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Research Article

Polyembryony in citrus: does the largest embryo in the seed develop a nucellar seedling?

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ABSTRACT: A generalized concept in the Citrus genus is that highly polyembryonic varieties produce only a small number of hybrids. Small zygotic embryos congregate primarily near the micropyle, while nucellar embryos organize near or away from the micropyle. In the present study, the authors determined the number of polyembryonic seeds, embryos per seed, and the largest embryo (LE) position in five citrus cultivars: C35 citrange, Volkamer lemon, Amblycarpa mandarin, Minneola tangelo, and Valencia orange. The percentage of nucellar seedlings obtained exclusively from the LE per seed was then calculated. The polyembryony percentage varied largely between genotypes, from 65 to 98 %, and the mean number of embryos ranged from 2.9 to 4.6. The chalaza contained up to 87 % of the LE. Out of 30 primers, 17 Simple sequence repeats [SSRs] (AG14, ATC09, CAT01, CCSM147, CCSM18, CCSM13, CCSM4, F2, F4, F6, GT03, TAA41, TAA45, TAA1, F7, F11 and TAA52) identified nucellar plants identical to the female parent (genetic similarity index [GSI] value ≥ 0.95). This study establishes for the first time the relationship between the sexual or asexual origin of seedlings derived from LE embryos isolated from seeds and the SSR primers described above. While the five citrus cultivars had high polyembryony levels, 30 % of the resulting plants differed from the female parent in C35 Citrange, 45 % in Volkamer lemon, 15 % in Amblycarpa mandarin, 15 % in Valencia orange, and 45 % in Minneola tangelo. The largest seedling is not always nucellar: in the five citrus species studied, the LE produces 55 to 85 % of the nucellar embryos.

Keywords: Apomictic *Citrus* genotypes, SSR, polyembryony, seedlings different from the female parent, embryo origin

Introduction

Polyembryony is a type of sporophytic apomixis commonly found in citrus species, except in *Citrus medica* L., *Citrus grandis* Tanaka¸ *Citrus clementina* ¨Tanaka, and in a number of mandarin hybrids (Aleza et al., 2010; Ribeiro et al., 1999). Some researchers have attempted to link the embryo characteristics to their sexual or asexual origin. For example, in the mature *Citrus reshni* Hort. ex Tanaka seed, the smallest embryos developing at the micropyle end are usually considered zygotic embryos (Andrade-Rodríguez et al., 2005), while the nucellar embryos may develop near or away from the micropyle (Kishore et al., 2012). However, in apomictic citrus, developing nucellar embryos were found to promote early embryogenesis and competition for endosperm nutrients among embryos (Wakana and Uemoto, 1988). Furthermore, the initial development of both zygotic and nucellar embryos is inhibited in the chalazal region (Koltunow et al., 1995; Wakana and Uemoto, 1988), and consequently, both embryos grow preferentially near the micropyle end.

Different researchers claim that cultivars showing high polyembryonic percentages are less likely to develop hybrid seedlings since their zygotic embryos will not survive under field conditions. In contrast, the largest nucellar embryos might produce vigorous seedlings (Aleza et al., 2010; Bastianel et al., 1998; Soares-Filho et al., 2000). Thus, the selection of polyembryonic genotypes is preferred for the promotion of vegetative or clonal propagation (Andrade-Rodríguez et al., 2004; Duarte et al., 2013; Passos et al., 2006). Additionally, the occurrence and germination of the two embryo types depend on multiple factors like genotype, climate, pollinators, fertilization, seed development stage, and other plant physiological conditions (Kishore et al., 2012; Rao et al., 2008; Yildiz et al., 2013).

Microsatellites (SSRs or repeated single sequences) are efficient molecular markers that identify nucellar and zygotic seedlings in citrus seeds obtained either through self-pollination or cross-pollination, even with low heterozygosity levels. SSRs have identified cross-hybrids between citrus species (Carrillo-Medrano et al., 2018; Ruiz et al., 2000; Rao et al., 2008; Yildiz et al., 2013). These researchres found no association between the sexual or asexual origin of the seedling and the embryo in the seed.

