Article

Embryogenesis and organogenesis of Mexican creole avocado

Anareli Quintero-Jiménez[1]
Elena Heredia-García[2]
César L. Aguirre-Mancilla[1]
Juan Carlos Raya-Pérez[1]
Juan Gabriel Ramírez-Pimentel[1]
Gabriel Iturriaga[1§]

[1]National Technological Institute of Mexico-Technological Institute of Roque (TecNM-IT Roque). Celaya-Juventino Rosas road km 8, Celaya, Guanajuato, Mexico. CP. 38110. (anareli@yahoo.com; ceaguirre@itroque.edu.mx; juraya@itroque.edu.mx). [2]Bajío Experimental Field-INIFAP. Celaya-San Miguel de Allende highway km 6.5, Celaya, Guanajuato, Mexico. CP. 38110. (elena_herediag@yahoo.com.mx).

[§]Corresponding author: gaiturriaga@itroque.edu.mx.

Abstract

The Drymifolia avocado variety (*Persea americana* Mill.) is generally used as rootstock, so it is important to implement somatic embryogenesis and *in vitro* organogenesis techniques for its genetic improvement, conservation and clonal propagation. In this work, direct and indirect somatic embryogenesis and organogenesis of the Drymifolia variety were evaluated. Germination of the mature somatic embryos was induced with 0.5 mg L^{-1} of 6-N-benzyl amino purine (BAP) and 1 mg L^{-1} of gibberellic acid (GA_3). Of the six accessions evaluated, Celaya 79, Comonfort 53, San Miguel, BG24, BG181 and Zutano, only San Miguel responded to the embryogenesis process. In direct embryogenesis, the highest regeneration efficiency was obtained with 0.2 mg L^{-1} of picloram (46%) and 10 mg L^{-1} of ANA (40%). In indirect embryogenesis, the San Miguel accession formed callus with 0.2 mg L^{-1} of picloram and had a regeneration efficiency of 45%, conserving its regeneration potential for up to six months. Regarding organogenesis, decapitated immature zygotic embryos were cultured in medium with or without growth regulators and all six accessions responded positively to both conditions. The Comonfort 53 accession had higher regeneration efficiency (54%) with growth regulators. This study carried out from 2016 to 2018, provides a new and promising approach for the regeneration and multiplication of *P. americana* var. Drymifolia through zygotic embryos.

Keywords: avocado, Drymifolia, *in vitro* regeneration, micropropagation.

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Introduction

The avocado (*Persea americana* Mill.) is one of the most important fruits in the world; it has been recognized for its health benefits and described as the most nutritious of all fruits, especially for the benefits of its fatty acids, which is why it has become an important part of the diet in many countries (Fonseca *et al*., 2016). According to FAO, Mexico is the largest avocado producer, which corresponds to 25% of world production (FAOSTAT, 2018).

The main factors that limit avocado production are pests, diseases (Ploetz *et al*., 2015; Sharma *et al*., 2017) and abiotic factors (Bonomelli *et al*., 2018). Incorporating new characteristics into avocado through genetic improvement is complicated, since it has a long vegetative period of approximately 6 to 8 years and self-pollination is difficult due to protogyny (Imbert, 1997; Gazit and Degani, 2002). Therefore, commercial avocado production is based on grafting varieties that confer tolerance to pathogens and cold stress (Whiley *et al*., 1990), tolerance to salinity (Álvarez-Acosta *et al*., 2018) or tolerance to root rot (Van den Berg *et al*., 2018).

Some of these rootstocks are hybrids of Guatemalan or Mexican races; for example, *P. americana* var. Drymifolia is a Mexican breed that is frequently used as a rootstock in orchards in our country (Rincón-Hernández *et al*., 2011). Therefore, it is of great importance to implement its clonal propagation, germplasm conservation and genetic improvement.

