Abnormal meiotic behavior in three species of *Crotalaria*

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Abstract – The objective of this work was to compare the meiotic behavior and pollen grain viability of three species of *Crotalaria*. Slides for meiotic analysis were prepared by the air-drying technique. Pollen grain viability was measured by three staining procedures (Alexander’s solution, tetrazolium chloride and fluorescein diacetate) and in vitro germination in a sucrose solution. Eight bivalents were observed, confirming previous reports on populations from other regions of Brazil, as well as from other countries. All species showed abnormal meiotic behavior as follows: in *Crotalaria micans*, cytomixis and abnormal chromosome pairing in diakinesis; in *C. spectabilis*, abnormal chromosome pairing in diplotene; in *C. zanzibarica*, shrunk nuclei in leptotene and zygotene. Pollen grains of all three species show low viability, which may be associated with the irregularities of the meiotic behavior.

Index terms: *Crotalaria*, cytogenetics, cytomixis, meiosis, pollen grain viability.

Comportamento meiótico anormal em três espécies de *Crotalaria*

Resumo – O objetivo deste trabalho foi comparar o comportamento meiótico e a viabilidade dos grãos de pólen de três espécies de *Crotalaria*. A análise meiótica foi realizada por meio da técnica de secagem ao ar. A viabilidade dos grãos de pólen foi avaliada por testes de coloração (corante de Alexander, cloreto de tetrazólio e diacetato de fluoresceína) e por teste de germinação em solução de sacarose. Foram observados oito bivalentes, confirmando relatos prévios em populações de outras regiões do Brasil e de outros países. As três espécies apresentaram comportamento meiótico irregular: em *Crotalaria micans*, citomixia e pareamento irregular na diacinese; em *C. spectabilis*, pareamento irregular no diplóteno; e em *C. zanzibarica*, núcleo fortemente condensado nas fases de leptóteno e zigóteno. A viabilidade dos grãos de pólen das três espécies é baixa, o que pode estar associado às irregularidades do comportamento meiótico.

Termos para indexação: *Crotalaria*, citogenética, citomixia, meiose, viabilidade do grão de pólen.

Introduction

*Crotalaria* L. belongs to the tribe Crotalarieae and is the third largest genus of the subfamily Faboideae (Fabaceae), comprising around 600 herbaceous and shrub species distributed in the tropics and subtropics (Polhill, 1982). In Brazil, there are 31 native and 11 introduced species of *Crotalaria* (Flores et al., 2006). Some are used in agriculture (nitrogen fixation in crop rotating systems and biological control of nematodes); in the paper and fiber industry, in landscaping (Polhill, 1982; Mendonça et al., 1999) and in phytoremediation (Pereira et al., 2002).

Although most of the species of *Crotalaria* are diploid (2n = 2x = 16), some are polyploid, with predominance of tetraploids (2n = 4x = 32), and a few are 2n = 2x = 14 (Mondin et al., 2007). Information on chromosome number, quantitative karyotype parameters, chromosome banding patterns and rRNA gene mapping have been used for phylogenetic and chromosome evolution inferences in *Crotalaria* (Oliveira & Aguilar-Perecin, 1999; Mondin, 2003; Tapia-Pastrana et al., 2005; Almada et al., 2006; Flores et al., 2006; Mondin et al., 2007).

However, little is known on the meiotic behavior of *Crotalaria* species. Verma & Raina (1980) evaluated twenty species and observed chromosome stability based on predominance of normal bivalents, but also related the occurrence of univalents, multivalents, and bridges with or without fragments in some species. These irregularities were considered as evidence for structural changes during the evolution of the genus.

Almada et al. (2006) observed regular meiosis in diploid and polyploid taxa of *Crotalaria*, with ring bivalents as the main configuration in diakinesis. Interestingly, even though some laggard chromosomes
and bridges without fragments were found in all taxa, meiosis was more regular in polyploids than in diploids. The authors considered that polyploid species are probably allopolyploids.

Increasing information on the meiotic behavior of Crotalaria species may give important insights on the numerical and structural chromosome changes involved in the evolution of the genus, as shown by Verma & Raina (1980) and Almada et al. (2006).

The objective of this work was to compare the meiotic behavior and pollen grain viability of three species of Crotalaria.

**Materials and Methods**

Flower buds from 25 accessions of three species of Crotalaria were collected between November 2007 and March 2008, in two municipalities of Minas Gerais state, Brazil. Vouchers were deposited at the herbarium of Universidade Federal de Lavras (ESAL): C. spectabilis Roth, five accessions from Ijaci (21º11'15"S, 44º56'15"W) (ESAL voucher number 22066); C. zanzibarica Benth, ten accessions from Ijaci (21º11'15"S, 44º56'15"W) (ESAL voucher number 22067); and C. micans Link, ten accessions from Lavras (21º14'43"S, 44º59'59"W) (ESAL voucher number 22070). Duplicates were identified at the herbarium of the Jardim Botânico do Rio de Janeiro. The buds were fixed in Carnoy (3 methanol:1 acetic acid), immediately after collection, and stored at -20°C.

