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In Vitro Propagation of 'Guayabo del país' (*Acca sellowiana* (Berg.) Burret)

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ABSTRACT

Acca sellowiana (Berg.) Burret, known as 'Guayabo del país' or 'Feijoa' is a medium-sized tree of the Family Myrtaceae, native to southeastern Brazil and Uruguay. It can be found in regions with different climate conditions, it is somewhat resistant to frosts, it exhibits precocious productivity, high nutritional value, good postharvest life and is adequate for fresh consumption as well as for the elaboration of manufactured products. This fruit tree has an excellent agronomical and commercial potential. However, the low success achieved to date with conventional vegetative propagation of selected materials is an important limitation for its production for commercial purposes. Under these circumstances micropropagation has been considered as an alternative for the production of high quality and pathogen-free plant material. Explants tested were nodal segments obtained from mother plants selected for productivity and fruit quality. Shoots were disinfected with 2% sodium hypochlorite for 20 min, washed three times with distilled water adding citric acid (0.1 $g.l^{-1}$) to the third wash. The culture medium used for introduction was WPM (Lloyd and McCown 1981) supplemented with MS vitamins (Murashige and Skoog 1962), 3% sucrose, 0.7% agar, 0.44 μM BAP (6-benzylaminopurine) and 0.054 μM NAA (1-naphthalenacetic acid). For multiplication of cultures, semi-solid medium with different combinations of auxins and cytokinins were evaluated. The best treatments were those with 9.8 and 19.6 μM 2iP (6-γ-γ-dymethylamino purine). Average multiplication rate was 2 shoots/explant. Due to the low multiplication rate observed in semi-solid media, we evaluated the performance of liquid cultures in permanent immersion bioreactors. Medium composition was WPM, MS vitamins, 3% sucrose supplemented with 4.9 μM 2iP. Phloroglucinol was used to promote tissue lignification and to minimize hyperhydricity. Rooting percentage obtained was 40% (both *in vitro* and *ex vitro*).

Keywords: bioreactors, liquid culture, micropropagation, phloroglucinol
Abbreviations: BAP, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **2iP**, 2-isopentyl adenine; **MS**, Murashige and Skoog; **NAA**, 1-naphthaleneacetic acid; **WPM**, Woody Plant Medium

INTRODUCTION

Acca sellowiana (Berg.) Burret, of the plant Family Myrtaceae, known as 'Guayabo del país' or 'Feijoa', is one of the numerous native fruit tree species that are currently not part of any industrial process of breeding, production and commercialization in Uruguay. However, commercial cultures of this species are exploited in Europe, New Zealand and United States, where some varieties with a narrow genetic base are being used.

'Guayabo del país' is well adapted to a wide range of climates throughout its distribution, it is resistant to frosts and has precocious productivity, high nutritional value, good postharvest life and is apt for fresh consumption as well as for the elaboration of different manufactured products (jam, juice, etc.) (Cunda 2006).

The fruit of *A. sellowiana* has excellent organoleptic properties, high iodine content (3 mg/100 g pulp) and significant amounts of vitamin C. Fruits can be stored for about a month at room conditions, without losing its characteristic flavor and aroma (Azam 1981). They are an important source of substances with antibacterial, antioxidant, anti-inflammatory, and antimutagenic properties, and therefore fruit extract might be used with medicinal purposes (Vuotto *et al*. 2000). Bontempo *et al*. (2007) demonstrated that the extract of *A. sellowiana* has important anti-cancer properties, therefore being potentially useful for the treatment of human diseases with minimal side effects.

Flowers are hermaphroditic but generally self-incompatible, and are cross-pollinated by birds and insects (Ducroquet 2000; Finardi 2002). The tree is relatively easy to propagate from seeds. However, the quality of fruits obtained from seed-propagated trees is highly variable from one seedling tree to the next (Thorp and Bieleski 2002). Propagation from seeds additionally has the disadvantage of a high growth and production variability and delayed fructification (Franzon 2004). Conversely, vegetative propagation would produce uniform orchards, with more homogeneous plant populations but at present there are no available methods for the propagation of the species based on cuttings or grafting (Franzon 2004). Besides, *A. sellowiana* is relatively difficult to propagate by these techniques, when compared with other fruiting plants (Thorp and Bieleski 2002).

