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Seeds

Number of seeds in fruits and frequency of hybrids obtained in crossings with IAC 2019Maria mandarin

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Abstract -The Citrus Breeding Program of the Citriculture Center at the Agronomic Institute (IAC) has developed a mandarin cultivar IAC 2019Maria from the crossing between Murcott IAC tangor (*Citrus reticulata* x *Citrus sinensis*) and Pera IAC sweet orange (*C. sinensis*). The present study aimed to assess the number of seeds in fruits of IAC 2019Maria mandarin and to identify zygotic embryos and triploid plants in the crossings between IAC 2019Maria mandarin, Pera IAC sweet orange, and Ponkan mandarin (*C. reticulata* Blanco), in addition to IAC 2019Maria mandarin in open and self-pollination. IAC 2019Maria flowers were self-pollinated, pollinated with pollen from Pera sweet orange and Ponkan mandarin, and had no pollination. The embryos were identified using microsatellite molecular markers and ploidy was assessed by flow cytometry. The results of the treatment with no pollination suggest the variety does not produce parthenocarpic fruits. The genotyping results showed that 100% of the populations consist of zygotic embryos, suggesting that IAC 2019Maria mandarin is a plant with low polyembryony. The ploidy analysis of the hybrids allowed identifying a triploid plant from an aborted seed from the crossing with Pera sweet orange and two tetraploids, one from the crossing with Pera sweet orange and one from self-pollination.
Index terms: Zygotic embryo, IAC 2019Maria, genetic improvement, triploidy.

Número de sementes em frutos e frequência de híbridos obtidos de cruzamentos com a tangerina IAC 2019Maria

Resumo - No Programa de Melhoramento de Citros do Centro de Citricultura do Instituto Agronômico (IAC), foi desenvolvida uma cultivar, a tangerina IAC 2019Maria, proveniente do cruzamento entre tangor Murcott IAC (*Citrus reticulata* x *Citrus sinensis*) e laranja Pera IAC (*C. sinensis*). O presente trabalho teve como objetivo avaliar o número de sementes em frutos da tangerina IAC 2019Maria e identificar embriões zigóticos e plantas triploides dos cruzamentos entre tangerina IAC 2019Maria e laranja Pera IAC e tangerina Ponkan (*C. reticulata* Blanco), tangerina IAC 2019Maria em polinização aberta e de autopolinização. As flores de IAC 2019Maria foram autopolinizadas, polinizadas com pólens de laranja Pera e tangerina Ponkan e sem polinização. Os embriões foram identificados utilizando marcadores moleculares microssatélites e a ploidia foi avaliada por citometria de fluxo. Os resultados do tratamento sem polinização sugerem que a variedade não produz frutos partenocárpicos. Os resultados da genotipagem demonstraram que 100% de todas as populações consistem em embriões zigóticos, sugerindo que a tangerina IAC 2019Maria é uma planta com baixa taxa de poliembrionia. A análise da ploidia dos híbridos permitiu identificar uma planta triploide de semente abortada do cruzamento com laranja Pera e duas tetraploides, uma do cruzamento com laranja Pera e uma de autopolinização.
Termos para indexação: Embrião zigótico, IAC 2019Maria, melhoramento genético, triploidia.

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Introduction

Brazil is one of the largest global citrus producers, particularly regarding processed fruit such as sweet oranges. However, when it comes to dessert fruits for consumption *in natura*, such as mandarins, Brazil still places behind China, Spain, and Turkey (FAO, 2022). The prevailing mandarin varieties in Brazil are Ponkan mandarin and Murcott tangor (BASTOS et al., 2014), however, those varieties do not meet the international market standards, which has greatly valued the consumption and development of seedless fruits (OLIVEIRA; SCIVITTARO, 2011). One example is Murcott tangor, with about 20 seeds per fruit, which keeps it from the foreign market (PIO et al., 2005). In addition, the varieties Ponkan mandarin and Murcott tangor are susceptible to Alternaria brown spot (ABS), caused by fungus *Alternaria alternata*, which severely affects Brazilian mandarin orchards, causing loss of productivity, an issue for citrus growers (AZEVEDO et al., 2010).

One of the main citrus germplasm banks in the country is located at the Sylvio Moreira Citriculture Center of the Agronomic Institute (IAC) in Cordeirópolis, SP. The Citriculture Center has been developing a breeding program that yielded the cultivar IAC 2019Maria mandarin, an F_1 hybrid from the crossing between Murcott IAC tangor (*C. reticulata* x *C. sinensis*) (female parent) and Pera IAC sweet orange (*C. sinensis*) (male parent). The female parent is a hybrid between sweet orange and mandarin that is resistant to citrus variegated chlorosis (CVC) and citrus leprosis, but susceptible to ABS. The male parent is resistant to ABS, but susceptible to CVC and citrus leprosis.

IAC 2019Maria is the first fully Brazilian mandarin cultivar, developed over 20 years of research at the IAC. It is the first citrus cultivar by IAC protected by the National Service for the Protection of Cultivars (SNPC) of the Ministry of Agriculture, Livestock, and Food Supply (MAPA), and licensed to nurseries. Given its resistance to ABS, the cultivar causes less environmental impact since it reduces or even eliminates the need for pesticide application, also lowering production costs (CCSM/IAC, 2000).

