

Obtaining Triploid Hybrids by Means of Open Pollinations and Controlled Crosses Involving Diploids Parental

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Abstract

The creation of triploid hybrids is an important genetic improvement strategy for the development of new commercial citrus scion varieties. The objective of this work was to quantify the frequency of triploids obtained from natural and controlled crosses of different mandarin varieties under varying environmental conditions in the state of Bahia. The experiments were conducted in the municipalities of Cruz das Almas (Recôncavo Baiano region) and Mucugê (Chapada Diamantina region). The first experiment was based on fruits from open pollinations of the varieties 'Page', 'Ortanique', 'Ellendale', 'Clemenules', 'Swatow', 'Piemonte', 'Fortune', 'South Africa', 'Montenegrina', 'Kincy', 'Span Americana', 'Fremont', 'Nova', 'Dancy' and 'Murcott', and the second involved fruits from controlled crosses of female parents 'Nova', 'Fortune' and 'Ortanique' and male parents 'Page', 'Montenegrina', 'Swatow', 'Fremont' and 'Kincy'. The seeds selected were inoculated in test tubes containing approximately 10 mL of Woody Plant Medium (WPM). When the plants reached circa 60 days of age, leaf samples were removed for quantification of the DNA by the flow cytometry technique. In Mucugê, three triploids were identified from open pollinations, 'Clemenules' (1) and 'Ortanique' (2), while in Cruz das Almas, no triploids were obtained. In the controlled crosses, triploids were only obtained in Mucugê: 'Ortanique' × 'Montenegrina' (4), 'Ortanique' × 'Kincy' (1) and 'Ortanique' × 'Swatow' (2). Based on the data, it is suggested that the environmental conditions of Mucugê favored the formation of triploids.

Keywords: *Citrus* spp., *in vitro* culture, hybridization, genetic improvement

1. Introduction

Citrus are among the most important group of fruit trees in Brazil, due to the nutritive value of the fruits and the socioeconomic role they play in the export (Knudsen, Almeida, Langer, Abreu, & Halberg, 2011). In this aspect, the expansion of the genetic base of Brazilian orchards, especially related to the varieties used for scion and rootstocks, including those with capacity to adapt to abiotic stresses, tends to contribute significantly to the achievement of economically superior incomes in the country (Syvertsen & Sanchez, 2014).

Although citrus presents great importance in the economy, Brazil has little expressive participation in the world exports of fresh orange and mandarin. In addition to phytosanitary issues, one of the reasons for the low Brazilian participation in this market is the lack of cultivars that meet the minimum quality requirements demanded by the external market of fresh fruit, being one of the main ones, the absence of seeds in the fruits (Latado, Carvalho, & Machado, 2007).

One of the strategies used to obtain seedless fruits is the cultivation of triploid genotypes, such as the acid lime tree 'Tahiti' [*Citrus latifolia* (Yu. Tanaka)], which has high commercial value and is highly productive (Silva, Bezerra, Bassan, Avilés, & Arthur, 2016). Triploid hybrids in citrus can be obtained from crosses of diploid (2×) parents with tetraploids (4×), hexaploids (6×) with tetraploids (4×) and diploids (2×) with diploids (2×) or open pollinated, in the case of 2× with 2×, the triploid embryos originate from the fertilization of non-reduced female

gametes (diploids), with reduced male gametes (haploids), being the natural frequency of obtaining triploids very low (close to 5%), varying according to the maternal genotype (Esen & Soost, 1971, 1973a).

Parthenocarpy is a fundamental peculiarity for the production of seedless fruits, and is a characteristic that is present in citrus germplasm (Aleza, Cuenca, Juárez, Pina, & Navarro, 2010a). Thus, the creation of triploid hybrids is an important genetic improvement strategy for the development of new commercial scion varieties (Ollitrault, Dambier, Luro, & Froelicher, 2008).

The objective of this study is to quantify the frequency of triploids obtained from natural crosses (open pollinations) and controlled crosses of different mandarin varieties in varying environmental conditions in the state of Bahia, in Brazil.

2. Materials and Methods

2.1 Agroclimatic Conditions of the Study Area

The experiments were conducted in two municipalities in the state of Bahia in Brazil. The first was Cruz das Almas, located in the Recôncavo Baiano region, with geographic coordinates 12°40'19" southern latitude and 39°06'23" eastern longitude, at an elevation of 226 m. The climate is hot and tropical with an average annual rainfall of 1170 mm, varying from 900 mm to 1300 mm, with the rainy season lasting from March to August and the dry season was from September to February. The average annual temperature is 24.5 °C and the relative humidity is 80%.

