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PLANT BREEDING - Article

Relationship between meiotic instability and fertility in F_2 generation Arabusta coffee plants

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ABSTRACT: Parental plants, an F_1 interspecific hybrid and the F_2 generation from Arabusta coffee plants were investigated for meiotic behavior and viability of pollen to understand part of their reproductive biology that affects their possible use in coffee breeding programs. Both parental plants (*C. canephora* var. Robusta 4x and *C. arabica* var. dihaploid Bourbon Vermelho) showed a meiosis diploid-like behavior, despite presenting a small percentage of irregularities, just as occurred for the F_1 Arabusta hybrid. On the other hand, all F_2 plants showed a higher frequency of anomalies that compromised pollen viability. The highest meiotic indices were registered for three analyzed plants of the F_2 generation, and the pollen viability tests revealed the highest values for staining (PVS) and germination in vitro tests (PVG) for three others different F_2 plants. The meiotic analysis and pollen viability tests may facilitate the selection of the best genetic resources, reducing the time needed for producing new hybrid cultivars. F_2 plants which have high meiotic indices and/or high pollen fertility could be used as pollen donors in crossbreeding programs when there is interest in their functional or morphological characteristics. In contrast, the F_2 plants that showed low pollen viability could be exploited as sterile male plants or discarded from a breeding program.

Key words: microsporogenesis, meiotic index, pollen viability, post-meiotic products.

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INTRODUCTION

The meiotic process is highly conserved in eukaryotes and plays a central role in the life cycle of all organisms with sexual reproduction because it maintains the chromosome number of the species and generates variability by genetic and chromosomal recombination. The normal and harmonious course of this process, especially proper segregation of chromosomes, is directly reflected in plant fertility. Atypical meiosis may alter basic mechanisms, including the lack of pairing of homologous chromosomes, non-occurrence of recombination and unbalanced chromosome segregation, which can affect gametic viability, resulting in reproduction problems (Pagliarini 2000).

Meiotic analysis and pollen viability tests are very important because they may reduce the time needed for producing new hybrid cultivars once hybrid plants with meiotic irregularities and/or unviable pollen grains are discarded, and thus allow the selection of more stable genotypes (Lavinscky et al. 2016). The evaluation of meiotic behavior is a prerequisite to select the best candidates for the introgression of genes from one species to another through hybridization. Gene introgression has been employed to increase the variability of individuals in breeding programs for the development of new varieties and cultivars.

In coffee breeding, one of the main goals has been to transfer the resistance from *Coffea canephora* (*C. canephora*) to *Coffea arabica* (*C. arabica*), since the former has proved to be more resistant to adverse conditions, particularly to various diseases and pests (Fazuoli et al. 2000; Herrera et al. 2002) *C. arabica* is a segmental allopolyploid (2n = 4x = 44) (Pinto-Maglio and Cruz 1998) that has a high beverage quality and accounts for nearly 70% of global production, whereas *C. canephora* is diploid (2n = 2x = 22), self-incompatible, usually more vigorous and high yielding and contributes to the remaining 30% of global production (FAO 2016; Gimase et al. 2014).

Hybrids between the species *C. arabica* and *C. canephora* have been obtained on different occasions, and they are called "Arabusta" (Mendes 1950). Because of its importance, meiotic behavior had already been studied to some F_1 hybrids and also to parental plants in order to explore the genetic resources of these hybrids (Mendes 1950; Medina 1952; Medina 1963; Mônaco and Medina 1965; Medina and Rijo 1969; Reddy and Narayan 1981; Owuor 1985; Boaventura and Cruz 1987; Boaventura 1990).

The F_2 Arabusta plants used in this study have very different morphological characteristics, as well as different levels of fruit production and have never had their meiotic behavior evaluated before. As these plants may be useful in coffee breeding programs aiming for the introgression of new genes, in this study a sample of Arabusta F_2 plants was investigated along with the two parents and their interspecific hybrid (F_1) for meiotic behavior and the viability of pollen to understand the part of their reproductive biology that affects the fertility of the plant to determine its possible use in breeding programs for coffee improvement.

MATERIAL AND METHODS

Plant Material

The material used in this study were the parent plants *C. canephora* var. Robusta 4n (Co 254) and *C. arabica* var. Bourbon Vermelho dihaploid (Co 667), the interspecific hybrid F_1 (CH2460) and their F_2 plants.

The donor parent of this hybrid F_1, *C. canephora* cv. Robusta, was obtained by doubling the number of chromosomes in a normal diploid (2n = 22) using colchicine treatment by Mendes (1947). The other parent, *C. arabica* cv. Bourbon Vermelho, was derived from a dihaploid (n = 22), in which the chromosomes were also doubled by a colchicine treatment (Mendes 1947). By crossing these plants, F_1 generation Arabusta interspecific hybrids was obtained. Therefore, all these plants have 2n = 4x = 44 chromosomes. The parent plants and the F_1 hybrid are localized in the Experimental Station at the Agronomic Institute at Campinas, SP, Brazil (Lat 22°54 S, Long 47°03 W and Altitude 854 m).

The F_2 plants were obtained by selfing of the F_1 Arabusta interspecific hybrid (CH2460), work carried out by the author Ramos and belong to lot 1 of a germplasm collection from the experimental field located at Mococa, SP, Brazil (Lat 21°28 S, Long 47°01 W and Altitude 665 m). The 30 F_2 Arabusta plants from which the flower buds were collected for meiotic and pollen analysis are part of this collection.

Floral buds in the early stages of development (~5 mm length) were collected 15–30 days before anthesis from all the plants analyzed. Approximately 100 floral →

buds were sampled per plant for all categories and fixed in Carnoy's solution (3:1 of ethanol: glacial acetic acid), then degassed with a vacuum pump for 5 min. The buds were stored for 24 h at room temperature and then at –20 °C (Pinto-Maglio and Cruz 1998; Iacia and Pinto-Maglio 2013) until they were used.