Slides for meiotic analysis were prepared from cell suspensions, according to Viccini et al. (2005), with modifications in the enzymatic digestion of anthers. This step was adjusted for Crotalaria using Pectinex Ultra SP-L (Novozymes), at 34°C, in a water bath, for four to six hours. Slides were air-dried, stained with 10% Giemsa solution and evaluated under light microscope (Leica DMLS). All meiotic phases were analyzed and representative figures were digitalized using a digital microcamera (Nikon Digital Sight DS-Fi1). The meiotic index: MI (%) = 100(number of normal tetrads/total meiocytes) was calculated from 5,000 meiocytes per species.

Pollen grain viability was evaluated using three staining methods: Alexander’s solution, for 24 hours with fixed and fresh material, at 4°C; 2,3,5-triphenyltetrazolium chloride in 5 (TTC 5%) and in 50% (TTC 50%) sucrose solution, for 2 hours with fresh material, at room temperature in the dark; and fluorescein diacetate (FDA) 6.25 μg mL⁻¹ in 25% sucrose solution, for 30 min with fresh material, at room temperature in the dark. Slides were prepared by squashing the anthers (fresh or fixed) in a drop of staining solution. In each method, percentage of viable pollen grains was obtained from 1,000 mature microsporocytes observed in five slides per species. Slides for the Alexander and TTC methods were evaluated under a light microscope (Leica DMLS), while those for FDA were evaluated under epifluorescence using an Olympus BX60 microscope (460–490 nm excitation and 515–550 nm emission filter). Fresh pollen grains were cultured in liquid media containing sucrose at 15, 20 and 50% for C. spectabilis, C. micans and C. zanzibarica, respectively, for 24 hours, in a humid chamber, at 28°C. All pollen grains from five slides per species were analyzed under a light microscope (Leica DMLS) to estimate germination percentage.

**Results and Discussion**

Synchrony of flower bud size and meiotic phases was observed. The best interval of flower bud size to obtain meiocytes was 6.5 to 7.0 mm for C. zanzibarica; 4.5 to 5.0 mm for C. micans and 5.5 to 6.0 mm for C. spectabilis. All three species showed n = 8 chromosomes (Figure 1), confirming previous reports

![Figure 1. Chromosome number (n = 8) of Crotalaria species. (A) Diakinesis in C. zanzibarica; (B) metaphase I in C. micans; (C) metaphase I in C. spectabilis; (D) rod (white arrow) and ring (black arrow) bivalents in C. zanzibarica; (E) bivalents in C. micans; (F) rod (black arrow) and ring (white arrow) bivalents in C. spectabilis. Bar: 5 μm.](image)
for C. zanzibarica (Atchinson, 1950; Yeh et al., 1986), 
C. micans (Almada et al., 2006; Flores et al., 2006) and 
C. spectabilis (Mondin, 2003; Almada et al., 2006).

Ring and rod bivalents were detected in 0.33% 
diakineses for C. zanzibarica (Figure 1 D) and in 
0.73% diakineses for C. spectabilis (Figure 1 F). In the 
latter species, ring pairing was more frequent as also 
observed by Almada et al. (2006). In C. micans, neither 
rod nor ring bivalents were observed (Figure 1 E), 
while Almada et al. (2006) described their occurrence.

All three species had irregular meiotic behavior with 
different kinds of abnormalities. In C. zanzibarica, 
approximately 14% of the meiocytes were irregular. Of 
these, 89.19% were in prophase I, presenting strongly 
condensed nuclei, different from the normal leptotene 
and zygotene pattern (Table 1 and Figure 2 A). Sticky 
chromosomes (Figure 2 B), laggaror lost chromosomes 
in metaphase I and prophase II (Figure 2 C and D), 
irregular meiotic spindle (Figure 2 E), and triads with 
micronuclei (Figure 2 F) were also observed.

In C. micans, ca. 8% of the meiocytes were irregular, 
presenting: sticky chromosomes (Figure 3 A); laggar 
or lost chromosomes in metaphase I (Figure 3 B); 
irregular meiotic spindle in metaphase II and 
asynchronic telophase II (Figure 3 C and D). Laggar 
or lost chromosomes and bridges in anaphases I and II 
were reported by Almada et al. (2006).

This species also showed migration of genetic 
material between cells, evidencing the occurrence 
of cytomixis (Figure 3 E to I), which corresponded 
to 16% of the total irregularities. Cytomixis was 
observed only in prophase I, corresponding to 11.08% 
of the irregularities in leptotene/zygotene and 4.92% 
of those in pachytene. Occurrence of cytomixis during 
prophase I, mainly in pachytene, has been reported in 
species of Fabaceae (Wang et al., 2002; Belluci et al., 
2003; Haroun et al., 2004; Sidorchuk et al., 2004).

