Chapter 16
Somatic Embryogenesis of Alpataco (*Prosopis alpataco* L.)

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16.1 Introduction

Prosopis alpataco Phil. (Fabaceae, Mimosoideae) is a native woody species from the arid region. It is normally found in the central *Monte* desert, *Argentina* (Vega Riveros et al. 2011; Catalano et al. 2008; Villagra and Roig 2002; Kiesling 1994) where great part of Patagonia steppe is included. It is commonly known as "alpataco", quechua word that means "tree with subterraneous branches". It is a multifunction species with great importance for soil support and wildlife refuge. The alpataco is also an economically valued species, being used as food, fuel wood and medicinal resource by the indigenous communities of the arid Patagonian (Ragonese and Martínez-Crovetto 1947; Ruiz Leal 1972; Portal 1996; Steibel 1997; Rapoport et al. 2003; Ladio and Lozada 2009; Boeri et al. 2016). Its wood is used for fuel and charcoal production. Currently, overgrazing and logging are degrading this important genetic resource.

The genus *Prosopis* is sexually produced, which is a disadvantage in the establishment of the seedlings, depending on the sites and the specie. The vegetative propagation of this genus has a great interest to advance the domestication, improvement, restoration and conservation of these multi-purpose plants. There are some reports on the micropropagation of different *Prosopis* species including

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P. tamarugo and *P. chilensis* (Jordan and Balboa 1985; Jordan 1987), *P. alba* (Jordan et al. 1985; Tabone et al. 1986; Castillo de Meier and Bovo 2000), *P. juliflora* (Nandwani and Ramawat 1991), *P. cineraria* (Goyal and Arya 1984; Shekhawat et al. 1993), *P limensis* (Minchala-Patiño et al. 2014), *P. glandulosa* (Rubluo et al. 2002) and *P. laevigata* (Buendía-González et al. 2007, 2012). However, there is only one protocol reported for plant regeneration via somatic embryogenesis in the genus *Prosopis* (which comprises about 44 species) (Buendía-González et al. 2007). There is no protocol available for in vitro plantlet regeneration of alpataco. This chapter describes the embryogenic callus induction, somatic embryo development and germination of *P. alpataco* from cotyledonary explants.

16.2 Materials and Methods

All the cultures steps must be incubated in a plant growth chamber under controlled conditions of temperature (25 ± 2 °C), under a 16 h light: 8 h dark photoperiod (provided by fluorescent tubes) and average photosynthetic photon flux of 50 µmol $m^{-2}s^{-1}$.

Basic medium composition is listed in Table 16.1. Required modifications for different culture stages are listed in Table 16.2. The pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min.

Table 16.1 Chemical composition of *Prosopis alpataco* basic culture medium (Murashige and Skoog media)

Ingredients	mg/L
Ammonium nitrate	1650.000
Boric acid	6.200
Cobalt chloride.6H_2O	0.025
Copper sulphate.5H_2O	0.025
EDTA disodium salt.2H_2O	37.300
Ferrous sulphate.7H_2O	27.800
Glycine (free base)	2.000
Magnesium sulphate	180.690
Manganese sulphate.H_2O	16.900
Molybdic acid (sodium salt).2H_2O	0.250
Myo-inositol	100.000
Nicotinic acid (free acid)	0.500
Potassium iodide	0.830
Potassium nitrate	1900.000
Potassium phosphate monobasic	170.000
Pyridoxine hydrochloride	0.500
Thiamine hydrochloride	0.100
Zinc sulphate.7H_2O	8.600

The pH was adjusted to 5.8–6.2 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min

Table 16.2 Formulations of *Prosopis alpataco* media

	Stage I Initiation explant preparation	Stage II Induction SE culture medium	Stage III SE proliferation	Stage IV Maturation	Stage V Germination
Sucrose	30,000	30,000	30,000	30,000	30,000
2,4-D	–	2	–	–	–
IBA	–	–	–	–	
BA	–	1.5	–		–
Activated charcoal	–	1000	–	–	–
Agar	8000	–	8000	8000	8000
Phytagel	–	2000	–	–	–
Basal salt mixture	1.0 × MS salts	1.0 × MS salts	1.0 × MS salts	0.5 × MS salts	0.5 × MS salts

All units are in mg/l
Basal salt mixture containing micro and macro elements with vitamins as described by Murashige and Skoog (1962)

16.2.1 Explant Preparation

Mature pods (containing the seeds) should be collected from adult *P. alpataco* trees growing in natural field conditions, during January and March (Argentina).