Chromosome preparation

For slide preparations, two or three whole anthers were squashed in a 2% aceto-carmine stain, according to Pinto-Maglio and Cruz (1998). Pollen mother cells (PMCs) with well individualized stages were chosen for the analysis of chromosome association and distribution. The meiosis stages analyzed were metaphase I, anaphase I, telophase I, metaphase II, anaphase II and telophase II of each genotype. Sixty cells of each stage were examined using a light microscope with a phase contrast condenser. Subsequently, the percentages of the numbers of cells in meiotic division (CMD), with and without anomalies, were calculated for each plant category (Parents, F_1, F_2). At metaphase I of F_2 plants, the numbers of univalents, bivalents, trivalents, tetravalents and polyvalents were evaluated. The plants that presented a meiotic index higher than 85% were considered meiotically stable according to Love (1951). The meiotic index was calculated based on the ratio of normal tetrads (four cells) and abnormal tetrads (dyads, triads, polyads), considering also the presence of microcytes.

Pollen viability

Tests of pollen viability were estimated by the staining method and in vitro germination. A basic Alexander's stain containing 2 mL of lactic acid was used for the analysis of pollen viability by staining (PVS) (Alexander 1980). Slides were prepared using all the anthers from one floral bud. Pollen grains were considered viable when they had cytoplasm content colored by Alexander's stain and non-viable when pollen had no coloring. Also was considered the ones that had irregular size or otherwise had reduced cytoplasm. Four slides were analyzed per plant.

For the pollen viability analysis by germination in vitro (PVG), a culture medium containing 50 $g{\cdot}L^{-1}$ sucrose, 20 $g{\cdot}L^{-1}$ agar, 0.1 $g{\cdot}L^{-1}$ boric acid, 0.3 $g{\cdot}L^{-1}$ calcium nitrate, 0.2 $g{\cdot}L^{-1}$ magnesium sulfate and 0.1 $g{\cdot}L^{-1}$ potassium nitrate was used (Neto et al. 2009). Pollen grains were removed from the anthers and spread on slides containing the culture medium. The slides were placed in a Petri dish with filter paper moistened with water, and then they were incubated at a temperature of 28 °C for four hours. Pollen grains were considered viable if they had a pollen tube germinated with a length greater than or equal to the diameter of the pollen grain.

Images

The images of the PMCs, tetrads and pollen grains were captured by an Olympus BX50 microscope with an Olympus Q-color 3 CCD digital camera and the software Image-ProPlus 6.0 – Media Cybernetics.

Statistical analysis

To compare percentages of anomalies and pollen viability among the parents and F_1 hybrid Arabusta, we performed the ANOVA test with a level of 95% ($p < 0.05$), followed by Tukey's test. Pearson correlation (r) analysis was used to look for an association between the rate of normal meiosis and the frequency of viable gametes.

RESULTS AND DISCUSSION

Meiotic analysis

A high meiotic irregularities index has been recorded in the 30 F_2 plants analyzed. Different kinds of abnormalities were observed, including the presence of multivalent and/or univalent, non-oriented chromosomes, laggard chromosomes, bridges and anomalies in the tetrads formation in the final stages of the division (Table 1; Fig. 1). Meiotic abnormalities were quite expected since F_2 plants were derived from an F_1 Arabusta interspecific hybrid. This condition often produces extensive gametic variations due to poor chromosome pairing and unbalanced chromosome segregation.

Parameters as meiotic index and percentages of bivalents paring at metaphase I have been used to evaluate the normality of the meiotic process and the ability of a plant in the production of normal male gametes. The meiotic process can be considered normal if the plant has a meiotic index higher than 85% (Love 1951). The F_2 plant →

73 showed the lowest meiotic index (5%) while the highest meiotic index (70%) was detected in five of the F_2 plants: 99, 143, 39, 74, 01 (Table 1). Therefore, considering Love (1951), we can verify that all F_2 plants are meiotically unstable and unsuitable for plant breeding based on hybridization. However, other parameters including total percentage of abnormalities and pollen viability were evaluated and were also considered.

The pairing of bivalents per cell, in the F_2 plants, ranged from 15.10 ± 3.23 in plant 124 to 19.50 ± 1.25 in plant 23 (Table 2), which indicates abnormal chromosome pairing, since the expected number should be 22 bivalents. The irregular meiotic behavior can be seen also through the total percentage of cells in meiotic division with anomalies that ranged from 51.1% for plant 94 to 93.3% for plant 143 (Table 1; Fig. 2).

In addition, the occurrence of cells with different numbers of chromosomes was found among plants of the F_2 population and even among cells of a single plant, which ranged from 2n = 40 to 2n = 48 (Table 3). Plant 89 showed the lowest percentage of cells with the normal number of 44 chromosomes (16.67%), and plants 40, →

Table 1. Percentages (%) of meiotic irregularities in the F_2 population of Arabusta coffee plants.

Plant	Uni	Tri	Tetra	Non-oriented chromosomes	Laggards	Dyads	Tryads	Polyads	Microcytes	Abnormalities total	Meiotic index
1	93.33	6.67	20.00	50.00	53.33	0.00	0.00	1.67	53.33	62.20	55.00
7	100.00	10.00	20.00	60.00	61.67	0.00	0.00	1.67	45.00	58.30	51.70
16	100.00	3.33	46.67	50.00	58.33	0.00	23.33	10.00	56.67	61.70	10.00
17	100.00	3.33	83.33	58.33	70.00	0.00	0.00	0.00	80.00	75.00	20.00
23	90.00	3.33	33.33	70.00	73.33	0.00	0.00	0.00	65.00	62.80	35.00
29	96.67	13.33	6.67	56.66	66.66	0.00	8.33	1.67	63.33	72.80	26.70
35			16.67	30.00	38.33	0.00	0.00	0.00	45.00	58.30	48.30
38	100.00	10.00		37.50	78.33	0.00	0.00	0.00	51.67	61.70	48.30
39	96.67	16.67	40.00	61.66	63.33	0.00	0.00	1.67	63.33	69.40	60.00
40	100.00	3.33	33.33	60.00	56.66	0.00	3.33	0.00	50.00	67.20	46.70
44	80.00	23.33	36.67	50.00	48.33	5.00	30.00	1.67	56.67	60.00	6.70
45	100.00	3.33	80.00	41.66	75.00	0.00	3.33	3.33	51.67	68.90	41.70
54	100.00	10.00	30.00	55.00	63.33	0.00	0.00	1.67	83.33	63.30	15.00
56	100.00	16.67	63.33	51.67	71.67	0.00	1.67	0.00	86.67	61.10	11.70
57	96.67	23.33	46.67	48.33	60.00	0.00	3.33	0.00	71.67	67.20	25.00
60	96.67	46.67	46.67	60.00	56.67	0.00	1.67	1.67	63.33	65.60	35.00
63	86.67	6.67	36.67	46.67	61.67	1.67	1.67	6.67	43.33	67.80	48.30
73	100.00	13.33	23.33	60.00	88.33	0.00	5.00	0.00	90.00	88.30	5.00
74	96.67	16.67	80.00	41.66	48.33	0.00	0.00	0.00	41.67	55.60	58.30
82	76.67	6.67	53.33	51.67	70.00	0.00	0.00	0.00	88.33	66.10	11.70
86	75.00	10.00	75.00	51.66	48.33	0.00	0.00	0.00	58.33	53.30	41.70
89	100.00	3.33	36.67	73.33	65.00	0.00	3.33	0.00	68.33	70.60	28.30
94	100.00	0.00	50.00	40.00	65.00	0.00	1.67	0.00	51.67	51.10	46.70
98	100.00	3.33	36.67	66.66	71.66	0.00	0.00	1.67	46.67	62.20	51.70
99	100.00	0.00	40.00	46.66	81.67	0.00	3.33	0.00	26.67	73.90	70.00
102	90.00	10.00	50.00	65.00	78.33	0.00	3.33	0.00	80.00	75.60	16.70
117	90.00	10.00	43.33	63.33	78.33	0.00	0.00	0.00	65.00	73.90	35.00
124	80.00	35.00	65.00	43.33	65.00	3.33	18.33	0.00	41.67	56.67	40.00
143	100.00	10.00	30.00	43.33	81.66	0.00	5.00	0.00	68.33	93.30	65.00
152	96.67	6.67	40.00	50.00	68.33	0.00	0.00	0.00	68.33	71.10	31.70

