Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Using *in vitro* culture for conservation of genetic resources: micropropagation of a monumental *Prunus dulcis* tree

Conchi Sánchez[1], Saleta Rico[1], Anxela Aldrey[1], Damián Dasilva[1], Jose María Rey-Benayas[2], Nieves Vidal[1]

[1] Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Avda. de Vigo s/n. Apdo 122. 15780 Santiago de Compostela, Spain (conchi@iiag.csic.es).
[2] Fundación Internacional para la Restauración de Ecosistemas – FIRE (www.fundacionfire.org). C/ Princesa 3 dpdo., Apto 703. 28008, Madrid, Spain.

Abstract
In this study we present the micropropagation of an ancient almond tree, probably 300- years-old, together with juvenile material proceeding from its seeds. Three types of material were used: 1) shoots flushed on the tree at the beginning of spring, 2) shoots forced to flush in a phytotron from branch segments collected in late winter, and 3) seeds collected in autumn and stored at a cool place for six months before being germinated *in vitro*. Murashige and Skoog medium supplemented with 0.5 mg L^{-1} N^6-benzyladenine and 0.05 mg L^{-1} indole-3-butyric acid was used for culture establishment and stabilization. Different combinations of plant growth regulators were evaluated for shoot proliferation and elongation. Roots formed spontaneously in the multiplication medium. Rooted shoots from cultures obtained from the mother tree and from lines that had been obtained from small seedlings were successfully acclimatized in the phytotron and the greenhouse.

Keywords: almond, biodiversity, education, divulgation, mature tree

Introduction

The need to conserve biodiversity has been granted increasing political and social attention in the last years (FAO 2011). Monumental or emblematic trees, both "wild" trees living in forests and century-old, "domesticated" agricultural trees, should be preserved *in situ* for their intrinsic value, their cultural legacy, the rich diversity of microhabitats they generate, and the quantity of organisms that depend on them for life (Moya and Moya 2013). Also, they should be preserved *ex situ* for the study and the conservation of their genetic resources, for educational issues and for reintroducing plant material of high quality in their natural areas, most of them currently degraded or threatened (Dulloo et al. 2010). Within *ex situ* conservation methods, *in vitro* culture presents special advantages in the case of emblematic trees, such as the small quantity of plant material needed to begin the micropropagation procedure and the possibility of implementing such long-term conservation techniques as cryopreservation (Postman et al. 2006, Pence 2013).

The aim of this study was to micropropagate mature material from an ancient almond tree, named "Gladiador" (Fig. 1A), located in Membrilla (Central Spain), together with juvenile material proceeding from its seeds. This monumental tree, probably 300-years-old, dominates a landscape formed by hundreds of olive trees, and has a special emblematic meaning for the population of the area, as has been recognized by the International Foundation for Ecosystem Restoration (FIRE).

Materials and Methods

For establishment of axillary shoot cultures, plant material was provided by the FIRE foundation. Three types of material were used: 1) shoots flushed on the tree at the beginning of spring (Fig. 1B), 2) shoots forced to flush in a phytotron from branch segments collected in late winter (Fig. 1C,D), and 3) seeds collected in autumn and stored at a cool place for six months before being germinated *in vitro* (Fig. 1E).

