

ZYGOTIC EMBRYO CULTURE AND MUTATION BREEDING IN AVOCADO (*PERSEA AMERICANA* MILL).

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ABSTRACT

Mutation induction and biotechnological techniques are current approaches used in plant breeding. At present work, both methodologies were combined in order to obtain a mutation-breeding model in avocado. *In vitro* germination and rooting of zygotic embryos, sprout multiplication and plantlets adaptation in Cuban avocado varieties were studied. Percentage of germinated entire embryos were higher using mature than immature embryos. Near of 80 % of entire plantlets obtained by embryo culture technique were adapted to greenhouse conditions. Based on inhibition of entire sprout fraction, radiosensitivity curves for Duke and Hass varieties were developed. Inhibition of entire sprout fraction was described by a second order polynomial equation. Fit of experimental data and theoretical model was equal to 0.96 and 0.95 for Hass and Duke radiosensitivity curves, respectively. LD₅₀ values defined as the dose, at which the 50 % of entire sprout fraction are inhibited, were determined in 27 and 28 Gy for Hass and Duke varieties, respectively. Gamma-rays mutagenic doses for zygotic embryos of both varieties were also established between 19 and 25 Gy. Applied mutagenic dose did not affect significantly plantlets development. However, leaf and root anomalies, atrophied and chlorophyll-deficient shoots and albinism were observed at doses higher than LD₅₀ values. The usefulness of the combined approach to improve avocado varieties was discussed. This *in vitro* methodology appears as an alternative to traditional breeding methods, particularly for improving agronomic characteristics as rot-root resistance and salt tolerance in avocado.

Key Words: Zygotic embryo culture, Mutation breeding, γ -rays, avocado.

INTRODUCTION

The avocado is an important fruit tree, which have been incorporated into dietary culture of many countries across the world. In spite of its wide acceptance, soil-borne disease *Phytophthora* root-rot and abiotic stress as salinity have limited its intensive production (Gallo-Llobet *et al.*, 1999; Zilberstaine, 1999; Kremer-Kohne and Duvenhage, 2000; Kremer-Kohne *et al.*, 2001).

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 doing breeding programs very expensive are among the main problems (Lavi *et al.*, 1991). In our country, breeding efforts have been limited to selection of varieties, its vegetative propagation and *ex situ* conservation.

Mutation induction techniques are alternative breeding methods, which have been widely used for genetic improvement of major crops, ornamentals and eventually perennial fruit crops (Przybyla, 1994; Maluszynski *et al.*, 2000). Mutation breeding in avocado is very recent. First efforts were made by Salvador Sánchez Colín CICTAMEX Foundation in Mexico in order to obtain dwarf and architectural improved genotypes (Sánchez-Colín *et al.*, 1990). These studies have demonstrated the usefulness of induced mutation using γ -rays to modify plant architecture, vegetative growth, flowering, fruit setting and certain changes on fruiting behavior in avocado (De la Cruz *et al.*, 1995a,b, 1998; Rubi *et al.*, 1995). Nowadays, FAO/IAEA Coordinated Research Project aimed at improving fruit crops by mutation induced and biotechnology have been established (Mohan, 2002).

The use of biotechnological techniques as tissue culture in mutation breeding schemes could be a more effective way to improve plant varieties by means of selection optimization, shorten breeding schemes and therefore diminishing cost of breeding effort (Maluszynski *et al.*, 1995). Tissue culture for different types of avocado explants have been established (Mohamed-Yasseen, 1993). The utility of these techniques to micropropagation and morphogenetic capacity restoration of rootstock (Pliego-Alfaro and Murashige, 1987; Barcelo-Muñoz *et al.*, 1999) and to breeding purposes (Sneke and Barlass, 1983; Bijzet, 1999; Witjaksono and Litz, 1999; Litz and Litz, 2002) in avocado have been also demonstrated.

The present study has hypothesized that zygotic embryo culture could be an effective model for mutation breeding in avocado. According to this, our objectives were: (1) to study *in vitro* germination and rooting of zygotic embryos, sprout multiplication and plantlets adaptation in Cuban avocado varieties; and (2) to develop radiosensitive curves of zygotic embryos of Duke and Hass varieties and to establish their γ -rays mutagenic dose.

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

Zygotic embryo culture

Seeds with different developmental state as those of fruits between 4-43 week-old from fruit-set were used. An embryo was considered as 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 sterilize. Aseptic seeds were divided by half 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 tube containing 5 ml of Murashige and Skoog (1962) salt medium, diluted to half strength (1/2 MS) supplemented with 30000 mg/L of sucrose, 100 mg/L of inositol, pH 5.7±0.1; except for multiplication experiments where 0.5 mg/L of benzilaminepurine (BA) and giberelic acid (GA₃) 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 (1/2) salt medium. This step resulted critical during material adaptation.