Uni = univalent; Tri = trivalent; Tetra = tetravalent; Meiotic index = normal tetrads/abnormal tetrads.

98, and 99 had the highest percentage of cells with 44 chromosomes (93.33%) (Table 3). The chromosome number of F_2 plants confirm their allopolyploid nature, as they were obtained by selfing of the F_1 Arabusta interspecific hybrid, which also shows cells with an inconstant number of chromosomes, ranging from 2n = 40 to 2n = 46. These →

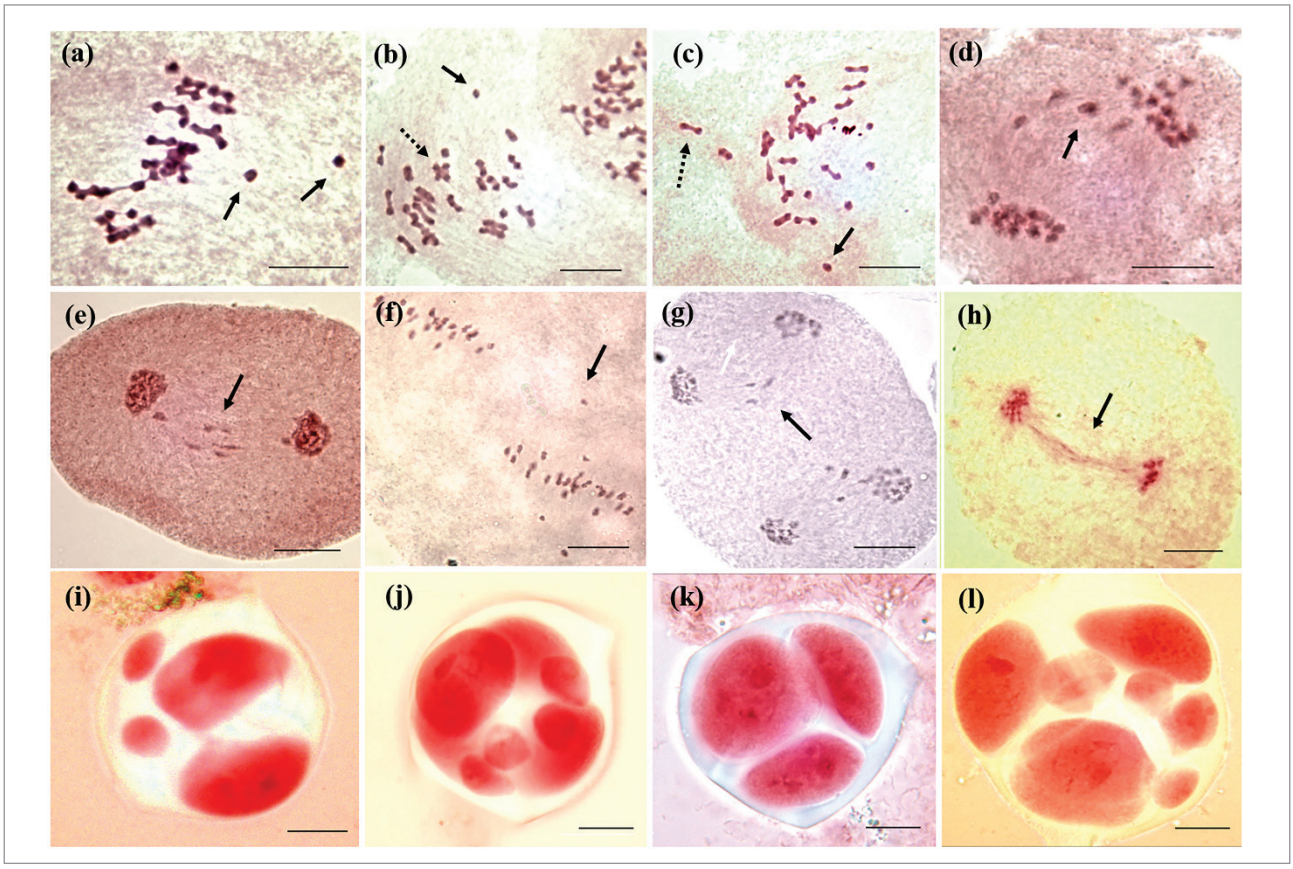
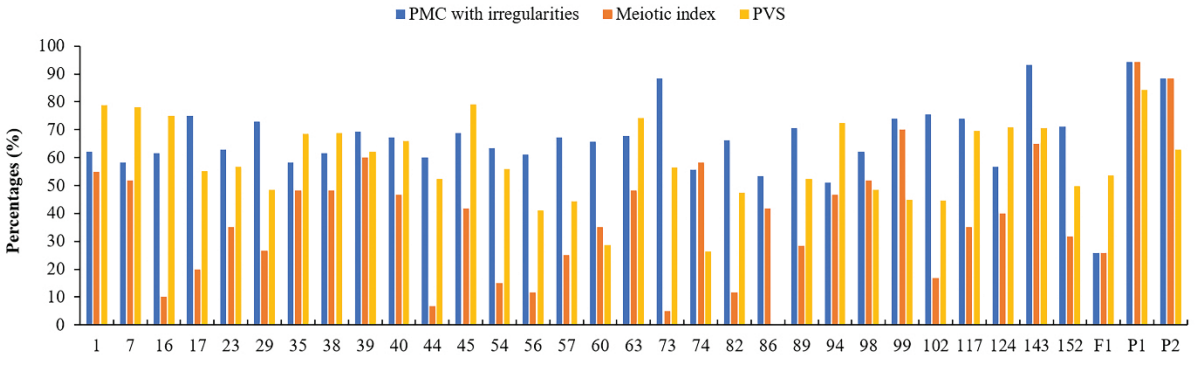


