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Reproductive biology and cytology of *Hypericum brasiliense* Choisy (Hypericaceae)[1]

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ABSTRACT – (Reproductive biology and cytology of *Hypericum brasiliense* Choisy (Hypericaceae)). This is the first study of reproductive biology and cytology carried out with *Hypericum brasiliense*, a species with medicinal properties and potential agronomic interest. Three populations of *H. brasiliense* collected at Southeastern Brazil were studied. The results indicate that *H. brasiliense* is preferentially allogamous, self-compatible, facultative apomitic and anemophilous. Male sterility was observed in about 50% of individuals from the three populations. Anatomical studies evidenced structural abnormalities in anthers of male sterile flowers, showing enlarged tapetal cells and thick secretion deposits on the tapetal cell surfaces that may cause nutritional deficit for pollen mother cells. In cytogenetic studies several haploid chromosome numbers were observed like n = 4, 8, 9, 11, 16 and 17, including the presence of multivalents and micronuclei in tetrads, indicating the occurrence of abnormalities in the meiotic process of *H. brasiliense*. Despite these meiotic abnormalities the pollen viability and *in vitro* pollen germination rate observed in fertile flowers may be considered high. The diploid chromosome number 2n = 16 was observed, and the chromosomes in metaphase were small and similar. Fluorochrome staining techniques using DAPI and CMA_3 were applied, with no positive bands observed.

Key words - anatomy of anthers, apomixy, irregular meiosis, male sterility

RESUMO – (Biologia da reprodução e citologia de *Hypericum brasiliense* Choisy (Hypericaceae)). Este é o primeiro estudo de biologia da reprodução e citologia realizado em *Hypericum brasiliense*, uma espécie com propriedades medicinais e de interesse agronômico. Três populações de *H. brasiliense* da região Sudeste do Brasil foram estudadas. Os resultados obtidos dos estudos indicam que *H. brasiliense* é uma espécie preferencialmente alógama, auto-compatível, apomítica facultativa e anemófila. Foi observada macho-esterilidade em 50% dos indivíduos estudados nas três populações. Estudos anatômicos evidenciaram anormalidades estruturais nas anteras de flores macho-estéreis, como células do tapete aumentadas, com grossas camadas de secreções depositadas em sua superfície que podem originar deficiências nutricionais para as células mães do pólen. Nos estudos citogenéticos diversos números haplóides foram observados, tais como n = 4, 8, 9, 11, 16 e 17, incluindo a presença de multivalentes e micronúcleos em tétrades, indicando a ocorrência de anormalidades no processo meiótico de *H. brasiliense*. Apesar das anormalidades meióticas a viabilidade polínica e o índice de germinação *in vitro* dos pólens observado para as flores férteis podem ser considerados altos. O número cromossômico diplóide registrado foi 2n = 16, com cromossomos metafásicos pequenos e similares entre si. Foram aplicadas técnicas de bandamento com fluorocromos DAPI e CMA_3, sem que bandas positivas fossem observadas.

Palavras-chave - anatomia de anteras, apomixia, irregularidade meiótica, macho-esterilidade

Introduction

The genus *Hypericum* L., included in Hypericaceae A. Juss., contains about 460 species among trees, shrubs and herbs with an almost worldwide distribution and characterized by the presence of translucent and black glands (Robson 2006). Extracts of *Hypericum* species have been target of studies due to their antidepressant and antitumoral properties (Bombardelli & Morazzoni 1995, Mendes *et al.* 2002).

Although *Hypericum perforatum* L. is the most studied species of the genus, some studies also included *Hypericum brasiliense* Choisy as a source of the same secondary metabolites and medicinal proprieties related to *H. perforatum* (Rocha *et al.* 1994, 1995, Abreu *et al.* 2004). To increase the knowledge of this Brazilian native species, several aspects of its biology are required to be studied, such as reproductive biology and cytology. These studies provide the basis to create and maintain germplasm banks and may indicate patterns for breeding programs.