Ducroquet *et al*. (2000) described various methods for the vegetative propagation of *A. sellowiana*, of which propagation from cuttings stands out as the most efficient with 4 to 76% rate of success in New Zealand, genotype being the main source of variation in productivity (Ivey 1979, cited by Ducroquet *et al*. 2000). Grafting on 1-2 year-old seedling rootstock of *A. sellowiana* is usually done by the end of winter in Santa Catarina state, Brazil, and the process takes at least 3 months to complete. Preliminary studies on the micropropagation of this tree based on organogenesis were developed from meristem explants and young leaves (Bhojwani *et al*. 1987), and also from shoot apical meristems and microcuttings (Dal Vesco and Guerra 1999). Similar studies showed low bud neoformation and high contamination and browning percentages, with both sources of explants (Oltramari *et al*. 2000). Protocols for *in vitro* propagation developed by Oltramari *et al*. (2000) showed that woody plant medium (WPM) (Lloyd and McCown

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1981) basic culture medium without plant growth regulators (PGRs) induced the best organogenic responses from nodal segments, both quantitative and qualitative; moreover, the organogenic response observed by them largely depended on genotype. Cutting-grown plants take longer to establish in the orchard and grafted plants produce many suckers from their seedling rootstocks, both situations could complicate management techniques. Micropropagated plants have not yet been field-tested (Thorp and Bieleski 2002).

Research with *A. sellowiana* in Brazil includes breeding, studies on genetic diversity, germplasm conservation, propagation and technology transfer (Franzon 2004). At present there are two cultivars of this species developed in Brazil, 'SCS 411-Alcantara' and 'SCS 412-Helena', obtained in breeding programmes. These cultivars are self-fertile, complementary in terms of maturation, and present good resistance levels to the main diseases that affect the plant in Brazil (Epagri, s.f.).

As mentioned above, one of the main constraints for the commercial propagation of *A. sellowiana* in Uruguay is the yet unsuccessful vegetative propagation of selected materials (Vignale and Bisio 2005). In this work we consider the study of micropropagation as an alternative to conventional propagation techniques in this species, to produce high quality plant material at a big scale and pathogen-free. The main objective of this research was to standardize a micropropagation protocol for *A. sellowiana*, evaluating different *in vitro* techniques, including liquid medium in bioreactors during the multiplication stage.

MATERIALS AND METHODS

Plant material

Mother plants were selected from the Minor Fruit Trees Programme of the Faculty of Agronomy, Universidad de la República, Uruguay (http://www.fagro.edu.uy). They were cultivated in a greenhouse. Specimens represented eleven genotypes, corresponding to four localities of origin (Florida, Tacuarembó, Salto and San José). Materials are identified as Ca 70, Ca 74, Ca 75, Ca 127 (four clones from San José), Ch 128, Ch 162, Ch 304, Ch 24, Ch 168, Ch 246 (6 clones from Salto and Tacuarembó where weather conditions are similar) and CºCh (one clone from Florida). Mother plants in the greenhouse were treated periodically with fungicide (Benlate®, 2 $g.l^{-1}$) and sprouting was induced by a cytokinin treatment of 4.4 mM 6-benzylaminopurine (BAP) (Sigma B-3408; Sigma-Aldrich). Basal sprouting was used as source of explants to introduce *in vitro*.

Disinfection of explants

Branches were washed with commercial detergent (Deterjane®) and tap water. Nodal segments were surface disinfected with 2% NaOCl for 20 min, washed three times with distilled water adding citric acid (0.1 $g.l^{-1}$) in the third wash.

Culture media

Basal medium used in the different stages was WPM supplemented with MS vitamins (Murashige and Skoog 1962) and 3% sucrose. The gelling agent employed was 0.7% agar (Sigma A-4550; Sigma-Aldrich, St. Louis MO, USA), and pH was adjusted to 5.8 prior to autoclaving in all cases. For the introduction stage, PGRs employed were 0.44 µM BAP (Sigma B-3408; Sigma-Aldrich) and 0.054 µM NAA (1-naphthalenacetic acid; Sigma N-0640; Sigma-Aldrich).