Figure 1. Anomalies present in cells in meiotic division of plants in F_2 Arabusta plants. (a) metaphase I with univalent (arrows); (b) univalent (full arrow) and tetravalent (dotted arrow); (c) metaphase I with non-oriented chromosomes (full arrow) and bivalent (dotted arrow); (d) anaphase I with non-oriented chromosomes (arrow); (e) anaphase I with laggard chromosome migration (arrow); (f) Metaphase II with non-oriented chromosomes (arrow); (g) telophase II with laggards chromosomes migration (arrow); (h) chromosomal bridge (arrow); (i) dyad with two microcytes; (j) polyad with irregular microcytes; (k) triad; (l) tetrad with several microcytes. Bar = 10 µm.



PMC = pollen mother cells; PVS = staining pollen viability; Meiotic index = Meiotic index = normal tetrads/abnormal tetrads. P_1 = *C. arabica*; P_2 = *C. canephora*.

Figure 2. Relation between meiotic anomalies and pollen viability in F_2 Arabusta plants, F_1 hybrid and parent plants. The data are show by percentages (%).

variations on chromosome number may be the result of the elimination of chromosomes, that normally occur when genomes with different chromosome numbers are hybridized (Sakai et al. 2007).

As previously stated, diverse kinds of abnormalities were observed for the F_2 plants (Table 1; Fig. 1) and the most common anomaly recorded was the presence of univalents at metaphase I (Fig. 1a), ranging from 75% for plant 86 to 100% of cells observed for plants 07, 16, 17, 38, 40, 45, 54, 56, 73, 89, 94, 98, 99, and 143 (Table 1). The univalent values recorded were larger than those found for the parents or F_1 plants. Its occurrence may be →

Table 2. Mean of bivalents, univalents, trivalents and tetravalents at metaphase I of the F_2 Arabusta plants.

G	Plants	Bivalent	Univalent	Trivalent	Tetravalent
F_2	1	19.27±1.67	3.77±1.68	0.07±0.25	0.23±0.50
	7	17.77±1.87	4.33±1.35	0.10±0.31	0.57±0.68
	16	16.37±2.11	4.67±1.74	0.03±0.18	0.20±0.60
	17	18.17±1.70	4.83±1.46	0.03±0.18	0.37±0.56
	23	19.50±1.25	4.59±1.62	0.03±0.18	0.10±0.40
	29	19.13±1.33	4.33±2.04	0.17±0.46	0.20±0.48
	35	*	*	*	*
	38	17.40±1.85	4.95±1.39	0.10±0.31	0.55±0.76
	39	18.43±1.70	4.53±1.81	0.20±0.48	0.47±0.73
	40	18.87±1.31	4.23±1.65	0.03±0.18	0.43±0.63
	44	15.67±2.64	3.53±2.32	0.37±0.76	1.17±0.79
	45	18.60±1.59	4.57±1.79	0.03±0.18	0.33±0.55
	54	16.57±2.54	5.47±1.93	0.10±0.31	1.07±0.92
	56	16.50±2.39	3.50±1.04	0.17±0.38	0.57±0.68
	57	17.73±2.12	3.90±1.63	0.40±0.81	0.47±0.51
	60	15.23±2.31	4.37±2.04	0.63±0.76	1.17±0.91
	63	18.43±1.79	2.80±1.77	0.07±0.25	0.43±0.68
	73	16.67±1.72	6.47±1.43	0.13±0.35	0.27±0.52
	74	16.20±2.98	3.27±1.55	0.23±0.63	1.60±1.19
	82	17.50±2.53	3.20±2.31	0.07±0.25	0.73±0.83
	86	16.65±2.25	3.45±2.46	0.10±0.31	1.15±0.88
	89	17.20±1.95	5.63±2.11	0.03±0.18	0.53±0.78
	94	17.63±1.87	3.53±1.50	0.00	0.77±0.90
	98	17.00±2.12	7.07±2.36	0.30±0.53	0.30±0.47
	99	19.13±1.66	3.80±1.71	0.00	0.43±0.57
	102	17.27±2.32	5.37±2.54	0.10±0.31	0.63±0.72
	117	16.43±2.03	5.83±1.53	0.23±050	0.60±0.77
	124	15.10±3.23	4.25±3.01	0.60±1.05	1.05±0.94
	143	17.27±2.20	5.63±2.08	0.13±0.43	0.40±0.67
	152	18.47±2.39	4.10±1.74	0.10±0.40	0.40±0.50
P_1	CH2460	21.77±0.57	0.13±0.51	0.00	0.07±0.25
P_2	Co 667	20.03±1.69	0.80±1.13	0.00	0.70±0.79
F_1	Co 254	18.97±2.31	4.88±2.77	0.00	0.20±0.41

G = Genotype; Data are representing as mean ± standard deviations; *Unvalued plant.

Table 3. Chromosome numbers for somatic cells analyzed in the Arabusta F_2 plants, F_1 hybrid and parents P_1 and P_2.