The second municipality was Mucugê, in the Chapada Diamantina region, with geographic coordinates 13°00'19" southern latitude, 41°22'15" western longitude and at an altitude of 983 m. The climate is characterized as semi-humid, with the rainy season lasting from October to March and the dry season from April to September. The average annual rainfall is about 1100 mm, with an average of 600 mm in the dry season and 1500 mm in the wet season, while the average yearly temperature is 20 °C.

2.2 Experimental Design

Two experiments were conducted, one involving fruits collected from open pollinated trees of two farms and the second one involving fruits collected from controlled pollinations.

2.3 Materials

2.3.1 Source of Planting Material

The planting material (seed) used for raising seedlings was collected from fruits raised in two experimental locations. The fruits from the open pollinations were collected in different months of the year 2015, the fruits of all the varieties were collected when they were ripe (with the coloration of the yellowish bark), the same collection criteria were used, both for fruits from open pollinations and from controlled crosses. The experiment was conducted in three locations: at the Citrus Active Germplasm Bank of the Embrapa Cassava and Fruits, located at the municipality of Cruz das Almas; and in two farms: (Alpercata farm and Lavoura and Pecúária Igarashi Ltda farm) both located in the municipality of Mucugê.

The sample collections were done in April, May and June 2015. A sample of thirty fruits were collected, from each of fifteen varieties open pollinated: 'Ortanique' tangor [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck]; 'Clemenules' mandarin (*C. clementina* hort. ex Tanaka); 'Montenegrina' mandarin (*C. deliciosa* Ten.); 'Nova' tangelo ['Clementina' mandarin (*C. clementina*) × 'Orlando' tangelo (*C. paradisi* Macfad.) × *C. tangerina* hort. ex Tanaka]; 'Piemonte' tangelo ['Clemenules' mandarin × 'Murcott' tangor (*C. reticulata* × *C. sinensis*); 'Page' tangelo ['Clementina' mandarin × 'Minneola' tangelo (*C. paradisi* × *C. tangerina*); 'Dancy' mandarin (*C. tangerina*); 'Fremont' mandarin ('Clementina' mandarin × 'Ponkan' mandarin *C. reticulata*); 'Ellendale' tangor (*C. reticulata* × *C. sinensis*); 'Fortune' mandarin (*C. clementina* × *C. tangerina*); 'Span Americana' mandarin (*C. reticulata*); 'Murcott' tangor (*C. reticulata* × *C. sinensis*); 'Kincy' mandarin ['King' mandarin (*C. nobilis* Lour.) × 'Dancy' mandarin]; 'Swatow' mandarin (*C. reticulata*); and 'South Africa' mandarin (*C. reticulata*).

The controlled pollinations were performed at the Citrus Active Germplasm Bank of the Embrapa Cassava and Fruits, located at the municipality of Cruz das Almas; and in two farms: (Alpercata farm and Lavoura and Pecúária Igarashi Ltda farm) both located in the municipality of Mucugê. The controlled crosses were carried out in the month of September of the year of 2015, involving the varieties Nova, Fortune and Ortanique as female parental, and the male parents 'Page', 'Montenegrina', 'Swatow', 'Fremont' and 'Kincy'.

The number of controlled pollinations varied according to the availability of flowers at the time of their realization. They were carried out in the period of greatest solar intensity and temperature, on dry days, between 10:00 a.m. and 4:00 p.m., to facilitate release and use of the pollen.

The pollen collected from recently opened male parents was used to pollinate the female parent flowers, which had been emasculated before anthesis. The emasculation was performed in the balloon stage (close to floral opening), by carefully eliminating the petals and anthers with tweezers and scalpel, avoiding contact with the stigma. The pollination was performed immediately after emasculation, while the stigma was receptive (moist).

The fruits were collected between four and five months after the pollinations. Those from both the natural pollinations and controlled crosses were taken to the Tissue Culture Laboratory. The seeds were removed by an incomplete transversal cut in each fruit, to assure integrity the same (Figures 1A and 1B). After extraction, the seeds were washed with neutral detergent and tap water (Figure 1C), counted per fruit and classified according to the development stage and size, as normal (seeds with well-formed tegument), underdeveloped (seeds with wrinkled tegument) and small (seeds with 1/6 to 1/3 of normal size), as shown in Figure 1D.