This study aimed to identify nucellar seedlings produced by female parents in seedlings obtained from the largest embryo in seeds for two citrus cultivars (Valencia orange and Minneola tangelo) and three rootstocks (C-35 citrange, Volkamer lemon, and Amblycarpa mandarin) by using 30 microsatellites as SSR markers.

Materials and Methods

Plant material

The plants studied included two citrus cultivars, Valencia orange (*Citrus sinensis* (L.) Osbeck) and Minneola tangelo (*C. paradisi* Macf. x *C. reticulata* Blanco), and three citrus rootstocks: C-35 citrange (*C. sinensis* x *Poncirus trifoliata*), Volkamer lemon (*C. volkameriana* Pasq.) and Amblycarpa mandarin (*C. amblycarpa* (Hassk.) Ochse). From two fruit production cycles, 2013–2014 and 2014–2015, 100 viable seeds of mature open-pollinated fruits were selected from each cultivar. These seeds were used to determine the percentage of polyembryony, the number of embryos per seed, and the largest embryo position in each seed. In the 2014–2015 cycle, the largest embryos were extracted from 20 seeds and germinated *in vitro* for molecular analysis. All trees grew under the humid tropical conditions of Cazones, Veracruz, Mexico (20°41′ N, 97°19′ W, 22 m.a.s.l.); 25 °C average temperature; 82 % average RH.

Polyembryonic seeds and their largest embryos

Ten mature fruits from the five cultivars were sampled at random from each harvest, and their seeds removed for washing in 2 % (v/v) sodium hypochlorite with five drops of liquid detergent followed by stirring for 15 min to remove the mucilage in the seed coat. Ten seeds per fruit were then selected at random to complete a 100 viable-seed sample. The seed coat was then removed from each seed, and their embryos separated with a dissecting needle, tweezers, and a 10x stereomicroscope. The number of embryos per seed and the percentage of polyembryonic seeds were recorded (Figure 1). The binary code proposed by Kishore et al. (2012) was used to classify the seeds as follows: '0' for monoembryonic seeds (one embryo per seed) and '1' for polyembryonic seeds (two or more embryos per seed).

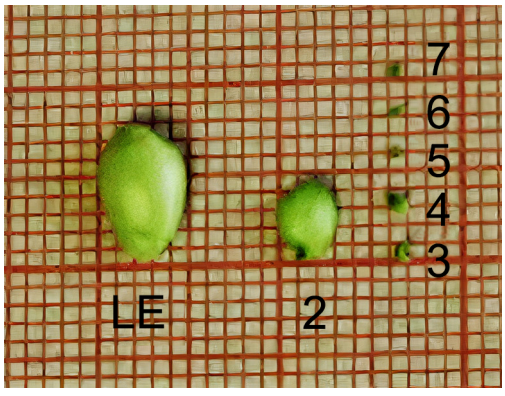

Figure 1 – Selection of the largest embryo (LE) within the seven embryos formed by an Amblycarpa mandarin fruit seed, shown on a millimetric scale. Ordered by size, the largest embryo was considered number one.

The largest embryo (LE) in each seed was separated, and when two embryos were of similar size, both were discarded to avoid uncertainty in the LE classification. The location of each LE was recorded with a binary code: '1' for the chalazal region (opposite the hilum) and '0' for the micropylar region.

The Shapiro-Wilk normality test on the number of polyembryonic seeds and the number of embryos per seed showed that data were not normally distributed. These statistical procedures were conducted using the PROC UNIVARIATE procedure in SAS (Statistical Analysis System, version 9.3). Thus, a non-parametric Mann-Whitney test was used for the purpose of comparing the two harvest cycles (2013–2014 and 2014–2015).