G	Plants	Chromosome number (2n)							
		40	41	42	43	44	46	47	48
		Cell number							
F_2	01	0	1	6	0	23	0	0	0
	07	2	2	4	0	21	1	0	0
	16	3	1	1	0	21	4	0	0
	17	3	2	4	0	21	0	0	0
	23	2	1	1	0	25	1	0	0
	35	*	*	*	*	*	*	*	*
	29	0	1	0	0	27	2	0	0
	38	5	0	2	0	23	0	0	0
	39	1	1	1	0	26	0	1	0
	40	0	0	2	0	28	0	0	0
	44	5	0	0	1	23	1	0	0
	45	3	0	5	0	17	5	0	0
	54	2	1	3	0	22	1	0	1
	56	4	0	7	0	17	2	0	0
	57	5	0	3	0	21	1	0	0
	60	0	2	0	0	20	3	3	2
	63	5	0	5	0	19	1	0	0
	73	9	0	0	0	18	1	0	2
	74	3	0	2	0	24	0	0	1
	82	4	1	4	0	21	0	0	0
	86	1	1	2	0	26	0	0	0
	89	5	0	14	0	5	4	0	2
	94	5	2	6	1	16	0	0	0
	98	2	0	0	0	28	0	0	0
	99	0	0	0	0	28	1	0	1
	102	1	2	1	0	26	0	0	0
	117	4	0	5	1	19	0	0	1
	124	7	0	3	0	16	2	0	2
	143	9	0	6	1	14	0	0	0
	152	5	0	5	0	20	0	0	0
P_1	CH2460	0	0	2	0	58	0	0	0
P_2	Co 667	1	0	5	0	48	6	0	0
F_1	Co 254	4	2	6	4	41	1	0	0

G = Genotype; N = 30 analyzed cells per planta; P_1 = *C. arabica*; P_2 = *C. canephora;* *Unvalued plant.

attributed to the lack of chromosomes pairing or precocious chiasma terminalization at metaphase I (Silva et al. 2011; Do Pico and Dematteis 2012). This result corroborates the previous discussion of a hybrid between *C. arabica* and *C. canephora* by Boaventura and Cruz (1987).

Multivalents were also observed in metaphase I, including trivalents and tetravalents (Fig. 1b). Only plants 94 and 99 did not present trivalents in their cells. In contrast, plant 60 had the highest percentage among F_2 plants, with 46.67% of the PMCs with trivalents (Table 1). This anomaly was peculiar from the F_2 plants, since the parents and F_1 hybrid plants did not demonstrate trivalents (Table 4). In relation to tetravalents, the percentages of the cells ranged from 6.67% in plant 29 to 83.33% in plant 17 (Table 1). This probably occurred because F_2 plants had an increase in the number of homologous and homeologous chromosomes in their complement that could pair at metaphase.

Multivalents may present pairing problems, as well as univalent chromosomes. Both chromosome forms tend not to orient themselves at metaphase I and metaphase II. This illegitimate pairing of multivalents and some eventual recombination results in low viability of the pollen grains, as was recorded by Melo et al. (2015), when studying *Passiflora* interspecific F_1 hybrids, and by Techio et al. (2006), in meiotic studies with elephant grass (*Pennisetum purpureum*), pearl millet (*Pennisetum glaucum*) and their interspecific hybrids.

Non-oriented chromosomes (Figs. 1c, 1d and 1f) also were observed in metaphase I and metaphase II, ranging from 30% in plant 35 to 73.33% for plant 89 (Table 1). This irregularity may have been caused by premature chiasmata disjunction since they have an essential function in ensuring proper chromosome segregation at the first meiotic metaphase, preventing precipitate disjunction, and helping to ensure that the kinetochores of the homologous chromosomes that comprise a bivalent are oriented towards opposite poles of the cell (Sumner 2003). The presence of univalents, which was highly observed in this study, may be attributed to the ocurrence of precocious chiasmata terminalization. However, evaluation of chiasmata number would be necessary to prove this in these materials.

If chiasmata are precociously terminalized, occur in small number or are absent, it can result also in laggard chromosomes (Figs. 1e and 1g). We observed a high frequency of lagging chromosomes in the PMCs in anaphase I, telophase I, anaphase II, and telophase II stages of the F_2 plants. Laggard chromosomes were observed in all plants and the percentages per cell ranged from 38.33% in plant 35 to 88.33 in plant 73 (Table 1). These irregularities were also found by Krug and Mendes (1940), Owuor (1985) and Boaventura and Cruz (1987) in this kind F_1 hybrid of coffee. Laggard chromosomes that failed to reach the pole and were not included in tetrad nuclei formation was also observed in the meiotic behavior and pollen fertility analysis of *Chrysolaena species* (Do Pico and Dematteis 2012).

Chromosomal bridges were also exclusively observed in the F_2 plants (Table 1; Fig. 1h). The occurrence of chromosomal bridges, observed mainly in plants 29, 45 and 94, may reflect a failure of pairing of homologous chromosomes, resulted of a missegregation of multivalent figures, as demonstrated after an investigation of meiotic chromosome segregation in maize (Murphy and Bass 2012). Besides that, chromosomal bridges can be formed also due to terminal breaks or formation of dicentric chromosomes as a result from ring chromosome or sister chromatid fusion events (Lopez et al. 2014).

Abnormal post-meiotic products such as polyads, dyads and triads resulting from the meiotic abnormalities were also recorded for many of the F_2 plants. The highest percentages of normal tetrads were in plants 99 (70%) and 143 (65%) (Table 1). Dyads (Fig. 1i) were observed in plants 44, 63, and 124, while triads (Fig. 1k) were observed in →

Table 4. Meiotic abnormalities of the parent plants *C. arabica* cv Bourbon Vermelho (P_1), *C. canephora* cv Robusta (P_2) and the F_1 Arabusta hybrid.

	Uni	Tri	Tetra	Non-oriented	Laggards	Bridge	Dyads	Tryads	Polyads	Microcytes	Abnormalities total	Meiotic index
P_1	6.67a	0.00a	6.67a	35.83a	34.17a	0.00a	1.67a	4.17a	0.00a	1.67a	31.10a	94.17a
P_2	36.67b	0.00a	53.33c	35.00a	45.00b	0.00a	0.00a	1.67a	2.50a	7.50a	38.33a	88.33a
F_1	86.67c	0.00a	20.00b	50.83b	80.00c	0.00a	0.00a	0.83a	0.00a	73.33b	81.39b	25.84b

Uni = univalent. Tri = trivalent. Tetra = tetravalent; Meiotic index = normal tetrads / abnormal tetrads; Data represent percentages of abnormalities of four biological repetitions. ANOVA test, *p< 0.05; colum wise values followed by different letters differ significantly at 95%.

