ZYGOTIC EMBRYO CULTURE IN AVOCADO (*Persea americana* Mill)

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ABSTRACT. Biotechnological techniques are current approaches used in plant breeding. At the present work, the utility of zygotic embryo culture as a method for breeding purpose in avocado was evaluated. *In vitro* germination and rooting of zygotic embryos, sprout multiplication and plantlet adaptation of Cuban avocado varieties were studied. Percentages of germinated entire embryos were higher using mature rather than immature embryos. Almost 80 % of entire plantlets obtained by embryo culture technique were adapted to greenhouse conditions. The usefulness of this approach to breeding purposes in avocado was discussed. This *in vitro* methodology appears as an alternative to traditional breeding methods, particularly for improving agronomic characteristics, such as root-rot resistance and salt tolerance in avocado.

Key words: *Persea americana*, plant embryos, plant developing stages, somatic embryo, micropropagation, plant breeding

RESUMEN. Las técnicas biotecnológicas son métodos usados actualmente en el mejoramiento de plantas. En el presente trabajo, se evaluó la utilidad del cultivo de embriones cigóticos, como un método con fines de mejoramiento en aguacate. Se estudió la germinación, el enraizamiento y la tasa de multiplicación de brotes *in vitro*, y la adaptación de plántulas de variedades cubanas de aguacates. El porcentaje de germinación de embriones enteros fue mayor usando embriones maduros que inmaduros. Cerca del 80 % de las plántulas enteras, obtenidas con la técnica de cultivo de embriones, se adaptaron en condiciones de invernadero. Se discutió la utilidad de esta metodología con fines de mejoramiento, la cual se ve como una alternativa a los métodos tradicionales de mejora genética, en particular para mejorar características como la resistencia a la pudrición de la raíz y a la salinidad en aguacate.

Palabras clave: *Persea americana*, embriones vegetales, etapas de desarrollo de la planta, embrión somático, micropropagación, fitomejoramiento

INTRODUCTION

The avocado is an important fruit tree, which has been incorporated into the dietary culture of many countries of the world. In spite of its wide acceptation, soil-borne disease *Phytophthora* root-rot and abiotic stress as salinity have limited its intensive production (1,2,3,4).

Genetic breeding in avocado using conventional hybridization methods is quite difficult. Therefore, only a few formal genetic studies have been reported. The long juvenile period and the large area required for growing trees through very expensive breeding programs are among the main problems (5). In our country, breeding efforts have been limited to selection of varieties, its vegetative propagation and *ex situ* conservation.

Biotechnology could be an alternative approach to conventional breeding methods, because it is a more effective way to improve plant varieties by means of selection optimization, shortening breeding schemes and therefore diminishing the cost of breeding efforts. Tissue culture for different types of avocado explants has been established (6). The utility of these techniques to micropropagation and morphogenetic capacity restoration of rootstock (7,8) and to improve agronomically important traits (9, 10, 11, 12, 13, 14) in avocado has been also demonstrated.

A genetic breeding program has been recently initiated (15), aimed to improve tolerance to biotic and abiotic stresses in Cuban avocado varieties, by combining the use of mutation induction and biotechnological techniques. Considering the eventual use of zygotic embryo culture technique in this program, the *in vitro* germination and rooting of zygotic embryos, sprout multiplication and plantlet adaptation are studied in the most important commercial avocado varieties in Cuba.

MATERIALS AND METHODS

Plant material. Fruits were obtained from open-pollinated trees of Duke, Hass, Suardía Estación, Catalina and Jaruco No. 1 varieties located at Güira de Melena station of the Tropical Fruit Research Institute (IIFT). Genotypes were selected on the basis of their relevance for breeding purposes in Cuba.

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Duke variety is used as rootstock for both *ex situ* conservation and production in our country, and the remaining varieties are among the most important cultivars in Cuba.

Zygotic embryo culture. Seeds with different developmental states as those between 4-43 week-old fruits from fruit-set were used. An embryo was considered mature when it was extracted from ripe fruits, which depended on the genotype. Seeds were dipped into 90 % (v/v) ethanol and flamed to surface sterilized as previously indicated (10). Aseptic seeds were divided by halves into separated cotyledons, excising the plumule-radicle axes together with 1 cm-thick sections of cotyledon, and transferring them into tubes of nutrient medium.

For all experiments, zygotic embryos were put on filter paper bridges into glass tubes containing 5 mL of Murashige and Skoog salt medium (16) diluted to half strength (½ MS) supplemented with 30 000 mg.L^{-1} of sucrose, 100 mg.L^{-1} of *i*-inositol, pH 5.7±0.1; except for multiplication experiments, where bencilaminepurine (BA) and giberelic acid (GA_3) at 0.5 mg.L^{-1} were also added. Four week-old entire plantlets were transferred to glass pots containing 10 mL of fresh medium without hormones and grown for eight more weeks. Cultures were grown in a climate-room with a relative humidity of 60 %, temperature of 25±2°C and light intensity of 2500 lx provided by Chiyoda lux fluorescent lamps and measured using a Yu116 Luxometer (Russia). A 16-hour light photoperiod was used.