Seeds were selected with priority that were 3 to 6 times smaller than the seeds containing diploid embryos, since these have strong potential to produce triploid individuals (Aleza, Juárez, Cuenca, Ollitrault, & Navarro, 2010b).

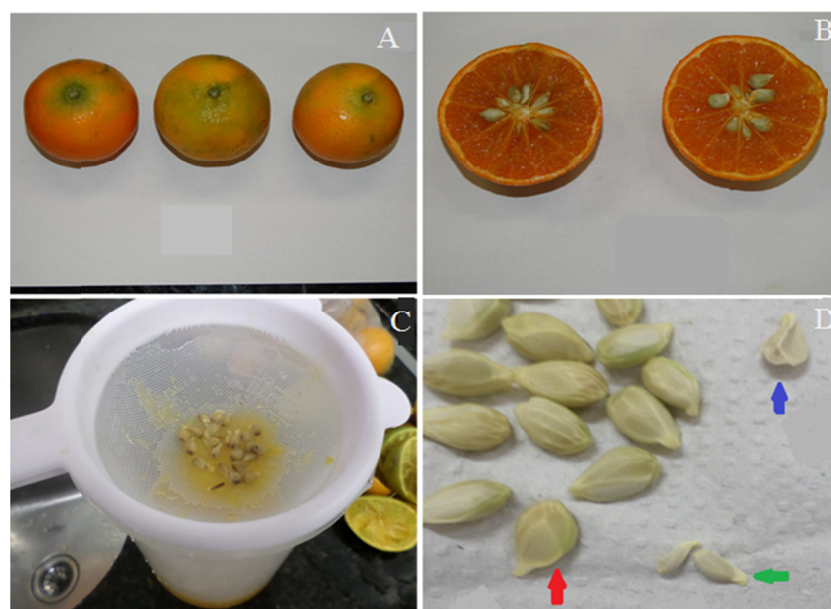


Figure 1. A: Fruits of the Page variety; B: fruits cut to remove the seeds; C: Seeds were washed with neutral detergent; D: Seeds classified as having normal size, indicated by the red arrow, underdeveloped, indicated by the blue arrow, and small, indicated by the green arrow

The tegument of all small seeds, undeveloped and 20 normal seeds has been removed, being that the number of small and underdeveloped seeds inoculated varied according to the quantity of seeds available. The seeds were disinfested under aseptic conditions in a laminar flow cabinet by immersion in 70% ethanol for 5 minutes, followed by a 0.5% sodium hypochlorite solution containing two droplets of Tween 20[®] for 20 minutes and three washings in autoclaved distilled water. The disinfested seeds were then inoculated in test tubes containing approximately 10 mL of Woody Plant Medium-WPM (Lloyd & McCown, 1980) and kept in a growth room at temperature of 27 ± 1 °C, with photon flux density of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 hour photoperiod.

2.3.2 Flow Cytometry Analyses

When the plants reached 60 days of age, leaf samples were removed for DNA quantification by flow cytometry according to the method described by Dolezel, Greilhuber, and Suda (2007), with the modification that tests were performed with the species *Crotalaria breviflora* DC for comparison with a citrus standard consisting of ‘Sunki Tropical’ mandarin (*Citrus sunki* (Hayata) hort. ex Tanaka), to define the DNA content. The analysis was performed using small citrus leaf fragments ($2C = 0.70$ pg of DNA) for comparison with the content of *Crotalaria breviflora* ($2C = 2.02$ pg of DNA). The samples were macerated in Petri dishes over ice in the presence of 1 mL of LB01 nuclear isolation buffer (Dolezel & Bartos, 2005), followed by addition of 0.20 μL of propidium iodide to stain the DNA. The DNA content (pg) of the unknown samples was determined according to Dolezel et al. (2007) with the formula:

$$\text{Sample 2C value (DNA pg)} = \frac{\text{Sample mean peak position} \times \text{Reference 2C value}}{\text{Reference 2C mean peak position}} \quad (1)$$

Flow cytometry analyses generate histograms that allow identifying the ploidy level of the individuals evaluated. Figures 2 and 3 contain the histograms generated by the flow cytometry method, to illustrate how the triploids were identified.