16 out of 30 plants studied, and plant 44 had the highest percentage (30%). Polyads (Fig. 1j) were also observed in 11 plants, and the highest percentage was in plant 16 (10%) (Table 1). Silva et al. (2012), evaluating the meiotic behavior and pollen viability of representative plants of wild Caricaceae species, argued that dyads and triads can produce unreduced gametes (1n), while the polyads generate unbalanced gametes due to the presence of micronuclei, which are derived from lagging chromosomes.

Microcytes (Fig. 1i, 1j and 1l) were observed in all F_2 plants with an average per plant of 60.83% (Table 1). The percentage of tetrads analyzed that contained microcytes ranged from 26.67% in plant 99 to 90% in plant 73. The elimination of the microcytes at the end of meiosis leads to the formation of aneuploid pollen grains, and their incorporation into nuclei leads to the formation of grains with increased numbers of chromosomes, such as 2n pollen. These pollen grains with a gain or loss of chromosomes was also registered by Risso-Pascotto et al. (2003) in *Brachiaria*, where the microcytes were formed by aggregation of micronuclei to the cell wall and kept isolated from other cells during cytokinesis, leading to the formation of microspores with an increased number of chromosomes. However, these microspores with an altered number of chromosomes result in grains with variation in size, even though they may still be viable. These considerations may explain the different number of chromosomes found in F_2 plants cells.

From the results of meiotic behavior for F_2 plants, it was verified that there are some correlations among the anomalies registered. The presence of univalents and unequal nuclei, in the final products of meiosis, showed a negative correlation (–0.54, $p < 0.05$), and this was also registered for univalents and dyads (–0.55, $p < 0.05$) (Table 5). In the same way, a negative correlation was registered between the presence of microcytes and the meiotic index (–0.74, $p < 0.01$). Positive correlations were verified for laggard chromosomes and total anomalies (+ 0.66, $p < 0.01$) and between dyads and triads (+ 0.75, $p < 0.01$) (Table 5).

Many of the irregularities reported in the process of meiotic division in this study were also found in other studies with coffee hybrids, such as *C. racemosa* and *C. arabica* (Medina 1963), *C. arabica* and *C. kapakata* (Mônaco and Medina 1965), *C. dewevrei* and *C. eugenioides*, *C. liberica* and *C. eugenioides* (Reddy and Narayan 1981), and *C. arabica* and *C. canephora* (4x) (Owuor 1985; Boaventura and Cruz 1987). Furthermore, some meiotic abnormalities observed in the F_2 population could also reflect the doubling of the single diploid *C. canephora* genome or incompatibility caused by genomic divergence between their parents.

Table 5. Significance Pearson correlation for 13 degrees of freedom at 0.05 and 0.01%

	b	c	d	e	f	g	h	i	j	k	l	m	n
a	-0.26	-0.21	0.06	0.32	0.19	-0.12	-0.54*	-0.55*	-0.27	0.07	-0.01	0.29	0.19
b		0.09	-0.07	-0.29	-0.19	-0.11	0.39	0.41	0.28	-0.11	0.07	-0.11	-0.15
c			-0.18	0.00	0.04	-0.24	0.20	0.04	-0.02	-0.07	0.04	-0.24	-0.07
d				0.28	-0.22	-0.10	0.04	-0.17	-0.12	-0.07	0.39	0.27	-0.28
e					0.10	-0.21	-0.21	-0.25	-0.18	-0.18	0.33	0.66*	-0.08
f						-0.08	0.04	-0.10	0.03	0.09	-0.11	-0.08	0.04
g							0.21	0.05	-0.12	0.26	-0.18	-0.07	0.23
h								0.14	0.04	-0.09	-0.11	-0.33	0.07
i									0.75**	0.12	-0.22	-0.21	-0.20
j										0.42	-0.13	-0.07	-0.39
k											-0.21	-0.1	-0.12
l												0.42	-0.74**
m													-0.08

*; **: significant Pearson correlation for 13 degrees of freedom at 0.05 and 0.01 % respectively; a = univalent; b = trivalent; c = tetravalent; d = non-oriented chromosome; e = laggard chromosome; f = stickiness chromosome; g = bridge; h = unequal nuclei; i = dyads; j = triads; k = polyads; l = microcyte; m = total of anomalies; n = meiotic index.

Both parental plants showed normal chromosome pairing for the great majority of the cells analyzed. For the *C. arabica* plant, the mean number of bivalents per cell was found to be 21.77 ± 0.57 (Table 2), which indicates a normal chromosome pairing and confirms it's tetraploidy. The normality in the *C. arabica* plant can also be seen by the total percentage of cells in meiotic division without anomalies, which is 68.90% (Table 4; Fig. 2). In relation to the parental *C. canephora*, 61.67% of the meiotic cells analyzed did not have anomalies (Table 4; Fig. 2) and the cells showed 20.03 ± 1.69 bivalents (Table 2). The parental *C. arabica* also demonstrated a normal meiotic behavior with the meiotic index equal 94.17 (Table 4), and the parental *C. canephora* exhibited a meiotic index of 88.33, showing a normal paring. These parental plants behave such as diploids; however, despite the meiose normality, some univalents, tetravalents, non-oriented chromosomes, lagging chromosomes, dyads, triads, and microcytes were observed for both in this study (Table 4).

Pinto-Maglio and Cruz (1998) had already showed that *Coffea arabica* is a segmental allopolyploid formed of two genomes with chromosome sets very similar morphologically to each other; despite this, the species has a typical disomic meiotic division with regular pairing of homologues, as was exposed in this study. A previous microsporogenesis analysis in one synthetic tetraploid *C. canephora* plant showed a high rate of irregularities in all phases of meiosis (Boaventura 1990).

The evaluation of meiotic cells in the Arabusta F_1 hybrid revealed a mean number of bivalents per cell of 18.97 ± 2.31 (Table 2). This resulted in 18.61% of cells without anomalies (Table 4; Fig. 2), which was different at 5% by the Tukey test compared to percentages of both parents. Nevertheless, we found an inconstant number of chromosomes, which ranged from 2n = 40 to 2n = 46 (Table 3), what may be explained by the chromosome elimination concept (Sakai et al. 2002), what is highly recorded in hybrids originated from parents with different chromosome numbers. In metaphase I, 86.67% of the cells contained univalent and 20% tetravalents (Table 4).