In vitro genetic conservation or improvement requires a complete plant regeneration system through somatic embryogenesis or adventitious organogenesis. In organogenesis sprouts can be formed directly from the explant or indirectly from callus. In contrast, somatic embryogenesis is a process that involves the formation of embryos from plant somatic cells and compared to organogenesis, it is a slower process and involves a risk of somaclonal variation (Fehér, 2019).

The regeneration efficiency of avocado plants by *in vitro* organogenesis from asexual tree tissues is low and depends on the variety (Bandaralage *et al*., 2017). Some reports of micropropagation were successful using nodal explants, juvenile tissues and axillary sprouts of avocado seedlings (Martínez-Pacheco *et al*., 2010). However, the obstacles to the in vitro establishment of avocado have been contamination by bacteria, fungi and the darkening of the explant (Nhut *et al*., 2008).

The regeneration of avocado by somatic embryogenesis has been described in different varieties such as Hass (Pliego-Alfaro and Murashige, 1988); Duke (Mooney and Van Staden, 1987), T372 (Witjaksono *et al*., 1998), Anaheim (Perán-Quesada *et al*., 2004), Duke 7 (Márquez-Martín *et al*., 2012) and Reed (Encina *et al*., 2014).

Different explants have been used to study the embryogenic regeneration pathway of avocado, such as immature zygotic embryos in the globular stage or nucela (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999; Suarez *et al*., 2006), but an embryogenesis system for the Drymifolia variety has not yet been reported. In this work we present an efficient method for the regeneration of avocado of this variety through organogenesis and somatic embryogenesis from immature zygotic embryos.

Materials and methods

Vegetal material

The plant material was obtained from the germplasm bank of the National Institute of Agricultural and Livestock Forestry Research in Celaya, Mexico during the spring-summer cycle. The Drymifolia variety avocado trees were six years old on average and these were fertilized annually with NPK, (200-200-300) and Ca, Mg, Fe and Zn (05-01-1.5). Immature avocado fruits of 5-8 cm in diameter were collected from the accessions, Celaya 79, Comonfort 53, San Miguel, BG24, BG181 and Zutano (Mexican race × Guatemalan race), they were washed with disinfectant soap (Dermocleen®) and rinsed with tap water; they were soaked in commercial sodium hypochlorite (Cloralex®) for 10 min and rinsed three times for one minute in sterile water. Cotyledonous stage zygotic embryos (EC) were used as explants.

Induction of direct embryogenesis in somatic embryos

The induction medium (I) consisted of modified Gamborg salts (GM) (Gamborg *et al*., 1968; Perán-Quesada *et al*., 2004) added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 1 g L^{-1} of activated carbon, 30 g L^{-1} of sucrose, 8 g L^{-1} of agar (Sigma-Aldrich), and different concentrations of 1-naphthaleneacetic acid (ANA) (2, 5 or 10 mg L^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D) (5 or 10 mg L^{-1}) or 4-amino-3,5,6-trichloropicolinic acid (picloram) (0.05, 0.1 or 0.2 mg L^{-1}). The pH of the medium was adjusted to 5.7, they were sterilized in an autoclave at 121 °C for 15 min, and 30 mL were poured per 100 × 25 mm Petri dish) under laminar flow hood conditions. The EC of all the accessions were cultured in medium I and kept in the dark at 30 °C until the induction of somatic embryos (ES).

Indirect embryogenesis

The callus induction medium (IC) consisted of GM salts added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 1 g L^{-1} of activated carbon, 30 g L^{-1} of sucrose, 8 mg L^{-1} of agar (Sigma-Aldrich), 0.2 mg L^{-1} of picloram and it was adjusted to pH 5.7. The EC were grown in IC and kept in the dark at 30 °C for the induction of embryogenic callus (EC).

Maintenance of embryogenic callus

The embryogenic callus (EC) was subcultured in IC every two weeks. In each Petri dish (100 x 15 mm) containing 30 ml of IC with 0.2 mg L^{-1} of picloram, 50 mg of EC were cultured and kept at 30 °C in the dark. 50 mg viable EC per Petri dish was subcultured weekly for nine months.