Identification of nucellar seedlings

In the 2014–2015 cycle, 20 mature seeds were selected at random and washed in 10 g L^{-1} of $Ca(OH)_2$ solution for 10 min. The seed coat was removed, and the seeds were disinfected with 2.6 % NaOCl (commercial sodium hypochlorite) for 5 min in a sterile environment. NaOCl was decanted, and the seeds rinsed three times with sterile distilled water. The integument was then removed, and the largest embryo (LE) per seed excised and sowed in test tubes (25 mm × 150 mm) containing 10 mL of culture medium (20 g L^{-1} sucrose and 6 g L^{-1} agar-agar, pH 5.7). The LE were incubated in darkness at 24–26 °C for two weeks.

After germination, successful seedlings were sub-cultured into a culture medium containing 3.0 mM NH_4NO_3, 3.0 mM KNO_3, 0.2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, and 0.1 mM $MgSO_4$. Incubation room conditions were kept constant for approximately two months: the temperature at 24–26 °C and 16 h of light at 30 µmol m^{-2} s^{-1} provided by white fluorescent tubes.

In vitro seedlings, 3 to 4 cm in length, were then grafted onto Volkamer lemon rootstocks to promote growth. Rootstocks were four to five months old. Both grafted parts were tied together with Parafilm, and the graft was covered with plastic bags to prevent dehydration. The plastic bag was removed four weeks later. Then grafted plants were stored in a greenhouse at 30–35 °C and 80–95 % relative humidity for ten months.

Young leaves were collected from the grafted plants and the female parent from each citrus genotype. Sampled leaves were frozen at –80 °C, freeze-dried at –47 °C for five days, and ground in a bead mill homogeniser. Approximately 100 mg of tissue was collected. Leaf DNA was extracted according to the protocol by Sudheer et al. (2008), with the following modifications: the extraction solution contained 1M β-mercaptoethanol and chloroform:octanol (24:1), and was used for organic phase separation. DNA quality was assessed in 1.0 % (w/v) agarose stained with ethidium bromide (10 mg m L^{-1}) for 15 min. The concentration and purity of DNA were quantified with an Ultraviolet-visible spectrophotomer at 260/280 nm absorbance.

The polymerase chain reaction (PCR) amplification of a specific segment of DNA by oligo sets was performed as recommended by Rao et al. (2008) in a thermocycler, without fluorescent markers. Table 1 shows the set of 30 microsatellites used in this research. The 'Touchdown' PCR program consisted of a cycle at 94 °C for 3 min for initial denaturation, followed by 10 cycles at 94 °C for 30 s, annealing temperatures as defined for each primer for 30 s (Table 1), and 72 °C for 45 s for the extension. Next, the PCR continued with 33 cycles at 94 °C for 30 s, followed at 51 °C for 30 s and 72 °C for 45 s. The final PCR extension was at 72 °C for 30 min. The PCR product was mixed with 6 µL of loading buffer (50 mM Tris, 5 mM EthyleneDiamine TetraAcetic acid (EDTA) pH 8, 25 % sucrose, and 0.2 % w/v bromophenol blue) and analyzed by electrophoresis in a 14 % polyacrylamide gel (acrylamide: N,N'-methylbisacrylamide, 29:1). The gel was stained with 0.2 % $AgNO_3$. A 1 kb molecular size marker was used. The running electrophoretic conditions were 190 V and 200 mA for 90 min.

Identification of nucellar seedlings required analysis of DNA polymorphisms by comparing the SSR markers in these seedlings against the SSR markers in the female parent. Different amplicons were recognized as polymorphisms. Seedlings classified as genetically different from the female parent could be separated from nucellar seedlings by the different banding patterns (Rao et al., 2007).

The polymorphism information content (PIC) of each marker was calculated as described by Novelli et al. (2006). PIC expresses the ability of each marker to differentiate the origin of the seedlings. Furthermore, the genetic similarity index or GSI was used to identify nucellar seedlings (Mannen et al., 1993). This index was determined for individuals of all citrus genotypes by the formula GSI = (2p + q)/2N, where p is the number of identical alleles between two genotypes, q the number of different alleles between two individuals (heterozygous or heterozygous with null alleles), and N the total number of alleles analyzed. The standard error and the confidence interval (IC 0.95) were calculated using an SAS software program (Statistical Analysis System, version 9.3). The sample size for each cultivar was estimated with a confidence level of 95 % (Holland, 2006; Wu et al., 2007).