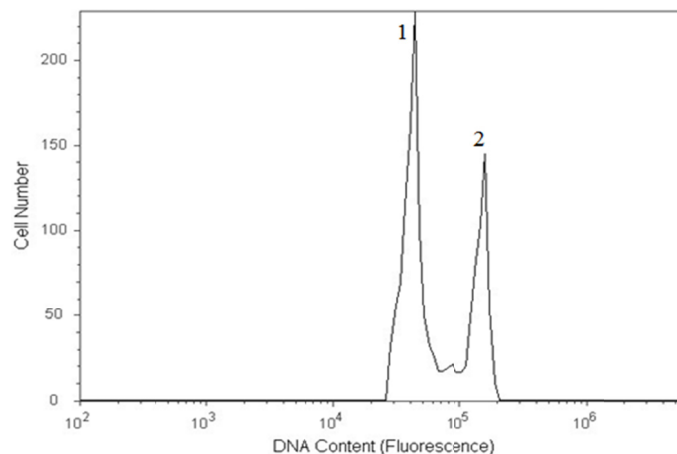


Figure 2. Histogram obtained by flow cytometry analysis of a diploid leaf sample of 'Cravo' mandarin (*Citrus reticulata* Blanco) (peak 1) and a sample of the diploid control, *Crotalaria breviflora* DC. (peak 2)

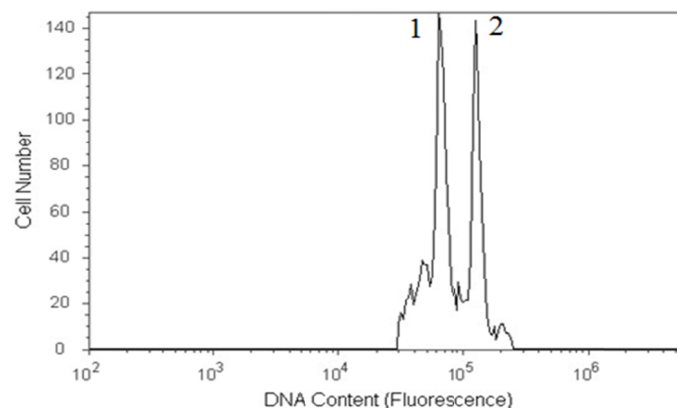


Figure 3. Histogram obtained by flow cytometry analysis of a leaf sample of a triploid 'Fortune' mandarin (*Citrus clementina* hort. ex Tanaka × *Citrus tangerina* hort. ex Tanaka) (peak 1) and a diploid control sample, *Crotalaria breviflora* DC (peak 2)

3. Results

3.1 Ploidy of the Citrus Plants Obtained From Open Pollinations, Measured by Flow Cytometry

The data related to the 15 mandarin varieties collected in Cruz das Almas and Mucugê, in April, May and June 2015, are reported in Table 1. In Mucugê, the frequency of obtaining triploids among the varieties was of 100% ('Ortanique' and 'Clemenules'). The frequency variations occurred in function of the percentage of germinated seeds, type of seed, environment and genotype. There was a low germinated seed rate due to losses caused by contamination. All the undeveloped and small seeds found in the fruits were placed to germinate. However, a low number of plants was obtained, because there was a lot of loss due to the contamination of seeds by endogenous bacteria and fungi, making it difficult to analyze a larger number of samples.

Table 1. Frequency of obtaining triploid hybrids with 15 mandarin varieties, in function of genotype, seed type (normal seed-N and underdeveloped seed-UD) and environment (Cruz das Almas-CZA and Mucugê-MCG). Fruits collected in April, May and June 2015

Seed	Variety ¹	Germination (%)		Plants obtained		Triploids (%)	
		CZA	MCG	CZA	MCG	CZA	MCG
N	South Africa	20	40	1	2	0	0
	Clemenules	40	20	2	1	0	0
	Dancy	40	40	2	2	0	0
	Ellendale	0	0	0	0	0	0
	Fortune	0	80	0	4	0	0
	Fremont	20	40	1	2	0	0
	Kincy	100	20	5	1	0	0
	Montenegrina	0	0	0	0	0	0
	Murcott	0	0	0	0	0	0
	Nova	100	40	5	2	0	0
	Ortanique	0	100	0	5	0	0
	Page	0	0	0	0	0	0
	Piemonte	0	0	0	0	0	0
	Span Americana	40	20	2	1	0	0
	Swatow	0	0	0	0	0	0
UD	South Africa	0	20	0	1	0	0
	Clemenules	0	20	0	1	0	1 (100%)
	Dancy	20	40	1	2	0	0
	Ellendale	20	0	1	0	0	0
	Fortune	0	0	0	0	0	0
	Fremont	0	0	0	0	0	0
	Kincy	0	0	0	0	0	0
	Montenegrina	0	20	0	1	0	0
	Murcott	0	60	0	3	0	0
	Nova	40	80	2	4	0	0
	Ortanique	0	40	0	2	0	2 (100%)
	Page	0	20	0	1	0	0
	Piemonte	20	0	1	0	0	0
	Span Americana	0	40	0	2	0	0
	Swatow	0	0	0	0	0	0