Somatic embryo maturation

To induce the formation of ES maturation, these were transferred to a maturation medium (M) containing GM salts added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 1 g L^{-1} of activated carbon, 30 g L^{-1} of sucrose, 16 g L^{-1} of agar (Sigma-Aldrich) and 0.2

mg L^{-1} 4-amino-3,5,6-trichloropicolinic acid (picloram). The pH of the medium was adjusted to 5.7, they were sterilized in an autoclave at 121 °C for 15 min and 30 ml were poured per Petri dish (100 × 25 mm) under laminar flow hood conditions and kept in the dark at 30 °C.

Germination of mature embryos

The germination medium (GE) consisted of GM salts (Gamborg *et al*., 1968) added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 50 g L^{-1} sucrose, 2.5 g L^{-1} Phytagel® (Sigma-Aldrich), 0.5 mg L^{-1} BAP, 1 mg L^{-1} AG_3; the pH of the medium was adjusted to 5.7. Mature ES in colitedonar stage with a diameter of approximately 5-10 mm were placed in Petri dishes (10 per box). The mature ES were subcultured in fresh medium every two weeks for a period of six to eight weeks until germination. The cultures were incubated in a growth chamber at 25 ±2 °C with 16 h of light and light intensity of 30 µmol m^{-2} s^{-1} provided by fluorescent lamps of cold white light of 40 W (Osram, Monterrey, Nuevo León, Mexico).

Induction of organogenesis

The apical meristems were dissected from the EC of the accessions used and cultured in the organogenesis induction medium (I) that consisted of GM salts, 30 mg L^{-1} of sucrose, 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 2.5 g L^{-1} of Phytagel® (Sigma-Aldrich), with growth regulators (RC) 0.5 mg L^{-1} of BAP and 1 mg L^{-1} AG_3 or without growth regulators (SRC). The pH of the medium was adjusted to 5.7 before its sterilization in an autoclave 121 °C for 15 min. 30 ml of medium were poured into 120 ml glass flasks and two explants were placed in each one. The cultures were kept in a growth chamber at 25 ±2 °C with 16 h of light.

Growth and rooting of seedlings and adventitious sprouts

The rooting medium (R) was composed of the GM salts added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 25 mg L^{-1} of indole acid. 3-butyric acid (AIB), 30 g L^{-1} of sucrose 2.5 mg L^{-1} Phytagel® (Sigma-Aldrich), the pH of the medium was adjusted to 5.7. 30 mL of it were poured into 120 mL glass flasks and one seedling was placed in each one. The germinated ES and the 2 cm long sprouts obtained by organogenesis were transferred to rooting medium for approximately 4-5 weeks.

Subsequently, the seedlings were placed in elongation medium (E) in Magenta® containers with a culture medium with half the concentration of MS salts (Murashige and Skoog, 1962), added with 1 mg L^{-1} of pyridoxine, 1 mg L^{-1} of thiamine, 100 mg L^{-1} of myo-inositol, 2.5 g L^{-1} Phytagel® (Sigma-Aldrich), adjusting its pH to 5.7.

The *in vitro* regenerated seedlings were transferred to pots with vermiculite (Termolita, Santa Catarina, Nuevo Leon, Mexico) and covered with plastic bags to favor their *ex vitro* adaptation. They were watered with half the concentration of the Murashige and Skoog (MS) medium every 3 days and cultured at 25 ±2 °C with 16 h of light in a growth chamber as described previously and kept under these conditions for 2 months approximately. Subsequently, the plastic bag was removed and they were acclimated under conditions in the greenhouse (25-28 °C and 170-285 mmol m^{-2} s^{-1} light intensity).

