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# Searching for mechanisms behind meiotic abnormalities in *Crotalaria spectabilis* Roth

## Desvendando mecanismos relacionados a anormalidades meióticas em *Crotalaria spectabilis* Roth

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**ABSTRACT**

The identification of epigenetic marks associated with problems in the meiotic process can enlighten the mechanisms underlying the irregularities and the impacts in the genetic constitution of gametes. Therefore, this study aimed to verify the relationship between the pattern of phosphorylation in serine 10 of histone H3 (H3S10ph), a (peri) centromeric epigenetic mark, with meiotic abnormalities in a wild population of *Crotalaria spectabilis* Roth. The main abnormalities observed were transfer of genetic material through cytoplasmatic connections, DNA elimination and abnormal spindle array. Different forms of elimination (chromatin fragmentation, ring formation, lagging chromosomes and micronuclei) were observed from the early phases until tetrad formation. The eliminated chromatin was either positive or negative for the immunosignal of H3S10ph, so it may be occurring elimination of acentric fragments, as well as of chromosomes with active or inactive centromeres. Therefore, dysfunctional centromere is not the only candidate cause for elimination. The transfer of genetic material and the abnormal spindle arrays are evidence that this population can produce aneuploid gametes and 2n pollen grains.

**Index terms:** Leguminosae; cytomixis; abnormal meiotic spindle; unreduced pollen grain; chromosome elimination.

**RESUMO**

A identificação de marcas epigenéticas associadas a problemas no processo meiótico pode esclarecer os mecanismos subjacentes às irregularidades e aos impactos na constituição genética dos gametas. Portanto, este estudo teve como objetivo verificar a relação entre o padrão de fosforilação na serina 10 da histona H3 (H3S10ph), uma marca epigenética (peri)centromérica, com anormalidades meióticas em uma população silvestre de *Crotalaria spectabilis* Roth. As principais anormalidades observadas foram transferência de material genético por meio de conexões citoplasmáticas, eliminação de DNA e arranjo anormal do fuso. Diferentes formas de eliminação (fragmentação da cromatina, formação de anéis, cromossomos atrasados e micronúcleos) foram observadas desde as fases iniciais até a formação de tétrades. A cromatina eliminada foi positiva ou negativa para o imuno sinal da H3S10ph, portanto pode estar ocorrendo eliminação de fragmentos acêntricos, bem como de cromossomos com centrômeros ativos ou inativos. Portanto, centrômero disfuncional não é a única causa candidata para eliminação. A transferência de material genético e os arranjos anormais de fusos são evidências de que essa população pode produzir gametas aneuploides e grãos de pólen 2n.

**Termos para indexação:** Leguminosae; citomixia; fuso meiótico anormal; grão de pólen não reduzido; eliminação cromossômica.

## INTRODUCTION

*Crotalaria* L. is one of the largest genus of the subfamily Papilionoideae (Leguminosae) with at least 700 species (Rockinger et al., 2017; World Checklist of Vascular Plants - WCVP, 2022). These species, mainly herbs and shrubs, are distributed throughout tropic and subtropical regions (Polhill, 1982), being found in forage areas and along roadsides. In Brazil, there are 42 *Crotalaria* species, including 31 natives and 11 introduced (Flores; Tozzi 2018). Among these, two species from India stand out, *Crotalaria juncea* L. and *Crotalaria spectabilis* Roth (Polhill, 1982). These species show economic value since

they can be used for green fertilization in agriculture (Leal et al., 2005), for soil coverage and prevention of erosion (Choi et al., 2008), and for the control of nematodes (Nascimento et al., 2020). All these characteristics have led to the interest in the development of new varieties to better exploit the agriculture potential of these species (Rovaris et al., 2021; Muli et al., 2021).

A faithful meiosis process is important for the success in plant breeding, since a regular meiosis is essential for the generation of viable gametes used in the hybrids production and seeds formation. Abnormalities in meiosis, such as univalent and multivalent in diakineses and bridges with and without fragments in anaphase, were described for some species of *Crotalaria* (Verma; Raina 1980; Almada et al., 2006). In *C. spectabilis*, irregular chromosome pairing and segregation, micronuclei in telophase II and low pollen grain viability were described (Ferreira et al., 2009). The authors consider that these irregularities may be a consequence of a pericentric inversion in chromosome I described by Mondin and Aguiar-Perecin (2011). However, in addition to chromosomal rearrangements, the mis-segregation of chromosomes can also be related to non-functional centromeres (Sanei et al., 2011; Barra; Fachinetti, 2018; Dumont et al., 2020).