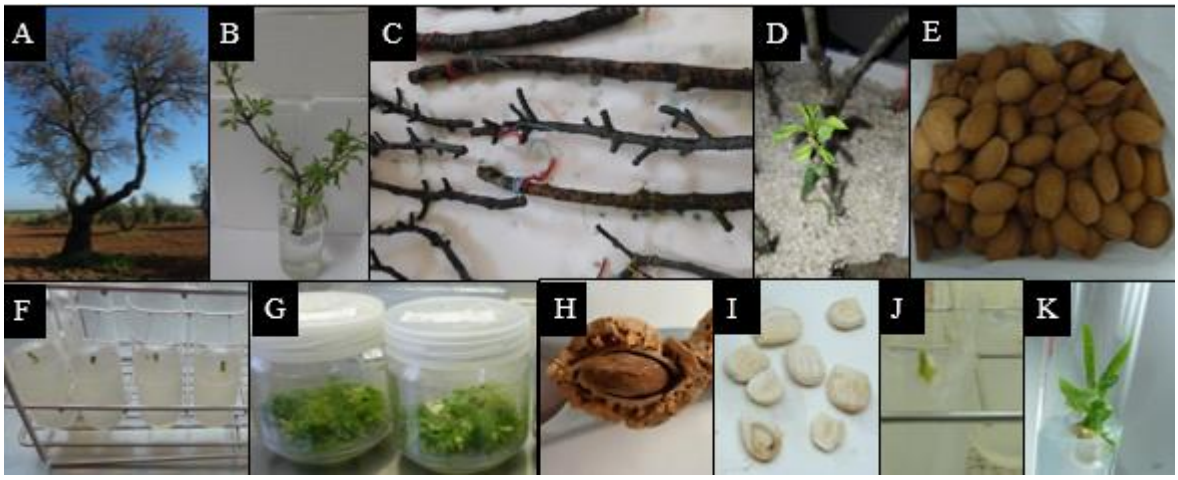

***Figure 1**. Plant material used for in vitro establishment. A) "Gladiador" almond tree. B) Shoots flushed on the tree at the beginning of spring. C,D) Shoots forced to flush in a phytotron from branch segments collected in late winter. E) Seeds. F) Nodal segments from the mother tree after sterilization. G) Shoots of mature origin cultured in jars. H) Cracked seed. I) Seeds before sterilization and embryo isolation. J) Embryo inoculated in a test tube. K) Germinating embryo used for the initiation of a juvenile line.*

For establishment of mature material originated from branches of the mother tree, the shoots were stripped of their leaves and surface sterilized by immersion for 40-50 s in 70% ethanol and for 15 min in a 10 g L^{-1} solution of sodium hypochlorite containing 2-3 drops of Tween 80®. The shoots were then rinsed three times in sterile distilled water. Nodal segments (10 mm) were cut from the shoots and inoculated in tubes with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L^{-1} N^6-benzyladenine (BA) and 0.05 mg L^{-1} indole-3-butyric acid (IBA), 3% sucrose and 0.7 % (w/v) Bacto agar (Fig. 1F). The medium was adjusted to pH 5.7 before being autoclaved at 121 °C for 20 min. Cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark. The explants were transferred every 2 weeks during the first 6 weeks after establishment and were then maintained in 50 ml of the above mentioned medium (multiplication medium; MM) in 300 ml glass jars (Fig. 1G) and were subcultured every 4-5 weeks.

For establishment of juvenile material, seeds collected in autumn under the canopy of the tree were stored in a cool chamber (8-12 ºC) for six months. Then, the shells were cracked (Fig. 1H) and the seeds (Fig. 1I) were washed with tap water before being sterilized following the same protocol as described for shoots, but with 60 s of ethanol treatment and 20-40 min in a 10-15 g L^{-1} solution of sodium hypochlorite. Embryos were isolated and inoculated in tubes with MS medium with 0.5 or 1 mg L^{-1} BA for *in vitro* germination (Fig. 1J). Shoots obtained from germinated embryos (Fig. 1K) were used to initiate separate juvenile lines, which were maintained in jars as described for mature material.
After 7-9 months of culture in MM for culture stabilization, the effect of different combinations of plant growth regulators (BA, IBA and Gibberellic acid (GA_3)), were evaluated for shoot proliferation, elongation and adventitious root formation. These treatments are described in Table 1. The explants (15 mm) were cultured in the proliferation medium (1 month), and the obtained clusters were transferred to elongation medium (1 month).

Rooted shoots from cultures obtained from the Gladiador mother tree and from lines originated from seeds were planted in pots with a commercial peat:perlite mixture (2:1) and acclimatized in a phytotron before being transferred to the greenhouse. For mid-term conservation, shoots from mature and juvenile origins were submitted to cold storage (4-6 ºC) under dim light.