Statistical analysis

For direct and indirect somatic embryogenesis, the experimental unit (UE) was a Petri dish with 5 EC, each with three repetitions for each accession (Celaya 79, Comonfort 53, San Miguel, BG24, BG181 and Zutano) and different concentrations ANA (2, 5 or 10 mg L^{-1}), 2,4-D (5 or 10 mg L^{-1}) or picloram (0.05, 0.1 or 0.2 mg L^{-1}). To evaluate the viability of the embryogenic callus over time, 50 mg of callus per Petri dish were used as UE with five repetitions and the regeneration was evaluated at month 1, 3, 6 and 9 after having been induced. The UE for organogenesis was a Petri dish with 10 explants of each accession and five replicas with growth regulators (0.5 mg L^{-1} BAP and 1 mg L^{-1} AG_3) or without them. A completely randomized design was used for each experiment. Significance was determined by analysis of variance with the SAS statistical package (SAS Institute Inc., 2012) and the Tukey test ($p \leq 0.05$) for the comparison of means.

Results and discussion

Direct embryogenesis

In previous works on *in vitro* regeneration of *Persea americana* var. Hass, immature fruits were used to extract zygotic embryos (ZE) in the globular stage (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999). In contrast, in this work immature fruits were used different accessions of *P. americana* var. Drymifolia and the hybrid Zutano (Figure 1A) to isolate ZE in the cotyledon stage (Figure 1B). All the CT showed leaf primordia, hypocotyl and root apex (Figure 1C). Only the ZE from the San Miguel accession responded to the different direct somatic embryogenesis induction treatments. This coincides with other authors (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999; Raharjo and Litz, 2005), who observed that the embryogenic response of explants depends on the genotype. The ZE formed ES in globular stage in the area of the radicle 15 days after its culture in medium I (Figure 1D).

Feher (2019) indicated that this may be due to the fact that the beginning of embryo formation can occur from stem cell-like pericycle cells and that embryogenesis may share initial steps with the formation of lateral roots. After 15 days in medium I, it was identified that the ES groups that were formed by explant developed synchronously in some explants (Figure 1E) and in other ZE the development phases were different (globular, torpedo and heart) (Figure 1F and G). The maturation of the ES occurred in the medium M with 0.2 mg L^{-1} of picloram and an indicator was the change in color, from translucent (Figure 1F) to opaque white (Figure 1 G), in addition to a change in the state of development. due to its transition to the cotyledon stage, which occurred approximately two weeks after culturing in medium M (Figure 1H).

At this stage of maturation, the ES were excised from the ZE (Figure 1H) and were kept in the same medium until they reached a size of 5-10 mm (2 weeks). After this time, the ES were transferred to GE medium (Figure 1I), where after 15 days in the presence of light, the mature embryos began to photosynthesize and germinated after 30 days of culture in GE medium (Figure 1J). This result contrasts with that reported by Encina *et al*. (2014), where twice the time was required to obtain similar results with the Duke, Hass, Anaheim and A10 avocado varieties through indirect embryogenesis and with suspension cultures.