There are studies of reproductive biology in *Hypericum* only for *H. perforatum*. Some researchers have related the occurrence of apomixy in *H. perforatum*, recognizing this species as a facultative apomictic one, due to the occurrence of sexual and aposporic processes

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in the same plants (Noack 1939, Matzk *et al.* 2001, Barcaccia *et al.* 2006). A study of reproduction pathways of *H. perforatum* identified 11 distinct mechanisms of seed formation and observed the occurrence of pseudogamy and apospory (Matzk *et al.* 2001). An analysis of 55 species of the genus *Hypericum* demonstrated a high plasticity of reproduction pathways (Matzk *et al.* 2003). Moreover, male sterility was also reported for the genus (Hoar 1931, Hoar & Heartl 1932), probably derived of abnormalities in the meiotic division. Apomixy excludes segregation and recombination during meiosis and fertilization (Koltunow *et al.* 2000) what may have an important role in agricultural development.

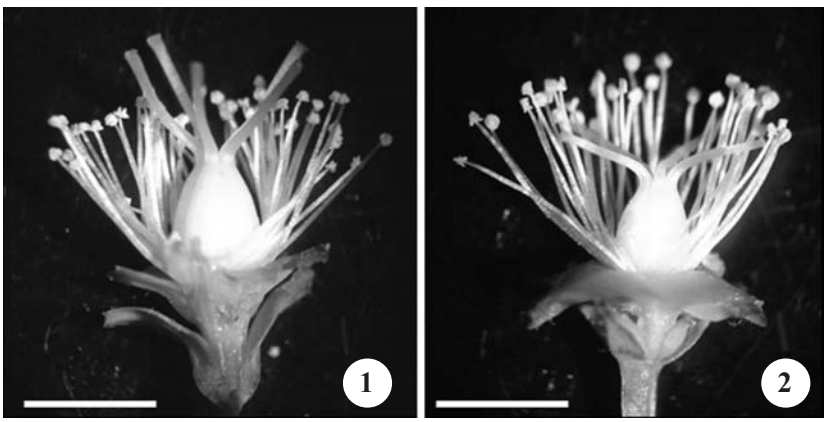
The objective of the present study is to characterize the reproductive biology and cytology of *Hypericum brasiliense*.

Material and methods

Seeds were obtained from three populations collected at Camanducaia-MG, Ibitipoca-MG and Ibiúna-SP, in Southeastern Brazil. In order to confirm the diploid chromosome number, root tips were pretreated with PDB (*p*-dichlorobenzene) in satured solution at 15 °C for 2 hours, fixed in Carnoy's solution (ethanol 3:1 acetic acid, v/v) for 24 hours and stored at -20 °C. For slide preparation roots tips were hydrolyzed in a solution of citrate buffer with 2% of cellulase (Sigma-Aldrich) and 20% of liquid pectinase (Sigma-Aldrich) for 70 minutes at 37 °C, squashed in 45% acetic acid and stained with 4',6-diamidino-2-phenylindole (DAPI) and chromomicin A_3 (CMA_3) (Schweizer 1976). For meiotic studies buds were collected and anthers squashed with acetocarmine 1.2%. Pollen viability was analyzed following Alexander (1980). *In vitro* pollen germination test followed Conger (1953). The floral observations were carried out from July to November of 2005 and 2006. Self-compatibility test followed Kho & Bäer (1968) protocols. Emasculated buds conserved in 2% agar solution were pollinated with their own pollen grains and observed after 6, 12, 24 and 48 hours. Emasculated buds were pollinated with pollen obtained from other individuals and used as control. Autogamy and apomixis were investigated in the two floral types indistinctly by tests carried out in greenhouse. For the first test flower buds in pre anthesis were bagged. For the second test flower buds in pre anthesis of the two floral types were emasculated and bagged. Freely pollinated flowers were used as control. The morphology of anthers was examined in histological sections of immature and mature buds. Flower buds were fixed in Carnoy's solution, dehydrated in an alcohol series and included in historesin (Gerrits 1991). The 6 µm thick sections were made in a microtome and stained with Toluidine Blue. All images were captured by an Optronics DEI-750 camera and processed in Image-Pro Plus 3.0 program.