1. Place the collected seeds at −20 °C to prevent attack of coleopteran (Bruchidae).
2. Store the seeds in paper envelopes under controlled conditions of humidity, light and temperature (0–5 °C).
3. Scarify the seeds mechanically by removing a small lateral fragment of the seed coat and disinfested according to Villagra and Roig-Juñent (1997) method modified as follows: immersion in ethanol 70% (v/v) for 5 min, followed by immersion in sodium hypochlorite 20% (v/v) (27 g Cl*/l).
4. Rinse the seeds five times with sterile double-distilled water.
5. Transfer the sterile seeds into the Petri dish with semisolid Murashige and Skoog (1962) medium (MS) supplemented with 3% sucrose (w/v), 0.8% (w/v) agar without plant growth regulators (Table 16.2).
6. The pH of the medium is adjusted to 5.8 before autoclaving at 120 °C for 25 min.
7. Incubate the seeds in a plant growth chamber under controlled conditions of temperature, humidity, and light (mixed light provided by fluorescent tubes and incandescent lamps). The germination events began on the second day.
8. Remove the cotyledon explants from 2 week-old seedlings as shown in Fig. 16.1.

Fig. 16.1 Cotyledonary explants used for the induction of somatic embryogenesis of *P. alpataco*

16.2.2 Callus Culture

Both cotyledonary nodes and embryonic axes can be used as explants for callus induction.

1. Put the explants onto a 10 cm plastic Petri dish (100 × 15 mm) with *Induction SE culture medium* (30 ml) (Table 16.2).
2. The onset of callogenesis is from the embryonic axis and the cut surface (Fig. 16.2).

16.2.3 Proliferation and Maturation of Somatic Embryos

1. After 60 days in the *Induction medium culture* embryogenic callus should be transferred into MS medium without plant growth regulators (Table 16.2).
2. Initially, the embryogenic callus looks dark brown and after 10 days localized greenish callus appears.
3. Globular somatic embryos appear first on the surface of callus, and then, embryos at different stages of development form (Fig. 16.3a–d).

The essential characteristics of the development of somatic embryos of alpataco, especially after the globular stage, were comparable to zygotic embryos of the specie (Fig. 16.4).

Fig. 16.2 Callogenesis from cotyledonary explants of alpataco

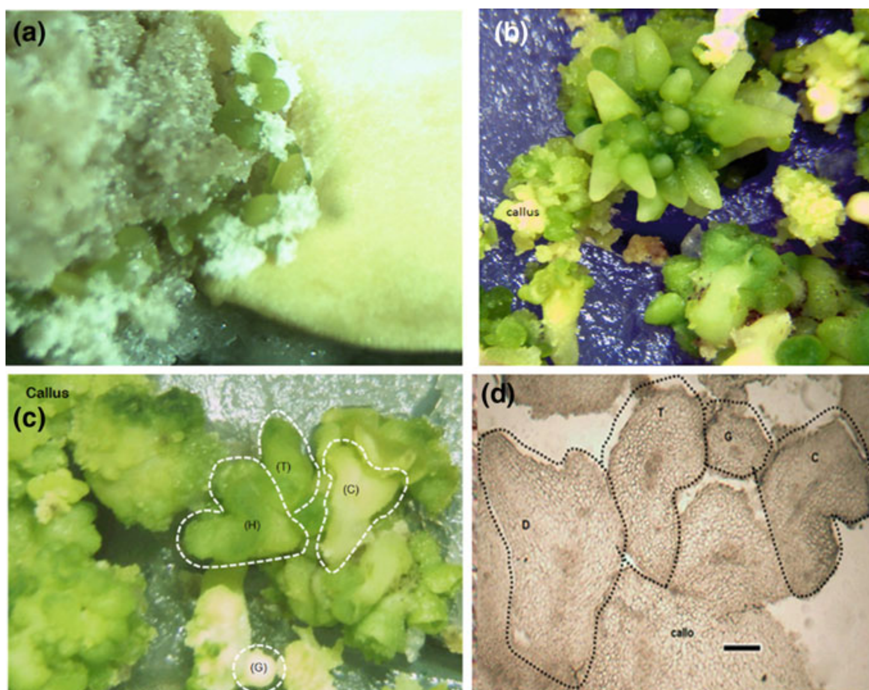

Fig. 16.3 **a** Somatic globular embryos from embryogenic callus. **b** Somatic embryos obtained at different developmental stages. **c** globular (G), heart (H), torpedo (T), cotyledonary (C). **d** Microscopy of callus: globular state (G), heart (C), torpedo (T) and cotyledonary, early phase (D)