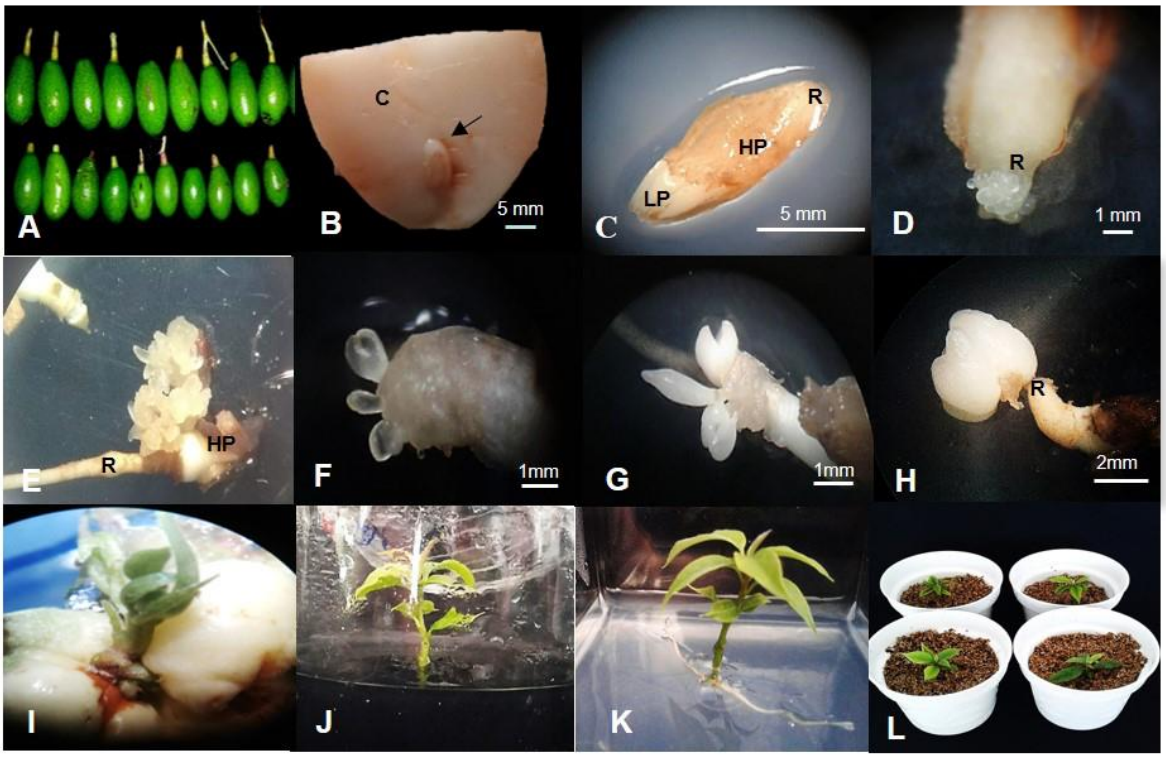

Figure 1. Direct somatic embryogenesis in *P. americana* var. Drymifolia. A) immature fruits 5-8 cm in diameter; B) zygotic embryo (EC) indicated by the arrow, cotyledon I; C) ZE, foliar primordia (LP), hypocotyl (HP), radicle I; D) somatic embryos (ES) in globular stage in the radicle I after one week in the middle of induction and maturation (0.2 mg L^{-1} picloram); E) ES after two weeks in the middle of induction and maturation; F) ES with translucent appearance in the ZE; G) ES in maturation; H) mature ES separated from radicle; I) germination of mature ES; J) sprouts in the middle of rooting; K) seedling in the middle of elongation; and L) plants acclimatized in greenhouse conditions (after 3 months).

Furthermore, it was observed that not all mature embryos germinated bipolarly (apex and root). This is probably due to the fact that the conversion of ES to a plant with bipolar germination occurs at a low frequency (Raharjo and Litz, 2003). Pliego-Alfaro and Murashige (1988) reported that meristems fail to organize in most avocado ES, and that this prevents bipolar germination.

For this reason, in the present study, germinated unipolar embryos with approximately 2 cm height (three weeks in GE approximately) were separated from the cotyledons and transferred to medium R (Figure 1J). Subsequently, they were transferred to medium E to promote elongation of the apical part and growth of the root system (Figure 1K). Finally, it took two months to adapt them to *ex vitro* conditions (Figure 1L). The treatments that promoted the highest percentage of explants that formed somatic embryos, contained 10 mg L^{-1} ANA (66.6%) or 0.2 mg L^{-1} picloram (46.6%), these same treatments induced the highest number of embryos per explant (Table 1).

Table 1. Somatic embryogenesis of *P. americana* var. Drymifolia accession San Miguel.

