Identification of zygotic and nucellar seedlings in polyembryonic mango cultivars

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Abstract – The objective of this work was to evaluate the occurrence of polyembryony in the mango cultivars Manila and Ataulfo, and to determine whether seedlings cultured in vitro are zygotic or nucelar. Percentage of polyembryony was calculated and the number of embryos in 100 seeds of each cultivar was recorded. 'Manila' exhibited 97% polyembryony with 3.4 embryos per seed, while 'Ataulfo' had 95% polyembryony with 3.2 embryos per seed. Later, 20 seeds of each cultivar were established in vitro, and it was analyzed those in which all embryos germinated (12 seeds from 'Manila' and 7 from 'Ataulfo'). DNA was extracted from seedling leaf tissue, and its origin was identified with 14 RAPD primers. The polymorphic markers recognized the seedlings of sexual origin in seven of nine 'Manila' polyembryonic seeds, and in four of seven 'Ataulfo' ones. Also, in polyembryonic seeds not all zygotic seedlings were produced by small embryos located at the micropyle.

Index terms: *Mangifera indica*, embryo culture, funiculus, molecular markers, polymorphism.

Identificação de plântulas zigóticas e nucelares em cultivares de manga poliembriônicas

Resumo – O objetivo deste trabalho foi avaliar a ocorrência de poliembrionia nas mangas 'Manila' e 'Ataulfo' e determinar se as plântulas cultivadas in vitro são zigóticas ou nucelares. A percentagem de poliembrionia foi calculada e o número de embriões em 100 sementes de cada cultivar foi determinado. 'Manila' apresentou 97% de poliembrionia com 3,4 embriões por semente, enquanto 'Ataulfo' teve 95% de poliembrionia com 3,2 embriões por semente. Posteriormente, 20 sementes de cada cultivar foram cultivadas in vitro, tendo-se analisado aquelas em que todos os embriões germinaram (12 sementes de 'Manila' e sete de 'Ataulfo'). O DNA foi extraído a partir de tecidos foliares das plântulas, e sua origem foi identificada com 14 iniciadores RAPD. Os marcadores polimórficos reconheceram o embrião de origem sexual em sete das nove sementes poliembriônicas de 'Manila' e em quatro das sete sementes de 'Ataulfo'. Além disso, nem todas as plântulas zigóticas foram produzidas por embriões pequenos localizados no micrófilo das sementes poliembriônicas.

Termos para indexação: *Mangifera indica*, cultura de embriões, funículo, marcadores moleculares, polimorfismo.

Introduction

Mexico is the world's fifth largest producer of mango and the second largest exporter (Servicio de Información Agroalimentaria y Pesquera, 2011). The best accepted cultivars in the domestic market are the polyembryonic ones, or yellow cultivars, such as Manila and Ataulfo. Mango plants are propagated mainly by seed and grafting. However, in polyembryonic cultivars the zygotic embryo is viable and may produce morphological and genetic diversity (Gálvez-López et al., 2010). To guarantee the variety and maximum uniformity, it is essential to graft both monoembryonic and polyembryonic cultivars onto polyembryonic rootstock (Galán Saúco, 2009). Sánchez S. et al. (2008) report that seed from 'Manila'-type, polyembryonic cultivars are used as rootstock.

In polyembryonic mango, there is one sexual embryo per seed and several somatic or nucellar ones, which share their entire genetic constitution with the mother plant (Cordeiro et al., 2006; Galán Saúco, 2009). Adventitious embryos are initiated directly from the maternal nucellar tissue, which surround the embryo sac containing a developing zygotic embryo (Aleza

et al., 2010). Therefore, in polyembryonic crops, the identification of the zygotic embryo has great importance (Villegas & Andrade, 2008). Moreover, nursery plant producers can use the nucellar embryos to propagate disease-free clone rootstocks (Soares Filho et al., 2003; Santos et al., 2010), or rejuvenate old clones that have lost their vigor through constant vegetative propagation (Batygina & Vinogradova, 2007; Villegas & Andrade, 2008).