When the GSI had a value ≥ 0.95, the seedlings obtained from the largest embryo in each seed were identified as identical to the female parent and thus were classified as nucellar plants. Moreover, when the GSI values were < 0.95, they were classified as plants different from the female parent.

Results

Polyembryonic seed and its largest embryo

The percentage of polyembryonic seeds differed in the two cycles for C35, Amblycarpa, and Minneola, while Volkamer and Valencia showed no statistical differences (Table 2). The cycle did not affect the number of embryos per seed in C35, Volkamer, Amblycarpa, and Valencia, and the embryo number increased only in Minneola tangelo for the 2014–2015 period (Table 2). The percentage of large embryos located at the chalaza (PEC) reached 100 % for Volkamer lemon, while PEC ranged between 87 and 93 % for the other genotypes (Table 2).

Identification of nucellar seedlings produced by the largest embryo in each seed of fruits harvested from the female parent

The degrees of genetic similarity (nucellar) between progeny seedlings for each SSR primer and between all SSRs primers were determined and compared to the SSR bands produced by the female parent (Figure 2). Seventeen molecular markers (Table 3) detected nucellar seedlings and genetically different seedlings from the female parent (GSI < 0.95). These 17 primers generated 137 polymorphic bands. PIC values greater than 0.5 allowed for selection of certain markers, such as TAA 41 and F4, by high discrimination capacity (DeWoody et al., 1995). The mean genetic similarity and standard error among progenies varied from 0.95 (SE = 0.03), 0.96 (SE = 0.03), and 0.96 (SE = 0.04) in C35 citrange, Amblycarpa mandarin, and Valencia orange, respectively, to 0.91 (SE = 0.04) in Volkamer lemon and Minneola tangelo. The sample size oscillated from ten in C35 citrange to 20 seeds for Minneola tangelo.

A unique, single SSRs primer could not identify all the seedlings. However, the discrimination capacity of the 17 selected primers allowed for the detection of seedlings different from the female parent as putative zygotic seedlings. The lack of the banding pattern of the male in the comparison prevented the identification of heterozygotic seedlings (thus named "putative zygotic seedlings"). In C35 citrange, 30 % of the seedlings were classified as being different from the female parent, while they represented 45 % in Volkamer lemon, 15 %

Table 1 – Annealing temperatures in 'Touchdown' PCR, with a decrease of 1 °C for each amplification cycle.

Annealing temperature	Primers
60-50 °C	CAGG9[a], CAC33[a], F2[d], F4[d], F6[d], F7[d], F9[d], F10[d], TAA1[a], TAA3[a], TAA15[a], TAA33[a]
58-48 °C	AG14[b], ATC09[b], CAC23[a], CAT01[b], CT02[b], F3[d], F11[d], TAA27[a], TAA45[a]
56-46 °C	CCSM4[c], CCSM17[c], CCSM18[c], GT03[b], TAA41[a], TAA52[a]
54-44 °C	CCSM6[c], CCSM13[c], CCSM147[c]

[a]Kijas et al. (1997), [b]Barkley et al. (2006), [c]Novelli et al. (2006), [d]Rao et al. (2008).

in Amblycarpa mandarin, 15 % in Valencia orange, and 45 % in Minneola tangelo. Furthermore, the GSI values for nucellar seedlings ranged from ≥ 0.95 to 1.0. The percentages per genotype for this type of seedlings were 70 % in C35 citrange, 55 % in Volkamer lemon, 85 % in Amblycarpa mandarin, 85 % in Valencia orange, and 55 % in Minneola tangelo (Table 4).