Results

Floral biology and anther anatomy − The flowers are bisexual, aromatics with 5 yellow petals and numerous stamens (35-50), ovary superior, parietal placentation, 5 carpels with numerous ovules per carpel and 5 subcapitate stigma. We observed a floral dimorphism between individuals, one pattern showing short filaments and translucent anthers, characterizing male sterility, and the other showing yellow anthers and long filaments (figures 1, 2). Each individual presented only one floral type with non observed numerical predominance of one type upon the other in the three populations. In the first floral type we found empty anthers and in the second abundant pollen production. The fruits are dehiscent capsules with 110-220 seeds. Flower anthesis begins around 5:00 AM, and these flowers remain open until around 2:00 PM when the senescence begins. Fruits became mature between 10 at 12 days later. No visitors were observed in the field.



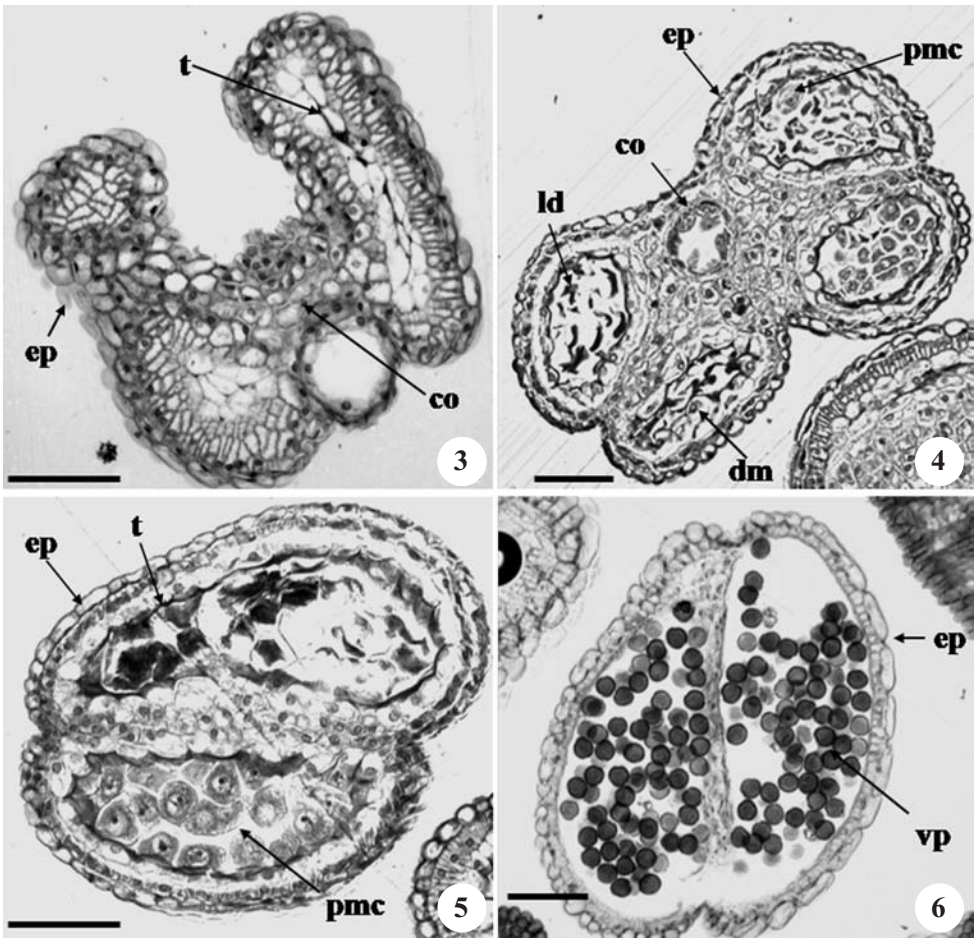
Figures 1-2. Floral dimorphism in *H. brasiliense*. 1. Male-sterile flower with translucent anthers and shorter stamen. 2. Fertile flower with none structural abnormality. Bar = 1 mm.

