

ABNORMAL BEHAVIOR OF SPINDLE DURING MICROSPOROGENESIS OF *Passiflora* (PASSIFLORACEAE)

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ABSTRACT: *Passiflora* genus, Passifloraceae family, has more than 500 species and 120 of them are native species of Brazil. All species produce fruits that are used as food, medicine and decoration. Floral buttons of five species were collected and fixed in a mixture of ethanol and acetic acid (3:1). The slides were prepared by squashing and staining with 1% propionic carmine. Results showed that during microsporogenesis there were few irregularities, mostly frequently related to chromosome irregular segregation as: precocious migration to poles in metaphase I and II, non-oriented bivalent chromosomes at metaphase I and II, and laggard chromosomes in anaphase I and II, forming micronuclei in telophases I and II and tetrad with microcyte. Another observed irregularity is related to the organization of spindle fibers in meiosis II as they organize themselves in T and V shapes and in sequential spindle. However, in the V-shaped spindle configuration, there was fusion between two nuclei that were close, forming triads instead of tetrads. Irregular chromosome segregation, abnormal spindles and irregularities in the cytokinesis process were responsible for the formation of monads, dyads, triads and polyads. However, the pollen grain viability was not harmed, presenting an 83.98% to 98.59% fertility variation.

KEYWORDS: *Passiflora*; Microsporogenesis; Abnormal spindle; Meiosis; Pollen viability.

COMPORTAMENTO ANORMAL DE FUSO DURANTE A MICROSPOROGÊNESE DE *PASSIFLORA* (PASSIFLORACEAE)

RESUMO: O gênero *Passiflora*, família Passifloraceae, apresentam mais de 500 espécies, havendo no Brasil aproximadamente 120 espécies nativas. Todas as espécies produzem frutos que são utilizados como produtos alimentícios, medicinais e ornamentais. Botões florais de cinco espécies foram coletados e fixados em etanol/ácido acético (3:1). As lâminas foram preparadas utilizando a técnica de esmagamento e coradas com carmim propiônico a 1%. Como resultado, observou-se que durante a microsporogênese poucas irregularidades foram encontradas, as mais frequentes estão relacionadas à segregação irregular dos cromossomos, tais como: migração precoce para os pólos em metafase I e II, bivalente não orientado em metafase I e II, e cromossomos retardatários em anáfase I e II, levando a formação de micronúcleos em telófases I e II, e micrócito em tétrades. Outra irregularidade observada esta relacionada a organização das fibras dos fusos em meiose II, que se organizam na forma em T, em V e fuso sequencial. Na configuração de fuso na forma de V ocorreu fusão entre dois núcleos que estavam próximos, formando triade ao invés de tétrade. A segregação irregular dos cromossomos, a formação de fusos anormais e as irregularidades no processo de citocinese foram responsáveis pela formação de mônades, díades, tríades e poliades como produtos final da meiose. Porém, a viabilidade dos grãos de pólen não foi comprometida, apresentado uma variação de 83,98% a 98,59% de fertilidade.

PALAVRAS CHAVE: *Passiflora*; Microsporogênese; Fuso anormal; Meiose; Viabilidade de pólen.

Introduction

There are over 600 registered species of the Passifloraceae family and it is believed that approximately 500 belong to the *Passiflora* genus and 200 of them are native to Brazilian regions (FERREIRA, 1994). Bernacci et al. (2005) report that due to the great incidence of this genus in Brazil this country can be considered one of its center of origin and diversification.

Passion fruit species stand out because of their medicinal properties, have decorative value due to their flowers; however, their main use is as human food as juice, sweets, jam, ice cream and liqueur. They are rich in vitamin A, calcium and phosphorus (MELETTI; MAIA, 1999; FALEIRO; JUN-

QUEIRA; BRAGA, 2006). Faleiro, Junqueira and Braga (2006) also report that few species are known and used as food, and the most utilized ones are the yellow or sour passion fruit (*P. edulis* f. *flavicarpa*) and the sweet passion fruit (*P. alata*). Peixoto (2005) explained that in European and North American countries, several varieties of passion fruit are used as ornamental plants; however, in Brazil, few species have their potential explored for this purpose.