Discussion

Polyembryonic seed frequency and largest embryo

The percentage of polyembryonic seeds (PS) varied between genotypes and between growing cycles. In Minneola, the PS increased 35 % from 2013 to 2015, while in C35 and Amblycarpa, it decreased 7 % over the same period. The fruit production cycle did not affect the number of embryos per seed for most genotypes. Minneola was an exception, possibly due to its genetic load (Andrade-Rodríguez et al., 2004; Kishore et al., 2012; Soares-Filho et al., 1995). Consequently, the differences observed between researchers might result from various factors such as crop management, pollination, seed development, genotype (genera and species), as polyembryony observations for more than ten years in

Table 2 – Characteristics of citrus polyembryony seed in five citrus cultivars sampled during the 2013-2014 and 2014-2015 fruit growing cycles.

Cultivars	Harvest	P	NE	PEC
C35 Citrange	2013-2014	83.0 b	3.9 a	93
	2014-2015	90.0 a	4.0 a	
Volkamer Lemon	2013-2014	88.0 a	3.6 a	100
	2014-2015	83.0 a	2.7 a	
Amblycarpa Mandarin	2013-2014	88.0 b	4.6 a	93
	2014-2015	95.0 a	5.2 a	
Valencia Orange	2013-2014	98.0 a	4.4 a	87
	2014-2015	93.0 a	4.6 a	
Minneola Tangelo	2013-2014	65.0 b	2.9 b	93
	2014-2015	90.0 a	4.0 a	

P = Polyembryony percentages, NE = number of embryos per seed, PEC = percentage of large embryos located in the chalaza. Means with different letters per cultivar in a column are statistically different, according to the non-parametric Mann-Whitney test ($p \leq 0.05$).

Table 3 – Seedlings (sample size = 20) obtained from the largest embryo per seed identified as different from the female parent by simple sequence repeats (SSR) primers in five citrus cultivars.

Cultivars	Seedlings different from the female parent	Molecular primer (allele size on par bases) with polymorphism information content (PIC) values greater than 0.5	
C35 Citrange	6 (30 %)	CCSM4 (87-125)	F4 (150-162)
		CCSM13 (72-83)	F6 (137-162)
		CCSM18 (202-275)	F7 (93-105)
Volkamer Lemon	9 (45 %)	CAT01 (137-200)	F11 (138-173)
		CCSM13 (72-83)	GT03 (150-210)
		CCSM147(113-123)	TAA41 (137-195)
			F4 (150-162)
Amblycarpa Mandarin	3 (15 %)	TAA1 (162-178)	TAA45 (131-143)
		TAA41 (137-195)	
Valencia Orange	3 (15 %)	AG14 (137-190)	ATC09 (177-210)
		TAA41 (137-195)	
Minneola Tangelo	9 (45 %)	CAT01 (137-200)	F7 (93-105)
		CCSM4 (90-120)	F11 (138-173)
		F2 (137-166)	TAA41 (137-195)
		F4 (150-162)	TAA52 (84-120)

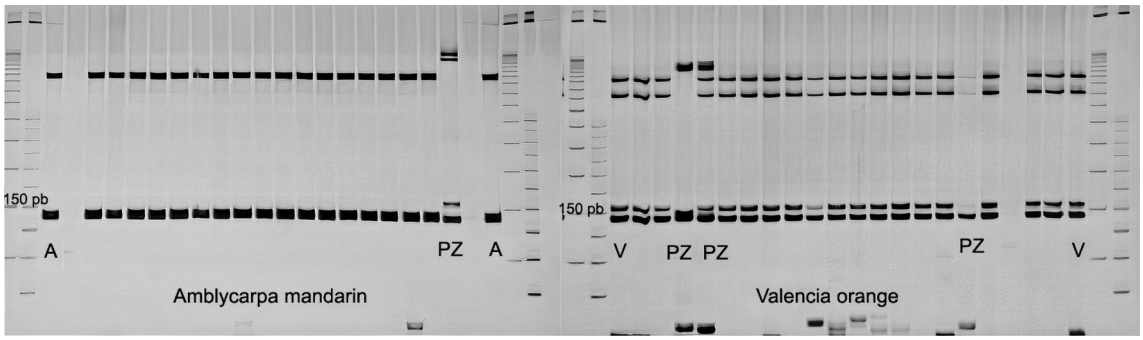

Figure 2 – Microsatellite F4 alleles banding pattern (150–162 base pair, bp) on 14 % polyacrylamide gel, 50 and 25 bp molecular size markers; A = Amblycarpa mandarin female parent; V = Valencia orange female parent; PZ = Seedling different from the female parent.

