Polyploidy and apomixis in accessions of *Senna rugosa* (G.Don)
H.S.Irwin & Barneby

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**Abstract:** *Senna rugosa* (G.Don) H.S.Irwin & Barneby (Leguminosae) is a species from the Brazilian Cerrado commonly used for medicinal purposes. Chromosome variability (*n* = 14 and 28) described for this species indicates the occurrence of chromosome races and recent polyploidization in its evolution history. The aim of this study was to verify the relationship between ploidy level and occurrence of polyembryony in 5 accessions of *S. rugosa*. Cytogenetic analysis revealed the type of interphasic nuclei, chromosome number and morphology, and nuclear DNA content. Frequency of polyembryony was estimated by germination test. Pollen grain viability was estimated by different techniques. There was variability among the accessions of *S. rugosa* for interphasic nucleus type (semireticulate and nonreticulate), genome size (2.57 to 2.80 pg/2C), and polyembryony frequency (from 2 to 4 embryos). Pollen grain viability was 2.5% to 20%, depending on the technique used. Numeric chromosome variability was found among meristematic cells from each individual (*2n* = 28 to *2n* = 112), with prevalence of *2n* = 56. In mitotic metaphases with *2n* = 56, all chromosomes were metacentric and of similar size. The data described, combined with previous descriptions of irregularities in chromosome pairing, suggest that *S. rugosa* is an autoploid facultative apomictic species, with interpopulation variability for level of apomixis expression.

**Key words:** Apomixis, DNA content, chromosome number, polyembryony, polyploidy

1. Introduction

*Senna* Mill. is one of largest genera in Caesalpiniioideae (Leguminosae Juss.), with about 300 species (Lewis, 2005). In Brazil, this genus consists of 80 species, 4 subspecies, and 55 varieties (http://floradobrasil.jbrj.gov.br/2012/FB023149). Many of these species are shrubs that occur in the margins of park savanna and in developing forests as pioneers; they are also common on road edges (Biondo et al., 2005). They might be used as medicine, ornamentation, or sources of wood and in restoration for degraded areas (Lorenzi and Abreu, 2002; Arato et al., 2003). In some species, the plants are severely damaged to remove the roots, stems, or bark for medicinal purposes. This is the case of *Senna rugosa* (G.Don) H.S.Irwin & Barneby, commonly known as black root. Its roots are used as a vermifuge and to treat snake bites (Rodrigues and Carvalho, 2001).

*Senna rugosa* is a widely distributed species in Brazil and shows interpopulation variability for chromosome number. Populations with *n* = 14 and *n* = 28 were found in accessions from São Paulo State (Coleman and Demenezes, 1980) and Paraná State (Biondo et al., 2005), respectively, while accessions from Minas Gerais State showed populations with both chromosome numbers (Marques-de-Resende et al., 2013). The existence of these cytotypes (likely diploid and polyploid) and the occurrence of irregularities in chromosome pairing (mainly quadrivalent formation) suggest that polyploidy is an important and recent event in the evolution of this species (Marques-de-Resende et al., 2013).

Polyploidy, being associated with the development of apomictic species, may affect sexuality, since a diploid or aneuploid gamete is required for the transmission of genes responsible for apomixis. Thus, while supplying duplicated genes, polyploidy may be the fuel of long-term diversification and evolutionary success. Apomixis, on the other hand, allows neopolyploids to maintain their heterogeneous structure in unfavorable reproductive conditions until apomictic individuals are able to break the reproductive barrier and establish themselves (Carman, 1997; Comai, 2005; Nassar et al., 2008). The relationship between polyploidy and apomixis has already been described in natural polyploids (Gustafsson, 1946; Firetti-Leggeri et al., 2013), in interspecific hybrids of cultivated species such as in the genus *Manihot* Mill. (Freitas and Nassar, 2013), and in experiments of artificial chromosome duplication of *Paspalum notatum* var. *saurae*.

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diploid individuals, which also present natural polyploids (Quarin et al., 2001).

The aim of this study was to verify the existence of variations in ploidy level and the occurrence of apomixis in 5 accessions of *Senna rugosa* from Minas Gerais State, Brazil.