Souza; Pereira and Vieira (2008) report that cytogenetic studies are poor when considering the great number of existing species, and that most of the studies are related to the counting of chromosome number. Cytogenetic is the area of genetics that studies cytological occurrences, mainly the ones related to the chromosome behavior; therefore, meiotic and

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mitotic processes are study subjects of cytogenetics (MONDIN; NETO, 2006). Soares-Scott et al. (2005) state that the lack of studies are justified by the difficulty to collect and germinate seeds and by the small chromosome size in the species of this genus.

Passiflora genus has been characterized by several basic chromosome numbers, like $x=3$, $x=6$, $x=9$, $x=10$ and $x=12$ (STOREY, 1950; MELO; CERVI; GUERRA, 2001; SOUZA; PEREIRA; MARTINS 2002, MELO; GUERRA, 2003). Souza; Pereira and Vieira (2008) report that $x=9$ is the observed number for most species of the *Passiflora* genus.

Melo and Guerra (2003) suggest that $x=6$ be the basic primary number and that $x=9$, $x=10$ and $x=12$ be secondary ones, that is, that the species with $x=6$ be originally diploid and that $x=9$, $x=10$ and $x=12$ have tetraploid origin with decreasing disploidy (12, 10, 9). As an example, they suggest that *P. foetida* had tetraploid origin with $2n=4x=24$ ($n=12$, $x=6$), varying to $n=10$ by a decreasing disploidy process where the loss of four chromosome occurred, resulting in $2n=20$.

The analysis of the meiotic behavior and the pollen viability has been done for some species of *Passiflora* and in general few irregularities have been observed (SOUZA; PEREIRA; VIEIRA, 2008). Considering the diversity of existing species, cytogenetic studies may contribute to a greater efficiency of genetic improvement programs by providing data for inferences of taxonomic relations and evolutionary patterns within *Passiflora* genus. Thus, the microsporogenesis analysis of five different species of the *Passiflora* genus aims to confirm the number of reported chromosomes, describe meiotic irregularities, and verify the fertility of pollen grains in order to select plants to be used in intra and interspecific crossings in genetic improvement programs of *Passiflora*.

Material and Methods

Seeds of *Passiflora* species from the germoplasm collection of the Agronomical Institute of Parana (Instituto Agronômico do Paraná -IAPAR), experimental station of Londrina, PR, Brazil, were sown in August 2008. The seedlings reached the ideal size to be transplanted to the field in the beginning of November, 2008 and were planted on November 3, 2008. Soil correction, management system (budding and fertilization) and weed control followed the recommendations by Meletti and Maia (1999).

Floral buttons were collected at different development stages from February to April 2009 and

fixed in ethanol/acetic acid (3:1 v/v) for 24 hours. After this period, the material was washed and transferred to alcohol at 70%, and stored under refrigeration. The slides were prepared by squashing and staining with 1.0% propionic carmine and analyzed under light microscopy. Photomicrographs were made with a digital camera DCE-2 Image Driving Soft Ware, and only adjusted for contrast and brightness.

Results and Discussion

Chromosome counting during microsporogenesis showed that *P. alata*, *P. amethystina*, *P. cincinnata* and *P. edulis* Sims species presented $2n=18$ chromosomes (Figure 1A) and *P. foetida* $2n=20$ chromosomes (Figure 1B). For *P. alata*, *P. amethystina*, *P. cincinnata* and *P. edulis* species, chromosome pairing was typical for diploid species, predominantly nine bivalents (Figure 1A). In *P. foetida*, the association of eight bivalent and one tetravalent was observed in most analyzed diakinesis (Figure 1B). The presence of chromosome in tetravalent association in *P. foetida* reinforces the theory of tetraploid origin suggested by Melo and Guerra (2003).

