Cytogenetics and Reproductive Biology of

*Bixa orellana* L. (Bixaceae)

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Summary Cytogenetic and reproductive assays were carried out on a population of annatto (*Bixa orellana*) of Southeastern Brazil. The reproductive data indicate that *B. orellana* is self-compatible but preferentially alogamous and entomophylous. No apomictic fruits were obtained from emasculated flowers. Fruits formed exclusively by self-pollination were smaller and had fewer seeds than those obtained by open pollination, highlighting the importance of pollination for annatto seed production. The meiotic process was analyzed and some abnormalities observed resulted in only some sterile pollen (1.9%) with no significant reduction of fertility. The cytogenetic studies showed the haploid chromosome number \( n = 7 \) and the diploid number \( 2n = 14 \), with a remarkable bimodal karyotype. FISH with 45S rDNA and 5S rDNA probes showed that these loci do not occur in synteny. Both probes hybridized in the proximal regions of the short arm of pair 1 (45S) and pairs 4 and 6 (5S). One pair of 5S rDNA sites presented larger signals in FISH than the other pair. These karyological features suggest that fusion of chromosomes and amplification of DNA segments played an important role in karyotype evolution of *B. orellana*.

Key words Annatto, FISH, Karyotype, Meiotic process, rDNA site.

*Bixa orellana* L. is a perennial tree native to the Neotropics (Rivera-Madrid et al. 2006), also known as annatto or achiote in Mexico and urucum in Brazil. *Bixa* is the only genus of Bixaceae and includes six species (Carvalho et al. 2005a), with *B. orellana* being the most important, used in the food industry as a natural red dye constituting of bixin and norbixin carotenoids found in the seed coat (Scotter et al. 1998). Mainly propagated by seeds (Rivera-Madrid et al. 2006), this culture has an estimated world consumption of 14,500 tons of seeds per year (Parimalan et al. 2008). Besides the food-industrial use, studies have pointed out some medicinal properties of its natural pigment such as antigenotoxic and pro-vitamin A—like activity (Kovary et al. 2005, Karchuli and Ganesh 2009).

*B. orellana* flowers are actinomorphic, hermaphodite, polystemone with unicarpelate and unilocular ovary (Decraene 1989). In the opened flower the stigma stands above the anthers. There is one report of protandry for a Colombian population of *B. orellana* (Gómez 1980) and one of synchrony between pollen and stigma maturation in a Brazilian population (Almeida and Pinheiro 1992). Joseph et al. (2012) presented studies for some reproductive characters of an Indian population of *B. orellana*, showing its pollen morphology and viability. There are no data for self-compatibility or apomictic fruit for *B. orellana* or for the other *Bixa* species.

There are a few cytogenetic studies for *B. orellana*, basically presenting chromosome numbers

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(Mukherjee 1975, Morawetz 1986) with chromosome number variation between them. Simmonds (1954) and Krishnan and Ayyangar (1987) reported $2n=16$ while Mukherjee (1975) reported $2n=14$. Almeida et al. (2006) proposed the diploid chromosome number $2n=14$, reporting the first cytomolecular data for the species with the chromosomal localization of 45S rDNA site. These authors presented a bimodal karyotype, with one pair of chromosomes significantly longer than the others. This longer pair of chromosomes presents the 45S rDNA site in paracentromeric position.

There are few papers regarding *B. orellana* and some of them attempted to analyze the seed development and germination (Amaral et al. 2000). Others looked for genetic improvement of the species (Carvalho et al. 2005a, 2005b, Rivera-Madrid et al. 2006). Carvalho et al. (2005a), based on Compton et al. (1996), aimed to produce polyploids of *B. orellana* for breeding programs. Rivera-Madrid et al. (2006) presented a methodology for artificial pollination in *B. orellana* and some data of floral biology. Due to its economic potential, the present study aims to characterize the reproductive biology and cytogenetics of *B. orellana*. Fluorochrome DAPI and CMA banding, which highlight regions rich in AT and GC bases, respectively, and FISH with 45S and 5S rDNA probes were used for cytogenetic characterization. These differential chromosome staining techniques are useful tools for karyotype characterization due to the possibility of unequivocal chromosome identification (Kang et al. 2008). Meiosis in anther cells and pollen viability were analyzed in order to establish the integrity of the male reproductive system that is central to successful fertilization (Suzuki 2009). Self-compatibility analysis, controlled pollination tests, seed production, fertility rate and germination assays completed the investigation of the reproductive processes.