Consumers of fresh fruits have emphasized the lack of seeds as a quality requirement (BARRY, 2004). To Oliveira and Scivittaro (2011), the international market considers as seedless the batches that have an average of up to two seeds per fruit. However, citrus species usually require seed development to produce fruits. When they self-pollinate, they have up to five seeds per fruit. Nonetheless, the presence of plants of other varieties close to the relevant ones may undesirably increase that number (GUARDIOLA, 1992). Azevedo and Pio (2002), when studying the influence of pollination in Murcott tangor, observed that, when flowers of that variety were pollinated with Valência and Natal sweet oranges, the number of seeds increased when compared with pollination with Ponkan mandarin, Pera sweet orange, and self-pollination. Some species, however, do not need fertilization to form fruits, a phenomenon called parthenocarpy, in which the fruits formed with no pollination and they have no seeds. Clementine varieties have a high rate of parthenocarpy and may produce commercial crops of seedless fruits (OLIVEIRA et al., 2004; OLIVEIRA; SCIVITTARO, 2011). In this case, its own pollen is incompatible and does not fertilize the ovules (DONADIO et al., 1998).

Triploid varieties are of great interest for citrus breeding as their fruits usually tend to be parthenocarpic, a desirable characteristic to obtain seedless fruits (OLIVEIRA, 2013). Varieties for commercial use such as Garbi and Safor have been selected from triploid hybrids (SOARES FILHO et al., 2013). Thus, the selection of triploid plants had been and still is a very interesting way of developing seedless cultivars (KHAN, 2007).

One way of obtaining seedless fruits is by producing triploid hybrids via crossing between diploid (2x) and tetraploid (4x) genotypes (REFORGIATO RECUPERO; RUSSO; RECUPERO, 2005), or between diploids (2x) and diploids (2x), in which the female parent contributes with nondisjunction gametes (ESEN; SOOST, 1971).

Zygotic embryos are usually identified based on morphological markers when the male parent has a dominant morphological marker. However, when neither parent has a dominant character, other methods must be used, such as using DNA polymorphisms (MACHADO et al., 2005).

Among DNA-based methods, the analysis with molecular markers such as SSR (Simple Sequence Repeat) microsatellites has proved efficient (CRISTOFANI et al., 2001; RUIZ; BRETO; ASINS, 2000).

SSR microsatellite molecular markers have been widely used in breeding programs and for genetic certification in citriculture and they help in the identification, differentiation, and characterization of varieties and hybrids (CRISTOFANI et al., 2001; CRISTOFANI et al., 2003; DEZOTTI et al., 2017; NOVELLI et al., 2006; PALMIERI et al., 2007); Rao et al. (2008), when using SSR-EST markers, were able to differentiate nucellar plantlets from zygotic plantlets in mandarin (*C. reticulata* Blanco) and pummelo (*C. maxima* Merr.) hybrids.

Thus, given the growing demand for seedless citrus varieties, it is indispensable to know the compatibility and cross-pollination rate of the main varieties grown in the country. That will allow proper planning of orchards, preventing varieties that can produce seedless fruits from being planted near orchards of compatible varieties (AZEVEDO; PIO, 2001).

This way, the present research aimed to understand the influence of cross-pollination, self-pollination, and pollination impediment in the formation of seeds in fruits of IAC 2019Maria mandarin, in addition to identify zygotic embryos and determine the ploidy level of the hybrids obtained from crossings of IAC 2019Maria mandarin with Ponkan mandarin (*C. reticulata* Blanco) and Pera IAC sweet orange (*C. sinensis*), self-pollination and open pollination.

Material and methods

Plant Material

The experiment was conducted at the Sylvio Moreira Citriculture Center of the Agronomic Institute (IAC) in the city of Cordeirópolis, SP, Brazil, located at 22°32' S and 47°27' W with 639 m altitude and Cwa climate according to the Köppen classification. The soil is typical dark-red dystrophic latosol with clayey texture (PIO; MINAMI; FIGUEIREDO, 2001).

The IAC 2019Maria mandarin plants [tangor Murcott IAC (C. reticulata x C. sinensis) and Pera IAC sweet orange (C. sinensis)] were grafted onto Rangpur lime (C. limonia) (Figure 1).

Figure 1. Standard of cultivar IAC 2019Maria grafted onto Rangpur lime. Plant was five years old (Botucatu, SP, Brazil).

Influence of Pollination on the Number of Seeds of IAC 2019Maria Mandarin

Between August and October 2018, five types of pollination were carried out in IAC 2019Maria mandarin, namely: cross-pollination with Ponkan mandarin, cross-pollination with Pera IAC sweet orange, self-pollination, pollination impediment, and open pollination.