Note. ¹Tangor 'Ortanique' [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck]; 'Clemenules' mandarin (*C. clementina* hort. ex Tanaka); 'Montenegrina' mandarin (*C. deliciosa* Ten.); 'Nova' tangelo ['Clementina' mandarin (*C. clementina*) × 'Orlando' tangelo (*C. paradisi* Macfad.) × *C. tangerina* hort. ex Tanaka]; 'Piemonte' tangelo ['Clemenules' mandarin × 'Murcott' tangor (*C. reticulata* × *C. sinensis*); 'Page' tangelo ['Clementina' mandarin × 'Minneola' tangelo (*C. paradisi* × *C. tangerina*); 'Dancy' mandarin (*C. tangerina*); 'Fremont' mandarin ('Clementina' mandarin × 'Ponkan' mandarin *C. reticulata*); 'Ellendale' tangor (*C. reticulata* × *C. sinensis*); 'Fortune' mandarin (*C. clementina* × *C. tangerina*); 'Span Americana' mandarin (*C. reticulata*); 'Murcott' tangor (*C. reticulata* × *C. sinensis*); 'Kincy' mandarin ['King' tangerine (*C. nobilis* Lour.) × 'Dancy' mandarin]; 'Swatow' mandarin (*C. reticulata*) e 'South Africa' madarin (*C. reticulata*).

With respect to the environmental effect on the formation of seeds with potential to generate triploid plants, the conditions in Mucugê were favorable. Three triploids were identified from this location, one of the 'Clemenules' variety and two 'Ortanique' (Table 1). For the locality of Cruz das Almas, triploids were not obtained.

Though the environment in Mucugê favored the formation of triploid in comparison to Cruz das Almas, mention should be made of the low seed germination rate in both environments and, consequently, the small number of plants obtained. But, all the plants produced by in vitro culture, regardless of the seed type and local, were evaluated by flow cytometry.

3.2 Ploidy of Citrus Plants Obtained From Controlled Crosses, Measured by Flow Cytometry

We performed 197 pollinations in Cruz das Almas (Table 2): 'Ortanique' × 'Kincy' (5); 'Ortanique' × 'Fremont' (19); 'Nova' × 'Kincy' (43); 'Nova' × 'Page' (71); 'Fortune' × 'Kincy' (10); and 'Fortune' × 'Page' (49). The fruit-setting rates varied from 4% ('Nova' × 'Page') to 40% ('Ortanique' × 'Kincy').

Table 2. Controlled crosses carried out in Cruz das Almas. Number of flowers pollinated (NP), number of fruits obtained after pollination (NF) and fruit-setting rate (SR)

Crosses ¹	NP	NF	SR (%)
'Ortanique' × 'Kincy'	5	2	40
'Ortanique' × 'Fremont'	19	-	-
'Nova' × 'Kincy'	43	15	35
'Nova' × 'Page'	71	3	4
'Fortune' × 'Kincy'	10	-	-
'Fortune' × 'Page'	49	-	-
Total	197	20	

Note. ¹Tangor 'Ortanique' [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck]; 'Kincy' mandarin ['King' mandarin (*C. nobilis* Lour.) × 'Dancy' mandarin (*C. tangerina* hort. ex Tanaka)]; 'Nova' tangelo ['Clementina' mandarin (*C. clementina* hort. ex Tanaka) × 'Orlando' tangelo (*C. paradisi* Macfad.) × *C. tangerina*]; 'Page' tangelo ['Clementina' mandarin × 'Minneola' tangelo (*C. paradisi* × *C. tangerina*)]; 'Fremont' mandarin ['Clementina' mandarin × 'Ponkan' mandarin (*C. reticulata*)]; 'Fortune' mandarin ('Clementina' mandarin × 'Dancy' mandarin).