Despite the relevance of several apomictic genera, such as *Euphorbia*, *Mangifera*, *Malus*, *Ribes*, *Beta*, *Panicum*, *Brachiaria*, *Dichanthium*, and *Pennisetum* (Bicknell & Koltunow, 2004), most studies on embryogenesis and polyembryony have been done on citrus; therefore, this genus has been considered the model for both processes (Batygina & Vinogradova, 2007). However, results obtained with citrus have been extrapolated to other species, leading to possible erroneous interpretations.

The relationship of morphological traits of polyembryonic species with the zygotic origin of the seedlings has been sought. Certain phenotypical aspects, however, are dominant traits determined by a single gene, which may be expressed after several years or modified by environmental factors (Sánchez-Damas et al., 2006; Das et al., 2007). When mango is propagated from polyembryonic seeds, farmers allow them to germinate and produce several seedlings (including sexually produced plantlets). They select those with desirable traits and assume that this selection guarantee the nucellar origin of the seedling (Gálvez-López et al., 2010). Nevertheless, it is common to find in the orchards, plants with different phenotypes due to the sexual origin of the seedling (Cordeiro et al., 2006; Gálvez-López et al., 2010).

Different morphological and biochemical markers have been used to distinguish nucellar from zygotic seedlings, but none is as efficient as molecular markers (Andrade-Rodríguez et al., 2004; Rao et al., 2008). The random amplified polymorphic DNA (RAPD) are molecular markers characterized by their abundance in the genome; they are highly polymorphic and simple to use, although, to guarantee their reproducibilty, amplification process should be optimized (Valadez Moctezuma & Kahl, 2000). This makes it possible to use RAPD to differentiate zygotic from nucellar seedlings in early stages, as reported by Cordeiro et al. (2006) for mango, and Andrade-Rodríguez et al. (2004, 2005) and Rao et al. (2008) for citrus.

The objective of this work was to evaluate polyembryony in the mango cultivars Manila and Ataulfo, and to determine whether seedlings cultured in vitro embryos are zygotic or nucellar.

Materials and Methods

The experiment was carried out with fruit from the municipality of Ixcuintla, state of Nayarit, Mexico. Mature, open-pollinated and healthy fruit were harvested from one 'Manila' and one 'Ataulfo' mother plant in the spring of 2010.

One hundred fruit per cultivar were collected, and their seeds removed (seeds with endocarp). For all 200 seeds, the following variables were evaluated: fresh matter weight (g), length (cm, from the peduncle base to farthest tip), and width (cm, the widest part perpendicular to length, including the curvature below the concave side). The endocarp and seed coat (testa with tegmen wrapping all embryos) were removed from each seed, and the number of embryos per seed was determined. We calculated the percentage of seeds having one to seven embryos, the percentage of polyembryony, and the number of embryos per seed (Andrade-Rodríguez et al., 2005).

Later, 20 seeds of each cultivar were established in vitro. Before that, the embryos were separated and numbered according to their position with respect to the funiculus. The embryo next to the point of insertion of seed funiculus in the seed coat was denominated ‘one’; the rest were arranged counterclockwise and then numbered. The funiculus was used as the reference, since in anatropous ovules, such as mango, it is next to the micropyle (Bachelier & Endress, 2009). Fresh matter weight (mg), length (mm, from the tip of the radicle to the opposite tip of the largest cotyledon) and width (mm, measured at the widest part, perpendicular to length, including the two cotyledons) were recorded for each embryo (64 embryos of 'Manila', and 54 embryos of 'Ataulfo'). Each of the cotyledons of the embryos was also weighed (mg).

A completely randomized design was used. For the polyembryony study, the experiment had two treatments (cultivars) and 100 replicates. For the analysis of embryo traits and origin, the other experiment had four treatments (embryo positions 1, 2, 3 and 4) and different

numbers of replicates (40, 36, 31 and 11, respectively). The variables were analyzed with the Kruskal-Wallis nonparametric method at 5% probability, using the R software (R Development Core Team, 2009). To correlate the seed and embryo trais, Pearson coefficient correlation was used in the statistical software SAS.