16.2.4 Germination and Conversion of Somatic Embryos

1. Transfer the somatic embryos to the *Germination culture medium* (Table 16.2) under the same environmental conditions (temperature and photoperiod). Somatic embryos germinate normally (Fig. 16.5). Separate somatic embryos from the callus without any further delay for preventing formation of secondary SE.

1. In order to obtain plants, place the germinated somatic embryos in Petri dishes (60 × 15 mm) with sterile vermiculite, moist with culture liquid half strength MS medium or on bridge filter paper in test tubes filled with 25 ml same culture medium.

16.2.5 Plant Establishment and Acclimatization

1. Rinse roots of the seedlings with sterile water to remove agar.
2. Transfer the normal plantlets to sterile vermiculite as substrate in glass containers.

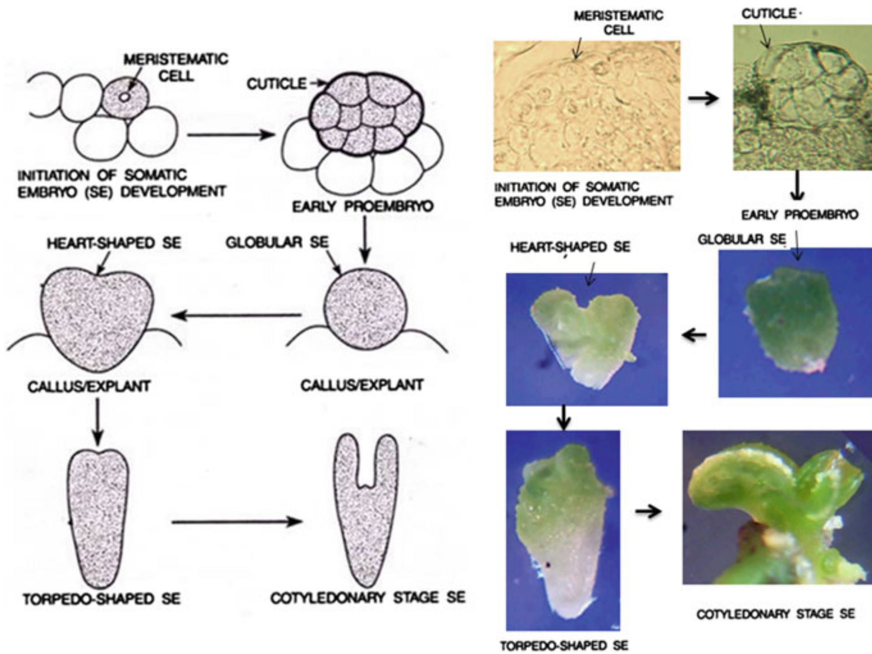

Fig. 16.4 Complete process of somatic embryogenesis from a surface cell of the explant. Figure left taken from S. Pujari (*Source* http://www.yourarticlelibrary.com/biotechnology/plant-tissues/regeneration-of-plants-shoot-regeneration-and-somatic-embryogenesis/33234/); right: somatic embryogenesis in *P. alpataco*

Fig. 16.5 Embling conversion from SE

Fig. 16.6 Plants from somatic embryos of *Prosopis alpataco* under greenhouse conditions

3. Cover the plantlets with nylon and keeps under the same environmental conditions.
4. After a week, open slowly the nylon wrapping. Transfer the plants to the growth chamber in controlled conditions of temperature (25 ± 2 °C), under a 16 h light: 8 h dark photoperiod (provided by fluorescent tubes) and average photosynthetic photon flux of 50 µmol $m^{-2}s^{-1}$.
5. Once acclimatized, transplant the plantlets to the containers with a mixture of 1:1 vermiculite–perlite under greenhouse.
6. During hardening, plantlets defoliate which is maintained during the first ten days in the greenhouse, and form new leaflets.
7. After 3 months, plants are transferred to natural conditions (Fig. 16.6).

