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Analysis of meiotic behavior in *Cordia ecalyculata* Vell. (Boraginaceae)

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Resumo

Análise do comportamento meiótico em *Cordia ecalyculata* Vell. (Boraginaceae). A espécie *Cordia ecalyculata* pertence à família Boraginaceae, é conhecida popularmente por café de bugre. Para fins medicinais é indicada como tônico, diurético, anti-inflamatório e inibidor do apetite. Visando contribuir para um melhor entendimento da espécie, inflorescências jovens de seis indivíduos foram coletadas e fixadas em solução de etanol e ácido acético (3:1) por 24 h. As lâminas foram preparadas utilizando a técnica de esmagamento e coradas com carmim acético a 1%. Durante a microsporogênese poucas irregularidades foram encontradas, as mais frequentes estão relacionadas à segregação irregular dos cromossomos, tais como: Ascensão precoce para os polos em metáfase I e II, bivalente não orientado em metáfase I e II, e cromossomos retardatários em anáfase I e II, levando a formação de micronúcleos. Outra irregularidade observada esta relacionada a organização das fibras dos fusos em meiose II, que se organizam na forma em T e V. Na configuração de fuso na forma de V ocorreu fusão entre dois núcleos que estavam próximos, formando tríade ao invés de tétrade, levando à formação de micrósporos 2n final da meiose. Entretanto, as irregularidades observadas não comprometeram a fertilidade da espécie em análise, uma vez que a viabilidade dos grãos de pólen variou de 95,42% a 100%.

Palavras-chave: *Cordia ecalycula*; Fuso irregular; Meiose; Microsporogênese

Abstract

Cordia ecalyculata belongs to the Boraginaceae family, and is commonly known as buggy coffee. It is indicated for medicinal use as a tonic, diuretic, anti-inflammatory and appetite suppressant. Young inflorescences of

six individuals were collected and fixed in a mixture of ethanol and acetic acid (3:1) for 24 hours. The slides were prepared by crushing and staining tissue with 1% acetic carmine. During microsporogenesis some irregularities were observed, mostly frequently related to irregular chromosome segregation. Irregularities included: precocious migration to poles in metaphase I and II, disoriented bivalent chromosomes at metaphase I and II, laggard chromosomes in anaphase I and II, and micronuclei formation. We also observed irregular spindle organization in meiosis II, leading to 'T' and 'V' shaped spindle configurations. In the V-shaped configuration, two nearby nuclei fused, forming triads instead of tetrads; this lead to formation of 2n microspore at the end of meiosis. However, pollen grain viability was not compromised, as pollen grain viability varied between 95.42% and 100%.

Key words: *Cordia ecalycula*; Irregular spindle; Meiosis; Microsporogenesis

Introduction

Cordia ecalyculata Vell, known commonly as buggy coffee, is an arboreal, evergreen, heliophytic species typical of humid and fertile soils in semideciduous forests (SOUZA; LORENZI, 2005). The wood can be used to make light boxes, matchsticks and toys, and is suitable for street afforestation. This species produces red fruit, similar to coffee. The fruits are appreciated by animals, and can be toasted and commercialized as a tea product (CRUZ, 1995; LORENZI, 2002). *Cordia ecalyculata* is also widely used for medicinal purposes, primarily by indigenous people, being indicated as a tonic, diuretic, anti-inflammatory agent and appetite inhibitor (LORENZI; MATOS, 2008).

Cytogenetics is a field focused on the study of cytological events, mainly those related to the behavior and genetics of chromosomes. Meiosis and mitosis are the basic events studied by cytogeneticists (MONDIN; NETO, 2006). During the meiotic process, changes may occur in various stages that can ultimately lead to formation of structural anomalies which compromise gamete viability (AULER et al., 2006). Despite the high diversity of native arboreal species in Brazil, little is known about the cytogenetic behavior of some of these species. Cytological reviews can enhance the efficiency of current conservation strategies, while improving the greater body of research regarding these species (AULER et al., 2006; SOUZA-KANESHIMA et al., 2010; KIIHL et al., 2011; GODOY et al., 2012).