Twenty seeds (seed with endocarp) per cultivar were disinfected with 2.6% sodium hypochloride, for 15 min, and rinsed with sterilized water. Then, seeds were kept in a cysteine solution (50 mg L^{-1}) for 10 min to prevent oxidation. After endocarp and seed case (testa with tegmen) were removed from each seed, each unseparated embryo was numbered and implanted in the culture medium of 20 g L^{-1} sucrose, 6 g L^{-1} agar and 1 g L^{-1} activated carbon, at pH 5.7. Cultures incubated in the dark at 24ºC±2º, for two weeks. Later, they were subcultured in the same medium to which 4 mL L^{-1} KNO_3 (1mol L^{-1}) and 4 mL L^{-1} Ca $(NO_3)_2$ (0.1mol L^{-1}) were added. Seedlings were kept under luminous intensity at 30 µmol m^{-2} s^{-1}, at 24ºC±2º with a 16 hour-light and 8 hour-darkness photoperiod, for one month.

Out of the 40 cultivated seeds, 36 germinated. Seeds were selected if all of their embryos emerged (complete series of embryos): three monoembryonic 'Manila' seeds, MeM-1, MeM-2 and MeM-3; nine polyembryonic 'Manila' seeds; and seven polyembryonic 'Ataulfo' seeds, which did not exhibit monoembryony. The polyembryonic seeds are represented with 'M' for 'Manila' or 'A' for 'Ataulfo', followed by a number indicating the seed, and another number indicating the embryo's position. Three seedlings were obtained from the monoembryonic and 26 seedlings from the polyembryonic 'Manila' seeds, while 18 seedlings were obtained from the polyembryonic 'Ataulfo' seeds.

Leaf samples (150 mg fresh matter weight) of the mother plant (PmM, 'Manila' mother plant; PmA, 'Ataulfo' mother plant) were collected. Young leaves of the in vitro seedlings were removed, and 100 mg samples were weighed and stored at -20ºC until DNA extraction.

Genomic DNA extraction was performed following the method described by Andrade-Rodríguez et al. (2005), modified to prevent production of phenols in the tissue and degradation of DNA: instead of chloroform:isoamylic alcohol (24:1), chloroform:octanol (24:1) were used. To evaluate the integrity of the extracted DNA, electrophoresis in 1.0% (w/v) agarose gel (Invitrogen, Carlsbad, CA, USA) was performed. The gel was dyed with ethidium bromide (10 mg mL^{-1} for 15 min) and observed under UV light transilluminator GVM20, (Syngene, Bangalore, Karnataka, India). DNA concentration and purity was determined with a spectrophotometer Nanodrop 2000/2000c, (Thermo Scientific, Waltham, Massachusetts, USA) at 260/280 nm absorbance readings.

Evaluations were done for sixty primers from Operon Technologies, series OPA 1-20, OPB 1-20 and OPC 1-20, (Invitrogen, Carlsbad, CA, USA), and eight primers specifically designed at the School of Agricultural Sciences, Universidad Autónoma del Estado de Morelos (Rodríguez-Rojas et al., 2012), for amplification of *Pouteria sapota* (SAP 1, 5' ATG CGA ACC G 3'; SAP 2, 5' GAC ACA TCG G 3'; SAP 3, 5' TGG GAC CTC C 3'; SAP 4, 5' GGA GCT ACC T 3'; SAP 5, 5' TAT AGG CCC T 3'; SAP 6, 5' CCT ACT CCA G 3'; SAP 7, 5' TGG GAA TCC C 3'; SAP 8, 5' GCC CCT ACT A 3'. Fourteen of the sixty-eight primers were selected based on the DNA amplification of seedlings obtained from the polyembryonic seeds.

The reaction mixture consisted of 10.0 µL dNTP Invitrogen (5 µmol L^{-1} of each dNTP), 2.5 µL buffer PCR (10X), 1.5 µL $MgCl_2$ (75 mmol L^{-1}), 0.3 µL Taq DNA polimerase Invitrogen (1.5 U), 2.0 µL primer (20 pmol mL^{-1}) and 4.0 µL template DNA (20 ng μL^{-1}); this was gauged to 25 µL with sterile de-ionized water. DNA amplification was performed through polymerase chain reaction (PCR) technique in a thermocycler TC-412, (Techne, Burlington, N, USA). The applied program was 94ºC for 4 min, followed by 36 cycles of 94ºC for 1 min, 36ºC for 1 min, and 72ºC for 1 min; the final extension was 72ºC for 10 min. PCR amplification products were separated in to 1.5% (w/v) agarose gel Invitrogen. The size of the fragments (pairs of bases) produced by RAPD was estimated with GeneTool version 3.6 (Synoptics, 2008); the molecular weight marker 1 kb DNA ladder (500 ng) (Gibco BRL, Gaithersburg, MD, USA) was used as a reference.