In Mucugê we carried out 734 pollinations (Table 3): 'Ortanique' × 'Kincy' (66); 'Ortanique' × 'Montenegrina' (86); 'Ortanique' × 'Page' (67); 'Ortanique' × 'Fremont' (17); 'Ortanique' × 'Swatow' (61); 'Nova' × 'Kincy' (28); 'Nova' × 'Montenegrina' (75); 'Nova' × 'Page' (81); 'Nova' × 'Fremont' (52); 'Nova' × 'Swatow' (122); 'Fortune' × 'Kincy' (16); 'Fortune' × 'Montenegrina' (11); 'Fortune' × 'Page' (22); 'Fortune' × 'Fremont' (12); and 'Fortune' × 'Swatow' (18). In this case, the fruit-setting rates varied between 7% in the 'Nova' × 'Page' cross and 39% for 'Ortanique' × 'Swatow'.

Table 3. Controlled crosses in Mucugê. Number of flowers pollinated (NP), number of fruits obtained after pollination (NF) and fruit-setting rate (SR)

Crosses ¹	NP	NF	SR (%)
'Ortanique' × 'Kincy'	66	15	23
'Ortanique' × 'Montenegrina'	86	33	38
'Ortanique' × 'Page'	67	22	33
'Ortanique' × 'Fremont'	17	4	24
'Ortanique' × 'Swatow'	61	24	39
'Nova' × 'Kincy'	28	6	21
'Nova' × 'Montenegrina'	75	18	24
'Nova' × 'Page'	81	6	7
'Nova' × 'Fremont'	52	14	27
'Nova' × 'Swatow'	122	39	32
'Fortune' × 'Kincy'	16	-	-
'Fortune' × 'Montenegrina'	11	-	-
'Fortune' × 'Page'	22	-	-
'Fortune' × 'Fremont'	12	-	-
'Fortune' × 'Swatow'	18	-	-
Total	734	181	

Note. ¹Tangor 'Ortanique' [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck]; 'Kincy' mandarin ['King' mandarin (*C. nobilis* Lour.) × 'Dancy' mandarin (*C. tangerina* hort. ex Tanaka)]; 'Montenegrina' mandarin (*C. deliciosa* Ten.); 'Page' tangelo ['Clementina' mandarin (*C. clementina* hort. ex Tanaka) × 'Minneola' tangelo (*C. paradisi* Macfad. × *C. tangerina*)]; 'Fremont' mandarin ['Clementina' mandarin × 'Ponkan' mandarin (*C. reticulata*)]; 'Nova' tangelo ['Clementina' mandarin × 'Orlando' tangelo (*C. paradisi* × *C. tangerina*)]; 'Swatow' mandarin (*C. reticulata*); 'Fortune' mandarin ('Clementina' mandarin × 'Dancy' mandarin).

In the controlled crosses performed in both Cruz das Almas and Mucugê, we used as female parents the varieties 'Nova', 'Ortanique' and 'Fortune', and as male parents 'Page', 'Swatow', 'Montenegrina', 'Fremont' and

‘Kincy’. However, the fruits resulting from the crosses involving the ‘Fortune’ variety as female parent did not set in either location, so it was not possible to evaluate the influence of this variety on the formation of triploids.

With respect to growing location, in Mucugê seven triploids were obtained, four from ‘Ortanique’ × ‘Montenegrina’ crosses, one from ‘Ortanique’ × ‘Kincy’ and two from ‘Ortanique’ × ‘Swatow’ (Table 4; Figure 4). The triploid frequency of the crosses varied from 50% (‘Ortanique’ × ‘Kincy’) to 100% (‘Ortanique’ × ‘Montenegrina’) and (‘Ortanique’ × ‘Swatow’).

Table 4. Controlled crosses carried out in Mucugê (MCG) and Cruz das Almas (CZA). Number of plants obtained from normal seeds (NS), number of plants obtained from underdeveloped seeds (UD), number of plants obtained from small seeds (SS), percentage of plants obtained (PO%) and percentage of triploids obtained (%)

Local	Female parent	Male parent	NS	PO%	Triploids%	UD	PO%	Triploids%	SS	PO%	Triploids%
MCG	‘Ortanique’	‘Kincy’	3	8	0	0	0	0	2	4	1 (50%)
		‘Montenegrina’	9	9	0	0	0	0	4	50	4 (100%)
		‘Page’	10	17	0	0	0	0	0	0	0 (0%)
		‘Swatow’	5	14	0	0	0	0	2	50	2 (100%)
		‘Fremont’	2	10	0	1	20	0	0	0	0 (0%)
MCG	‘Nova’	‘Swatow’	6	5	0	0	0	0	5	33	0 (0%)
		‘Kincy’	1	5	0	0	0	0	0	0	0 (0%)
		‘Fremont’	5	25	0	0	0	0	0	0	0 (0%)
CZA	‘Nova’	‘Montenegrina’	2	10	0	0	0	0	0	0	0 (0%)
		‘Kincy’	26	1	0	5	31	0	0	0	0 (0%)
CZA	‘Ortanique’	‘Page’	4	40	0	0	0	0	0	0	0 (0%)
		‘Kincy’	28	76	0	0	0	0	0	0	0 (0%)