16.3 Conclusions and Future Prospects

The present report describes the first protocol (Fig. 16.7) plantlet regeneration via somatic embryogenesis of *Prosopis alpataco*, which is one of the most important native woody species in Patagonia. However some protocol modifications are being developed. The somatic embryos must be separated manually, which is very difficult. Poor germination has to be improved. This shows that focus is needed to increase the rate of conversion of somatic embryos for field deployment. Without this, the somatic embryogenesis technology will have a very limited impact. Automation of synthetic seed production is our final goal. Including advanced tissue culture technology, bioreactors, somatic embryo encapsulation, and development of appropriate synthetic seed coating material requires further study to meet the goal of producing millions of synthetic seeds in a short time and cutting the cost of seed production.

Propagation of alpataco through seeds has limitations, because of hard seed coat. The seeds hardly germinate without any treatment under nursery conditions. The development and implementation of in vitro methodologies for the culture of *P. alpataco* not only allows the ex situ conservation but also massive production for this native species with regional interest.

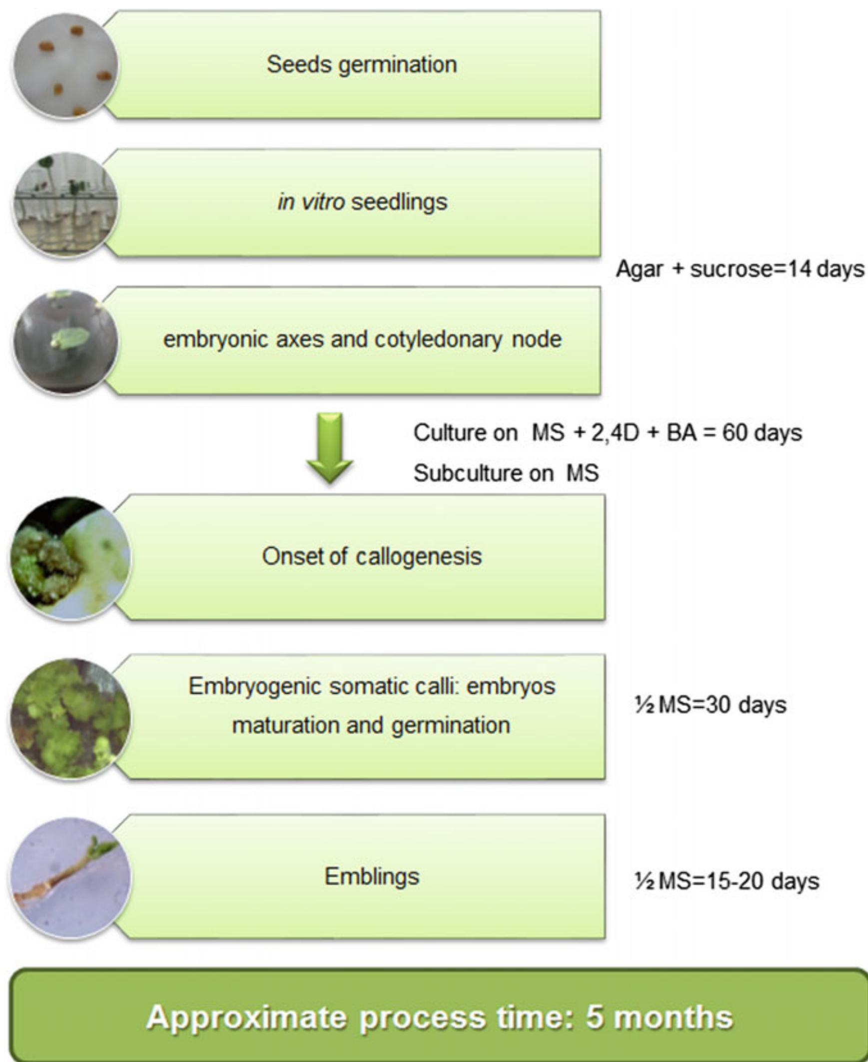

Fig. 16.7 Complete process for obtaining *P. alpataco* seedlings via somatic embryogenesis. Stages of alpataco in vitro regeneration system, specific PGR and time

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