Plants exhibiting amplification patterns different from the mother plant were considered zygotic. Amplified products were recognized as polymorphic based on the presence or absence, in the different samples, without considering differences of intensity. Plantlets were considered totally polymorphic if different in comparison to the female parent with more

than 50% of final primers used or, at least, three primers (Cordeiro et al., 2006). A zero-one matrix was created to estimate the Dice coefficient of similarity (Nei & Li, 1979), and with the UPGMA method (unweighted pair group method arithmetic average) a dendrogram was constructed with the software NTSYSpc 2.2 1h (Rohlf, 2009). The indexes of genetic similarity were analyzed with the statistical software SAS.

Results and Discussion

'Manila' exhibited 97% polyembryony and 'Ataulfo', 95%. Both cultivars had two to four embryos in more than 80% of their seeds (Table 1). According to Soares Filho et al. (2003) and Santos et al. (2010), if cultivars have polyembryony higher than 80%, the possibility of obtaining nucellar plants increases, making it possible to have a uniform rootstock.

The traits of mango embryos have not been considered in previous studies, despite their importance for germination capacity (Andrade-Rodríguez et al., 2004). In our work, embryos 3 and 4 weighed more and were longer, while embryos 1 and 2 were smaller (Table 2). There were no statistical differences for width among embryos. Villegas & Andrade (2008) reported that, in orange, the size of the embryos decreases as their position nears the micropyle tip of the seed. The embryo size is an important characteristic in polyembryony, since smaller ones are generally not viable, lacking sufficient food reserves, or because they dehydrate in mature seeds (Andrade-Rodríguez et al., 2005).

In citrus, the larger the number of embryos per seed, the smaller the size of all embryos (Soares Filho et al., 2003; Andrade-Rodríguez et al., 2004). This was not observed in the present study, in which only the weight, length and width of embryo 3 was significantly affected by the number of embryos per seed (Table 3): r = -0.70, -0.77, and -0.78, respectively ($p \leq 0.01$). Moreover, unlike citrus embryos of mature seed, which are easily separated and individually cultured in vitro (Andrade-Rodríguez et al., 2004, 2005), mango embryos of mature seed cannot be separated without causing lesions. Consequently, it is possible that, during seed formation, embryos develop under different dynamics in the two species, especially if we consider apomixis is a type of facultative reproduction that responds to the conditions to which they are adapted (Andrade-Rodríguez et al., 2005; Batygina & Vinogradova, 2007).

In 'Manila' and 'Ataulfo', a positive correlation was found between seed weight with endocarp (r=0.54) and number of embryos per seed (r=0.80). In the former cultivar, seeds with endocarp weighing between 13 and 18 g had larger number of embryos, while, in 'Ataulfo', seeds weighing≥19 g had more embryos (Table 4). Determining the relationship between seed traits and polyembryony is important to predict which seed may contain more embryos, since larger number of embryos in the seed increases their competitiveness and the possibility that the zygotic embryo degenerates (Andrade-Rodríguez et al., 2004; Costa et al., 2004).

Differences in weight of up to 90% were found between the pairs of cotyledons for all the evaluated embryos (64 'Manila' embryos, and 54 'Ataulfo'

Table 1. 'Manila' and 'Ataulfo' seeds with different number of embryos[(1)].

Cultivar	Number of embryos per seed							Polyembryony	Embryos
	1	2	3	4	5	6	7	(%)	per seed[(2)]
Manila	3	23	30	30	10	2	2	97	3.4a
Ataulfo	5	25	30	25	15	0	0	95	3.2a

[(1)]Means followed by equal letters in the columns are not significantly different, by Kruskal-Wallis test, at 5% probability. [(2)]Mean number.