Abnormal anthers were also observed. Enlarged and unorganized tapetal cells and a thick deposit of sporopollenin were observed in immature anthers from sterile flowers (figure 3). Early released secretion of lipidic precursors of the pollen surface and degenerated microspores were observed in locules of sterile anthers (figure 4). Fertile flowers presented organized tapetum and viable pollen mother cells (figures 5, 6).

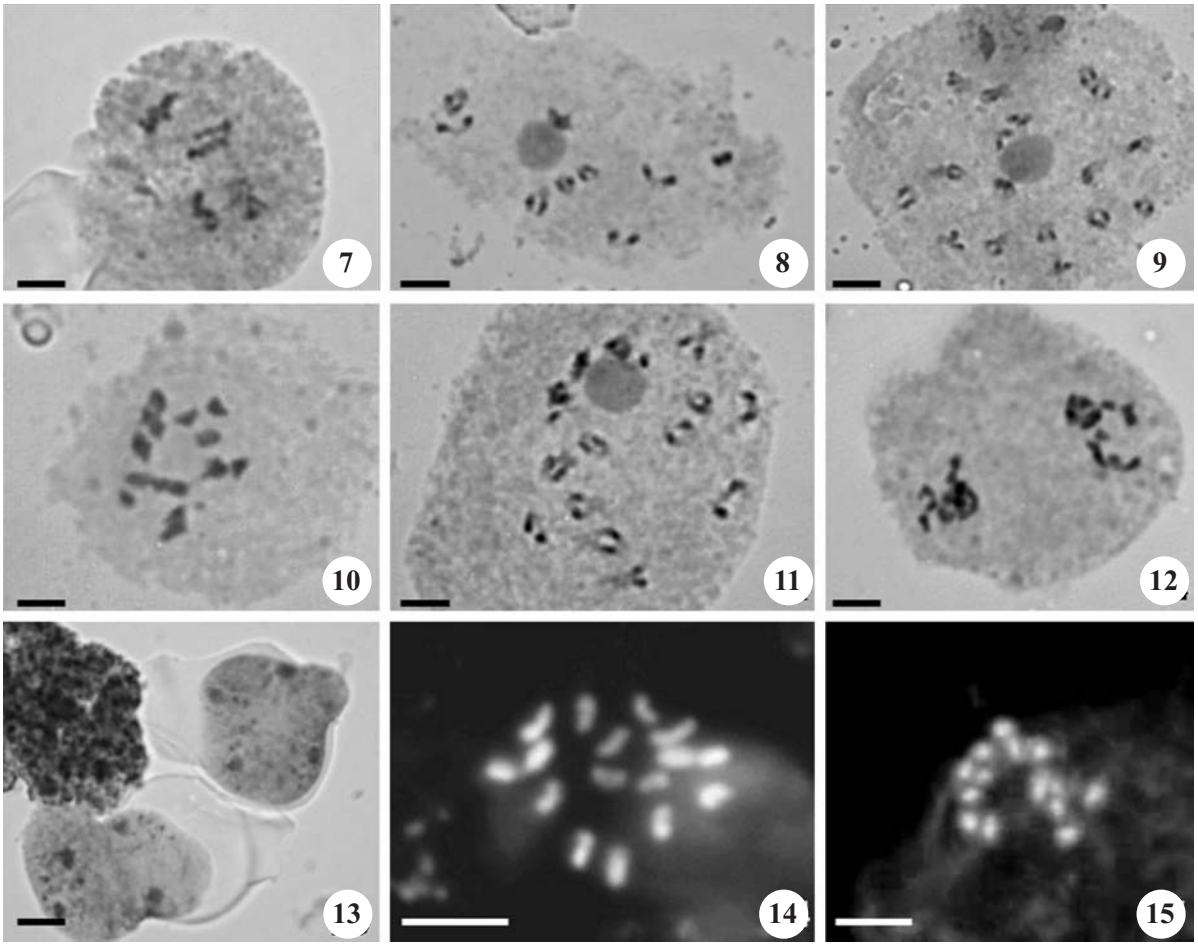
Cytology and reproductive biology − The diploid chromosome number observed was 2n = 16. The most observed haploid chromosome number was n = 8, although n = 4, 9, 11, 16 and 17 were also registered. Normal plates of anaphase, telophase and tetrads were observed, although some abnormalities were encountered in meiotic process, such as polyvalents on metaphase I and chromosome fragments in tetrads, indicating that meiosis is irregular in this species. The DAPI/CMA_3 staining did not show positive bands (figures 7-15).