In diakinesis, two chromosome pairs are associated to the nucleolus in 100% of observed cells, that is, the presence of the active nucleolus organizer region in two chromosome pairs in *P. amethystina* and *P. cincinnata* species. This result corroborates the studies done by Cuco et al. (2005) who compared the Karyotype of three species of *Passiflora*, *P. edulis*, *P. cincinnata* and *P. amethystina*, using molecular markers and detected the presence of secondary constriction and satellite in chromosomes 8 and 9, and in *P. amethystina* a region of 45S rDNA was also detected in chromosome 1, but it seems to be inactive.

Few irregularities were observed in the microsporogenesis process of the studied species (Table 1). Meiotic abnormalities observed in these species are related to chromosome irregular segregation, spindle organization and irregularities in the cytokinesis process, generating abnormal post-meiotic products.

The precocious chromosome ascending towards the poles observed in metaphase (Figure 1C) and the laggard chromosome in anaphase (Figure 1D) were observed in *P. alata*, *P. amethystina*, *P. cincinnata* and *P. foetida*, in the phases of meiosis I and meiosis II. Another irregularity related to the chromosome segregation observed metaphase I, was the presence of non-oriented bivalents in the equatorial plate, as it can be verified in Table 1. Anaphase

I is characterized by the segregation of homologue chromosome. The presence of univalent chromosome in metaphase I occurs because of the absence or precocious termination of chiasm. Chiasmata are responsible for the maintenance of bivalents and originate in prophase I after the crossing-over. The precocious

chromosome ascending is a very common irregularity in many plant species, mainly among polyploids, and may result in chiasm low frequency, chiasm precocious termination or the presence of assynaptic genes (*as*) and dissynaptic genes (*dy*) in prophase I (PAGLIARINI, 2000).

Table 1: Number of cells analyzed and percentage of abnormal cells in each meiotic phase in five analyzed species of *Passiflora*.

Phase Abnormalities	No. of analyzed cells (% of abnormal cells)				
	<i>P. alata</i>	<i>P. amethystina</i>	<i>P. cincinnata</i>	<i>P. edulis</i>	<i>P. foetida</i>
Metaphase I	108 (2.77)	135 (5.92)	165 (7.27)	161 (0.00)	197 (4.06)
Precocious migration	-	5 (3.69)	12 (7.27)	-	-
Non-oriented bivalent	3 (2.77)	3 (2.22)	-	-	8 (4.06)
Anaphase I	103 (5.82)	164 (8.53)	116 (8.62)	133 (0.00)	93 (7.52)
Laggard chromosome	6 (5.82)	14 (8.53)	10 (8.62)	-	7 (7.52)
Telophase I	114 (3.50)	192 (4.68)	210 (0.00)	151 (0.00)	130 (0.76)
Micronuclei	4 (3.50)	9 (4.68)	-	-	1 (0.76)
Prophase II	154 (1.94)	160 (4.37)	104 (0.00)	132 (0.00)	139 (0.00)
Micronuclei	3 (1.94)	7 (4.37)	-	-	-
Metaphase II	122 (9.83)	174 (19.54)	153 (10.45)	134 (14.92)	155 (10.32)
Precocious migration	3 (2.46)	5 (2.87)	5 (3.26)	-	6 (3.87)
Irregular spindle	9 (7.37)	29 (16.67)	11 (7.19)	20 (14.92)	10 (6.45)
Anaphase II	109 (9.17)	146 (21.90)	123 (17.07)	113 (29.20)	119 (36.13)
Laggard chromosome	-	8 (5.47)	7 (5.69)	-	5 (4.20)
Irregular spindle	10 (9.17)	24 (16.43)	14 (11.38)	33 (29.20)	38 (31.93)
Telophase II	140 (9.28)	138 (23.18)	239 (10.87)	148 (16.89)	161 (32.91)
Micronuclei	-	11 (7.97)	9 (3.76)	-	4 (2.48)
Irregular spindle	13 (9.28)	21 (15.21)	17 (7.11)	25 (16.89)	49 (30.43)
Tetrad	165 (4.24)	172 (26.72)	244 (3.27)	197 (5.07)	169 (9.51)
Microcyte	2	19 (11.04)	8 (3.27)	-	4 (2.36)
Triad	3	13 (7.55)	-	5 (2.54)	9 (5.32)
Dyad	2	3 (1.74)	-	3 (1.52)	2 (1.83)
Monad	-	6 (3.48)	-	2 (1.01)	-
Polyad	-	5 (2.91)	-	-	-
Micrósporos	348 (4.01)	312 (14.41)	567 (1.41)	387 (2.84)	325 (2.76)
unbalanced	6 (1.72)	31 (9.93)	8 (1.41)	-	3 (0.92)
2n	8 (2.29)	10 (3.20)	-	8 (2.07)	6 (1.84)
4n	-	4 (1.28)	-	3 (0.77)	-