Results and discussion

Seventeen lines of almond were successfully established, 12 corresponding to mature material originated from different branches of the mother tree (Fig. 2A,B) and 5 obtained from seeds (Fig. 2C).

***Figure 2**. Shoots of almond regenerated from mature material obtained from the mother tree (A, B) and from juvenile material obtained from seeds germinated in vitro (C).*

As the micropropagation of plant material derived from mature zones of ancient tree frequently presents more problems than the culture of juvenile material derived from seeds (Sánchez and Vieitez, 1991; Hackett and Murray, 1993), our first efforts were focused on the development of a reliable protocol for proliferation and rooting of the mature lines. Four treatments, combining two proliferation media and three elongation media, were tested. Briefly, the explants were cultured in MS BA 1 IBA 0.1 GA_3 0.1 (mg L^{-1}) or in MS BA 0.5 AIB 0.05 (mg L^{-1}) for one month, and then transferred to the same medium or to a half strength MS with GA_3 1 mg L^{-1} for another month (Table 1).

***Table 1**. Proliferation and elongation media used for culturing almond shoots. The concentration of plant growth regulators is expressed in mg L^{-1}.*

Treatments	Proliferation medium	Elongation medium
T1	MS BA 1 IBA 0.1 GA_3 0.1	MS BA 1 IBA 0.1 GA_3 0.1
T2	MS BA 1 IBA 0.1 GA_3 0.1	MS ½ GA_3 1
T3	MS BA 0.5 IBA 0.05	MS BA 0.5 IBA 0.05
T4	MS BA 0.5 IBA 0.05	MS ½ GA_3 1

The explants cultured in MS BA 1 IBA 0.1 GA_3 0.1 and transferred to the same medium (T1) produced more and longer shoots than the other treatments (Fig. 3A,B). However, T3 was overall more efficient, as 50% of the clusters developed spontaneous roots during the elongation step. The transfer of the cultures to half strength MS with GA_3 1 mg L^{-1} as the only plant regulator decreased the length of the shoots compared to the length of the shoots of cultures transfered to the original medium. This reduction was especially evident between T1 and T2 treatments. A sharp decrease in the number or rooted shoots was also observed, and T2 did not produce rooted shoots. The medium with GA_3 1 mg L^{-1} was used for elongation of other *Prunus* (Iacona and Muleo 2010) but it did not improve the propagation of our almond material.

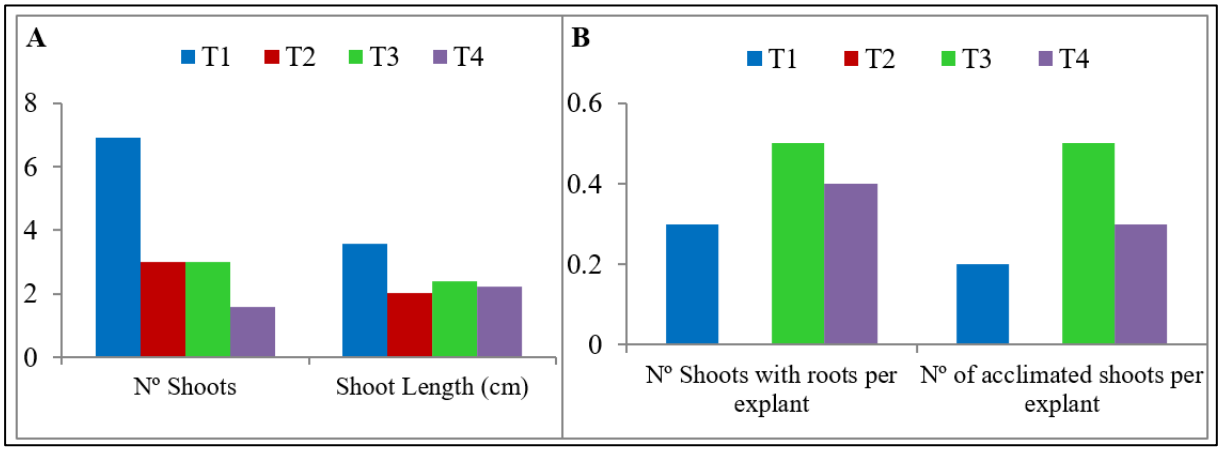

***Figure 3**. Effect of the combination of different proliferation and elongation media on the erformance of mature almond shoots. T1 to T4 treatments are described in **Table 1**.*