### Materials and methods

#### Plant material and chromosome preparations

Samples were obtained from the Aromatic Plants Germplasm Bank of the Instituto Agronômico de Campinas (IAC) in Southeastern Brazil. For the meiosis analyses, flower buds were collected and fixed in a solution of ethanol and acetic acid (3:1, v:v) for 24h at room temperature and stored at −20°C. Flower buds at different stages of development were squashed in a drop of 1.2% acetocarmine. The meiotic index was estimated by the percentage of normal tetrads. For the estimation of the pollen viability, we followed Alexander’s staining protocol (1980). For mitotic analyses, seeds were collected and germinated in Petri dishes with distilled water at 25°C. The root tips were excised and pre-treated with a saturated solution of p-dichlorobenzene (PDB) at 16°C for 3 h. The material was fixed in ethanol:acetic acid (3:1, v:v) for 24h and stored at −20°C. For slide preparations, root tips were digested in a solution of citrate buffer with 2% of cellulase and 20% of liquid pectinase for 1h at 37°C and squashed in a drop of 45% acetic acid, and then the coverslips were removed after an immersion in liquid nitrogen. For karyomorphological analysis, slides were stained with 2% Giemsa. Chromosomes of five well-spread cells were measured, paired and their total lengths and arm lengths were obtained. The chromosome idiogram was created based on the average lengths of each pair of chromosomes, including sections representing chromosome bands and rDNA sites observed in the present study.

#### Reproductive biology assays

For in-field floral biology analysis, fluorescent specimens of *Bixa orellana* were observed from 6:00 to 19:00h for two weeks. The flower visitors were noted and photographed and the time of visiting registered. For *in vivo* assays, flower buds in pre-anthesis of three individuals were collected, emasculated and preserved in agar 2% solid medium following Martin (1959) for the self-compatibility assay. Pollen grains of these flowers were collected, dried for 30min and dispersed on the stigma of the same flowers. Pollen grains from the same flowers were dispersed over the
stigmas of flowers collected from different individuals for control. These buds were fixed in F.A.A 70% solution after intervals of 6, 14, 24 and 48 h, treated with 1 M NaOH for 1 h and stained with aniline blue. Observations were made in a fluorescence photomicroscope. For apomixis tests, 15 flowers from six individuals were emasculated and bagged in the field during 15 days. For fruit formation tests, 30 flowers in pre-anthesis of three random individuals (10 flowers each) were bagged during 110 days. Their fruits were harvested 90 days after anthesis. The seeds were counted. As a control, 30 flowers in pre-anthesis of the same individuals were identified, open pollinated in the field and the resulting fruits harvested. For fertility rate estimation based on seed/ovule ratio, ovules of nine flowers from three individuals (three flowers each) were counted. For germination assays, seeds were collected from fruits formed by open pollination and by self-pollination (211 seeds from the first assay and 28 seeds from the second assay). These seeds were germinated in different Petri dishes with distilled water–imbibed filter papers, and the germination rate was registered based on the number of germinated seeds per total seeds of the assay. The assays were carried out between the summer (February) and winter (August) in Brazil.

Fluorochrome staining and in situ hybridization

The protocols of Schweizer (1976) were used for DAPI (4′,6-diamino-2-phenylindole) and CMA (chromomicin A3) banding. The protocol of Pendas et al. (1993) was used for fluorescent in situ hybridization (FISH). The probes used were pTa71 which contains one unit of 45S rDNA (9 kb) isolated from common wheat (Gerlach and Bedbrook 1979), and pScT7, with an insert of 5S rDNA with 300–500 pb length, which was isolated from Secale cereale (Lawrence and Appels 1985). The probe pTa71 was labeled with biotin-16-dUTP, and pScT7 with digoxigenin-11-dUTP (Boerhinger Mannheim) by nick translation, following the manufacturer’s instructions. The hybridized sites were detected with avidin-rhodamine and IgG-FITC, respectively. The slides were counter-stained with DAPI as shown by Heslop-Harrison (1991) and mounted with Vectashield anti-fading (Vector Laboratories). Slides were examined with an Olympus BX50 epifluorescent microscope. Chromosome images were acquired using an Olympus Q-color 3.0 camera and processed in Image-Pro Plus 6.0 program (Media Cybernetics) using only contrast and lightning adjustments, which are functions that affect the whole image equally.