The aim of this work was to study the meiotic behavior of *Cordia ecalyculata* Vell using the microsporogenesis technique to estimate the viability of the pollen grains, and to thereby obtain information that may assist in the maintenance and conservation of the species.

Material and Methods

Six individuals (*Cordia ecalyculata* Vell) were collected in Caiuá Ecological Station in Diamante do Norte, Paraná State, Brazil (22°41'S and 52°55'W) for analysis of young inflorescences. To identify the species of the individuals, herbarium specimens were mounted according to standard techniques (FIDALGO; BONONI, 1989), and were deposited in the State University of Maringá – UEM herbarium, under the following registration numbers: HUEM 16283, HUEM 16417, HUEM 21970, HUEM 23888, HUEM 23897 and HUEM 25385.

Inflorescences in different stages of development were collected and fixed in a solution of ethanol and glacial acetic acid (3:1) for a period of 24 h at room temperature, after which they were washed in 70% ethanol and stored in 70% ethanol at 4°C until use. Microscope slides were prepared by crushing and staining it with 1% acetic carmine. For analysis of meiotic behavior, at least 1000 cells from each individual were analyzed in various meiotic phases. The staining intensity and size of approximately 300 microspores and pollen grains of each individual were analyzed and classified following the criteria described by Caetano-Pereira et al. (1999) in order to estimate pollen grain viability.

Analyses were performed via optical microscopy, and the cells were photographed using a digital camera (Celestron, Model 44420), changing only contrast and brightness between sample visualization.

Results and Discussion

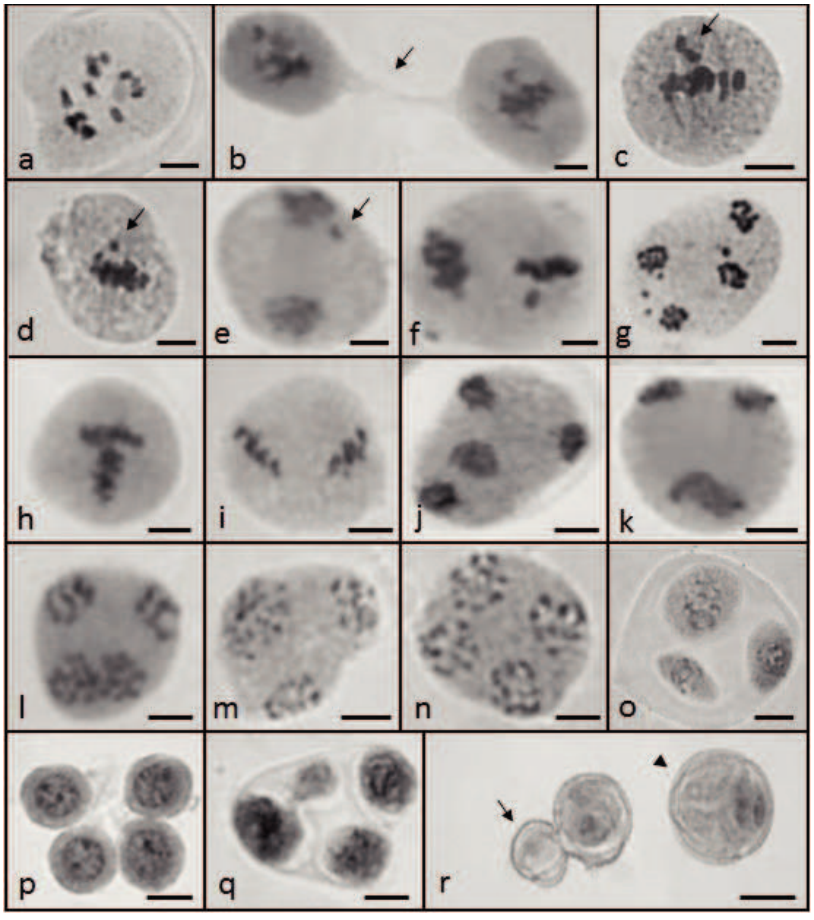
Cytological analyses of meiocytes in prophase I revealed 12 chromosomes in bivalent association, for a total of 24 chromosomes in diakinesis (2n = 24, Figure1a).