Non-oriented chromosomes were observed in metaphase I and metaphase II, with a frequency of 50.83% (Table 4). Laggard chromosomes were also common in hybrid F_1 and they were recorded in 80% of the PMCs of anaphase I and II (Table 4). There were also 0.83% triads and 73.33% tetrads, and both anomalies contained microcytes (Table 4). The F_1 hybrid showed an unstable meiosis with a low meiotic index of 25.84% (Table 4; Fig. 2). Boaventura and Cruz (1987) have already demonstrated that the hybrid Icatu, also derived from the crossing of *C. arabica* and a *C. canephora* tetraploid, had a high percentage of univalents and multivalents at metaphase I, in addition to irregular segregation in anaphase I and II, which led to the formation of irregular tetrads.

We can see that all F_2 plants had a higher frequency of anomalies, compared to the values from the parents and the F_1 hybrid. Abnormalities of F_2 plants showed that only 6.66% had a higher percentage of abnormalities than the F_1 hybrid. Furthermore, 70% of F_2 plants had values of meiotic index higher than that of the F_1 hybrid, which indicates that the F_2 plants have more regular meiosis than the F_1 hybrid (Fig. 2). However, the parental plants *C. arabica* and *C. canephora* had lower percentages of anomalies and higher meiotic indices than F_2 plants (Fig. 2), corroborating the fact that the parent plants have a more regular meiotic division than hybrids.

Pollen viability

Tests for pollen viability are widely used to monitor pollen fertility and mainly to make the crossing between possible economically important genotypes safer (Souza et al. 2002). The pollen viability was determined in this study using two different techniques, pollen viability by staining (PVS) and pollen viability by germination (PVG). Both tests have advantages and disadvantages, but both are valuable tools to verify the pollen fertility degree. PVS is a simplified method, but in some cases, the pollen grains can be distinguished, because the pollen grain wall can be sufficiently transparent and the aborted pollen grains become only very pale green and not colorless (Alexander 1980; Peterson et al. 2010). In its turn, PVG has been considered a more accurate method because it shows only the pollens grains that have the germination capacity of the tube, however, it is also not completely safe since it is performed in a culture medium and the pollen grains may undergo plasmolyze or dehydration rather than germination (Neto et al. 2009).

Through PVS test, the F_2 plant 74 showed the lowest percentage of viable pollen (26.24%), and plant 45 presented the highest percentage (79.16%) (Table 6; Fig. 2). On the other hand, through the PVG test, the F_2 plant percentages →

ranged from 3.40% in plant 98 to 38.38% in plant 29 (Table 6). This variation of pollen viability between F_2 plants, revealed by PVS and by PVG tests, showed that some F_2 plants (7, 16, 45, 63, 94, 124 and 143) had higher percentages of viability in one or in both tests when compared to parental *C. canephora* and F_1 hybrids (Table 6). The existing difference between PVS and PVG tests in this study can be justified because the time of incubation.

Table 6. Pollen viability of F_2 plants, F_1 Arabusta hybrid and parents *C. arabica* and *C. canephora*.

Genotype	Plants	PVS (%)	PVG (%)
F_2	**1**	78.96	10
	7	77.97	9.85
	16	74.96	36.51
	17	55.04	4.22
	23	56.64	7.7
	29	48.37	38.38
	35	68.53	21.11
	38	68.81	21.27
	39	62.23	21.43
	40	66.02	7.48
	44	52.24	7.38
	45	79.16	31.85
	54	55.95	16.86
	56	40.99	12
	57	44.24	7.78
	60	28.57	6.76
	63	74.25	28.54
	73	56.53	*
	74	26.24	*
	82	47.53	8.55
	86	*	*
	89	52.34	3.83
	94	72.49	15.9
	98	48.39	3.4
	99	44.74	*
	102	44.54	15
	117	69.57	10.7
	124	70.82	9.63
	143	70.67	16.19
	152	49.75	7.26
F_1	**CH2460**	53.70	6.25
P_1	**Co 667**	84.21	38.6
P_2	**Co 254**	62.86	12.27

*Unvalued plant.

However, though in the pollen germination tests in vivo the pollinic tubes of *Coffea* plants need 4 to 6 hours to enter the style and 20 hours to reach ovaries (Conagin and Mendes 1961), these incubation times would be impracticable in vitro tests, since in the culture medium there are no biological components that maintain the growth performance of the tube for a long period.

In addition, the F_2 plants 16, 17, 44, 54, 56, 57, 73, 74, 82 and 143 showed different sizes of pollen grains (Fig. 3). The F_2 plant 57 had grains with extreme sizes, some very large and others quite small, which may indicate the existence of pollen grains with different chromosome numbers, or aneuploid pollen grains. The largest ones could have additional chromosomes and the smaller ones fewer than the expected 22 chromosomes.

Certainly, the pollen viability of the F_2 plants was affected by the irregularities that occurred during the meiotic division process reported in this work, which generated unbalanced gametes, compromising, consequently, the viability of the pollen. It may be justified by the fact that these F_2 plants are derived from crosses among species with similar genomes, as is the case of the hybrid Arabusta, and tend to have high rates of pairing among homoeologous chromosomes. This kind of paring may impact on the meiosis recombination process and lead to the formation of aberrant gametes, what have direct relation with reduction of pollen fertility. Despite this, based on the results of PVS and PVG, we can say that F_2 plants 16, 45 and 63 have good reproductive capacity (Table 6) and could be used in breeding programs depending on the interest in their other characteristics.