The immunolocalization of the centromeric histone H3 (CENH3 or CENPA) is traditionally used to study the centromere function and chromosome segregation. Besides, centromeric activity can also be tracked by others epigenetic marks including the phosphorylation in serine 10 of histone H3 (H3S10ph) (Houben; Demidov; Karimi-Ashtiyani, 2013). The immunolocalization of this mark, in different species, showed its association with chromatid cohesion (Kaszas; Cande, 2000; Brasileiro-Vidal et al., 2005; Feitoza; Guerra 2011a; Paula et al., 2013) and functional centromere (Houben et al., 1999; Han; Lamb; Birchler, 2006; Fu et al., 2012; Houben; Demidov; Karimi-Ashtiyani, 2013). It was demonstrated that phosphorylation in serine 10 of histone H3 is crucial for centromere/kinetochore structure and for the mechanical stability of centromeres during chromosome movement (Houben et al., 2007; Feitoza; Guerra 2011a, b). In this way, this epigenetic mark can enlighten the mechanisms underlying the meiotic irregularities and the impacts in the genetic constitution of the gametes.

Therefore, this study aimed to verify the relationship between the (peri)centromeric epigenetic mark revealed by H3S10ph with meiotic abnormalities in a wild population of *Crotalaria spectabilis* Roth.

## MATERIAL AND METHODS

Anthers were collected from a wild population of *Crotalaria spectabilis* Roth located in Lavras (Minas Gerais, Brazil) and fixed for 40 min in 4% paraformaldehyde solution in PBS buffer (Phosphate Buffer Saline), at room temperature. After washes in PBS, the anthers were digested with an enzymatic mixture (4% pectinase; 2% cellulase; 5% cytohelicase) for 6 h and 30 min, followed by PBS washing. Slides were prepared by squash technique in PBS + 1% Triton X-100.

Protocol for immunolocalization of H3S10ph was adapted following Manzanero et al. (2000). Preparations were covered with 50 µL of blocking solution (3% BSA and 0.1% Triton X-100 in PBS buffer) for 20 min, at room temperature. Blocking solution was replaced by 25 µL of solution of H3S10ph antibody (Rabbit polyclonal IgG, Sta Cruz Biotechnology, USA) diluted (1:100) in blocking solution. Slides were incubated for 24 h, at 4 ºC. After washing with PBS, preparations were incubated with 25 µL of solution containing secondary antibody conjugated with FITC (Goat anti-rabbit IgG, Santa Cruz Biotechnology, USA) diluted (1:100) in blocking solution, for 2 h, at 37 °C, in dark humid chamber. After washing with PBS buffer, slides were mounted in Vectashield H-1000 containing DAPI (4',6-diamidino-2-fenilindol).

Slides were analyzed under an epifluorescence microscope (Nikon eclipse E400) using excitation/emission filters for DAPI (358/461) and for FITC (495/515). Images were recorded using software NIS Elements BR and processed in Adobe Photoshop CS3. Frequency of abnormalities per meiotic phase were calculated from a sample of 775 cells. The meiotic index (normal tetrads/total tetrads) was estimated according to Love (1951). Non-reduced gametes frequency was estimated by the formula 2n gametes = (2D + Tr) / (2D + 3Tr + 4T), where D=dyads; Tr=triads and T=tetrads, according to Yan et al. (1997).

## RESULTS AND DISCUSSION

Several types of meiosis abnormalities were observed in 29.63% of the meiocytes (Table 1). This frequency is higher than that reported for *Crotalaria spectabilis* Roth by Almada et al. (2006) and by Ferreira et al. (2009). In the former, meiosis was considered regular, with low frequency of lagging chromosomes in anaphase I and II. Ferreira et al. (2009) reported about 12% of abnormalities, with the predominance of multivalents configurations, and other abnormalities in a lower frequency, such as chromosome stickiness, lagging chromosomes, micronuclei and irregular spindle.