Auxins	Concentration (mg L^{-1})	Explants with somatic embryogenesis (%)	Average number of somatic embryos per explant	Germinated mature embryos (%)	Regeneration efficiency Plants/explant (%)
ANA	2	6.6 ±0.67 c	0.53 ±0.9 c	37.5 ±0.37c	13.33 ±0.31 c
	5	26 ±0.8 b	1.2 ±2.1 b	77.0 ±0.2 a	26 ±0.37 b
	10	66.6 ±0.64 a	3 ±2.9 a	53.8 ±0.33 a	40 ±0.37 a
2,4-D	5	20 ±0.81 b	0.86 ±1.7 c	20.0 ±0.34 c	-
	10	6.6 ±0.67 c	0.13 ±0.22 d	50.0 ±0.34 c	-
Picloram	0.05	20 ±0.81 b	1.4 ±2.6 b	71.4 ±0.24 b	26 ±0.37 b
	0.1	20 ±0.81 b	1.46 ±2 b	20.0 ±0.35 c	6 ±0.22 d
	0.2	46.6 ±0.45 a	3.2 ±2.9 a	81.8 ±0.14 a	46 ±0.38 a

The highest number of plants per explant was obtained with the treatments of 10 mg L^{-1} of ANA and 0.2 mg L^{-1} of picloram (46 and 40%, respectively) (Table 1). The regeneration system by direct somatic embryogenesis in avocado had not been previously described and implies an optimization of the time to obtain complete plants. The complete process to obtain plants from ES induction to acclimatization in the present work took approximately six months, while in the most recent report of avocado regeneration it took up to 14 months (Encina *et al*., 2014).

Indirect embryogenesis

The ZE of the San Miguel accession cultured in 0.2 mg L^{-1} of picloram developed embryogenic callus (EC) (Figure 2A). 50% of the callus formed were kept in IC medium and the other 50% were transferred to M medium. Of every 50 mg of EC in M medium (Figure 2B), the maximum regeneration potential was obtained between the first and third month after induced and no significant differences were found; on average 10.2 and 10 embryos matured (Figure 2C) and of these 66 and 60% germinated respectively (Figure 2D), of which 45 and 44% of complete plants were obtained (Table 2). In the sixth month the number of plants decreased by 55%, while in the ninth, the mature embryos lost their ability to germinate (Table 2).

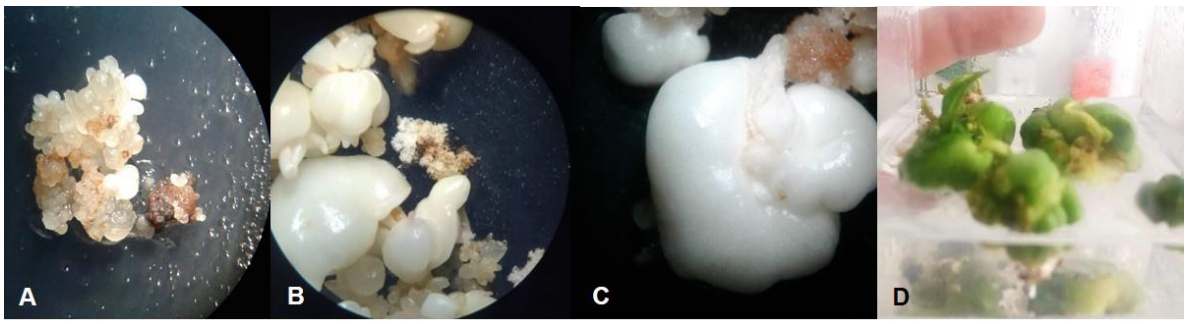

Figure 2. Indirect somatic embryogenesis in *P. americana* var. Drymifolia. A) embryogenic corns; B) maturation of somatic embryos (ES); C) ES mature in the middle of germination; and D) seedlings in the process of growth.

Table 2. Regeneration of embryogenic callus of *P. americana* var. Drymifolia accession San Miguel.

Months of subculture after callus induction	Average number of mature embryos/50 mg callus	Germination of mature embryos (%)	Regeneration efficiency No. plants/No. mature embryos (%)
Month 1	10.2 ±2.6 a	66 ±2.31 a	45 ±2.1 a
Month 3	10 ±1 a	60 ±1.09 a	44 ±2.2 a
Month 6	4 ±141 b	32 ±0.12 b	25 ±0.44 b
Month 9	2.2 ±20 c	-	-

Witjaksono and Litz (1999) mention that the embryogenic potential under maintenance conditions depends on the variety and can vary from three months to more than a year. This route of regeneration by indirect embryogenesis has been reported for other avocado varieties (Witjaksono and Litz, 1999; Encina *et al*., 2014) and in the present study the first evidence for the Drymifolia variety is shown.