Radiosensitivity curves

Glass tubes containing mature zygotic of Duke and Hass varieties were irradiated in a dose range between 15-50 Gy, then the embryos were immediately transferred to new tubes with fresh medium. Irradiation was conducted in a Russian PX-γ-30M ⁶⁰Co irradiator at 35°C. The rate dose values were between 38-46 Gy/min, estimated by Fricke dosimeter.

Percentage of entire sprout induction was used as criterion in order to know varieties sensibilities to gamma rays. This indicator was calculated for each treatment (radiation dose) as induced entire sprout / total embryo number. At least, three experiments were developed for each treatment and a minimum of 20 embryos was used in each experiment. Survival embryo data were compute for polynomial fit analysis according Origin-PC package (Microcal Software, Inc.).

RESULTS AND DISCUSSION

In vitro response of cultivated zygotic embryos is shown in Table 1. For all genotypes, the percentage of germinated entire embryos were higher using mature than immature embryos in accordance with a previous study (Rodriguez *et al.*, 1997). Between 16 and 34 % of immature embryos did not germinate, while this percentage ranged between 2 and 7 in the case of mature embryos. Near of 80 % of entire plantlets obtained by embryo culture techniques could be adapted to greenhouse conditions.

| | Varieties | | | | |
|---|-----------|------|------------------|----------|-------------|
| | Duke | Hass | Suardia Estación | 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 mature 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 | 84 | 81 | 89 | 80 |
| Percentage of germinated incomplete embryos | 26 | 12 | 4 | 9 | 10 |
| Percentage of contaminated cultures | 1 | 12 | 5 | 4 | 3 |

(>) Based on number of germinated entire embryos

According to the literature, germination percentage of *in vitro* propagated avocado depended on the used genotype, explant kind, salt medium and hormones concentration. Thus, *in vitro* propagated embryos in $1/2$ MS medium supplemented with 0.5 mg/ml of BA of Fuerte variety showed a germination percentage between 4-66 % depending on embryo maturity (Skene and Barlass, 1983). Rodríguez *et al.* (1997), using identical experimental conditions observed that embryo germination of Hass, Suardia Estación and Catalina varieties was near to 30 %. However, when the medium was supplemented with 0.5 mg/ml of BA and GA₃ it was increased until 46 %.

Zygotic embryo culture was limited by the number of germinated incomplete embryos and by contamination. The percentage of germinated incomplete embryos per genotype was always higher when immature embryos were used, except for Duke variety, whose mature embryos had too small size and therefore it were difficult to manipulate. In these cases, the plumule-radicle axis was easily broken when it was excised from cotyledons. This was a serious limitation, which have been observed during experimental procedure. On the other hand, culture contamination depended on genotypes ranging between 1 and 14 %. It has been shown than *in vitro* contamination could be high when axillary buds are used as explants in avocado micropropagation (Cooper, 1987; Capote del Sol *et al.*, 2000). In these studies, fungicide application and strict control of humidity during culture were necessary for control of contamination. Our results indicated that the application of ethanol at 95 % as disinfectant agent was effective to obtain sterile sprouts from zygotic embryos.

In order to establish a useful micropropagation method, *in vitro* multiplication rate of three avocado genotypes was studied (Table 2).

| | Varieties | | |
|---|-----------|-----------|------------------|
| | Duke | Hass | Suardia Estación |
| Immature embryos | | | |
| Percentage of culture with multiple sprouts | 31 | 46 | 55 |
| Number of sprouts per cultivated embryo | 3,3 ± 0,6 | 6,0 ± 2,0 | 6,3 ± 3,2 |
| Mature embryos | | | |
| Percentage of culture with multiple sprouts | 80 | 83 | 80 |
| Number of sprouts per cultivated embryo | 2,0 ± 1,0 | 4,1 ± 1,8 | 3,2 ± 2,2 |

Basal medium: $\frac{1}{2}$ MS medium, (1962)

Multiple sprout induction depended on studied genotype. Duke variety showed lower multiplication response. In general, the mean number of induced sprouts was lower for mature (ranging between 2-4) than for immature cultivated embryos (ranging between 3-6), in accordance with a previous study developed by us (Rodríguez *et al.*, 1997). Plantlets derived from subculture of these sprouts showed poor rooting and adaptability capacities.

Sneke and Barlass (1983) have shown that mature embryos cultivated in $\frac{1}{2}$ MS medium supplemented with 0.5 mg/ml of BA produce between 4-5 sprouts per embryo. Multiplication rate of cultivated axillary buds in MS medium supplemented with 0.65 mg/ml of BA was between 2.3 and 3.7 (Pliego-Alfaro *et al.*, 1987; Barcelo-Muñoz *et al.*, 1988). This index was 3 when buds were cultivated in Woody Plant Medium without hormones, but it was zero in presence of 1 mg/l of BA (Cooper, 1987). Biasi *et al.*, (1994) have also demonstrated a linear dependence between the concentration of cytokinin and multiplication rate of cultivated axillary buds in range between 0 and 4 mg/ml. However, BA or kinetin concentration equal or higher than 4 mg/ml produced tissue vitrification in accordance with previous studies (Pliego-Alfaro *et al.*, 1987; Schall, 1987). All these studies, including our data, evidence that the practical utility of these methods is still limited and could be optimized.