Pollination was performed manually following the protocol by Azevedo et al. (2013). First, 50 still closed flower buds were collected from Pera sweet orange and Ponkan mandarin, which were stored in Petri dishes in a grow room with 16 h photoperiod until opening of the anthers. Closed flower buds of IAC 2019Maria mandarin, prior to anthesis, with receptive stigmas, were emasculated and the pollen of the stored flower buds was brushed onto the stigma of IAC 2019Maria mandarin flowers, which were then stored in paper bags (CAMERON; FROST, 1968; AZEVEDO et al., 2013). In self-pollination, the closed flower buds were only protected by paper bags. To prevent pollination, the buds were emasculated and bagged, while for open pollination, the flowers were simply let free for natural pollination to occur (FERRARO; PIO; AZEVEDO, 2006). In total, five treatments were studied: 1) IAC 2019Maria mandarin x Pera sweet orange with 163 flowers pollinated; 2) IAC 2019Maria mandarin x Ponkan mandarin with 51 flowers pollinated; 3) self-pollination of 763 flowers of IAC 2019Maria mandarin; 4) emasculation of 312 flowers of IAC 2019Maria mandarin, and 5) IAC 2019Maria mandarin in open pollination.

Percentage of Fruits Harvested and Number of Seeds per Fruit

All fruits, whether resulting from the pollinations or not, were harvested in May 2019. The percentage of fruits harvested was obtained by comparing the number of fruits with the number of flowers pollinated. The number of seeds of ten fruits was obtained by direct counting after the fruits were opened.

The seeds collected from crossings were peeled and sterilized with 70% alcohol and in 25% sodium hypochlorite solution (2% active principle), then washed three times with autoclaved distilled water in a sterile environment to be inserted in tubes containing MS culture medium (MURASHIGE; SKOOG, 1962). The samples were kept in a grow room with 16 h photoperiod and temperature at 24 °C. The germinated plantlets were transplanted in tubes with commercial substrate and grown in a greenhouse.

Identification of Zygotic Embryos

DNA was extracted using the methodology described by Murray and Thompson (1980) with adaptations by Machado et al. (1996). The extracted DNA was dissolved in 80 µL Milli-Q H_2O with 10 ug/uL RNAse.

Nine SSR marker pairs were used, which were developed from information of citrus expressed sequence tags (ESTs) – CitEST (PALMIERI et al., 2007) and genomic sequences (NOVELLI et al., 2006) previously described by Dezotti et al. (2017), which are presented in Table 1.

Table 1. SSR locus, repetition type, forward and reverse sequence, size, number of alleles, observed heterozygosity (Ho), and expected heterozygosity (Hexp) of nine SSR-EST markers.

SSR	Repetition	Forward (5'-3')	Reverse (5'-3')	Estimated size (bp)	No. of alleles	(Ho)	(Hexp)	PIC
CCSM-EST-60	(ATC)8	cttggaggaaacagcagagg	cgaattggaatcaaaggcat	100 to 200	2	0.457	0.360	0.294
CCSM-EST-64	(GAA)10(n)21 (GAA)7	atctgcagggacaaaaccag	tcatcttcactcactcggca	200 to 300	2	0.347	0.287	0.245
CCSM-EST-89	(ATA)7	acttatcttgcacccgacga	gaggtctcgaagtcacggag	200 to 400	2	0.309	0.398	0.318
CCSM-EST-169	(ATGATC)4	acgtcgctagatcctgtgct	catacaccaaacaccgtcca	200 to 300	2	0.244	0.215	0.191
CCSM-EST-159	(TTCTTG)4	tgggtcattgatgttgtgct	cacagatgcagaaggggatt	<100	2	0.663	0.444	0.345
CCSM-EST-161	(TTTTTA)4	gaggaggacgaatgaaagca	gaacagaagagctggccaat	200 to 300	2	0.102	0.097	0.092
CCSM-EST-164	(TC)11	gagaagcccgtctgcactta	acgagagcggaaacaagaga	< 150	2	0.693	0.478	0.363
CCSM-EST-191	(CAG)9	gagggagtggctatgcaaga	tcgagattcaattgctgcac	100 to 200	3	0.388	0.468	0.402
CCSM-EST-234	(GGC)7	aatgcgtgggcaataacttc	ttcaatatcggcccaaactc	200 to 300	3	0.286	0.359	0.305

Source: Dezotti et al. (2017).

DNA amplification (PCR) was conducted using 100 ng DNA in a total volume of 15 µL containing 0.3 µL of each primer at a concentration of 10 µM (forward and reverse), 1.5 µL reaction buffer (Buffer 10X containing 16 mM $MgCl_2$), 0.3 µL Taq polymerase (5 U/µL), 1.2 µL dNTP at a concentration of 25 mM, 1.5 µL DNA, and autoclaved distilled water to complete the volume. Amplification was performed in Vereti 96-Well Thermal Cycler devices.

The amplified DNA was visualized in 3% agarose gel with ethidium bromide. Finally, for gel visualization, an imaging system was used for the analyses.

The zygotic plantlets were identified by comparing the patterns of DNA fragments from individuals of the progeny and of the parents. Those exhibiting DNA polymorphism in relation to IAC 2019Maria mandarin were considered zygotic, i.e., the ones with a different pattern than the mother plant.