Note. ¹Tangor ‘Ortanique’ [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck]; ‘Kincy’ mandarin [‘King’ mandarin (*C. nobilis* Lour.) × ‘Dancy’ mandarin (*C. tangerina* hort. ex Tanaka)]; ‘Montenegrina’ mandarin (*C. deliciosa* Ten.); ‘Page’ tangelo [‘Clementina’ mandarin (*C. clementina* hort. ex Tanaka) × ‘Minneola’ tangelo (*C. paradisi* Macfad. × *C. tangerina*) × ‘Dancy’ mandarin]; ‘Fremont’ mandarin (‘Clementina’ mandarin × ‘Ponkan’ mandarin *C. reticulata*); ‘Swatow’ mandarin (*C. reticulata*); ‘Nova’ tangelo [‘Clementina’ mandarin × ‘Orlando’ tangelo (*C. paradisi* × *C. reticulata*)].



Figure 4. Triploid plants from *in vitro* culture of seeds from controlled crosses between the parents: ‘Ortanique’ tangor [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck] × ‘Montenegrina’ mandarin (*C. deliciosa* Ten.); ‘Ortanique’ mandarin × ‘Swatow’ mandarin (*C. reticulata*)

Regarding seed type, all the triploids came from small seeds, and of the 13 plants obtained from these seeds, seven were triploids (54% frequency). However, no triploid plants were obtained in Cruz das Almas (Table 4).

In general, although the environment conditions in Mucugê promoted a higher frequency of triploids than in Cruz das Almas, where no plants were obtained with this ploidy from the controlled crosses, the number of plants obtained in both environments was low and directly proportional to the seed germination rate, which was also low in the majority of the varieties evaluated.

4. Discussion

Triploid citrus hybrids can be obtained by crossing diploid parents through production of unreduced gametes by the female parent, where the frequency of these gametes depends on the genotype (Esen & Soost, 1971; Luro, Maddy, Jacquemond, Froelicher, Morillon, Rist, & Ollitrault, 2004). Triploid embryos are more often produced by small seeds, between 1/6 and 1/3 of normal size. Generally these seeds do not germinate under normal greenhouse conditions, so it is necessary to rescue and culture these embryos *in vitro* to attain high germination rates (Aleza et al., 2010b).

Cameron and Frost (1968) observed that the frequency of obtaining natural triploid plants in interspecific or intraspecific progenies of *Citrus* originating from pollinations between diploid parents was generally low (near 5%), while according to Essen and Soost (1971), the rate varies in function of the maternal genotype. However, Aleza et al. (2010b), investigating controlled $2x \times 2x$ hybridizations involving monoembryonic and non-apomictic female parents of citrus, and using embryo rescue and *in vitro* culture techniques, achieved a 92% rate of triploid plants.

Esen and Soost (1971, 1973a, 1973b) concluded that triploid plants are formed from small seeds, which can be obtained via $2x \times 2x$ crosses, but the frequency of this event is generally low. In this context, the authors proposed the hypothesis that embryos formed from polyploid endosperm was the factor responsible for the small seed size, because all the seeds containing triploid embryos presented pentaploid endosperm.

In citrus, the endosperm of seeds that contain diploid embryos is naturally triploid. However, histological studies performed by Esen and Soost (1971, 1973a) demonstrated that small seeds contain embryos with pentaploid endosperm, which grows more slowly and develops prematurely, indicating that this is the factor responsible for reducing the size of the seeds that contain triploid embryos.

Cytogenetic analysis carried out by Aleza, Cuenca, Hernández, Juárez, Navarro, and Ollitrault (2015) revealed that triploid citrus embryos are associated with pentaploid endosperm, and there is strong evidence that these embryos result from fertilization of unreduced ovules by normal haploid pollen. Finally, they observed that depending on the genotype, the frequency of duplication between female gametes varied from less than 1% to more than 20%.

Aleza, Juárez, Cuenca, and Ollitrault, (2012a), working with $2x \times 4x$ hybridizations of citrus, obtained 1061 plants from normal seeds, of which 279 were triploids (26%), and found that in hybridizations using diploid (2n) female parents with tetraploid (4n) male parents, it is possible to obtain triploid plants from both normal and underdeveloped seeds.