Cytoplasmic connections joining pollen mother cells (PMC) were observed in four of the six analyzed individuals (HUEM 16283, HUEM 16417, HUEM 21970 and HUEM 23888) (Table 1). The connections disappeared in early meiosis while still in prophase I, and only HUEM 21970 displayed cytoplasmic connections during metaphase I (Figure 1b).

Gates (1908), analyzing an *Oenothera* species, described the cytoplasmic connections as thin, delicate, threadlike connections, resembling those that connect adjacent pollen mother cells. Heslop-Harrison (1966) suggested that cytoplasmic channels are formed in early prophase I connecting pre-meiotic cells, which plays a crucial role in cellular synchronization; these channels are disrupted resulting in complete separation of meiocytes during meiosis.

Wang et al. (2002), while working with *Lilium davidii*, observed that cytoplasmic channel formation occurred in the beginning of prophase I in the zygotene phase, and disappeared at the end of pachytene due to callose deposition.

FIGURE 1: Meiotic Abnormalities in *Cordia ecalyculata*. a) Diakinesis with 12 bivalent chromosomes; b) metaphase I with cytoplasmic channel joining two meiocytes (arrow); c) metaphase I with non-congressed chromosome (arrow); d) metaphase I with precociously migrating chromosomes (arrow); e) telophase I with micronucleus (arrow); f) metaphase II with both irregular spindle and non-congressed chromosome; g) anaphase II with both irregular spindle and lagging chromosomes; h) metaphase II with T-shaped irregular spindle; i) metaphase II with V-shaped irregular spindle; j) telophase II with T-shaped irregular spindle; k) telophase II with V-shaped irregular spindle; l) Late telophase II with V-shaped irregular spindle, showing nucleus proximity; m) telophase II with refunded nucleus; n) normal telophase II; o) triad; p) normal tetrad; q) tetrad with unbalanced microspores; r) unbalanced final product (arrow) and 2n (arrow head). Bar: 10 µm.



Irregularities related to the chromosomal segregation process were observed at low frequency in *C. ecalyculata*. In five of the six analyzed individuals, we observed the presence of non-congressed chromosomes during metaphase I (Figure 1c, Table 1).

Non-congressing chromosomes, also called disoriented chromosomes, may arise from spindle fiber connection errors in the chromosome kinetochore (ADAMOWSKI et al., 2000).

TABLE 1: Aspects of microsporogenesis in *C. ecalyculata* Vell.