Table 2. Embryo traits according to their position in the seeds of polyembryonic mango 'Manila' and 'Ataulfo'[(1)].

Embryo position	Fresh weight (mg)	Length (mm)	Width (mm)
1 (n = 40)	2087.30bc	26.40b	15.73a
2 (n = 36)	1256.50c	21.56b	14.59a
3 (n = 31)	4598.30a	38.80a	20.08a
4 (n = 11)	3658.20ab	36.30a	16.10a
CV (%)	102.00	47.21	74.33

[(1)]Means followed by equal letters do not differ by Kruskal-Wallis test, at 5% probability. Embryo position 1 is the embryo next to the point of insertion of the seed funiculus in the seed coat; the other embryo positions are arranged counterclockwise and numbered according to their position with respect to the funiculus.

Table 3. Pearson's correlation coefficient between polyembryonic embryo traits.

Embryo position	Fresh weight	Length	Width
1 (n = 40)	-0.111[ns]	-0.373[ns]	-0.156[ns]
2 (n = 36)	-0.168[ns]	-0.76[ns]	-0.262[ns]
3 (n = 31)	-0.703**	-0.766**	-0.783**
4 (n = 11)	-0.526[ns]	-0.488[ns]	-0.492[ns]

[ns]Non significant. * and **Significant at 5 and 1% probability, respectively.

embryos). In embryos close to the funiculus, one or both cotyledons were paper-thin. Citrus embryos also exhibit differences in shape and size between the two cotyledons of a pair (Villegas & Andrade, 2008), but not at with the same magnitude occuring in mango. In polyembryonic seeds, the difference between cotyledons is probably due to compaction during embryos growth, impeding their simultaneous development (Sánchez-Damas et al., 2006). Thus, assessment of polyembryony should be specific for each genus, species or cultivar.

Out of the 60 primers evaluated, 14 were selected because they amplified the largest number of sharply defined bands (8 to 17 bands): OPA-1, OPA-2, OPA-4, OPA-11, OPA-18, OPB-6, OPB-7, OPB-10, OPB-12, OPB-18, OPC-14, OPC-19, SAP-1 and SAP-4. These primers amplified 135 polymorphic bands for 'Manila', with 9.6 average bands per primer, and 95 polymorphic bands for 'Ataulfo', with 6.8 average bands per primer. These results also indicate genetic differences between the two cultivars (Gálvez-López et al., 2010).

Primer OPA-4 amplified the highest polymorphism for 'Manila' (94.4% of the bands, or 17 out of 18), as primer SAP-01 did for 'Ataulfo' (69.6%, 16 out of 23). No single primer by itself could identify all the zygotic seedlings, as Rajwana et al. (2008) had reported. However, the set of primers OPA-02, OPA-04, OPA-11, OPB-07, OPB-10, OPB-12, OPC-14 and SAP-04 together detected the zygotic embryos of both 'Manila' and 'Ataulfo' cultivars.

With regard to the position of embryos in the seed (embryo location with respect to the funiculus), zygotic seedlings were found in the positions 1, 2, 3 and 5 in 'Manila', and positions 1, 2 and 3 in 'Ataulfo' (Table 5). Moreover, zygotic seedlings were found mainly in the micropyle region (positions 1 and 2) in 66.6% of 'Manila' polyembryonic seeds, and 57.1% of 'Ataulfo' polyembryonic seeds. Our results coincide with those of Cordeiro et al. (2006).

Table 4. Average number of embryos in 'Manila' and 'Ataulfo' seeds according to their different weights with endocarp.

Seed weight (g)	Number of embryos per seed	
	'Manila'	'Ataulfo'
≤6.0	2.0	1.5
7.0–12.0	3.3	3.0
13.0–18.0	3.4	3.0
≥19.0	-	3.2

Of the three monoembryonic seedlings from 'Manila' seeds (MeM-1, MeM-2 and MeM-3), only the seedling from the MeM-3 seed was recognized as zygotic by 10 primers (OPA-01, 02, 04, 11, 18, OPB-06, 07, 10, 12 and SAP-04). Seedlings MeM-1 and MeM-2 were identified as nucellar, since they exhibited the same banding pattern as the mother plant. In these seeds, it is possible that the zygotic seedling was not detected because it degenerated, leaving the nucellar embryo to develop freely in the entire seed loculus (Batygina & Vinogradova, 2007). Another probable explanation is pointed out by Andrade-Rodríguez et al. (2005) for monoembryonic seeds of *Citrus reshni* and "Robinson" (*C. clementina* x Tangelo Orlando): they may have been zygotic, but identical to or differing little from the mother plant due to self-pollination.