Partial or complete chromatin migration involving 
cytomitic cells was observed (Figure 3 G), with some 
acting as donor and others as receptor meiocytes 
(Figure 3 H and I). Some authors suggested that 
chromatin and chromosome migration is not random 
but directional. For example, Falistocco et al. (1995) 
observed that, in Dactylis, cytomixis always occurred 
from a donor to a receptor cell and that, in several cases, 
complete or almost total genetic material migration 
ocurred not only between two cells, but also amongst 
several cells at the same time.

Table 1. Percentage of irregularities observed per 
meiotic phase of Crotalaria species.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Abnormal meiocytes (%)</th>
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<tbody>
<tr>
<td></td>
<td>C. zanzibarica</td>
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<tr>
<td>Leptotene/Zygotene</td>
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<tr>
<td>Pachytene</td>
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<td>Diplotene</td>
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<tr>
<td>Tetrads</td>
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<tr>
<td>Triads</td>
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</tr>
<tr>
<td>Undefined</td>
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</tr>
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</table>

Figure 2. Meiotic abnormalities in Crotalaria 
zanzibarica (n = 8). (A) Condensed nuclei; (B) sticky 
chromosomes; (C) metaphase I; and (D) prophase II 
with laggar or lost chromosomes; (E) irregular meiotic 
spindle; (F) triad with micronucleus. Bar: 5 μm.
Cytomixis in meiosis can have important implications in evolution, as it can lead to the formation of unbalanced gametes, including non-reduced ones. If these gametes are viable, they will originate aneuploid or polyploid plants. This was suggested by Belluci et al. (2003) working with *Medicago sativa*. High pollen grain viability (87.1 to 95.1%) of *Dactylis* plants showing cytomixis was reported by Falistocco et al. (1995), evidencing that cytomixis can be potentially important in the production of non-reduced pollen grains (2n).

In *C. spectabilis*, approximately 12% of the meiocytes were irregular. There was a high percentage of irregular diplotene (64.28%) showing multivalents (Figure 4 A). There were also: sticky chromosomes (Figure 4 B); early chromosome segregation in metaphase I (Figure 4 C); laggard chromosomes in prophase II (Figure 4 D); irregular meiotic spindle in metaphase II (Figure 4 E) and telophase II, as well as chromosome asynchronism and micronuclei (Figure 4 F). In populations of *C. spectabilis* from Argentina, Almada et al. (2006) found laggard or lost chromosomes and bridges, but all tetrads were normal.

Each species showed a predominant type of irregularity. Most of the abnormal meiocytes in *C. zanzibarica* had strongly condensed nuclei in prophase I. *Crotalaria spectabilis* and *C. micans* presented multivalents and, in the latter, cytomixis was also observed (Table 1). However, meiotic index values were high: *C. zanzibarica*, 92.19%; *C. micans*, 91.32%; and *C. spectabilis*, 88.18%.

Pollen grain germination in the analyzed species was low. Similar results were obtained in the pollen viability tests using fluorescein diacetate (FDA) and

**Figure 3.** Meiotic abnormalities in *Crotalaria micans* (*n* = 8). (A) Sticky chromosomes; (B) metaphase I with laggard chromosomes; (C) metaphase II and (D) asynchronic telophase II with irregular spindles; (E) cytomixis between meiocytes at the beginning of pachytene; (F) meiocytes in zygotene with simple chromatin bridge; (G) total chromatin migration from one meiocyte to another in prophase I; (H–I) chromatin migration among three or more cells in prophase I, with micronuclei. Bar: 5 μm.

**Figure 4.** Meiotic abnormalities in *Crotalaria spectabilis* (*n* = 8). (A) Diplotene with multivalents; (B) sticky chromosomes; (C) early chromosome segregation in metaphase I; (D) laggard chromosomes in prophase II; (E) irregular spindle in metaphase II; and (F) asynchronic telophase II, including micronucleus. Bar: 5 μm.
2,3,5-triphenyltetrazolium chloride (TTC) – 50%, indicating low pollen viability (Table 2 and Figure 5). Staining tests using Alexander’s solution, with both fixed and in natura materials, showed high percentage of viable pollen grains (Figure 5), followed by the test using TTC – 5% (Table 2). This could be explained by the incapability of Alexander’s solution and TTC – 5% to detect the low pollen viability shown in the germination test. These results show that FDA and TTC – 50 % are reliable tests for pollen grain viability studies in *Crotalaria*.

Low pollen grain viability could be either associated to the observed meiotic irregularities, since they have potential for chromosome number alteration, or to post-meiotic events. Still, considering that these species produce large amounts of seeds, both in natural populations and under cultivation, such low viability must be compensated by the large number of pollen grains.

**Conclusions**

1. *Crotalaria zanzibarica*, *C. micans* and *C. spectabilis* have irregular meiotic behavior, with predominance of shrunk nuclei in leptotene and zygotene in *C. zanzibarica*, cytomixis and abnormal chromosome pairing in *C. micans* and abnormal chromosome pairing in *C. spectabilis*.

2. Abnormal meiotic behavior does not affect the tetrad production.

3. Viability of pollen grains of *C. zanzibarica*, *C. micans* and *C. spectabilis* is low.

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**References**