Based on inhibition of entire sprout fraction radiosensitivity curves for Duke and Hass varieties were developed (Figure 1). Inhibition of entire sprout fraction depended on radiation dose according to the equation $Y(x) = a + b_1x + b_2x^2$, where $Y(x)$ is the logarithm of entire sprout fraction, x is the radiation dose; and a , b_1 and b_2 are the equation parameters (Table 3). The fit of experimental data and theoretical model was equal to 0.96 and 0.95 for Hass and Duke radiosensitivity curves, respectively.

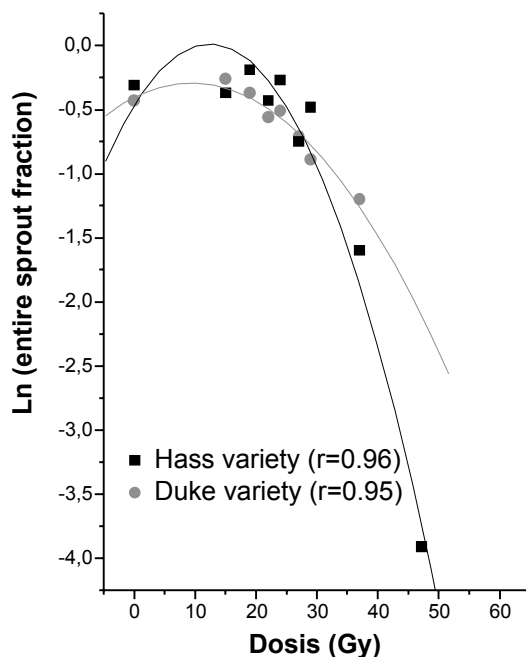


Figure 1. Effect of γ -rays on survival of avocado embryos of Duke and Hass varieties.

| Varieties | R-Square | Equation parameters | | |
|-----------|----------|---------------------|--------|--------|
| | | a | b1 | b2 |
| Duke | 0.95* | -0.3943 | 0.0166 | 0.0009 |
| Hass | 0.96* | -0.4711 | 0.0775 | 0.0004 |

(*) Significant for $p < 0.0001$

Based in the equation parameters, LD_{50} (defined as the dose at which the 50 % of entire shoot fraction is inhibited) was calculated. LD_{50} values of 27 and 28 Gy were obtained for Hass and Duke varieties, respectively. This result suggested a similar sensibility to γ -rays for both varieties, however; doses higher than LD_{50} evidenced that Hass variety was more sensible to lethal effect of radiation.

In general, doses lower than LD_{50} value were not toxic for both varieties and did not change significantly variety performance in culture (Table 4). *In vitro* germination, rooting and contamination levels were very similar for non-irradiated and irradiated avocado embryos. However, other qualitative indicators as leaf and root anomalies, atrophied and chlorophyll-deficient shoots and albinism were observed at doses higher than LD_{50} values.

Based on loss of grafting capacity of irradiated avocado scions, LD_{50} values between 20 and 40 Gy for a group of avocado ecotypes have been previously established (Sánchez-Colín *et al.* 1990). According to this criteria, an LD_{50} value of 30 Gy for Hass variety has been also reported (De la

Cruz *et al.* 1993). The results here obtained with Hass variety suggested that zygotic embryos are slightly more sensible to gamma rays than scions, maybe due to higher moisture content than that present in scions. The effect of moisture content on radiosensitivity of avocado varieties has been previously demonstrated (Rubi *et al.*, 1993).

Table 4. *In vitro* response of irradiated embryos[>] of Duke and Hass varieties cultivated in 1/2 MS medium.

| | Varieties | |
|---|-----------|------|
| | Duke | Hass |
| Total number of cultivated embryos | 698 | 317 |
| Percentage of germinated entire embryos | 52 | 76 |
| Percentage of non-germinated embryos | 7 | 3 |
| Percentage of germinated incomplete embryos | 35 | 9 |
| Percentage of contaminated cultures | 6 | 12 |

(>) Considering only irradiated embryos at doses lower than LD₅₀ values

Visser (1973), indicated that doses within LD₆₀-LD₇₀ range were useful for mutagenesis in fruit trees. According to this, we have calculated mutagenic doses between 19 and 26 Gy for Hass and Duke varieties. The results here found using zygotic embryos as experimental model agree with those obtained by De la Cruz *et al.* (1993) irradiating scions of Hass variety.

The present study has optimized a micropropagation method in avocado. The usefulness of the combined approach using mutation and embryos culture techniques to improve avocado varieties is evident. This combined 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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