For the multiplication stage of the genotypes introduced, six treatments differing in type and concentration of PGRs were evaluated against a control free of PGRs. Treatments were as follows:

T1) BAP 0.44 µM + NAA 0.054 µM
T2) BAP 0.88 µM + NAA 0.054 µM
T3) BAP 1.76 µM
T4) 2iP (6-γ-γ-dymethylamino purine) 9.8 µM
T5) 2iP 9.8 µM + NAA 0.108 µM
T6) 2iP 19.6 µM

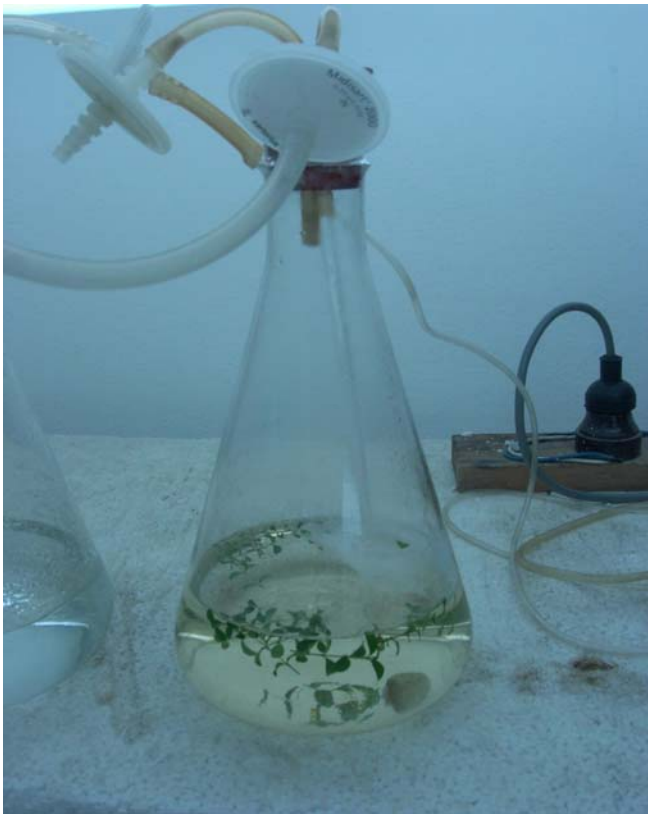

Fig. 1 Bioreactor employed for multiplication of *Acca sellowiana* in liquid culture.

We also evaluated the performance of the liquid medium supplemented with 9.8 µM 2iP (Sigma D-7674; Sigma-Aldrich) alone, and with the addition of 40 $mg.l^{-1}$ phloroglucinol (Sigma P-1178; Sigma-Aldrich) to prevent hyperhydricity by promoting lignification of shoots.

Bioreactors

Evaluation of liquid cultures was done in permanent immersion bioreactors (**Fig. 1**). Bioreactors were built with a 2-l Erlenmeyer, rubber lids with two ports for inlet and outlet filtered air (0.2 µm Millipore filters), and aeration was supplied by a Resun Air-Pump (AC-9803).

Growth conditions

Cultures were incubated at 20 ± 2°C, with a photon flux of 30 $\mu mol.m^{-2}.s^{-1}$, and a 16-h photoperiod.

Rooting

Two rooting conditions were evaluated for the microshoots obtained: *in vitro* and *ex vitro.*

In vitro, MS media supplemented with 2 $mg.l^{-1}$ IBA (indol-3-butyric acid) (Sigma I-5386; Sigma-Aldrich) for 1 week and then transfer to MS media devoid of auxin, was compared against a control without IBA. Rooted explants were transferred to pots for acclimation prior to transfer to the greenhouse.

Ex vitro, the base of the microshoots were immersed in IBA solution (500 ppm) for 1 min, and then placed in plastic trays with sterile substrate.