2. Materials and methods

Vouchers from 5 accessions of *Senna rugosa* (G.Don) H.S.Irwin & Barneby, each from a different location, were deposited in the Herbarium ESAL, in the Federal University of Lavras, Minas Gerais State, Brazil (Table 1). Duplicates from these samples were sent to the Herbarium Jardim Botânico do Rio de Janeiro (RB), where they were identified by Dr Haroldo Cavalcante de Lima.

Meristematic cells from root tips obtained from germinated seeds were analyzed for characterization of interphasic nucleus and karyotype description. Seeds were treated with 100% sulfuric acid for 20 min to break dormancy. After germination (in a wet chamber, 28–30 °C), 5- to 8-mm roots were harvested, treated with 2 mM 8-hidroxiquinolein at 30 °C for 1.5 to 1.75 h, and fixed in Carnoy’s solution (3 ethanol:1 acetic acid). Root tips were digested in pectinase and cellulase solution (100 and 200 U) at 37 °C for 10 to 20 min. Meristems were excised and compressed in 45% acetic acid under a cover slip. After removing the cover slips with liquid nitrogen, slides were air-dried and stained with 10% Giemsa solution in phosphate buffer (pH 6.8) for 5 min and were then analyzed with a bright-field microscope (Leica DMLS), equipped with a microcamera (Nikon Digital Sight DS-Fi1).

For each accession, at least 15 metaphases were selected for chromosome counting. Measures of chromosome short (s) and long (l) arms were obtained using the program Image Tool 3.0. These measures were used to calculate the total length of chromosome i (Cti = 1 + c), total length of haploid lot (CTLH = ΣCti), arm relations (RB = 1/c), and centromeric index (IC = c/Cti × 100). Chromosome morphology was determined through RB and IC values, following Guerra (1986).

Nuclear DNA from 3 specimens of each accession was quantified. Samples of 20 to 30 mg of young leaves from the accession and from *Pisum sativum* (internal reference standard) were ground in Marie buffer solution (50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM EDTA Na₂, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2), and 5 µL/mL 2-mercaptopetanol) for nuclei isolation (Marie and Brown, 1993). After filtering, 25 µL of propidium iodide (1 mg/mL) and 2.5 µL of RNase (50 µg/mL) were added.

The analysis of at least 10,000 nuclei per sample was made in a FACSCalibur flow cytometer (BD Biosciences, USA); histograms were obtained in Cell Quest software (Becton Dickinson and Company, USA) and analyzed in WinMDI software 2.8. The nuclei DNA content (pg) was estimated by comparing the G1 nuclei peak position of the sample with *Pisum sativum* using the relation $Q = (E/S) \times R$, in which Q is the amount of DNA in the sample (pg/2C), E is the position of the G1 peak in the sample, S is the position of G1 peak in the reference standard, and R is the content of DNA of the standard (9.09 pg/2C). Data were submitted to analysis of variance and to the Scott–Knott probability test (α = 0.05).

The viability of 1000 pollen grains bulked from the 5 accessions was verified through staining techniques: Alexander’s solution for 24 h with fixed flowers at 4 °C; 1% 2,3,5-triphenyltetrazolium chloride in 5% sucrose solution (TTC 5%) for 2 h with fresh flowers at room temperature in the dark; fluorescein diacetate (FDA) in 25% sucrose solution (6.25 µg mL –1 ) for 30 min with fresh flowers at room temperature in the dark; and in vitro germination test (IVG) in liquid medium (15% sucrose and 0.01% boric acid).