The result for pollen viability test, which was carried out exclusively with floral buds with normal anthers, was 83.5%. *In vitro* pollen germination test presented 43.7% of germination. Test of flower emasculation for apomixy presented 100% of fructification in male sterile plants and no fructification in fertile individuals. Self-pollination test presented 70% of fructification and free pollination tests in both floral types presented 100% of fructification.

In vitro test of pollinic tube growing in stigma showed that *H. brasiliense* is a self-compatible species.



Figures 3-6. Anatomic slides of anthers of *H. brasiliense* stained with Toluidine Blue in different stages of development. 3. Transversal section of an immature anther from a sterile flower presenting enlarged, unorganized tapetal cells and a thick deposit of sporopollenin. 4. Locules of a sterile anther with deposits of lipidic precursors of the pollen surface on tapetal cells and some secretion released early into the locule and degenerated microspores. 5. Anther of a fertile flower in transversal section with organized tapetum and viable pollen mother cells. 6. Mature fertile anther with normal development and pollen grains apparently viable, before the dehiscence. Arrows show epidermis (ep), connective tissues (co), tapetum (t), lipidic deposit (ld), pollen mother cells (pmc), degenerated microspore (dm) and viable pollens (vp). Bar = 200 μm.



Figures 7-15. Cells of *H. brasiliense* in division. Figures 7 to 13 show cells in meiotic division presenting several haploid numbers. 7. Multivalents in metaphase I. 8, 9 and 11. Diakinesis with different chromosome numbers. 10. Metaphase I with n = 11. 12. Telophase I apparently normal. 13. Tetrads with micronuclei. 14. Cell in mitotic division stained with DAPI (2n = 16). 15. Cell in mitotic metaphase stained with CMA_3 (2n = 16). Bar = 5 μm.

However the pollen tubes in cross-pollination reached the ovary in 24 hours, earlier than those of self-pollination that were observed only after 48 hours.

Discussion

The chromosome numbers registered here represented the first report for *H. brasiliense*. The diploid number 2n = 16 agrees with the basic number x = 8 related by Robson (1977) for the section *Spachium*, to which *Hypericum* belongs. There are several reports of intraspecific chromosome number variation in the genus *Hypericum* such as *H. elodes* with n = 16 (Al-Bermani *et al.* 1993) and 2n = 20 (Gibby 1981) and *H. perforatum* with n = 16, 17, and 18, and 2n = 32, 48 (Reynaud 1986). Hoar (1931) and Hoar & Haertl (1932) studied meiosis in the genus *Hypericum* and reported the tendency of *H. perforatum* chromosomes to clump difficulting chromosome number counting. The same difficulty was found in the present chromosome study of *H. brasiliense*. We could not observe additional information about *H. brasiliense* karyology due to the reduced length of its chromosomes and the tendency to clump cited above. The absence of positive bands in the DAPI/CMA_3 staining assays may be a consequence of these features.

Despite the remarkable smell exhaled by the flowers of *H. brasiliense*, floral visitants were not observed, what suggests that these flowers are anemophilous. According to Faegri & Pijl (1979) adaptations on floral morphology for pollen transference include adaptations also on pollen grain. For these reasons pollens of anemophilous plants tends to be smaller, dry, and are produced in higher amounts if compared to entomophilous plants pollen

grains. These pollen features were also observed in the three populations of *H. brasiliense* studied.