**Table 1:** Frequency (%) of meiotic abnormalities in a total of 775 meiocytes of *Crotalaria spectabilis*. Number in brackets means number of abnormal cells.

| Abnormalities | Stages of meiosis | | | | | |
|---|---|---|---|---|---|---|
| | Lep/Zig | Pachytene | Telophase I | Metaphase II | Telophase II | Tetrads* |
| Ring chromosome | 1.80 (14) | - | - | - | - | - |
| Micronucleus/Fragments | - | 5.67 (44) | - | - | 1.93 (15) | |
| Cytomixis | 6.19 (48) | - | - | - | - | - |
| Asynchrony | - | - | 0.90 (7) | - | - | - |
| Parallel spindle | - | - | - | 1.80 (14) | - | - |
| Tripolar spindle | - | - | - | 0.12 (1) | - | - |
| Lagging chromosome | - | - | - | 0.51 (4) | - | - |
| Triad | - | - | - | - | - | 3.74 (29) |
| Microcyte | - | - | - | - | - | 7.09 (55) |
| Total per stage | 7.99 (62) | 5.67 (44) | 0.90 (7) | 2.31 (19) | 1.93 (15) | 10.83 (84) |

*Total of normal tetrads = 486 cells.

Meiotic index (MeI) was around 85%, similar to the 88.18% reported by Ferreira et al. (2009). According to Love (1951), wheat plants with MeI higher than 90% can be considered cytologically stable for breeding purposes. Considering that the population studied here never went through any kind of artificial selection, this value can be taken as promising for use of these plants in crosses.

Cytomixis was identified in leptotene and zygotene (Figure 1A, Table 1). Cytomixis is a phenomenon that involves the migration of nuclei fragments or whole nuclei between cells through intercellular channels (cytomictic channels), mainly in plant male meiosis (Mursalimov; Deineko, 2018). Due to this transference of genetic material to the gametes, this phenomenon has a potential evolutionary significance. Indeed, cytomixis has been reported in different plant species (Páez et al., 2021), including in *Crotalaria* (Ferreira et al., 2009), with impacts in the fertility of the pollen, production of non-reduced gametes or adjustment of unbalanced genomes (Lattoo et al., 2006; Singhal; Kumar 2008; Mursalimov; Sidorchuk; Deineko, 2013). However, despite its importance, the causes and consequences of cytomixis are still unclear, mainly because the difficulties to develop a robust methodology to directly track this phenomenon (Mursalimov; Deineko, 2018).

Here, we observed that the chromatin fibers shared by different meiocytes was either positive or negative for the immunosignal of phosphorylated H3S10. Therefore, transfer of chromosomes/fragments with active and inactive centromeres may be occurring (Figure 1A). In tobacco male meiocytes, it was also observed that in the shared chromatin epigenetic marks like phosphorylation in serine 10 and 28, threonine 11 of histone H3 and in threonine 121 of histone H2Adoes not differ from the chromatin in intact microsporocytes (Mursalimov et al., 2015). This demonstrates that cytomixis does not affect chromatin epigenetic marks (Mursalimov et al., 2015).

Fragmentation of chromatin/micronuclei (Figure 1B) and the presence of ring chromosomes in the earlier phases (Table 1, Figure 1C) can be taken as evidences for elimination of genetic material in the beginning of meiotic process in *C. spectabilis.* Most of these fragments and rings never showed phosphorylation of H3S10, indicating elimination of acentric fragments or chromosomes with inactive centromere, which can result in the formation of microcytes observed in final meiosis phases (Figure 1D-E).

The presence of ring chromosomes in the pachytene can be linked to chromosome break in the telomeric region (Sybenga, 1992) and rearrangements such as centric fission (Perry; Slater; Choo, 2004) or intrachromosomal translocations (Lysak; Schubert, 2013). Centric fission can be a mechanism behind the formation of ring chromosomes when it is followed by a fusion end to end between centromere and telomere or by an illegitimate recombination between these regions (Perry; Slater; Choo, 2004). In the reciprocal intrachromosomal translocations, a brake in both arms produce both ring chromosome and acentric fragment (Lysak; Schubert, 2013). The ring chromosome, mainly the tiny ones, can be stably inherited for some cycles (Murata et al., 2008), but both products are unstable and eventually lost. In our results,

the absence of phosphorylated H3S10 in the rings suggests that they are either acentric fragments or with inactive centromeres. The micronuclei were either positive or negative for phosphorylated H3S10 (Figure 1F), indicating that may be occurring loss of acentric fragments and whole chromosomes with active or inactive centromere. Therefore, centromeric inactivity is a candidate mechanism underlying DNA elimination but not the only one in this population of *C. spectabilis*.