To verify polyembryony occurrence, seeds from all 5 accessions were germinated in a wet chamber at 28–30 °C after chemical scarification. The number of seedlings germinated from each seed was evaluated in 80 to 200

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Location</th>
<th>Denomination</th>
<th>ESAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vila Rica District</td>
<td>VR</td>
<td>26.573</td>
</tr>
<tr>
<td>2</td>
<td>Highway BR 265</td>
<td>MEX</td>
<td>26.574</td>
</tr>
<tr>
<td>3</td>
<td>Community of Serrinha</td>
<td>SER</td>
<td>26.575</td>
</tr>
<tr>
<td>4</td>
<td>Eldorado District</td>
<td>LE</td>
<td>26.576</td>
</tr>
<tr>
<td>5</td>
<td>Cascalho District</td>
<td>LVR</td>
<td>26.577</td>
</tr>
</tbody>
</table>

Table 1. Accessions of Senna rugosa (G.Don) H.S.Irwin & Barneby and their respective locations in the city of Lavras (21°14′43″S, 44°59′59″W), denominations, and registration numbers in the Herbarium ESAL of the Federal University of Lavras, Minas Gerais State, Brazil.
seeds per accession. Analysis of the completely randomized design was done using a binomial generalized model. The predictors of linear means from each genotype were compared using Student’s t test. All analyses were made in R software (http://www.R-project.org).

3. Results
Intraspecific variation for type of interphasic nucleus was detected in the accessions of *Senna rugosa* (G.Don) H.S.Irwin & Barneby, since accession VR presented semireticulate interphasic nuclei, with intense coloring of the diffuse chromatin (Figure 1a), while the other accessions presented nonreticulate interphasic nuclei with subtle granulation and light coloring of the diffuse chromatin (Figure 1b).

High variation in the number of chromosomes was observed in cells from all accessions (Table 2). This total variation also reflects the variation of chromosome number present in each meristem (Figures 1c–1f). In all accessions, tetraploid cells (2n = 56) were the most frequent (Figure 1d; Table 2).

Based on the measure of a mitotic metaphase with 2n = 56 chromosomes, *Senna rugosa* presented metacentric chromosomes with similar sizes and symmetric karyotypes (Figure 2). Mean total chromosome length (CTi) was 2.08 ± 0.39 µm, with the larger chromosome measuring 3.03 µm.

### Table 2.
Variation in chromosome number, nuclear DNA amount (pg), and polyembryony frequency among the accessions of *Senna rugosa* (G.Don) H.S.Irwin & Barneby. NT = Total number of evaluated cells. P% = Percentage of polyembryony.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Frequency of cells within each chromosome number</th>
<th>NT</th>
<th>DNA&lt;sup&gt;1&lt;/sup&gt; pg/2C</th>
<th>P%&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28</td>
<td>36</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td>MEX</td>
<td>11</td>
<td>5</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>LVR</td>
<td>-</td>
<td>12</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>LE</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>VR</td>
<td>-</td>
<td>15</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>SER</td>
<td>-</td>
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</tbody>
</table>

<sup>1</sup>Means followed by the same letter are not statistically different by the Scott–Knott test (α = 0.05).

<sup>2</sup>Means followed by the same letter are not statistically different by Student’s t-test to a 5% probability.
µm and the smallest 1.40 µm. Total length of haploid batch (CTLH) was 58.21 µm.

Analysis of the interphasic nuclei by flow cytometry for estimating the amount of genomic DNA of the accessions of *Senna rugosa* presented coefficients of variation between 0.44 and 0.72. The data on amount of DNA showed a distinction of 2 groups, the first formed exclusively by MEX, with the largest genome, and the other formed by all the other accessions (Table 2).

In all accessions of *S. rugosa* there were seeds with 2 to 4 embryos (Figures 3a–3d, accessions with 2 embryos; Figure 3e, accession with 3 embryos; Figure 3f, accession with 4 embryos) and they were statistically different for polyembryony frequency (Table 2). Pollen grain viability was low in all techniques used (Alexander’s solution, ~2.84%; TTC 5%, ~2.46%; FDA, ~20.1%, and IVG, ~3%), indicating that all 5 accessions may face problems in male gamete formation and sexual reproduction.