Table 4 – Genotype similarity index (GSI) values between seedlings obtained from the largest embryo end the female parent in five polyembryonic citrus cultivars. GSI was calculated with 30 simple sequence repeats (SSR) primers.

Replication	Cultivars				
	C35 Citrange	Volkamer Lemon	Amblycarpa Mandarin	Valencia Orange	Minneola Tangelo
	GSI	GSI	GSI	GSI	GSI
1	0.94	0.76	1.00	0.99	0.88
2	0.95	0.71	0.99	0.74	0.85
3	0.99	0.97	0.98	0.71	0.99
4	0.86	0.99	0.99	0.93	0.76
5	0.87	0.96	0.99	1.00	0.81
6	1.00	0.97	0.98	0.99	0.95
7	0.95	0.87	0.99	0.96	0.95
8	0.98	0.91	0.98	1.00	0.98
9	0.86	1.00	0.97	1.00	0.77
10	0.84	1.00	0.98	1.00	0.97
11	0.99	0.96	0.98	1.00	0.88
12	1.00	0.97	0.97	1.00	0.79
13	0.97	0.78	0.99	1.00	0.80
14	0.98	0.82	0.99	0.99	1.00
15	0.94	0.96	0.85	1.00	0.99
16	0.98	0.96	1.00	0.99	1.00
17	0.98	0.88	0.99	0.96	0.99
18	0.99	0.91	0.99	1.00	1.00
19	1.00	0.96	0.79	1.00	0.80
20	1.00	0.94	0.81	0.99	0.99
Nucellar seedlings[a]	70 %	55 %	85 %	85 %	55 %

[a]GSI values ≥ 0.95.

citrus and mango have revealed (Andrade-Rodríguez et al., 2004, 2005; Martínez-Ochoa et al., 2012).

Studies on immature seeds have shown that the endosperm promotes embryo development near the micropyle end. Similarly, nucellar embryo growth is favored as it starts earlier (Koltunow et al., 1995; Wakana and Uemoto, 1988). Consequently, the largest embryo in the seed should be the one that developed earliest. The largest embryo in each seed was located at the chalaza in at least 87 % of the cases. By considering the location (Table 3) and the asexual origin (Table 4), we identified that 65 % of the embryos in the chalaza were nucellar in C35 citrange, 55 % in Volkamer lemon, 79 % in Amblycarpa mandarin, 74 % in Valencia orange and 51 % in Minneola tangelo. Therefore, the chalaza is not an indicator of the location of the nucellar embryo.

Identification of nucellar seedlings when derived from the largest embryo

From previous experiences on molecular marking (Andrade-Rodríguez et al., 2005; Martínez-Ochoa et al., 2012), we used 30 microsatellites (Barkley et al., 2006; Kijas et al., 1997; Novelli et al., 2006; Rao et al., 2008) associated to the GSI analytical–method to identify seedlings derived from the nucella.

Our results showed that 17 SSRs were the most useful molecular markers for detecting nucellar seedlings in citrus plants and identifying polymorphism levels depending on the number of seedlings that were different from the female parent (Table 3). TAA41 was the most informative primer for identifying seedlings different from the female parent in the five cultivars studied (orange, lemon, mandarin, tangelo, and citrange). However, previous research classified this primer as poorly informative for several citrus crosses (Carrillo-Medrano et al., 2018; Yildiz et al., 2013). Therefore, only one SSR primer or a small primer set is insufficient for determining the embryo sexual origin in different citrus cultivars.