Results

Reproductive biology

During reproductive assays several pollinators were observed visiting B. orellana flowers in the field, mainly bumble bees (Bombus sp.) and ants. The Bombus bees presented high visiting activity since the early morning (6:00 a.m.) until the end of the afternoon (6:00 p.m.). No nocturnal visitors were observed. The average number of ovules per flower was 50.55±3.7. The viable pollen index was 98.1%. The rate of fruit formation, average number of seeds per fruit and fertility rate are presented in Table 1. No apomictic fruits were formed in emasculated flowers. The growth of pollen tubes in vitro (Figs. 1–3) in the self-pollination test was similar in velocity and amount of tubes to the control test, although the number of growing tubes was not counted in both assays. After 24 h pollen tubes of self-pollinated pollen grains reached the ovules in the ovary (Fig. 3).

<table>
<thead>
<tr>
<th>Tests</th>
<th>F.F%</th>
<th>S.F</th>
<th>F.R</th>
<th>S.G%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-pollinated</td>
<td>13.3</td>
<td>8.5±3.6</td>
<td>0.16</td>
<td>85.7</td>
</tr>
<tr>
<td>Open-pollinated</td>
<td>53.3</td>
<td>44.6±3.1</td>
<td>0.88</td>
<td>54.5</td>
</tr>
</tbody>
</table>
Although fruits obtained from bagged flowers were smaller and with less seeds than the open pollinated fruits (Fig. 4), the seeds were similar in length and shape. The indexes of seed germination were estimated for both assays and are presented in Table 1.

Cytogenetic studies

The haploid chromosome number $n=7$ was observed. We observed on a diakinesis plate (Fig. 5) the longer pair of chromosomes linked to the nucleolus, showing the localization of the nucleolus organizer region (NOR). The presence of seven bivalents was observed in a metaphase 1 cell (Fig. 6). The percentage of normal tetrads (meiotic index) was 96.8%. Among the abnormalities observed at the end of meiotic process, triads represented 1.28% of all the tetrads observed, dyads 0.55% and monads 1.28% (Figs. 7, 8).

We observed in mitotic cells the diploid chromosome number $2n=14$ (Fig. 9). The karyotype of the species is bimodal, with one pair of chromosomes remarkably longer (3.2 μm) than the other.

Figs. 1–12. Results of reproductive biology and cytogenetic assays carried out on Bixa orellana. 1. Pollen germinated on stigma after in vitro self-pollination observed after 6 h of pollination. 2. After 24 h. 3. After 48 h. 4. Fruits collected from the field assays showing at the top a fruit from free-pollination (control) and at the bottom a fruit of spontaneous self-pollination. 5. Diakinesis ($n=7$), with arrow showing longer bivalent associated to nucleolus. 6. Metaphase 1 ($n=7$), with arrow showing a double-sized bivalent. 7. Regular tetrads. 8. Monad (Figs. 5–8 bar=5 μm). 9. Chromosomes stained with Giemsa. 10. Chromosomes stained with CMA3, with arrows showing positive bands. 11. Chromosomes counter-stained with DAPI with arrows showing 45S rDNA sites stained with FITC. 12. Chromosomes counter-stained with DAPI with arrows showing 5S rDNA sites stained with FITC (Figs. 9–12 bar=1 μm).
pairs (1.4–1.9 μm). The DAPI banding technique did not show positive results. A metaphasic cell stained with CMA₃ showed positive bands in pair 1 (Fig. 10). Two 45S rDNA sites in the paracentromeric position of the longer chromosomes were observed in FISH assay, with no DAPI band observed (Fig. 11). The hybridization with the 5S rDNA probe identified four sites located in intercalary positions of two pairs of distinct small chromosomes, with one pair with a stronger signal than the other (Fig. 12). The chromosome idiogram shows one submetacentric and six metacentric pairs of chromosomes (Fig. 13). This idiogram was drawn based on average length values (Table 2).

**Discussion**

**Biology of reproduction**

In the present paper we present some data of reproductive biology for *Bixa orellana*, which is a self-compatible, preferentially alogamous and entomophylous species. The pollen tubes reached the ovules in the *in vitro* self-pollination assay (Fig. 3) showing that *B. orellana* is self-compatible. This fact also reinforces the existence of maturing synchrony between stigma and pollen proposed by Almeida and Pinheiro (1992), although we may not discard the protandry presented by Gómez (1980) due to the increased genetic variability presented by Carvalho *et al.* (2005c) for *B. orellana* populations.

The formation of fruits in flowers bagged in the field indicates that the species are not totally dependent of pollinator agents, although the number of formed fruits and fertility rates presented a significant decrease with the absence of external pollination.