Phase / Abnormalities	**Individuals analyzed**					
	HUEM 16283	HUEM 16417	HUEM 21970	HUEM 23888	HUEM 23897	HUEM 25385
METAPHASE I	**104**	**136**	**107**	**140**	**140**	**158**
Cytoplasmic channels	–	–	02 (1,87%)	–	–	–
Precocious migration	04 (3,85%)	04 (2,94%)	02 (1,87%)	06 (4,29%)	06 (4,28%)	07 (4,43%)
Non-congression	–	12 (8,82%)	14 (13,08%)	01 (0,71%)	04 (2,86%)	04 (2,53%)
ANAPHASE I	**97**	**112**	**87**	**61**	**128**	**89**
Lagging	04 (4,12%)	08 (7,14%)	03 (3,45%)	01 (1,64%)	02 (1,56%)	–
TELOPHASE I	**106**	**160**	**152**	**172**	**157**	**137**
Micronuclei	02 (1,89%)	11 (6,87%)	06 (3,95%)	–	02 (1,27%)	03 (2,19%)
PROPHASE II	**152**	**164**	**182**	**162**	**112**	**175**
Micronuclei	–	08 (4,88%)	04 (2,20%)	02 (1,23%)	–	–
METAPHASE II	**166**	**174**	**116**	**166**	**121**	**166**
Non-congression	–	05 (2,87%)	09 (7,76%)	11 (6,63%)	–	–
Precocious migration	14 (8,43%)	–	–	–	–	–
T-shaped spindle	08 (4,82%)	05 (2,87%)	19 (16,38%)	11 (6,63%)	–	05 (3,01%)
V-shaped spindle	16 (9,64%)	21 (12,07%)	17 (14,66%)	09 (5,42%)	–	08 (4,82%)
ANAPHASE II	**128**	**144**	**133**	**104**	**72**	**93**
Lagging	04 (3,12%)	–	03 (2,25%)	04 (3,85%)	–	–
T-shaped spindle	06 (4,69%)	04 (2,78%)	09 (6,77%)	07 (6,73%)	–	02 (2,15%)
V-shaped spindle	11 (8,59%)	16 (11,11%)	21(15,79%)	08 (7,69%)	–	03 (3,22%)
TELOPHASE II	**153**	**174**	**121**	**169**	**144**	**176**
Micronuclei	03 (1,96%)	–	02 (1,65%)	02 (1,18%)	–	02 (1,14%)
T-shaped spindle	–	15 (8,62%)	09 (7,44%)	08 (4,73%)	–	03 (1,70%)
V-shaped spindle	03 (1,96%)	05 (2,87%)	14 (11,57%)	09 (5,33%)	–	05 (2,84%)
TETRAD	**186**	**171**	**403**	**234**	**182**	**188**
Triad formation	–	–	06 (1,49%)	04 (1,71%)	–	04 (2,12%)
Total	**1092**	**1235**	**1301**	**1208**	**1056**	**1182**

Precocious migration of chromosomes in metaphase I was observed in all six individuals analyzed (Table 1), in which some homologous chromosomes became separated and migrated to opposite poles early (Figure 1d). According to Kiihl et al. (2010), early migration to the poles may occur due to absence or early terminalization of chiasmata. Chiasmata are formed after crossing-over during prophase I, having the function of keeping the homologous chromosomes together until the onset of anaphase I (ALBERTS et al., 2010). Pagliarini (2000) suggested that precocious migration of the chromosomes to the poles may be due to the presence of asynaptic (as) and desynaptic (dy) genes. Thus, during prophase I synapse absence results in lack of chiasma formation.

Until early anaphase I, the homologous chromosomes are held together by chiasmata, which are formed in prophase I. At this stage the disruption of centromeres and the shortening of the spindle fibers causes each chromosome to be pulled to opposite poles. Chromosomes that remain delayed (in relation to the majority) in migrating to the poles during segregation are

called 'lagging' chromosomes. Pagliarini and Pozzobon (2005) suggest that the cause of lagging chromosomes in anaphase I may be late terminalization of chiasmata.

Early-migrating chromosomes in metaphase I, and laggards in anaphase I cannot be incorporated into telophasic nuclei to form micronuclei (Figure 1e). The micronucleus constitutes a small nuclear mass, and may have one or more chromosomes, delimited by the membrane and separate from the main core (FENECH, 1997). Micronuclei were observed in both telophase I and prophase II.

Following meiosis II, disoriented chromosomes arising in metaphase II, and lagging chromosomes in anaphase II were visualized (Figure 1f, g) (Table 1). The irregular segregation of chromosomes in meiosis I and II can lead to the formation of micronuclei at the end of meiosis, and after cytokinesis, may either remain in microspore tetrads or may be eliminated in the form of microcytes for an additional cytokinesis. In this case, the formed tetrads will be genetically unbalanced, and the resulting unbalanced microspores will be unviable.