Ploidy Analyses via Flow Cytometry

The ploidy of the plants derived from each crossing was assessed by the flow cytometry method cited by Latado et al. (2007). A PAS II-III flow cytometer (Partec Gmbh, Germany) equipped with a 100 W HBO bulb and kg1, BG 38, and CG 435 filters was used. The samples were assessed in the software CyView (Partec Gmbh, Germany) with calibration of Gain = 600 and Low level (LL) = 0.70 to produce histograms. The samples whose coefficients of variation were above 5% were discarded (LATADO et al., 2007). The ploidy of the hybrid plants was determined in comparison with samples of diploid and triploid control plants. Only the samples identified as being polyploid and those from the diploid and triploid controls were analyzed with three replicates per plant (ROCHA, 2014). The IAC 2019Maria mandarin cultivar was used as diploid control (2x) while the Tahiti lime (C. latifolia Tanaka) cultivar was used as triploid control (3x).

Results and discussion

Of the treatments carried out in this work, pollination with Pera sweet orange had the highest number of set fruits (85 or 52%), whereas pollination with Ponkan mandarin yielded eight fruits (16%). The self-pollinated flowers produced 43 fruits (6%) and the treatment with no pollination resulted in one set fruit (0.3%) (Table 2). Similar results were observed in other studies. Azevedo and Pio (2002) reported in their research with Murcott tangor that no fruit setting took place in flowers with no pollination, which suggests the variety does not produce parthenocarpic fruits. Thus, it can be suggested that this variety also does not have the capacity of producing fruits without pollination. A possible explanation for such result is that the lack of pollination may cause a reduction in gibberellin levels in the ovaries, leading to a likely drop of the fruits (BEN-CHEIKH et al., 1997). In the case of self-pollination, the result may have been because a small amount of pollen grains reached the stigma, or perhaps due to pollen unviability, with it possibly becoming unviable before the stigma was receptive (WALLACE; LEE, 1999).

Table 2. Number of pollinations carried out (NPC), number of set and harvested fruits (NFH), and percentage of fruits harvested (PFH) per treatment in IAC 2019Maria mandarins (Cordeirópolis, 2019).

Treatments	NPC	NFH	PFH
Self-pollination	763	43	6%
No pollination	312	1	0, 3%
Pollination - Pera sweet orange	163	85	52%
Pollination - Ponkan mandarin	51	8	16%
Total	1,294	137	

The mean number of seeds in IAC 2019Maria mandarin from pollination with Ponkan mandarin (21.38) was higher when compared with pollination with Pera sweet orange (16.19) and with self-pollination (14.79), which shows that cross-pollination leads to more seed formation than pollination with pollen from the flower itself. However, regarding seed viability, the difference was more evident per treatment. The fruits obtained from pollination with Pera sweet orange had higher percentage of viable seeds (74%) and lower percentage of aborted seeds (26%), fruits from pollination with Ponkan mandarin also had higher percentage of viable seeds (68%) and lower percentage of aborted seeds (32%), whereas the fruits of self-pollination contained more aborted seeds (67%) and fewer viable seeds (33%) (Table 3 and Figure 2). Seed formation is one of the conditions for fruit setting in the plant, as was verified in this research, indicating that fruits from cross-pollination had a higher number of seeds and higher setting percentage (OLIVEIRA; SCIVITTARO, 2011).

Table 3. Mean number of seeds (MNS), percentage of viable (PVS) and aborted (PAS) seeds per fruit of IAC 2019Maria mandarin obtained from self-pollination and controlled pollination (Cordeirópolis, 2019).

Treatments	MNS	PVS	PAS
Pollen - Pera sweet orange	16.19	74	26
Self-pollinated	14.79	33	67
Open pollination	22.88	47	53
Pollen - Ponkan mandarin	21.38	68	32

In the self-pollination treatments, such result may have been due to a low amount of grains of pollen reaching the stigma or due to pollen unviability, which may have become unviable before the stigma was receptive (WALLACE; LEE, 1999), or even due to incompatibility. Carvalho et al. (1997) considered in their study with Sunki mandarin that self-pollination possibly prevented post-zygotic compatibility, leading to seeds ceasing development.

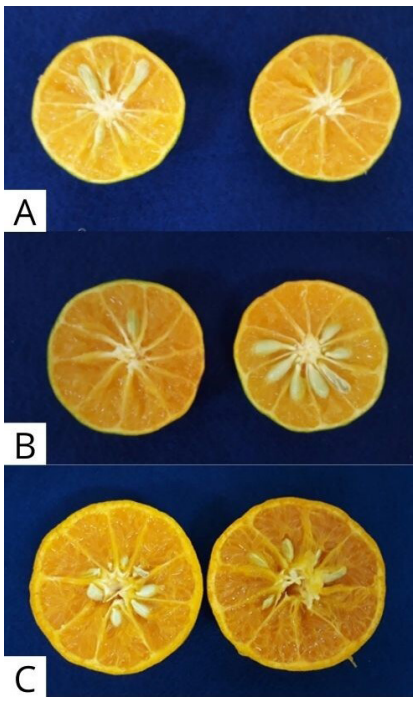

Figure 2. Harvested fruits from controlled crossings of IAC 2019Maria mandarin. Fruit from self-pollination (A); Fruit from cross-pollination with Pera sweet orange pollen (B); Fruit from pollination with Ponkan mandarin (C).