Statistical analysis

The experiments were repeated at least 5 times, 30 explants per treatment when using semi-solid medium and 15 explants per bioreactor in experiments with liquid culture medium. Comparisons were done using one-way analysis of variance and least significant difference (LSD) means separation with a confidence level of $P = 0.05$. STATISTICA® software was used.

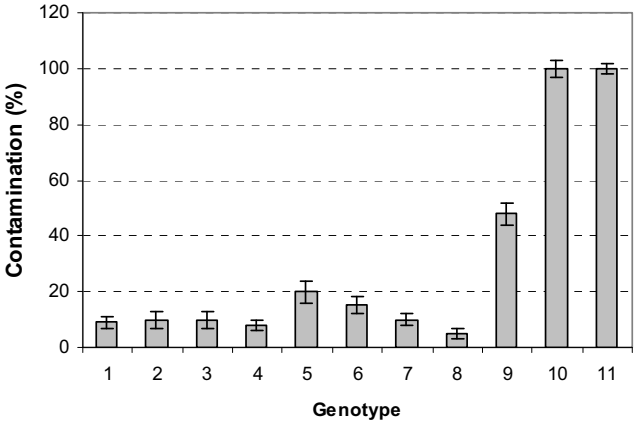

Fig. 2 Contamination of explants of *Acca sellowiana* during stage I of introduction, for the 11 genotypes evaluated. Numbers correspond to the following genotypes: 1) CºCh, 2) Ca70, 3) Ca74, 4) Ca75, 5) Ca127, 6) Ch304, 7) Ch128, 8) ChG24, 9) Ch162, 10) Ch168 and 11) Ch246 (see text for abbreviations).

RESULTS AND DISCUSSION

Establishment

The main limitation found at this stage was fungal contamination, with strong differences according to genotype (**Fig. 2**).

Browning due to oxidation of phenolic compounds was not significant, as it would be expected for a woody species.

In some genotypes, proliferation of callus around the axillary buds was observed, which prevented buds from developing.

Multiplication on semi-solid media

Fig. 3 shows the multiplication rate of three genotypes evaluated. Multiplication rate of Ca 75 material did not show significant differences for any of the PGR treatments. Cº Ch and Ca 74 showed higher proliferation rates in all cases, highest when 2iP was used.

The average multiplication rate for the different materials is shown in **Fig. 4**, highest when 2iP was the cytokinin used. Similar results were reported by Oltramari *et al.* (2000) in *A. sellowiana*, probably due to the low stability of this hormone (George 1993).

Progress in multiplication with bioreactors

Preliminary experiments employing bioreactors in stage 2 showed a significant increment in the multiplication rate of the genotypes evaluated.

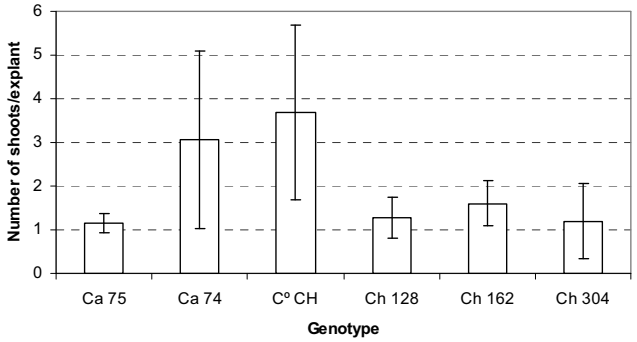

Fig. 4 Average multiplication rate for each genotype of *Acca sellowiana* in different semi-solid media.

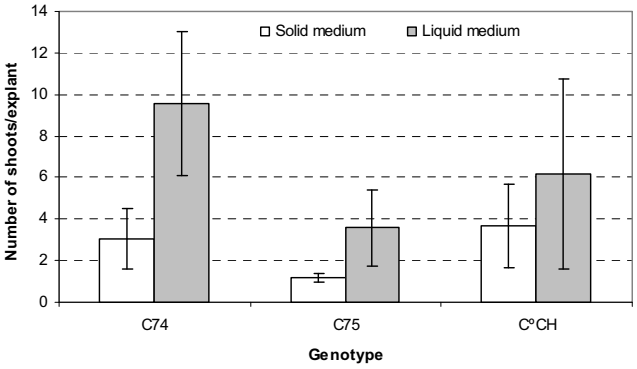

Fig. 5 Multiplication rate of three plant materials (Ca74, Ca75 y Cº Ch) of *Acca sellowiana* in liquid and semi-solid media.