Three month-old plantlets were transferred to pots containing a mix of soil, organic matters and charcoal breeze at 1:1:0,4 ratio for acclimatization, before these were transferred to normal greenhouse conditions. At this acclimatization state, plants were covered using transparent nylon for two weeks and watered three times weekly. First watering was made using MS (½) salt medium. This step resulted critical during material adaptation.

Statistical analysis. The percentages of germinated entire embryos, non-germinated embryos, germinated incomplete embryos, culture with multiple sprouts, adapted plantlets and contamination, obtained in cultures of immature and mature zygotic embryos, were compared using the *t* student test to comparison of percentage. Additionally, mean number of sprouts per cultivated embryo to each variety and their standard errors were calculated using a Kolmogorov-Smirnov test. Variance homogeneity was estimated by the F maximum test. Mean values for each variety were compared among them using a *t* student test.

RESULTS AND DISCUSSION

In vitro response of cultivated zygotic embryos is shown in Table I. For all genotypes, the percentages of germinated entire embryos were significantly higher using mature rather than immature embryos. In accordance to this, the percentage of incomplete embryos was significantly lower using mature rather than immature embryos, except Duke variety. Between 16 and 34 % of immature embryos did not germinate, while this percentage ranged between 2 and 7 in the case of mature embryos. Not significant differences were founded when the percentage of adapted plantlets obtained from immature and mature embryos were compared. Nearly 80 % of entire plantlets obtained by embryo culture techniques could be adapted to greenhouse conditions (Figure 1).

According to literature, germination percentage of *in vitro* propagated avocado depended on the genotype used, kind of explants, salt medium and hormone concentration. Thus, *in vitro* propagated embryos in ½ MS medium supplemented with 0.5 mg.mL^{-1} BA of Fuerte variety showed a germination percentage between 4-66 %, depending on embryo maturity (9). Other authors (10), using identical experimental conditions, observed that embryo germination of Hass, Suardia Estación and Catalina varieties was nearly 30 %. However, when the medium was supplemented with 0.5 mg.mL^{-1} of BA and GA_3, it increased until 46 %.

Table I. *In vitro* response of avocado zygotic embryos cultivated in ½ MS medium

	Varieties				
	Duke	Hass	Suardia station	Catalina	Jaruco No. 1
Immature embryos					
Number of cultivated embryos	70	51	50	49	65
Percentage of germinated entire embryos	44	38	40	45	33
Percentage of non-germinated embryos	16	34	29	23	31
Percentage of adapted plantlets†	83	80	83	85	86
Percentage of germinated incomplete embryos	26	22	28	38	33
Percentage of contaminated cultures	14	6	5	4	3
Mature embryos					
Number of cultivated embryos	203	99	55	81	63
Percentage of germinated entire embryos	71***	71***	83***	81***	80***
Percentage of non-germinated embryos	2***	5***	8***	6***	7***
Percentage of adapted plantlets†	80 ns	84 ns	81 ns	89 ns	80 ns
Percentage of germinated incomplete embryos	26 ns	12***	4***	9***	10***
Percentage of contaminated cultures	1***	12 ns	5 ns	4 ns	3 ns

(***) Significant with p<0.001 in *t* student test respect to immature embryo data; (ns) not significant; (†) Based on number of germinated entire embryos