Abnormalities in the orientation of the spindle fibers during meiosis were also observed, including parallel spindles (Figure 2A), and tripolar segregation (Figure 2B) which originate triads (Figure 2C, Table 1). Both types of spindle mis-orientation can generate non-reduced gametes (2n) (Pagliarini, 2000; Blasio et al., 2022). The production of unreduced gametes through these mechanisms has already been reported in *Solanum* L. (Andreuzza; Siddiqi, 2008; Tomé et al., 2009), *Arabidopsis thaliana* (L.) Heynh (D'Erfurth et al., 2008), *Medicago* L. (Barcaccia et al., 1995) and *Saccharum* L. (Bhat; Gill, 1985). In some species, these spindle abnormalities are controlled by specific genes, like *Atps1* in *Arabidopsis thaliana* (D'Erfurth et al., 2008), *ps* and *pc* in *Solanum phureja* Juz. & Bukasov (Mok; Peloquin, 1975b, a; Peloquin; Boiteux; Carputo, 1999).

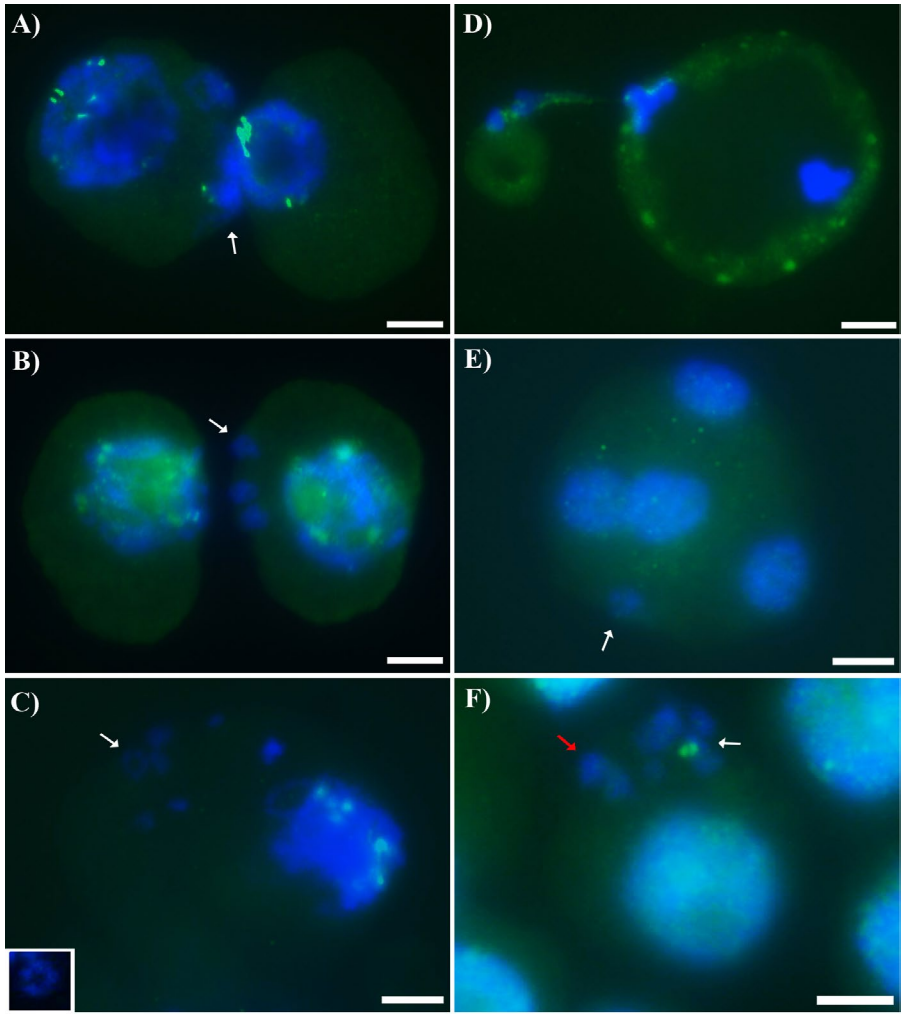


**Figure 1:** Abnormalities in meiosis of *Crotalaria spectabilis*. Blue indicates DNA and green indicates presence of phosphorylated H3S10. (A) Cytomixis in early prophase I (arrow). (B) Fragmented chromatin/micronuclei in early prophase I (arrow). (C) Fragmented chromatin/nuclei (arrow) and ring chromosome in early prophase I (highlighted). (D) Elimination of chromosome fragments in telophase I. (E) Tetrad with microcyte (arrow). (F) Fragmented chromatin/micronuclei with (white arrow) and without (red arrow) immunosignal. Bar = 5µm.