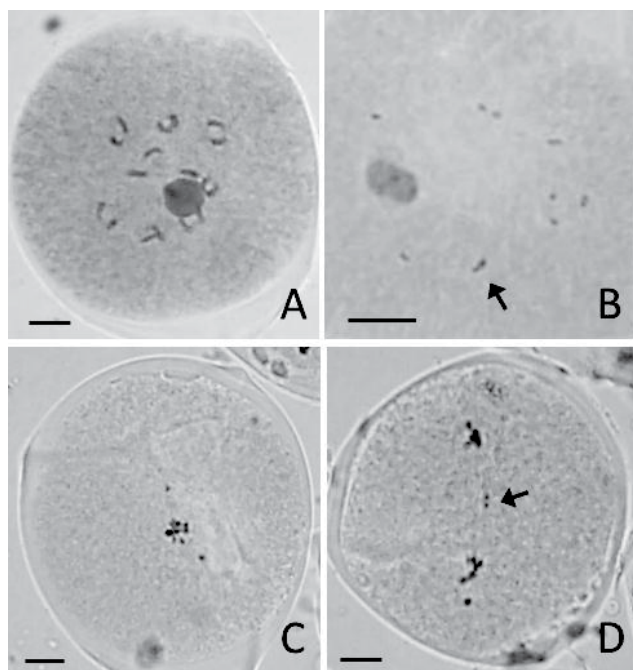


Figure 1: Aspects of *Passiflora* meiotic behavior. A) Diakinesis of *P. amethystina*, with nine bivalents, B) Diakinesis of *P. foetida*, showing eight bivalents and a tetravalent (arrow), C) Metaphase I with precocious chromosome migration, D) Anaphase I with laggard chromosomes (arrow). Bar = 10 μ m.

The presence of univalent chromosomes was not observed in diakinesis of the studied species and due to the small size of the chromosome, the number of chiasm was not verified. In studies carried out by Souza, Pereira and Martins (2002), the total average number of chiasm frequency observed in species of *Passiflora* com $x=9$ is 10.5, predominantly interstitial. However, the presence of precocious chromosome migration in metaphase I, observed in *P. amethystina* and *P. cincinnata* species, may have originated because of the occurrence of a single terminal chiasm, where the termination happened precociously and homologue chromosome reached metaphase I already separated. Similar suggestions have been described by Adamowski, Pagliarini and Batista (2000) in a study on *Paspalum maritimum* and by Baptista-Giacomelli, Pagliarini and Almeida (2000) on *Avena sativa*, where the number of precocious chromosome migration in metaphase I is much bigger than the number of univalent in diakinesis.

The chromosomes that keep delayed during the segregation in anaphase I and II are characterized as laggard (Figure 1D). According to Ricci (2006), this delay may occur due to the late terminalization of chiasm. Precocious chromosome migration in metaphases and laggard in anaphases can not be included in telophasic nuclei and form micronuclei in telophases I and II. Formed micronuclei may stay in tetrad microspores or be eliminated as a microcyte in

an extra cytokinesis, forming polyads (Figure 3C). Consequently, microspores (Figure 3G) and pollen grains of different sizes were formed in the end of meiosis. Souza, Pereira and Martins (2002) reported that the formation of genetically unbalanced and sterile pollen grains due to a failure in the process of chromosome segregation during the microsporogenesis of *P. edulis* Sims f. *flavicarpa*, known as yellow passion fruit. Similar behavior was also reported in studies carried out with different species of the *Passiflora* genus (SOUZA; PEREIRA; LAM-SANCHEZ, 1996; SOUZA et al., 2003; 2004), in interspecific hybrid of decorative *Passiflora* (ABREU et al., 2007).