Rooted shoots which were at least 25 mm high were chosen for transfer to pots (Fig. 4A,B). Almost all these plantlets were successfully acclimated (Fig. 3B, Fig. 4C). T3 treatment was chosen for subsequent experiments, as it provided a good number of relatively long shoots that easily formed adventitious shoots spontaneously. The protocol was applied to juvenile material with similar results (Fig. 4D).

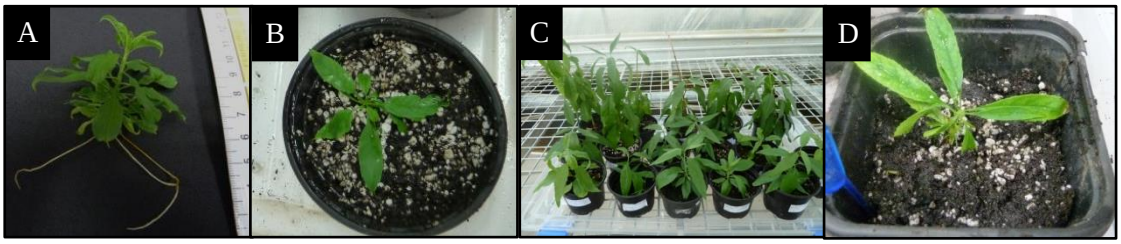

***Figure 4**. A-C) Rooted shoots of mature origin. (A,B) Rooted plant before (A) and after being transferred to a pot (B). C) Acclimated plantlets in the greenhouse. D) Juvenile plant during the acclimation step.*

So far, we have obtained 128 acclimated plantlets, 122 corresponding to clonal material from the mother tree and 6 corresponding to clonal material from seeds. Experiments are in progress to propagate more plants of juvenile origin, and both shoots from mature and juvenile origin were submitted to cold-storage at 4-6 ºC for mid-term conservation.

Acknowledgements: This research was partly funded through the contract FIRE-CSIC Ref. 20175906. We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance and Carlos Torres for plant material collection.

References

Dulloo ME, Hunter D, Borelli T (2010) *Ex situ* and *in situ* conservation of agricultural biodiversity: major advances and research needs. Not Bot Hort Agrobot Cluj 38:123-135

FAO (2011) Biodiversity for food and agriculture. Contributing to food security and sustainability in a changing world (www.fao.org/3/a-i1980e.pdf)

Hackett WP, Murray JR (1993) Maturation and rejuvenation in woody species. In: Ahuja MR (ed), Micropropagation of woody plants. Kluwer Academics Publishers, The Netherlands, pp. 93–105

Iacona C, Muleo R (2010) Light quality affects *in vitro* adventitious rooting and *ex vitro* performance of cherry rootstock Colt. Sci Hortic 125:630-636

Moya B, Moya J (2013). Monumental trees and mature forests threatened in the Mediterranean landscapes. Imelsa. Valencia (Spain)

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Pence VC (2013) *In vitro* methods and the challenge of exceptional species for Target 8 of the Global Strategy for Plant Conservation. Ann Missouri Bot Gard 99:214–220

Postman J, Hummer K, Stover E, Krueger R, Forsline P, Grauke Li, Zee F, Ayala-Silva T, Irish B (2006) Fruit and nut genebanks in the US National Plant Germplasm System. HortScience 41:1188-1194

Sánchez MC, Vieitez AM (1991) *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnut. Tree Physiol 8:59-70