In 'Huami Wuhegonggan' (HMWG) citrus (*C. sinensis* x *C. reticulata*), embryo abortion was the greatest cause of a lack of seeds and not self-incompatibility. However, the low viability and germination frequency of the pollen, and the consequent low fertility, may have contributed to the lack of seeds (QIN et al., 2015). In 'Zigui shatian' pomelo (*C. grandis* Osbeck), its self-sterility is due to the irregular development of post-zygotic embryos and not due to self-incompatibility, shown by the presence of wilted seeds (CHAI et al., 2011). Therefore, it is suggested that IAC 2019Maria mandarin orchards be planted isolated so as to prevent cross-pollination and avoid a considerable increase in the number of seeds in the fruits.

Five populations were obtained from the crossings, namely: population originated from self-pollination of IAC 2019Maria mandarin; population originated from IAC 2019Maria mandarin in open pollination (unknown male parent); population of IAC 2019Maria mandarin X Ponkan mandarin; population of plants originated from IAC 2019Maria mandarin X Pera sweet orange; and plants of aborted seeds of IAC 2019Maria mandarin X Pera sweet orange (Table 4).

Table 4. Characterization of plants of five crossing populations, number of plants obtained per crossing (NP), number of zygotic plants (NZ), number of nucellar plants (NN), and percentage of zygotic plants obtained by crossing (PZ).

Populations	N P	N Z	N N	P Z
IAC 2019Maria mandarin x Ponkan mandarin	11	11	0	100
IAC 2019Maria mandarin (open pollination)	21	21	0	100
Self-pollinated IAC 2019Maria mandarin	33	33	0	100
IAC 2019Maria mandarin x Pera sweet Orange	258	258	0	100
IAC 2019Maria mandarin x Pera sweet orange (aborted seeds)	11	11	0	100
TOTAL	334	334	0	100

Of the 27 SSR markers tested (DEZOTTI et al., 2017), nine were considered more informative and were used to genotype the populations, namely CCSM-EST-159, CCSM-EST-161, CCSM-EST-164, CCSM-EST-191, CCSM-EST-234, CCSM-EST-169, CCSM-EST-60, CCSM-EST-64, and CCSM-EST-89 (Table 1).

The markers that enabled visualizing two or more alleles (DNA fragments) in the mother plant were considered informative for the IAC 2019Maria mandarin self-pollination population, which allowed determining the self-pollination hybrid individuals by the lack of one of the bands present in the female parent.

For individuals derived from crossings, the markers considered informative were those with two or more alleles for at least one of the parents. For example, marker CCSM-EST-159 (Figure 3-A) allows visualizing only one allele for IAC 2019Maria mandarin, which is not informative for the self-pollination population, but in Ponkan mandarin and Pera sweet orange, two alleles could be visualized in each of the individuals, showing clear polymorphism between the parents. Marker CCSM-EST-164 (Figure 3-D), in contrast, had a pattern of the same two alleles for IAC 2019Maria mandarin and Pera sweet orange, enabling the identification of hybrid individuals by the lack of one of the bands in the progeny, which are present in both parents.

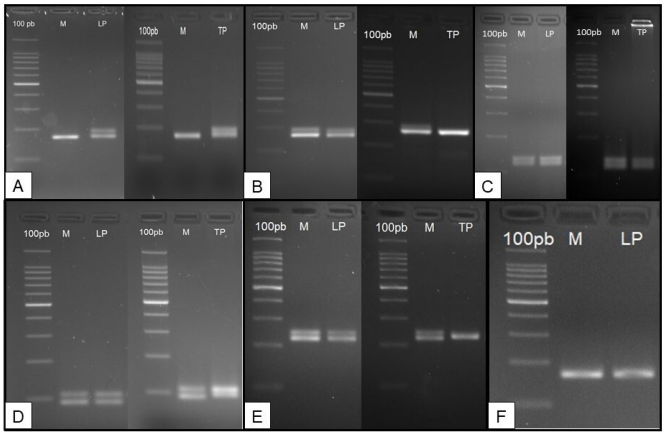

Figure 3. 3% agarose gel electrophoresis. DNA amplification of IAC 2019Maria mandarin (M), Pera sweet orange (LP), and Ponkan mandarin (TP) using SSR marker: A) CCSM-EST-159; B) CCSM-EST-161; C) CCSM-EST-191; D) CCSM-EST-164; E) CCSM-EST-234; and F) CCSM-40.

The markers considered non-informative were the ones that were monomorphic for both parents, not enabling the visualization of clear differences between them. For instance, genomic marker CCSM40 (Figure 3-F) was monomorphic for the IAC 2019Maria x Pera sweet orange population; in addition, since it had a pattern of only one band (one allele) for IAC 2019Maria mandarin, it was not considered informative for the population originated from self-pollination.

Meanwhile, markers CCSM-EST-159, CCSM-EST-161, CCSM-EST-164, CCSM-EST-191, CCSM-EST-234 (Figure 3), CCSM-EST-169, CCSM-EST-60, CCSM-EST-64, and CCSM-EST-89 amplified informative alleles that had clear polymorphism among IAC 2019Maria mandarin, Ponkan mandarin, and Pera sweet orange (Figure 3-A, B, C, D, E), hence they were used for genotyping the populations.