The degree of genetic similarity among seedlings was determined by comparing the absence or presence of fragments in agreement with the mother plant banding pattern. The average genetic similarity among 'Manila' seedlings was 0.972 ± 0.034, and 0.977 ± 0.040 among 'Ataulfo' seedlings, with coefficients of variation of 0.034 and 0.041, respectively. This indicates that the seedlings of both cultivars, regardless of the origin of

Table 5. Localization of zygotic seedling in the polyembryonic mango 'Manila' and 'Ataulfo'.

Seed	Number of seedlings	Zygotic position[1]
M 1	2	1, 2
M 2	5	5
M 3	3	0
M 4	3	1, 2, 3
M 5	2	0
M 6	5	2, 5
M 7	2	2
M 8	3	2
M 9	2	1, 2
MeM 1	1	0
MeM 2	1	0
MeM 3	1	1
A 1	3	1, 2, 3
A 2	3	1
A 3	4	0
A 4	2	2
A 5	2	0
A 6	2	0
A 7	2	2

[1]Zygotic position 1 is the embryo next to the point of insertion of the seed funiculus in the seed coat. The other embryo positions were arranged counterclockwise and numbered according to their position with respect to the funiculus. M, 'Manila'; A, 'Ataulfo'; MeM, monoembryonic 'Manila' seeds.

the embryo, are highly similar with a low coefficient of variation (Rao et al., 2008). Considering these results and the low probability of embryos in the positions 1 and 2 (generally zygotic) to germinate, 'Manila' and 'Ataulfo' cultivars would frequently produce nucellar seedlings from seeds, as already noted by Rao et al. (2008).

'Manila' and 'Ataulfo' seedlings formed the two major groups described in the dendogram (Figure 1). This expressed their differences as cultivars, although 'Ataulfo' mangoes were closely related to 'Manila' (Gálvez-López et al., 2010). Also, two groups of nucellar embryos of both cultivars can be clearly observed. Although it has been pointed out that Anacardiaceae, such as the genus *Mangifera*, have one ovule per ovary (Bachelier & Endress, 2009), 12 zygotic embryos were found in the seven 'Manila' polyembryonic seeds, in which zygotic embryos were identified, and six were found in the four 'Ataulfo' polyembryonic seeds.

In the present work 44.4% of the 'Manila' seeds and 14.3% of the 'Ataulfo' seeds had two to three zygotic embryos. For example, 'Manila' seed 4 had three zygotic (M4-1, M4-2 and M4-3) and no nucellar embryos. There are no previous reports for mango with more than one zygotic embryo per seed, although more than one has been found in other fruit species, such as *Prunus dulcis* (Mill.) (Martínez-Gómez & Gradziel, 2003), and in *Citrus* (Das et al., 2007; Aleza et al., 2010).

When more then one zygotic embryos per seed were found, they were very similar genetically (Figure 1). These results suggest the possibility of fertilization by different microgametes, as reported by Medina Filho et al. (1993) for citrus. Traditionally, apomictic processes are referred to as polyembryonic, and