Another frequently observed irregularity was the occurrence of irregular spindle. The five analyzed species presented problems in the spindle organization during the second meiotic division. Irregular spindles were observed since metaphase II until telophase II (Figure 2B, C, D, F, G, I, J, K), where the most affected species were *P. foetida* and *P. amethystina* (table 1). The most frequent configuration in the irregular organizations were transversal spindle, in T-shape (Figure 2C, J); followed by the tripolar spindle, in V-shape (Figure 2B, F, I, J, K); and sequential spindle, observed in lower frequency (Figure 2D, G). The meiotic division process is controlled by several genes and the fuse orientation is genetically controlled, a gene mutation that controls and organizes the spindle makes that a T-shaped tetrad is formed instead of a tetragonal tetrad (SHAMINA; DORAGOVA; TRUNOVA, 2000). A great number of t-shaped telophase II was observed, but after cytokinesis there was no gene unbalance in the microcytes; therefore, this abnormality was not considered in this study.

The formation of T-shaped or V-shaped telophase II is caused by the irregular organization of spindles that instead of arranging themselves in a parallel way, do it transversely or convergently, presenting a T or V configuration, respectively. In the convergent shape, a fusion between two closer nuclei may occur forming a final product in the end of the meiosis in the shape of a triad instead of a tetrad (ENDOW, 1999). *P. amethystina*, *P. edulis* and *P. foetida* species presented V-shaped telophase II (Figure 2K, L) and triads were observed with frequency ranging from 2.54% to 7.55% (table 1). Similar result was detected from *Brachiaria humidicola* where there was fusion of converging nuclei leading to the formation of restitutional nucleus ($2n$) in telophase II (BOLDRINI; PAGLIARINI; VALLE, 2006). There were several studies of several species where the tripolar spindle configuration has resulted in the formation of microspores $2n$ by the formation of restitutional

nucleus (CAETANO-PEREIRA et al., 1998; BIONE; PAGLIARINI; TOLEDO, 2000; RISSO-PASCOTTO; PAGLIARINI; VALLE, 2005; RICCI et al., 2007).

Abnormal spindles were also reported by Souza et al. (2003) in a study of fourteen different species of the *Passiflora* genus, and by Santos and Souza (2005) in *P. misera*. In both studies, the irregularities appeared in meiosis II and were responsible for irregular post-meiotic products like monads, dyads, triads and polyads. As a consequence of the irregular segregation of chromosomes in meiosis I and II and the abnormal organization of spindles in meiosis II, the cytokinesis pattern was also irregular and the post-meiotic products were abnormal like monads, dyads, triads and polyads (Figure 3C, D, E), were observed, resulting in unbalanced microspores and restituted nuclei (2n) (figure 3G) and tetranuclei (4n) (Figure 3H).

Cytokinesis has a secondary role that first depends on the segregation of chromosomes and the positioning of spindles (LORSON; HORVITZ; HEUVEL, 2000). Brown and Lemmon (1992) also reported that the cytoplasm cleavage follows a pattern that is determined by the position and the number of nuclei. This model suggests that the spindle alignment is determined by the position of chromosomes.