Regarding the most informative markers, CCSM-EST-159 and CCSM-EST-191 allowed the visualization of two alleles in the population, while markers CCSM-EST-161, CCSM-EST-164, and CCSM-EST-234 allowed visualizing three different alleles in the populations. Ponkan mandarin, for example, had only one band for marker CCSM-EST-234 of different molecular weight than the two bands of IAC 2019Maria mandarin and Pera sweet orange (Figure 3-E). For marker CCSM-EST-161 (Figure 3-B), of the two bands present in the female parent, only one was present in Ponkan mandarin. Marker CCSM-EST-164 (Figure 3-D) allowed the visualization of two bands in Ponkan mandarin, one of which in common with the female parent and the other of different molecular weight.

For the male parents, marker CCSM-EST-159 (Figure 3-A) allowed the visualization of two alleles, only one of which in common with IAC 2019Maria mandarin, while for the other markers the allele pattern of the male parent and IAC 2019Maria mandarin was the same.

The genotyping results of F_1 populations are shown in Tables 4 and 5. It can be seen that all individuals analyzed were considered hybrid as they exhibited clear polymorphism (different DNA fragment patterns) in relation to the mother plant (IAC 2019Maria mandarin).

The self-pollination treatment featured 763 pollinations, which yielded 43 fruits with about 172 seeds, from which 33 plants were obtained. In this experiment, all plants (100%) were identified as hybrid, which indicates IAC 2019Maria mandarin is a self-compatible plant. In this case, the identification of zygotic plants was verified via the joint analysis of four markers (Figure 4).

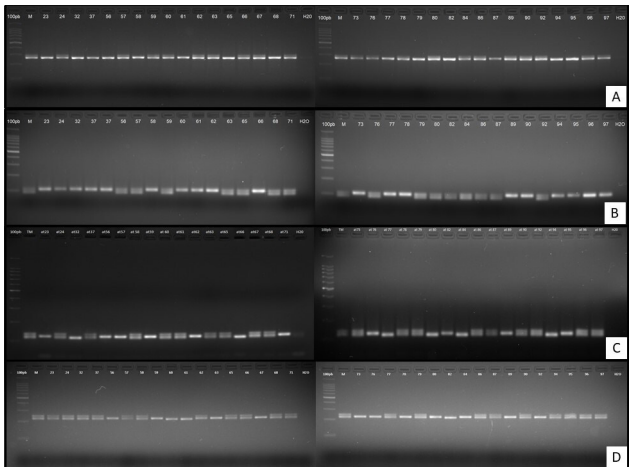

Figure 4. Genotyping in 3% agarose gel, with four markers, of hybrids of self-pollinated IAC 2019Maria mandarin: A) CCSM-EST-161; B) CCSM-EST-164; C) CCSM-EST-191; D) CCSM-EST-234.

From the open pollinations in IAC 2019Maria mandarin, 21 fruits were obtained with about 319 seeds and 21 plants, which were all identified as hybrids by genotyping with five markers. As well as self-pollinated ones, the plants whose DNA fragment pattern differed from the mother plant were considered hybrids as the male parent is unknown for comparison.

In the crossing of IAC 2019Maria mandarin with Ponkan mandarin, 51 pollinations were performed, from which eight fruits were obtained with 116 seeds and 11 plants, 100% of which were found to be hybrid when analyzed by five markers.

In the crossings between IAC2019Maria mandarin and Pera sweet orange, 163 pollinations were performed, from which 85 fruits were obtained with approximately 961 seeds and 258 plants, 100% of which were identified as deriving from zygotic embryos. Of the seeds obtained in this crossing, 348 were aborted and, when germinated, originated 11 plants, all of which hybrids. Both in the crossings with Ponkan mandarin and with Pera sweet orange, the analysis was performed considering as zygotics those that had alleles inherited from the male parent or that had a lack of maternal alleles.

The efficiency of each marker varied for each population (Table 5). Marker CCSM-EST-159 enabled identifying 89% of the hybrids of the entire population of IAC 2019Maria mandarin X Pera sweet orange and 91% of hybrids in plants from aborted seeds. In the open-pollination population, only 19% of the plants were identified as zygotic and, in crossings with Ponkan mandarin, 58%. In the study by Dezotti et al. (2017), that was the only specific marker identified for IAC 2019Maria mandarin (TM x LP 281).

Marker CCSM-EST-161 had informative alleles for 58% of IAC 2019Maria x Ponkan mandarin, 38% in open pollination, 41% in self-pollination, and 42% in crossings with Pera sweet orange. Marker CCSM-EST-164, in turn, ranged from 83% to 38%. Marker CCSM-EST-234 also varied a lot at 83% to 36%. Marker CCSM-EST-191 was not as informative for the populations, identifying less than 50% in all crossings.

The gel electrophoresis results of the samples of IAC 2019Maria mandarin progeny showed that 100% of the plants are hybrids, since, of the 334 plants assessed with five SSR markers, only six were not identified as zygotic, which required genotyping them with other markers (CCSM-EST-60, CCSM-EST-64, CCSM-EST-89, CCSM-EST-169). As seen in Figure 5, plants 51, 427, and 05 were certified as hybrids by marker CCSM-EST-169. Plants 388 and 86 were identified as hybrids by markers CCSM-EST-64 and CCSM-EST-89, while CCSM-EST-60 allowed confirming that plants 361 and 427 were hybrids.