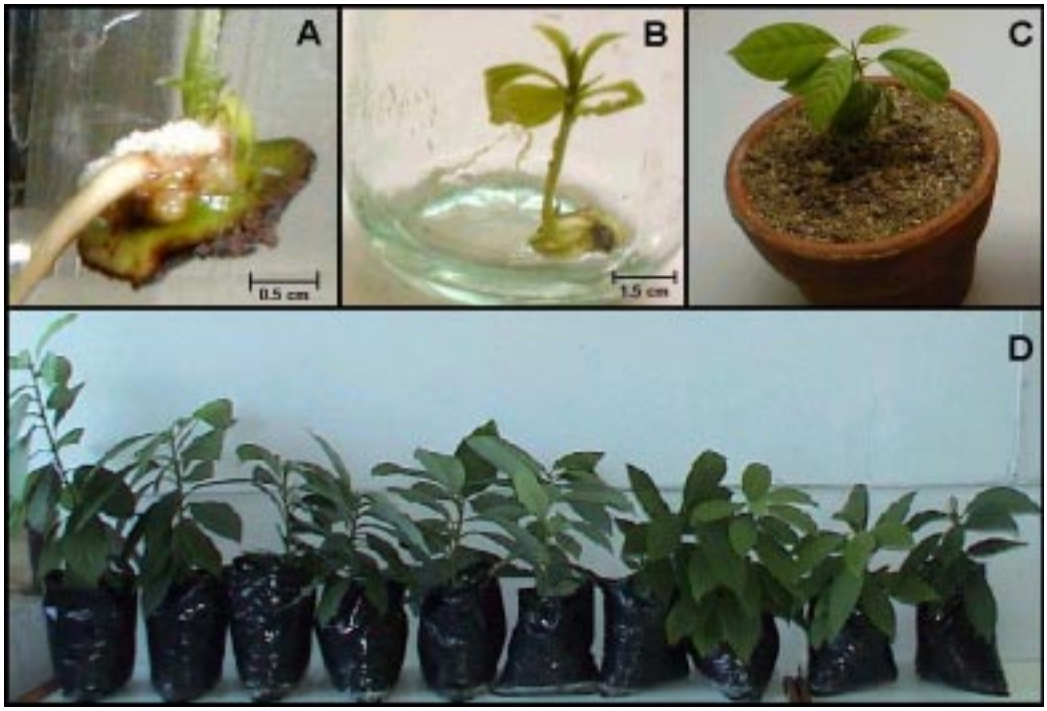

Figure 1. A-B, Avocado embryos cultivated on 1/2 MS salt medium supplemented with 30 g.L^{-1} of sucrose, 0.1 g.L^{-1} *i*-inositol. C, Three month-old plantlets of Duke variety adapted on organic substrate. D, Six month-old plantlets of Duke variety adapted to greenhouse conditions as indicated in materials and methods

Zygotic embryo culture was limited by both the number of germinated incomplete embryos and contamination. The percentage of germinated incomplete embryos per genotype was always higher when immature embryos were used, except for Duke variety, whose mature embryos were too small so that they were difficult to handle. In these cases, the plumule-radicle axis was easily broken when it was excised from cotyledons. This was a serious limitation, which has been observed during the experimental procedure.

On the other hand, culture contamination depended on genotypes ranging between 1 and 14 %. It has been shown that *in vitro* contamination could be high when axillary buds are used as explants in avocado micropropagation (17,18). In these studies, fungicide application and strict control of humidity during culture were necessary for control of contamination. These results indicated that the application of ethanol at 95 % as disinfectant agent was effective to obtain sterile sprouts from zygotic embryos, but it was not surprising for us because avocado seeds are a cleaner type of explants than axillary buds.

In order to establish a useful propagation method to breeding purpose, *in vitro* multiplication rate of three avocado genotypes was studied (Table II). Percentages of culture with multiple sprouts were significantly higher using mature rather than immature embryos ($p < 0.001$, in *t* student test). By the contrary, the number of sprouts per cultivated embryo was significantly lower for mature (ranging between 2-4) than for immature cultivated embryos (ranging between 3-6), in accordance with a previous study developed here (10). Multiple sprout induction also depended on the studied genotype. Duke variety showed lower multiplication response. Plantlets derived from subculture of these sprouts showed poor rooting and adaptability capacities.

Table II. *In vitro* multiplication response of avocado zygotic embryos cultivated in ½ MS medium supplemented with the hormones BA and GA_3 at 0.5 mg.L^{-1}

	Varieties		
	Duke	Hass	Suardia station
Immature embryos			
Percentage of culture with multiple sprouts	31	46	55
Number of sprouts per cultivated embryo†	3.3±0.1	6.0±0.3	6.3±0.4
Mature embryos			
Percentage of culture with multiple sprouts	80***	83***	80***
Number of sprouts per cultivated embryo†	2.0±0.1***	4.1±0.2***	3.2±0.3***

(***) Significant with $p<0.001$ in *t* student test; (n.s) not significant;
(†) Mean values and standard errors are presented
In all cases, three independent experiments with 15 replicate each were developed

Sneke and Barlass (9) have shown that mature embryos cultivated in ½ MS medium supplemented with 0.5 mg.mL^{-1} BA got between 4-5 sprouts per embryo. Multiplication rate of cultivated axillary buds in MS medium

supplemented with 0.65 mg.mL^{-1} BA was between 2.3 and 3.7 (19,20). This index was 3 when buds were cultivated in Woody Plant Medium supplemented without hormones, but it was zero in presence of 1 mg.L^{-1} BA (17). Other authors (21) have also demonstrated a linear dependence between the concentration of cytokinin and multiplication rate of cultivated axillary buds ranging between 0 and 4 mg.mL^{-1}. However, BA or kinetin concentration equal or higher than 4 mg.mL^{-1} got tissue vitrification in accordance with previous studies (19, 22). All these studies, including our data, make evident that the practical utility of these methods is still limited and could be optimized.

The present study has identified zygotic embryo culture as a potential method to breeding purpose in avocado. The usefulness of this approach using embryo culture techniques to improve avocado varieties is evident. This methodology appears as an alternative to traditional breeding methods to improve important characteristics as root-rot resistance and salt tolerance in avocado, where *in vitro* selection methods could be determinant.

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