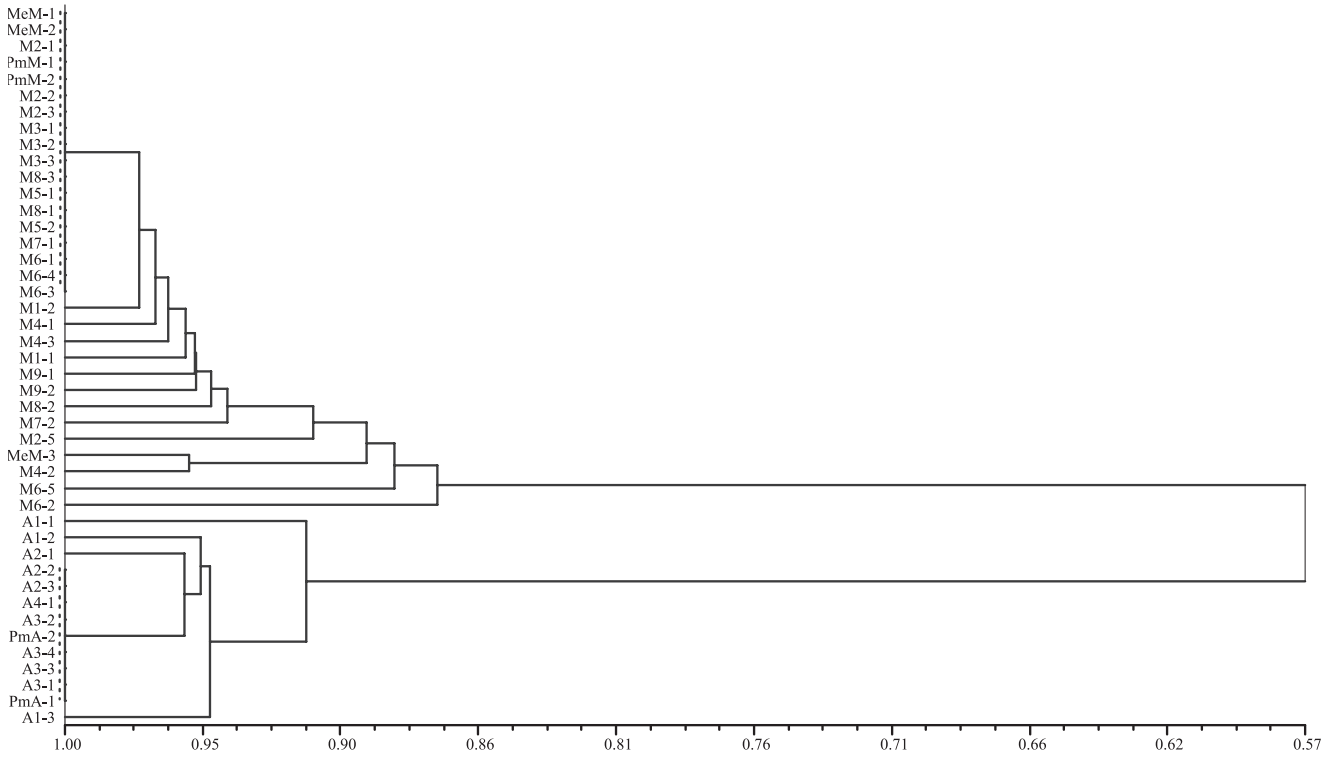

Figure 1. Dendrogram of zygotic and nucellar (dashed line on the y axis) seedlings obtained from nine 'Manila' (M) and seven 'Ataulfo' (A) seeds, based on Dice genetic distance (Nei and Li, 1979), using the UPGMA clustering method. The numbers beside the letters M and A indicate the seed and the position of the embryo. Embryo position 1 is the embryo next to the point of insertion of the seed funiculus in the seed coat. The other embryo positions were arranged counterclockwise and numbered according to their position with respect to the funiculus (1 to 5). 'Manila' (PmM) and 'Ataulfo' (PmA) mother plants are also included, as well as three monoembryonic 'Manila' (MeM) seeds.

nonapomictic processes as monoembryonic, although these terms may cause confusion, when attempting to distinguish nucellar embryony from sexual twinning (Aleza et al., 2010).

Conclusions

1. Mango cultivars Manila and Ataulfo show polyembryony in more than 80% of their seeds, and the possibility of obtaining nucellar plants from them is high.

2. Seed weight with the endocarp is an indicator of the number of embryos per seed.

3. 'Manila' (44.4%) and 'Ataulfo' (14.3%) seeds had two to three genetically similar, zygotic embryos, and the primers OPA-02, OPA-04, OPA-11, OPB-07, OPB-10, OPB-12, OPC-14 and SAP-04 are recommended to identify the largest number of zygotic seedlings in mango.

4. Zygotic seedlings are not always produced by small embryos located at the micropylar end of the seed.

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