*Bombus* bees, which were registered in our field observations, are considered by Abak *et al.* (2000) to be efficient pollinator agents due to their intense activity and increased vibration. These authors showed that *Bombus* pollination increased fruit size and number of seeds per fruit in eggplant.
Bentley (1977) showed that ants visiting extrafloral nectaries of *B. orellana* increased fruit production, probably by supplying protection for floral tissues against herbivores. The difference in fruit set observed here between bagged and unbagged flower buds cannot be explained by the absence of ant activity in bagged buds. The bags used during the in-field self-pollination assays preserved the flower bud’s integrity from herbivory avoiding abortion by injuries. Even the fruit size was different between the two assays, probably due to the number of seeds formed inside the fruits. Fruit size and seeds per fruit variation is an event reported for several species. Kang and Primack (1991) presented for *Celidonium majus* (Papaveraceae) a fruit size and seed number variation related to temporal variations. The size and shape of seeds observed in the two assays were similar, showing that for *B. orellana* the agent of pollination does not interfere in the quality of the seed formed, but only in the number of seeds and size of fruit. The rates of seed germination observed here agree with the 64–95% presented by Yogeesha *et al.* (2005) for seeds of annatto at different maturity stages. The seed germination rate variation between self- and free-pollinated fruits observed here is probably due to the different time of seed maturation applied to the assays.

Although the viable pollen index and the meiotic index estimated were high, some abnormal tetrads were observed. There were a small percentage of triads, dyads and monads although no meiotic abnormalities were observed, such as anaphasic bridges and irregular chromosome disjunctions. There are no data in the literature about meiotic irregularities for the genus *Bixa*. These abnormalities do not considerably affect the success of sexual reproduction in annatto. The high index of pollen viability observed here (98.1%) agrees with the 85% viability reported by Rivera-Madrid *et al.* (2006) and the 95% reported by Joseph *et al.* (2012), and explains the success of open pollination that produced one fruit for every two buds in the field.

The absence of fruit formation in emasculated flowers does not allow us to discard apomixy. This result indicates that there is no adventive embryony in *B. orellana*, which would produce apomictic embryos by autonomous development of somatic cells (Talent 2009). However, there are other processes that might produce parthenocarpic fruits and could not be investigated by emasculating tests, such as pseudogamous diplospory and apospory (Bhat *et al.* 2005). In order to identify all the possible reproductive systems occurring in *B. orellana*, new studies assaying seed progeny for polymorphic genetic markers and fruit ontogeny shall be done.

**Cytogenetic studies**

The haploid and diploid numbers observed and the karyomorphological data obtained agree with those presented in the literature (Hanson *et al.* 2001, Almeida *et al.* 2006). The mitotic chromosomes observed were small (1.4–3.2 μm) and mainly metacentric. Almeida *et al.* (2006) presented chromosomes ranging from 1.2 to 3.8 μm with five metacentric and two submetacentric chromosome pairs (5m+2sm) instead of the 6m+1sm presented here. Intraspecific karyomorphological variation is quite common in plants. Zhao-Yang *et al.* (2002) presented several karyotype variation among varieties of *Spiraea japonica*, mainly in chromosome size and organization. Besides this, the different measurement processes used by the cytogeneticists may lead to divergent karyomorphological data.

The length of the longest pair observed here (pair 1) is two times longer than the average length estimated for the other chromosome pairs. In this longest pair of chromosomes was found the nucleolus organizer region (NOR). This is confirmed by the diakinesis plate (Fig. 5). The result of FISH with 45S rDNA probe also confirms this location, as previously reported by Almeida *et al.* (2006). The localization of the two CMA positive bands presented here agrees with the localization of 45S rDNA and NOR. The correlation between sites of repetitive DNA sequences and heterochromatin bands is quite common in plant cytogenetics (Guerra 2000, Carvalho and Guerra 2002).
The observed results provide data to infer some events that may have taken place in karyotype evolution of *B. orellana*. The bimodal karyotype observed for *B. orellana* may be related with chromosomal fusion. We infer this karyotype evolution based on the paracentromeric position of 45S rDNA site in chromosome pair 1. This site is related with NOR and is often associated with the distal secondary constriction of the chromosome. The paracentromeric localization of NOR on the double-sized chromosome pair allows us to point out a chromosome fusion such as the Robertsonian translocation in *B. orellana* karyotype derivation, as suggested by Almeida *et al.* (2006). The presence of two pairs of 5S rDNA sites with different lengths is probably due to divergences in the number of repetitive sequences between the loci. This variation may be related with amplifications or deletions of sequences. The cytomoolecular data presented here for *B. orellana* reinforces the importance of chromosome mapping using banding techniques and *in situ* hybridization for cytogenetic characterization, as well as phylogenetic and evolutionary studies.

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