	0.125
Cupric sulfate	0.00625	0.0125	0.0125
Cobalt chloride	0.00625	0.0125	0.0125
2,4-Dichlorophenoxyacetic (2,4-D)	5		
6-Benzylaminopurine (BA)	3		
Putrescine		100	
Sucrose	60[a]	30[a]	30[a]
Myoinositol	250	50	50
Thiamine	2.5	0.05	0.05
Pyridoxine	0.5	0.25	0.25
Nicotinic acid		0.25	0.25
Agar	8[a]	8[a]	
pH	5.7	5.7	5.7

[a]g/L

3.2 Induction of Embryogenic Callus from Zygotic Embryos

1. In the laminar flow hood, under aseptic conditions, extract the zygotic embryo from the seeds with forceps and a scalpel, with the aid of a stereoscopic microscope (*see* **Note 2**).
2. Establish 10 zygotic embryos per Petri dish containing ECIM, and seal with parafilm or plastic wrap.
3. Incubate in the dark at 25 ± 2 °C in the growth room for 30 days (*see* **Note 3**).
4. Transfer callus to fresh ECIM.
5. Incubate in the dark at 25 ± 2 °C in the growth room for additional 30 days (*see* **Note 4**).

3.3 Expression and Maturation of Somatic Embryos

1. Transfer embryogenic callus to SEMEM.
2. Incubate in the dark at 25 ± 2 °C in the growth room for 30 days (*see* **Note 5**).
3. Transfer callus to fresh SEMEM (*see* **Note 6**).
4. Incubate in the dark at 25 ± 2 °C in the growth room for additional 30 days (*see* **Note 7**).

3.4 Germination and Growth of Somatic Embryos

1. Transfer scutellar stage somatic embryos to the twin bottle temporary immersion system plant jar and SEGGM medium (*see* **Notes 8**, **9**, and **10**).
2. After medium sterilization, it was added to different concentrations (0, 100, and 200 mL/L) of biosynthesized silver nanoparticles from *Agave cupreata* to stop microbial growing (*see* **Note 11**).
3. Program the PLC to have an immersion frequency of 2 min every 8 h.
4. Incubate at 25 ± 2 °C with a photoperiod of 16 h in the growth room, for 45 days.

4 Notes

1. It is necessary to keep the seeds in sterile distilled water to favor the softening of the testa.
2. Extract the entire zygotic embryo, without causing mechanical damage (*see* Fig. 2a).
3. Callus is induced in the zygotic embryo after 3 days in ECIM medium. Auxins, mainly 2,4-D, are important for the acquisition of embryogenic capacity and for the initial stimulation of somatic embryogenesis (*see* Fig. 2b).
4. The callus proliferates and is maintained in the same ECIM medium (*see* Fig. 2c).