Surprisingly, the meiotic index obtained for the F_2 plants is not always related to the percentages of meiotic abnormalities and pollen viability, or vice versa. The plants with the highest meiotic index (74 and 60) presented the lowest percentages of pollen viability in the PVS test (Fig. 2). In the opposite direction was the case of plant 16, which presented the lowest meiotic index but the highest percentage of pollen viability in the PVS test (Fig. 2). Maich et al. (1999) and Tian et al. (2015) verified in wheat that there is not always a significant relationship between a high meiotic index value and high fertility. This contradiction could be explained by some mechanisms that act in the final phases of meiosis, meaning that univalents and laggards are inserted in the nuclei of tetrads instead of forming micronuclei, thus increasing the frequency of quartets of tetrads with normal nuclei (Gaur et al. 1983). According to →

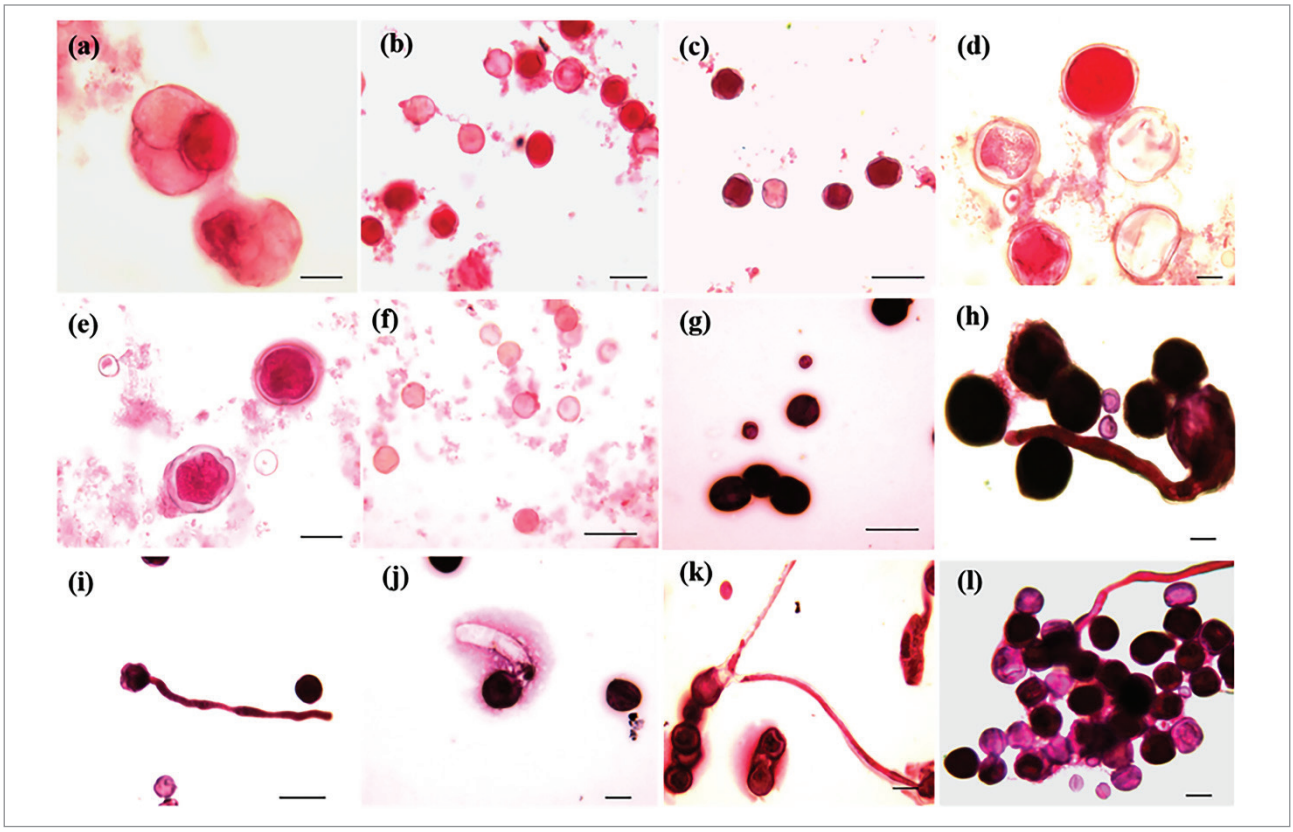


Figure 3. Overview of the pollen grains of plants of the F_2 Arabusta plants with Alexander's staining (PVS) and germination in vitro testing (PVG). (a) Malformed tetrad; (b), (c), (d) and (e) viable pollen grains (colored) and non-viable pollen grains (no color); (f) non-viable pollen grains; (g) grains with different diameters; (h)) pollen of different sizes and at different germinative stages; (i) germination of a normal pollen tube; (j) empty germinative tube; (k) pollen grain with two germinative tubes; (l) pollen of different sizes and at different germinative stages. Bar = 50 µm.

these authors, the degree of reintegration of these univalent and laggards may not be the same for all plants in a species.

The analysis of pollen viability by PVS and PVG for the parental plants and F_1 hybrid also reflected the different rates of viability for post-meiotic products and indirectly also for meiotic irregularities. The PVS analysis showed that *C. arabica* (P_1) had a maximum percentage of viability of 84.21% and uniform pollen grains regarding size and color. Already in *C. canephora* (P_2), 62.86% of pollen grains were viable (Table 6), and some grains were relatively larger in size than others. The F_1 hybrid had 53.70% viability and some pollen grains with different sizes (Table 6). In its turn, PVG analysis showed 38.6% of viability for *C. arabica* (P_1), 12.27% for *C. canephora* (P_2) and 6.25% of pollen viability for the F_1 hybrid (Table 6). These results for pollen viability of the parental and F_1 hybrid plants were similar to those registered by Mendes (1950) to plants of *C. canephora* with a duplicate chromosomal complement (4x). The author observed that only 21% of the pollen grains were viable in the germination tests, which is to be the expected for a plant with number of chromosomes synthetically duplicated. The results obtained for the hybrid derived from *C. arabica* and *C. canephora* also resembled those registered by Boaventura and Cruz (1987), showing a viability of 30.7% of pollen grains in the germination tests.

Based on our results, we suggest that a fertility assessment for plants such as F_2 plants should be based not only on these measured traits but also on the evaluation of fruit production, which would complement the effectiveness of pollen viability. In this case, the fertility of the female part of the plant should also have the fertility assured so that the results of the viability of the pollen can be trusted. Still, from the results here obtained, we can suggest also that the plants with the highest values for PVS and PVG tests (16, 45 and 63) and the highest MIs (99, 143 and 39) could be used as pollen donors in breeding programs. In contrast, plants with very low pollen viability, such as the F_2 plants 60 and 74, could →

be used when there is interest to sterile male plants or else discarded of the breeding program, depending of the interest.

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