A strong effect of genotype was observed, confirming the tendency observed on semi-solid media; Ca 74 was the plant material with the highest multiplication rate in permanent immersion in liquid media (**Fig. 5**).

The higher proliferation rate was associated to the immersion of the explant in the medium, probably because of a better contact of the plant tissue with the medium and thus optimal nutrient and supply of PGRs. In addition, aeration of the medium resulted in permanent movement of the explants which produced a gradual loss of apical dominance and promoted the growth of several axillary buds (**Fig. 6**).

The addition of phloroglucinol to the liquid culture medium to prevent hyperhydricity via promotion of lignification of the explants avoided the occurrence of plant malformations. Phloroglucinol can be used as an alternative precursor in the lignin biosynthesis pathway, and has been successfully employed to prevent hyperhydricity in other woody species in our laboratory (Ross 2006).

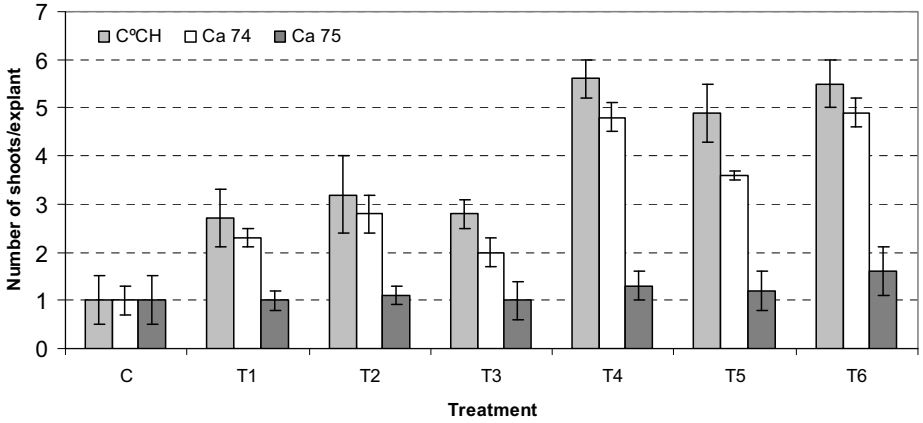

Fig. 3 Multiplication rate of *Acca sellowiana* on semi-solid media for the genotypes CºCh, Ca74 y Ca75. Data correspond to the media of three repetitions.

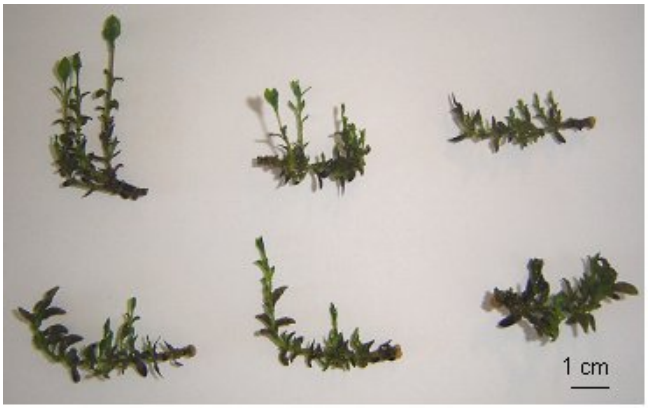

Fig. 6 Explants of *Acca sellowiana* after 30 days in the bioreactor, showing axillary bud development.

Table 1 Average rooting percentages and number of roots per explant for all the genotypes evaluated. (T1: MS without growth regulators, T2: MS plus IBA (9.8 µM) for 7 days).