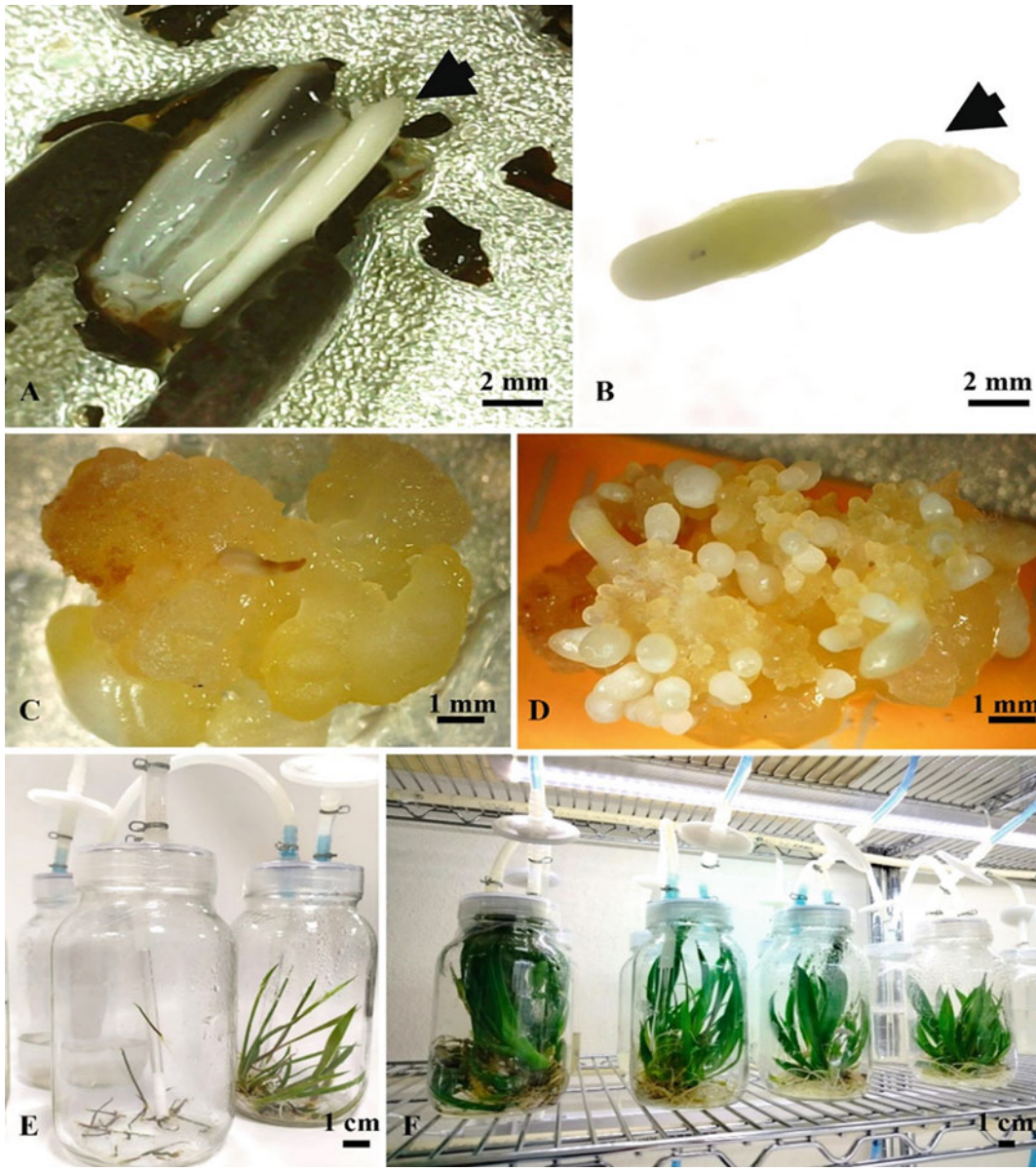

Fig. 2 Somatic embryogenesis process in a twin vial temporary immersion system. (**a**) Extraction of the zygotic embryo from the seed (arrow), (**b**) callus (arrow) obtained from the zygotic embryo after 3 days in ECIM medium, (**c**) callus obtained after 60 days in ECIM medium, (**d**) somatic embryos obtained after 60 days in SEMEM medium, (**e** and **f**) regenerated plantlets of *A. cupreata* from somatic embryos in a twin flask temporary immersion system

5. Expression of somatic embryos is observed after 15 days in the SEMEM medium.
6. This promoting action of putrescine on somatic embryogenesis may be due to efficient conversion of competent cells into embryos [14].

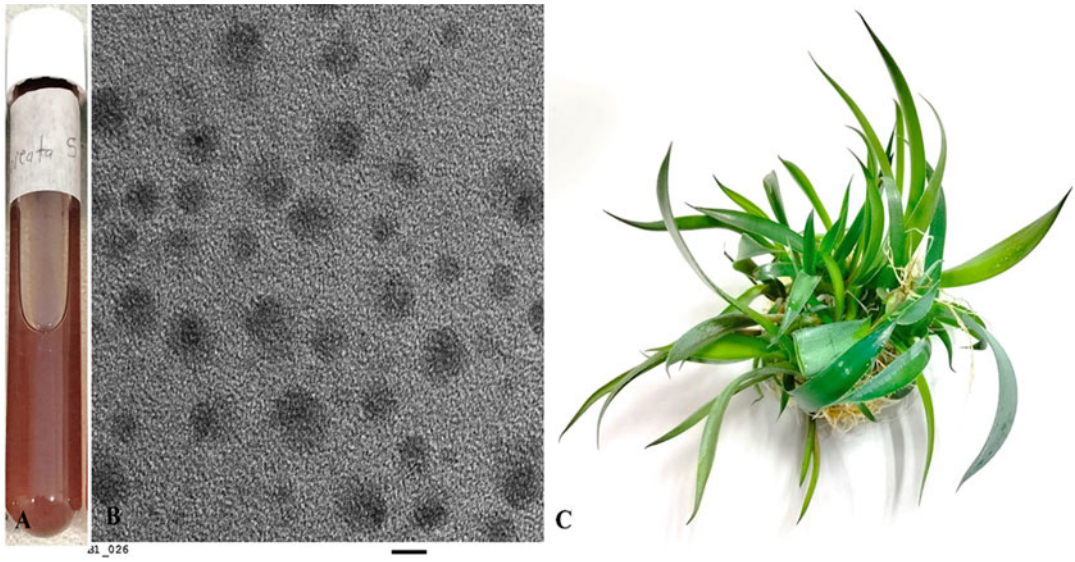

Fig. 3 Effect of silver nanoparticles (AgNP) biosynthesized from *Agave cupreata* extract on the germination and growth of *A. cupreata somatic embryos* in TIS. (**a**) AgNP biosynthesized from *A. cupreata* extract, (**b**) AgNP image obtained from a transmission electron microscope (TEM 2010 JEM at 200 kV acceleration in brightfield mode) (Bar = 20 nm), (**c**) seedlings regenerated from somatic embryos in a TIS supplemented with biosynthesized AgNP

7. Mature embryos can be obtained at 45–60 days on SEMEM medium (*see* Fig. 2d).
8. Selection the somatic embryos in the scutellar stage with whitish coloration.
9. The twin flask temporary immersion system is efficient in somatic embryo germination (*see* Fig. 2e).
10. Germination of the somatic embryos is a similar process that those happened in zygotic embryos; thus, in somatic embryogenesis, an apical part and a root part are generated without the need for a rooting stage (*see* Fig. 2f).
11. Silver nanoparticles biosynthesized from *Agave cupreata* extract reduce contamination in TIS, and with a concentration of 200 μL/L, they completely inhibit its growth (*see* Fig. 3).
12. The effectiveness of AgNP in the stop growing microbial contaminants from in vitro cultures depends on AgNP size, shape, and type of coating [15].
13. AgNP favor the germination percentage of somatic embryos, without affecting their growth rate.
14. Silver nanoparticles are more effective in a liquid medium; however, using them in a semisolid medium makes their diffusion difficult [6].

15. The use of polyethylene foam as a support in the TIS promotes hyperhydricity of plants; therefore, it is convenient to place a mesh in the glass tube of the container of plant material, to prevent somatic embryos from being transferred to the container of medium.
16. Using this protocol, high-quality, easy-to-handle plantlets are regenerated, and the oxygenation of the medium and gas exchange through the stationary medium are kept under control.

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