Irregularities related to the chromosomal segregation process, such as disoriented chromosomes, precociously migrating chromosomes, laggard chromosomes and micronuclei formation, have been reported in several different plant species, including: *Psychotria carthagenensis* (CORRÊA; FORNI-MARTINS, 2004), *Brachiaria brizantha* (MENDES-VIEIRA et al., 2006.), *Meconopsis aculeata* (SINGHAL; KUMAR, 2008), *Brachiaria bovonei* (RISSO-PASCOTTO et al., 2009), *Passiflora serrato-digitata* (KIIHL et al., 2011), Meliaceae species (GROSSI et al., 2011), *Alchornea triplinervia* (GODOY et al., 2012), and *Psychotria myriantha* (ALONSO-PEREIRA et al., 2013).

Another irregularity observed was the occurrence of irregular spindles in meiosis II. Five of the six individuals analyzed showed changes in the organization of the spindles, observed from metaphase II to telophase II. The individual HUEM 21970 showed the highest frequency of affected cells (Table 1). When irregular organization occurred, the most common conformations observed were a T-shaped transverse spindle formation (Figure 1h, J), followed by the V-shaped formation of the convergent zone (Figure 1i, k). According to Shamina et al. (2000), the conformation of the spindles is under genetic control, and a mutation in the gene can change their orientation. During polymerization, rather than arrange themselves in a parallel form, the zones are organized transversely or convergently, with a T-shaped or V-shaped configuration, respectively.

When oriented convergently, the resulting V-shaped spindle can lead to convergence of telophasic nuclei which may then become fused, generating a 2n nucleus, (i.e., a restitution nucleus) that forms a triad instead of tetrad after cytokinesis (ENDOW, 1999). HUEM 16283, HUEM 16417, HUEM 21970, HUEM 23888 and HUEM 25385 all presented V-shaped telophase II (Figure 1l, m). Triads (Figure 1o) were observed only HUEM 21970, HUEM 23888 and HUEM 25385, often ranging from 1.49% to 2.12% (Table 1). A triad consists of two microspores, one n and one 2n, leading to formation of 2n gametes. The formation of 2n microspores (Figure 1r) and 2n pollen grains was observed in 0.87% and 1.81% of post-meiotic products analyzed, respectively (Table 2).

TABLE 2: Final products of meiosis in *C. ecalyculata* Vell.

Phase Abnormalities	**Individuals analyzed**					
	HUEM 16283	HUEM 16417	HUEM 21970	HUEM 23888	HUEM 23897	HUEM 25385
MICROSPORE	**378**	**317**	**440**	**318**	**410**	**320**
Unbalanced	08 (2,11%)	–	–	–	–	–
2n	–	03 (0,95%)	02 (0,45%)	09 (2,83%)	–	05 (1,56%)
POLLEN GRAIN	**356**	**320**	**457**	**393**	**390**	**233**
Unbalanced	–	03 (0,93%)	05 (1.09%)	08 (2,04%)	–	02 (0,85%)
2n	–	05 (1,56 %)	04 (0.87%)	10 (2,54%)		02 (0,85%)

Similar studies (e.g., SOUZA et al., 1999, 2003; PASCOTTO-RISSO et al., 2005; RICCI et al., 2007; SHEIDAI et al., 2009; KIIHL et al., 2010; SOUZA-KANESHIMA et al., 2010; GROSSI et al., 2011; GODOY et al., 2012) correlate telophasic nucleic fusion and subsequent formation of 2n microspores with T-shaped convergent spindle formation.

The observed irregularities in this study did not compromise the fertility of the species in question, as viability of pollen grains ranged from 95.42% to 100%. Therefore, the process of microsporogenesis in *C. ecalyculata* seems well adapted, and may be included in future breeding programs involving the species.

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