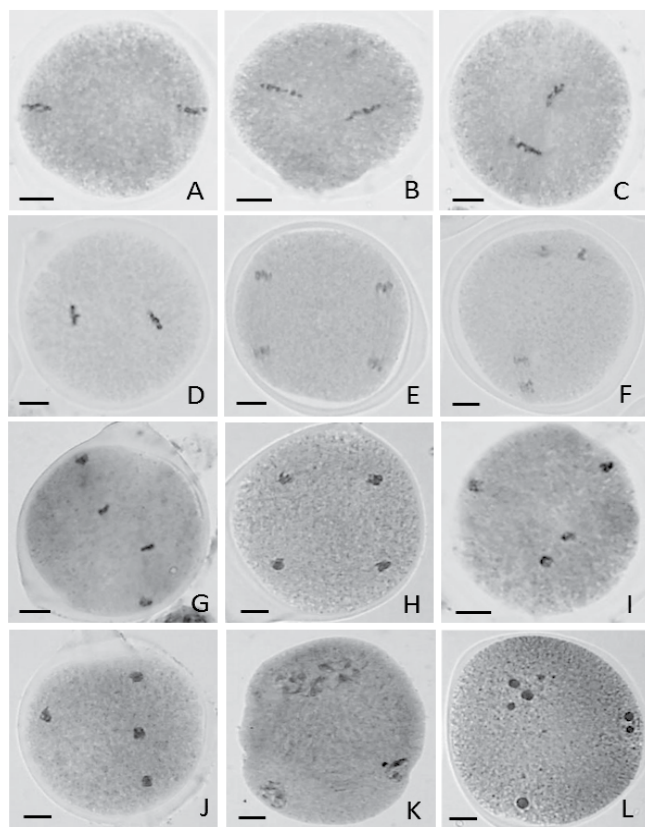


Figure 2: Irregular aspects of spindle organization. A) Metaphase II showing parallel spindle, B) Metaphase II with tripolar spindle, C) Metaphase II with transversal spindle, D) Metaphase II with sequential spindle, E) Anaphase II with parallel spindle, F) Anaphase II with tripolar spindle, G) Anaphase II with sequential spindle, H) Telophase II with parallel spindle, I) Telophase II with tripolar spindle, J) Telophase II with transversal spindle, K) Telophase II with tripolar spindle, and L) Telophase II with tripolar spindle and restitution in one of the poles. Bar = 10 μ m.

quential spindle. E) Anaphase II with parallel spindle F) Anaphase II with tripolar spindle, G) Anaphase II with sequential spindle, H) Telophase II with parallel spindle, I) Telophase II with tripolar spindle, J) Telophase II with transversal spindle. K) Telophase II with tripolar spindle, and L) Telophase II with tripolar spindle and restitution in one of the poles. Bar = 10 μ m.

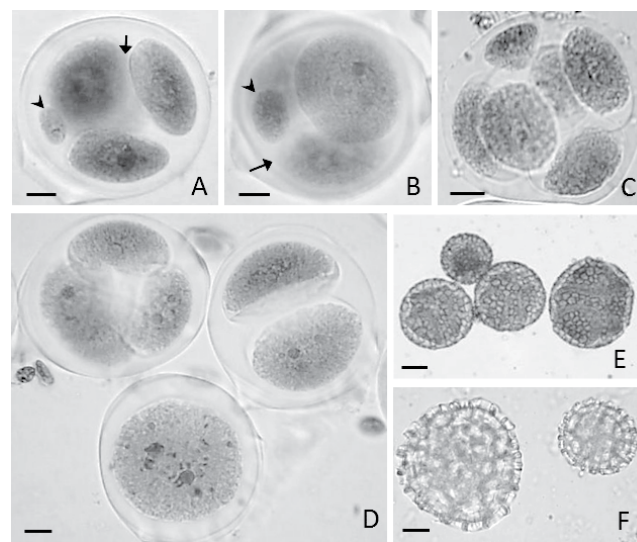


Figure 3: Meiotic products of *Passiflora*. A) Tetraedric tetrad, arrow shoes one of the microspores in the back, with microcyte (arrow head), B) Triad with microcyte (arrow head), observe microspores in the back (arrow), C) Polyad with six unbalanced microspores, D) Presence of tetrad, dyad and monad, E) Meiotic products with microspores of different sizes, normal, unbalanced and 2n, F) Normal pollen grain and pollen grain 4n. Bar = 10 μ m.

Conclusions

The observed irregularities did not compromise the fertility of analyzed species once the viability of pollen grains ranged from 83.98% to 98.59%. Therefore, regarding the microspore genesis, these species may be part of programs of *Passiflora* genetic improvement as pollen grain donors.

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