In this research study, we evaluated only the plant produced by the largest embryo in each seed, whereas other authors have evaluated all the embryos from the seed (Novelli et al., 2006; Rao et al., 2008; Carrillo-Medrano et al., 2018). Consequently, we measured plants related to the embryo that gave rise to them. Instead, Rao et al. (2007) used expressed sequence tag-derived simple sequence repeat markers (EST-SSRs) to differentiate hybrids of *C. reticulata* Blanco x *C. maxima* (Burm.) Merr., by planting whole seeds with all their embryos.

Out of 204 hybrids, they identified 73 % as zygotic. The use of morphological markers (for trifoliate leaf), Soares-Filho et al. (2000) recognized 39 % of zygotic plants from large embryos (≥ 5 mm) in the 'Sunki tangerine; however, these authors did not separate the individual embryos of each seed, since they studied a set of large embryos. In our study individual evaluations of each embryo and its sexual origin allowed us to generate reliable conclusions related to location, size and the probability of nucellar embryo origin only of viable embryos capable of producing seedlings.

One specific band (either by presence or absence) indicated a polymorphism in our study. The polymorphisms observed in SSRs derived from differences in the number of repeats of the motif caused by polymerase strand-slippage in DNA replication or by recombination errors (Carneiro et al., 2016). Furthermore, different alleles may exist at an SSR locus (mutations that may have evaded correction by the DNA mismatch repair system), implying that SSRs are more informative per locus than other molecular markers, including single nucleotide polymorphisms (SNPs) (Carneiro et al., 2016). Consequently, we considered an SSR polymorphism a marker good enough to differentiate the female parent from identical or different seedlings.

On the other hand, high seedling homogeneity (uniformity) is necessary as the rootstock might affect the grafted cultivar development (Andrade-Rodríguez et al., 2004; Passos et al., 2006). Therefore, it is essential to determine the type of seedlings, nucellar or zygotic, originating from seeds on a commercial scale (Passos et al., 2006). In previous studies on citrus polyembryony, researchers have claimed a preference for polyembryonic

rootstocks for apomictic propagation. For example, for *C. reshni* evaluated across three cycles, Andrade-Rodríguez et al. (2005) estimated between 79 and 90 % of polyembryonic seeds and 83 % of nucellar seedlings. On the other hand, for *C. volkameriana* rootstock, García et al. (1999) reported that only 78 % of nucellar seedlings derived from open-pollinated seeds, while we registered 55 % nucellar seedlings for the same citrus species. In the 2014–2015 season, all the cultivars had more than 83 % of polyembryony (Table 2). However, only Amblycarpa mandarin and Valencia orange had high levels of apomictic propagation with 85 % of nucellar embryos (Table 4).

Our results show the high probability of reproducing plants different from the female parent (not nucellar plants) from the LE from seeds of the five polyembryonic citruses studied. Considering that the percentage of plants different from the female parent ranged between 15 % and 45 %, vegetative propagation could only be achieved by selecting nucellar plants utilizing molecular markers. Therefore, future research on citrus polyembryony should explore the relationship between the origin of the individual embryo with its size and position in the seed. Such information might provide insights into the probability embryos in positions 2, 3, 4, 5, 6, and 7 (Figure 1) have for nucellar plant propagation.

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Authors' Contributions

Conceptualization: Martínez-Ochoa, E.C.; Villegas-Monter, A.; Alarcón-Zúñiga, B. **Data acquisition:** Martínez-Ochoa, E.C.; Villegas-Velázquez, I.; **Data analysis:** Martínez-Ochoa, E.C.; Villegas-Monter, A.; Alarcón-Zúñiga, B; González-Hernández, V.A.; **Design of methodology**: Martínez-Ochoa, E.C.; Villegas-Monter, A.; Alarcón-Zúñiga, B.; Villegas-Velázquez, I.; **Writing and editing:** González-Hernández, V.A.; Martínez-Ochoa, E.C.; Villegas-Monter, A.; Villegas-Velázquez, I.

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