	Rooting (%)	Number of roots/explant
In vitro T1	22.7	2.4
In vitro T2	44.4	2.0
Ex vitro	48.0	2.0

Rooting and acclimation

All the rooting conditions evaluated promoted root differentiation. Our results did not show significant differences for *ex vitro* and *in vitro* rooting with AIB, neither in number of microshoots which differentiated roots nor in the number of roots per explant or root length, as is shown in **Table 1**. Explants that rooted *in vitro* survived the acclimation stage without significant losses.

Fig. 7 shows rooting conditions evaluated, and acclimated growing plants obtained in the greenhouse.

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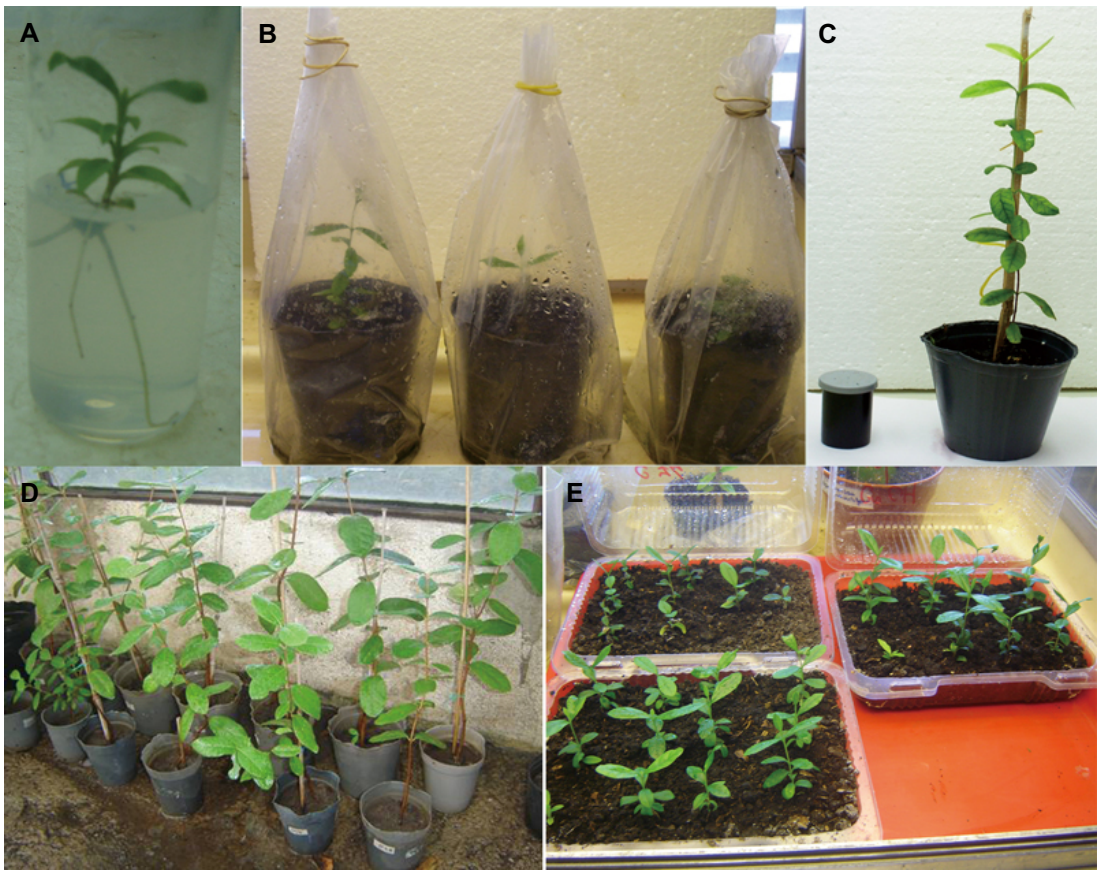

Fig. 7 Rooting of *Acca sellowiana*. (**A**) Plant rooted *in vitro* (T2). (**B**) Acclimation of *in vitro* rooted plants. (**C**) and (**D**) Plants obtained, rooted *in vitro*. (**E**) *Ex vitro* rooting conditions.

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