Organogenesis

Multiple sprout formation was induced four weeks after the decapitated ZE (Figure 3A) remained in the medium with growth regulators (RC) (Figure 3B), while the ECs that were cultured in the medium without growth regulators (SRC) required six weeks for sprout development, although with less efficiency in all accessions (Table 3). Until now, the use of decapitated ZE to induce organogenesis has not been described in avocado, although it has been described in other species (Quintero-Jimenez *et al*., 2010; Singh and Tiwari, 2012).

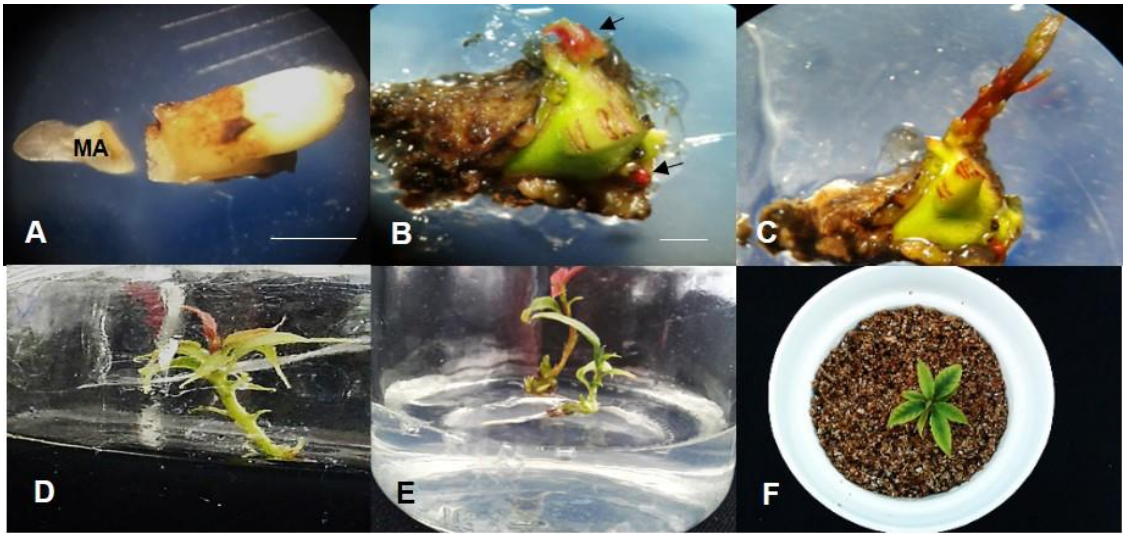

Figure 3. Regeneration of *P. americana* var. Drymifolia by organogenesis. A) Zygotic embryo without meristematic apex (AM); B) Regenerated sprouts after four weeks in the middle of organogenesis induction; C) Two-month sprouts in the middle with growth regulators; D) Sprouts in the middle of rooting; E) Seedlings in elongation medium after three months of starting the cultivation; and F) Plant acclimated in greenhouse conditions for two months.

In general, all the avocado accessions used in the present study had an organogenic response (Table 3). The Comonfort 53 accession had the best response in the RC medium with respect to the number of explants that formed sprouts (86%), while the BG181 and BG24 accessions showed the lowest values for this variable when they were cultured in a RC medium (14 and 26%, respectively) (Table 3).

Table 3. *In vitro* regeneration by organogenesis of different avocado accessions (*Persea americana* var. Drymifolia).