Table 5. Percentage of zygotic plants identified by pair of SSR markers separately: 1) CCSM-EST-159; 2) CCSM-EST-161; 3) CCSM-EST-164; 4) CCSM-EST-191; 5) CCSM-EST-234.

Populations	159	161	164	191	234
IAC 2019Maria mandarin x Ponkan mandarin	58%	58%	83%	25%	83%
IAC 2019Maria mandarin (open pollination)	19%	38%	38%	48%	62%
Self-pollinated IAC 2019Maria mandarin		41%	47%	47%	41%
IAC 2019Maria mandarin x Pera sweet orange	89%	42%*	54%*	46%	53%
IAC 2019Maria mandarin x Pera sweet orange (aborted seeds)	91%		45%	36%	36%

*Percentage of zygotic embryos identified in 57 individuals of IAC 2019Maria mandarin x Pera sweet orange.

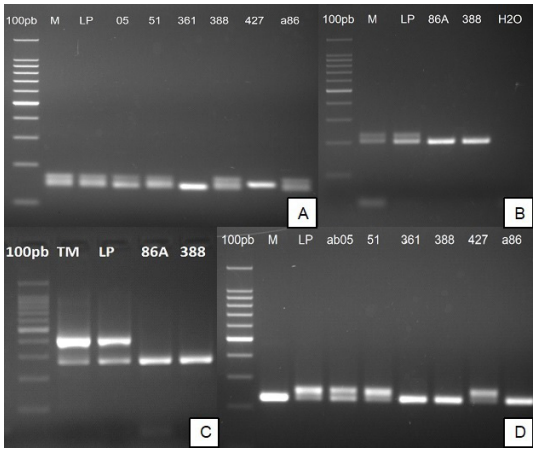

Figure 5. 3% agarose gel electrophoresis. DNA amplification of IAC 2019Maria mandarin (M), Pera sweet orange (LP), and six hybrids using SSR marker: A) CCSM-EST-60; B) CCSM-EST-64; C) CCSM-EST-89; D) CCSM-EST-169.

These results show a frequency of 100% of hybrids identified in all progenies analyzed. In comparison with isoenzyme methods, Ruiz, Breto and Asins (2000), when using microsatellites markers for the selection of nucellar and zygotic plantlets in crossings, concluded they are more efficient to identify plantlets of sexual origin given their high level of polymorphism.

According to Cristofani-Yaly (personal communication), the average number of embryos per IAC 2019Maria mandarin seed was 1.11, ranging from 1 to 2 embryos per seed, with a polyembryony percentage of 10.7%. Soares-Filho et al. (2000), when observing polyembryony of the Clementine and Sunki varieties, obtained an average of 1 and 1.3 embryos per seed, classifying them, respectively, as monoembryonic and low polyembryony.

Regarding the flow cytometry results, the histograms enabled identifying the ploidy of the plants obtained from crossings of IAC 2019Maria mandarin with Ponkan mandarin and Pera sweet orange, self-pollination, and open pollination (Figure 6).

The mean relative values of nuclear volume of the samples of diploid control plants (parents) were between 35 and 37, whereas the mean value of the triploid control plant was 53 (Table 6).

In the flow cytometry analyses, the minimum and maximum values found for the diploid hybrid plants were 31 and 44 with an average of 37. That means a haploid (x) plant would have an approximate value of 18, 37 for diploid (2x), 55 for triploid (3x), and 74 for tetraploid (4x).

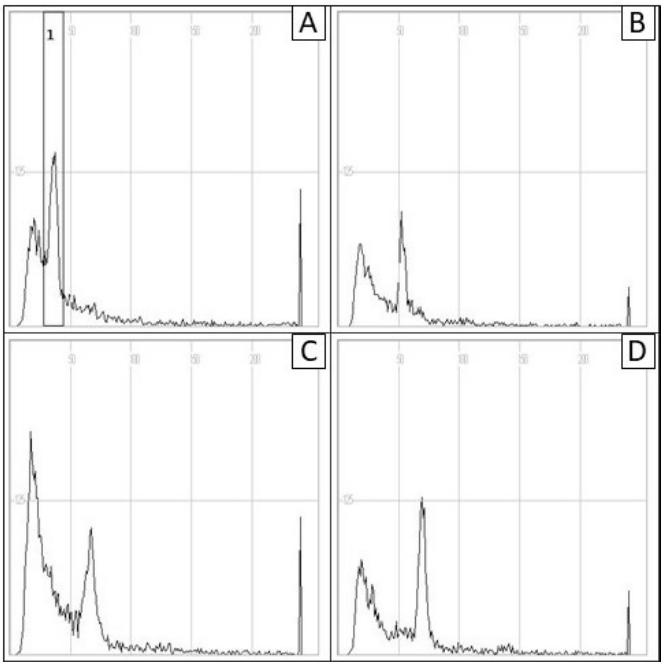

Figure 6. Histograms of leaf samples of plants of: A) diploid control (2x) IAC 2019Maria mandarin; B) 05 triploid (3x) hybrid; C) tetraploid (4x) hybrid from self-pollination 62; and D) tetraploid (4x) hybrid 72.