In the case of $2x \times 2x$ hybridizations, triploid hybrids are obtained from the union of the 2n megagamophyte with haploid pollen (Esen & Soost, 1971; Esen & Soost, 1973a; Geraci, Esen, & Soost, 1975). Luro, Costantino, Terol, Argout, Allario, Wincker, Talon, Ollitrault, and Morillon (2008) proposed that the restitution of the second meiotic division is the control mechanism for development of the diploid megagamophyte in the 'Clementina' variety, while Chen, Lyon, O'malley, Federici, Gmitter, Grosser, Chaparro, Roose, and Gmitter (2008) proposed that the mechanism is restitution of the first meiotic division in sweet oranges.

Some studies have shown that the aspects of the male parent and environmental conditions influence the production of triploid citrus plants (Aleza et al., 2010b; Luro et al., 2004; Vilorio & Grosser, 2005). The influence of environmental conditions on plants' reproductive success is well documented, by affecting the production and size of pollen and the growth of the pollen tube (Young & Stanton 1990). Besides this, the temperature during the progamic phase is one of the most important environmental factors that affect the performance of pollen. However, the response to temperature during the reproductive phase depends on the genotype (Hedhly, Hormaza, & Herrero, 2005).

Aleza, Froelicher, Schwarz, Agusti, Hernández, Juárez, Luro, Morillon, Navarro, and Ollitrault (2011), Hussain, Curk, Ollitrault, Morillon, and Luro (2011), Otto and Whitton (2000), and Saleh, Allario, Dambier, Ollitrault, and Morillon (2008) all have proposed the hypothesis that different environmental conditions, such as temperature, can have an effect on chromosome duplication events. Barrett and Hutchison (1978) identified

polyploidy rates of 0.75% and 0.90% from seeds of citrus plants grown in Florida and California, respectively, and associated this difference with temperature variation. The effect of low temperature on polyploidization events appears to be a general rule, both in plants and animals (Otto & Whitton, 2000; Ramsey & Schemske, 1998). Besides the adaptive advantage of polyploids, the larger rate of polyploidization events in a colder climate could be one of the factors related to the increased frequency of polyploid species in the Northern Hemisphere (Asker & Jerling, 1992).

Aleza et al. (2011) reported that the overall frequency of tetraploid seedlings among 30 *Citrus* varieties cultivated in Valencia (Spain) and Corsica (France) was from 1% to 8% (varying from 0% to 9% according to the cultivar and environmental conditions). Besides this, they confirmed the importance of the environmental conditions on the rates of tetraploids, studying the same genotype ('Carrizo' citrange) harvested in different countries. They found higher rates in the Mediterranean than in tropical areas and significant negative correlations between the rates of tetraploids and mean temperatures during the floral induction and flowering period. Based on this, they suggested that colder growing conditions favor tetraploidization events in citrus nucellar cells.

Temperature affects the efficiency of producing citrus hybrids based on $2x \times 4x$ hybridizations. The production of triploid hybrids is influenced by the fecundation success of the pollen, which is dependent on the pollen quality. In turn, this is determined not only by the male genotype, but also the environmental conditions, level of compatibility between the male and female parents, and environmental effects on the germination and growth of the pollen tube (Viloria & Grosser, 2005; Young & Stanton, 1990).

Aleza et al. (2012b) studied the effect of parents and environmental conditions on the production of citrus triploid hybrids in $4x \times 2x$ sexual hybridizations during five years. The hybridization results indicated the existence of an interaction between male parents and environmental conditions on the production of triploids. Besides this, the results of two previous studies indicated that environmental conditions also affect the production of triploid hybrids in $2x \times 2x$ hybridizations (Aleza et al., 2010b; Luro et al., 2004).

Aleza et al. (2010b), investigating hybridizations using $2x \times 2x$ crosses over a period of 10 years (1996 to 2006), observed that triploid hybrids were produced by seeds that had between 52% and 62% of the size of normal seeds, and that embryo rescue and flow cytometry are two essential techniques for genetic improvement programs aiming to obtain triploids in citrus plants based on hybridizations between diploid parents.

5. Conclusions

The frequency of obtaining triploids varied according to the genotype (the Ortanique variety showed the greatest potential to produce triploids); of seed size (small and underdeveloped seeds presented ability to generate triploids); and the environment (triploids were obtained only in the Mucugê environment).

However, further studies must be done, to better understand the effect of environmental conditions on the formation of triploid individuals.

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