The causes of the floral dimorphism here observed are probably related to cytoplasmic male sterility (CMS). Some mutations in mitochondrial genes lead to CMS, which has been described for about 150 plant species and would force out-crossing and thus contribute to genetic diversity in natural populations (Linke & Börner 2005). Although CMS causes abortion of the male gametophyte, it does not affect female gametophytic development (Levings 1990, Chiavarino *et al.* 2000) as observed here for *H. brasiliense*. The innermost cell layer in the locule of anther is the tapetum, which surrounds the developing pollen grains (Levings 1990) and is adjacent to the sporogenous tissue from the inside of the anther and to the middle layer from the outside of the anther (Chiavarino *et al.* 2000). Tapetal cells supply developing pollen by exporting nutrients and other molecules needed for pollen formation (Levings 1990). The anatomical analysis of *H. brasiliense* anthers presented abnormal deposits of secreted lipids like sporopollenin in the tapetal cells surface and early disorganization in tapetum layer. As pointed out by Shivanna & Johri (1985) the tapetum does not seem to play any direct role in microsporogenesis until the completion of meiosis. This early degradation of the tapetal cells here observed would lead to failure to nourish developing pollen grains and their consequent abortions, as observed by Levings (1990) in a CMS line of *Zea mays* L. and by Fei & Sawhney (2001) in *Arabidopsis thaliana* (L.) Heynh. The mutations that lead to CMS lines are involved with several features observed in male sterile species. Besides the tapetum abnormalities, we also observed short stamen and non-dehiscent anthers in *H. brasiliense*, which occurred simultaneously. These events added to some abnormalities in meiotic process such as chromosome mispairing with formation of multivalents and irregular disjunctions with micronuclei formation which were the sources of male sterility found in *H. brasiliense*. The presence of all these events in one species seems to be frequent. Sanders *et al.* (1999) presented a rich collection of male sterile mutants in *Arabidopsis thaliana* recognizing nine general classes of problems. These problems usually occur simultaneously due to the fact that they probably have the same origin, which is a mutation in one or a group of mitochondrial genes involved with CMS determination (Chase 2006).

The biology of reproduction assays indicate that *H. brasiliense* is auto-compatible and there are no barriers to self-pollination in this species, but presents preferentially cross-pollination. Although some individuals presented vestigial anthers, the tests carried out in flowers with normal anthers revealed high index of pollen viability. Variation between viable pollen index and pollen germination rate presented here is probably due to some inefficiency of *in vitro* germination technique. The auto-compatibility assay showed that induced self-pollination was well succeeded and the pollen tubes found no resistance to grow. However, the development of pollen tubes in cross-pollination control test was faster than that observed in self-pollination tests. The more rapid development of pollen tubes in cross-pollination results in an earlier ovule fecundation and allows to infer that *H. brasiliense* is preferentially alogamous.

The floral dimorphism observed is probably related with the reproduction pathway found in each type. Apomixis is probably the preferential mode of reproduction in populations with predominant sterile males with vestigial anthers, like that from Ibitipoca, MG. The fact that apomixis was observed only in male sterile plants characterizes *H. brasiliense* as a facultative apomitic species.

Several causes can lead to male sterility such as abortion, abnormalities in meiotic division and tetrad separation, and tapetum cells changes. Problems in exine are the mostly related responsible for male sterility in some species (Bhandari 1984). Male sterility may be associated with pseudogamy, a type of apomixis in which pollen grain is necessary only in the initial stage as a stimulus for seed formation. Possibly the apomict individuals have the male sterile pattern of anther.

The results obtained in the present study indicate high complexity and diversity of reproductive biology in *H. brasiliense*. Phenomena like these are possibly associated with apomixis. For better understanding the male sterility of *H. brasiliense*, ontogenic studies of anther are still necessary in order to explain the various events that lead to this condition.

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