Table 6. Readings of nuclear suspensions of leaves from hybrid triploid and tetraploid plants and diploid and triploid control plants from crossings of IAC 2019Maria mandarin stained with DAPI fluorochrome and assessed by flow cytometry.

Genotype	Mean reading	Estimated ploidy
IAC 2019Maria mandarin	37	2x
Tahiti lime	53	3x
Self-pollination hybrid 62	71	4x
Hybrid 72 (MM x PSW)	70	4x
Hybrid 05 (aborted seed)	55	3x

Of the population of plants resulting from self-pollination, 33 individuals were analyzed by flow cytometry, which showed that only one plant (3%), hybrid 62 (Table 6, Figure 6-C), had a peak at 71, thus being considered a tetraploid (4x) plant. Meanwhile, in the 11 plants from aborted seeds of IAC 2019Maria mandarin x Pera sweet orange, only hybrid 05 (Figure 6-B) was considered a triploid (3x), as it had an average of 55. The analyses of the histograms of the 11 plants from crossings of IAC 2019Maria mandarin x Ponkan mandarin and open pollination had only diploid plants (2x). The population of 258 plants of IAC 2019Maria mandarin x Pera sweet orange had only a single polyploid individual (0.4%), hybrid 72 (Figure 6-D), which was considered a tetraploid with mean value of 70.

Of the 334 hybrids obtained from crossings and assessed by flow cytometry, 331 (99.1%) were considered diploid and three (0.9%) were considered polyploid, with two tetraploids and one triploid. The triploid was obtained from an aborted (smaller) seed from the crossing of IAC 2019Maria mandarin x Pera sweet orange.

Regarding tetraploids, hybrid 62 was obtained from self-pollination, which shows IAC 2019Maria mandarin contributed with two 2x gametes. Hybrid 72 supposedly inherited a 2x gamete from each of the parents (IAC 2019Maria mandarin x Pera sweet orange). Several authors have reported low frequency of naturally obtaining triploid plants in *Citrus* progenies from 2x x 2x crossings (CAMERON; FROST, 1968; ESEN; SOOST, 1971; ROCHA, 2014).

Similar results were obtained in other works. Rocha (2014), when using the same method, observed similar values, with mean value of nuclear volume of 34 for diploids, 51 for triploids, and 68 for tetraploids, and a total of 6.2% of triploid plants in the entire population.

To Esen and Soost (1971), the percentage of triploid hybrids obtained from crossings between diploid citrus plants ranged from 24.0 to 0.41% of the plants depending on the female parent used.

Esen and Soost (1971) suggest that triploidy is a product of the union of a diploid (2x) and a monoploid (x) gamete, a situation in which, theoretically, either parent may contribute with the gamete with a non-reduced number (2x). However, those authors showed that the nondisjunction takes place in the female parent. That is confirmed when a tetraploid plant is obtained from self-pollination, as is the case of hybrid 62.

Aleza et al. (2010) found triploid hybrids in seeds that were 52 to 62% smaller than normal ones. To Esen and Soost (1971), the size of seeds with triploid embryos was reduced by 1/3 to 1/6 the size of diploid seeds, which is possibly related to lower development of the pentaploid endosperm or is due to its development ending earlier. As seen in this experiment, plant 05 (3x) was obtained from a smaller seed in relation to the others, which is correlated with what the authors propose. Aleza et al. (2010) obtained frequencies of 58 to 98.4% of triploid plants in hybrid mandarin populations when using prior selection method and *in vitro* sprouting of only the smaller seeds.

To Aleza et al. (2009), tetraploid plants of non-apomictic genotypes are of great interest for triploid breeding programs as female parents since they enable the production of large populations. According to Navarro et al. (2003), 4n x 2n crossings were more efficient in producing triploid plants, which shows the need to obtain tetraploid plants.

In the present study, 0.9% (three plants) of the 336 plants assessed were considered polyploid, which shows that crossings with IAC 2019Maria mandarin may potentially yield more polyploid individuals.

When pollinated with Pera and Ponkan varieties, the number of fruits set and the number of seeds per fruit of IAC 2019Maria mandarin increased, which suggests planting it near those varieties should be avoided in order to produce fruits with fewer seeds.

IAC 2019Maria mandarin, within the conditions under which this experiment was conducted, was not able to produce parthenocarpic fruits, which indicates pollination is a key factor for fruit formation.

Conclusion

IAC 2019Maria mandarin was self-compatible when self-pollinated and had a lower number of seeds in that situation. Thus, plating it isolated may be a strategy to produce fruits with a lower number of seeds.

The combinations of SSR markers allowed verifying that 100% of the plants regenerated were hybrids, which allows suggesting that IAC 2019Maria mandarin is a plant with low rate of polyembryony that may be widely used in breeding programs to obtain hybrids.

Through the flow cytometry technique, three polyploid hybrids could be identified, one triploid and two tetraploids, in all populations analyzed. The three polyploid plants will be propagated for the selection of possible parthenocarpic varieties.

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