**Figure 2.** Idiogram of the haploid complement of *Senna rugosa* (G.Don) H.S. Irwin & Barneby.

**Figure 3.** Occurrence of multiple seedlings per seed in 5 accessions of *Senna rugosa* (G.Don) H.S.Irwin & Barneby. Two seedlings in the following accessions: (A) MEX, (B) LVR, (C) LE, and (D) VR. (E) Three seedlings in accession VR. (F) Four seedlings in accession SER.
4. Discussion

Our data show high incidence of mixoploidy in the root tip meristematic tissue of plants from different accessions of *Senna rugosa* (G.Don) H.S.Irwin & Barneby. There are no previous reports on the variety of chromosome numbers among cells from the same meristem for any species of *Senna*. There are only reports of *Caesalpinia* (Leguminosae, Caesalpinioideae, Caesalpiniae) individuals, considered diploids, presenting cells with doubled numbers of chromosomes (Alejandra and Bernardello, 2005). On the other hand, chromosome number variation between populations of *S. rugosa* can be taken into account, since Coleman and Demenezes (1980) described accessions from the state of São Paulo with n = 14, and Biondo et al. (2005) identified a tetraploid accession (n = 28) in the state of Paraná. Marques-de-Resende et al. (2013) also related findings of 14 or 28 bivalents, as well as the occurrence of quadrivalents. These results indicate the existence of chromosomal races with different levels of ploidy.

The polyploid nature of *S. rugosa* was demonstrated by the prevalence of cells with 2n = 56 chromosomes, followed by the ones with numbers closer to 56 than to 28 chromosomes (Table 2). The high symmetry of the karyotype, revealed by similar chromosome sizes and centromere positions (Figure 2), corroborates the autopolyplody hypothesis raised by Biondo et al. (2005) and discussed by Marques-de-Resende et al. (2013).

In many species, the presence of more than one embryo suggests that some asexual reproduction mechanism (apomixis) is involved in the species propagation (Cruz et al., 1998). This turns out to be very important to maintain species with barriers for sexual reproduction, such as the low pollen viability presented by the accessions of *S. rugosa* (2.5% to 20%). The multiple seedlings per seed in all accessions of *S. rugosa* (Figure 3; Table 2) indicate that this species shows the adventitious embryony type of apomixis. According to Carneiro and Dusi (2002), this type of apomixis is found mainly in tropical and subtropical plants. In interspecific hybrids of *Manihot* Mill., Freitas and Nassar (2013) found a relation between polyploidy and the increase in the percentage of adventitious embryony. A positive relationship was also found between polyploidy and polyembryony in *Anemopaegma* Mart. ex Meisn. (Bignoniaceae) (Firetti-Leggieri et al., 2013).

Adventitious embryony can be identified by multiple seedlings per seed with 2 or more fused ovaries, by multiple stigmas, and by multiple ovules by flowers (Koltunow, 1993; Koltunow et al. 1995; Koltunow and Grossinklaus, 2003). The adventitious embryos can originate from individual cells from either nucellus or inner integument tissues (Lakshmanan and Ambegaokar, 1984).

When both sexual and asexual embryos are formed, the development of the fertilized ovule starts later than that of the adventitious embryo (Koltunow, 1993). Randell (1970) described a time lapse between the development of the apomictic and the sexual embryos in pseudogamic apomictic *Cassia* species in the Australian arid zone. This was observed in all the evaluated accessions, since multiple states of maturation were observed in the seedlings from the same seed (Figure 3a–3f). According to Randell (1970), this reproductive system in polyploids may represent an adaptation to floating environmental conditions, as is the case of the Australian arid zone, since sexual reproduction and hybridizations enhance the level of recombination and produce new genotypes for varying environmental conditions; asexual reproduction preserves the adapted biotypes produced.

Characteristics such as polyploidy, determined by either cytogenetic analysis or flow cytometry (Carman, 1997); meiosis abnormalities (Valle et al., 1994); and variable degree of abortion and pollen grain sterility (Asker and Jerling, 1992) are typical of apomictic species. These features, described in the literature for *S. rugosa*, in association with the data of this work (chromosome number variation, polyploidy, and low pollen viability), suggest that this is an apomictic species showing adventitious embryony.

Therefore, polyploidization is an important phenomenon in the evolution process of *Senna rugosa* (G.Don) H.S.Irwin & Barneby, which is a facultative apomictic species, with interpopulation variability for expression degree of apomixis.

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References