Accession	Growth regulators	Explants with sprouts (%)	Average number of sprouts per explant	Total number of plants	Regeneration efficiency Plants/explant (%)
Celaya 79	BAP+AG	66 ±0.42 b	1.93 ±1.23 b	21 ±1.21 a	42 ±0.31 b
	SRC	50 ±0.68 c	1.64 ±0.92 c	12 ±1.38 c	24 ±0.21 c
BG24	BAP+AG	26 ±0.55d	1.15 ±1.12 d	5 ±0.4 d	10 ±0.11 d
	SRC	10 ±0.51 e	1.4 ±2.6 c	1 ±0.13 d	2 ±0.12 d
BG181	BAP+AG	14 ±0.59 e	1.14 ±1.72 d	2 ±0.11 d	4 ±0.23 d
	SRC	12 ±0.5 e	1.16 ±1.23 d	1 ±0.13 d	2 ±0.12 d
Zutano	BAP+AG	56 ±0.77 b	2.35 ±1.91 b	13 ±0.8 c	26 ±0.35 c
	SRC	30 ±0.43 d	2.06 ±0.8 b	4 ±0.31 d	8 ±0.24 d
San Miguel	BAP+AG	62 ±0.76 b	3.29 ±0.75 a	19 ±1.09a	38 ±0.1 b
	SRC	40 ±0.46 c	3.05 ±0.95 a	11 ±0.52 c	22 ±0.14 c
Comonfort 53	BAP+AG	86 ±1.02 a	2.53 ±1.02 a	27 ±1.61 a	54 ±0.15 a
	SRC	68 ±0.8 b	2.38 ±1.04 b	16 ±0.97 b	32 ±0.26 b

BAP= 6-benzylaminopurine; AG= gibberellic acid; SRC= no growth regulators.

The San Miguel and Comonfort 53 accessions formed the highest percentage of explants with sprouts, although there were no significant differences in the average number of sprouts per explant (3.29 and 2.53, respectively) (Table 3). In contrast, Zulfiqar *et al*. (2009) observed in avocado of cv. Fuerte 2.5 sprouts per explant with axillary buds compared to 1.58 in the apical ones. On the other hand, in the present work it was observed that Comonfort 53 and Celaya had a lower average number of sprouts per explant (2.53 and 1.93, respectively) compared to San Miguel (3.29); however, the number of plants per explant of Comonfort 53 and Celaya was higher (54 and 42% respectively) than San Miguel (38%). This indicates that the initial induction does not always correspond in the same proportion to the differentiation and development of whole plants (Table 3).

Once the sprouts reached 2-3 cm in length after two months of culture in the RC medium, they were excised from the original explant (Figure 3C), before culturing them in the MR medium, in which the emergence of the root after 2 weeks (Figure 3D) and subsequently transferred to medium E (Figure 3E). When the seedlings reached 10 cm long, they acclimatized (Figure 3F). Comonfort 53 had the highest regeneration efficiency (54%), followed by Celaya 79 (42%) and San Miguel (38%) (Table 3). This is similar to that described by Martinez-Pacheco *et al*. (2010)

who showed an efficiency of regeneration via organogenesis of 57.5% for the Drymifolia variety. The results of this work showed that the *in vitro* regeneration protocol for the Drymifolia avocado variety through the organogenesis pathway with ZE is functional for the different accessions and the Zutano hybrid.

Conclusions

A reproducible and efficient protocol was established for the *in vitro* regeneration of avocado plants of the Drymifolia variety using six accessions. This protocol allowed the regeneration of plants from zygotic embryos in two ways: somatic embryogenesis and organogenesis. The use of picloram (0.82 µM) or ANA (53.7 µM) induces the formation of somatic embryos through direct embryogenesis, while picloram (0.82 µM) promotes the formation of embryogenic callus by indirect embryogenesis. *In vitro* propagation through organogenesis was established using decapitated zygotic embryos. San Miguel was the only one with a somatic embryogenic response, while Comonfort 53 had the best organogenic rate. By both regeneration routes, complete plants were obtained that adapted to greenhouse conditions in half the time of previous protocols.

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