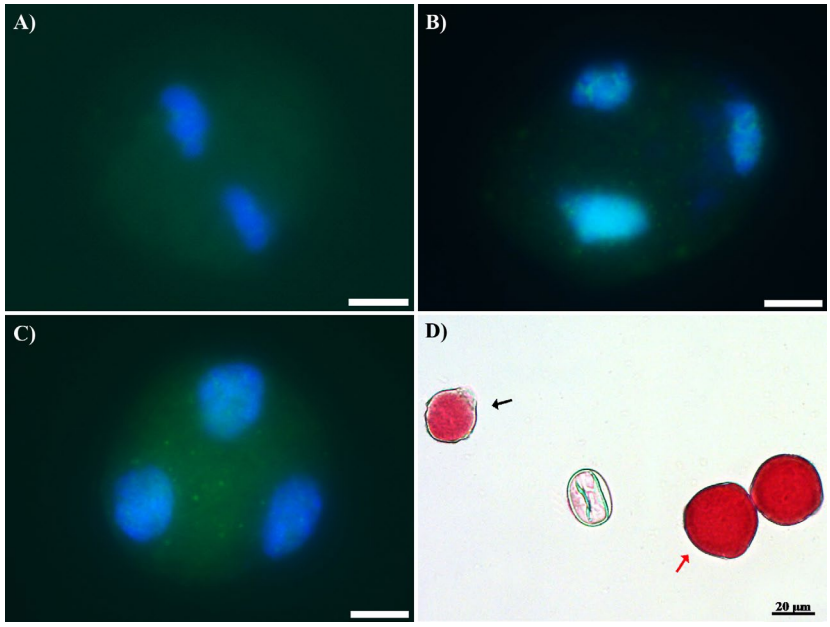


**Figure 2:** Abnormalities related to chromosome segregation during meiosis of *Crotalaria spectabilis*. (A) Metaphase II with parallel spindles configuration. (B) Tripolar segregation. (C) Triad. Scale bar: 5µm. (D) Large pollen grains (red arrow), probably with increased DNA content.

The abnormalities in the meiotic spindle verified in this *C. spectabilis* population indicates its potential for unreduced gametes production. Another evidence is the presence of large pollen grains, which is likely related to more genetic material than expected (Figure 2D). Our estimation of this potential, based on the proportion of dyads and triads (Yan et al., 1997), shows that 1.43% of its pollen grains may be unreduced.

The production of 2n gametes in diploid populations is widely recognized as the main event that originates polyploid plants, with dramatic consequences for the evolutionary history of a genus (Bretagnolle; Thompson 1995; Otto; Whitton 2000; D'Erfurth et al., 2008). In *Crotalaria*, polyploidy is considered as an important event for the speciation within the section Calycinae (Flores et al., 2006) in America, last region colonized by *Crotalaria* species. It is not known if polyploid species are auto or allopolyploids. In this last case, production of unreduced gametes could have played a key role in the evolution of the genus.

## CONCLUSIONS

We showed that the meiosis in the wild population of *Crotalaria spectabilis* presented cytomixis, elimination of genetic material throughout the whole cycle, either with or without active centromere. The immunolocalization of H3S10ph evidenced that centromeric inactivity is not the only putative mechanism underlying genetic material elimination. Moreover, the population of *C. spectabilis* used in this study has potential to produce unreduced gametes.

## AUTHOR CONTRIBUTIONS

Conceptual Idea: Braz, GT; Techio, VH; Torres, GA; Methodology design: Braz, GT; Resende, KFM; Paula, CMP; Techio, VH; Data collection: Braz, GT; Data analysis and interpretation: Braz, GT; Resende, KFM; Paula, CMP; Techio, VH; Torres, GA and Writing and editing: Braz, GT; Resende, KFM; Paula, CMP; Techio, VH; Torres, GA.

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