REPRODUCTIVE STUDIES ON IPECAC (*Cephaelis ipecacuanha* (BROT.) A. RICH; RUBIACEAE): MEIOTIC BEHAVIOR AND POLLEN VIABILITY

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ABSTRACT

Reproductive studies were carried out on Brazilian accessions of ipecac, *Cephaelis ipecacuanha*. Meiotic behavior was studied using the squashing technique. Irregular chromosome segregation in meiosis I and II, many sets of chromosomes in telophase II, micronuclei, incorrect cytoplasm division, incomplete cytokinesis and anomalous post-meiotic products, mainly polyads, were observed. The mean meiotic index was lower than 72%. Pollen viability was analyzed using Alexander solution, and the percentages ranged between brevistylous and longistylous floral morphs (85.3 to 93.1%), and among different localities (82.5 to 92.6%) analyzed. The size of pollen ranged between viable and sterile, and empty and shrunken sterile. In its natural habitat, this species is known to propagate by vegetative multiplication, but sexual reproduction seems to be as important as the vegetative propagation to this species.

Keywords: ipecac, heterostyly, meiosis, microsporogenesis, microgametogenesis, pollen viability.

RESUMO

Estudos reprodutivos em poaia (*Cephaelis ipecacuanha* (Brot.) A. Rich; Rubiaceae): comportamento meiótico e viabilidade polínica

Estudos reprodutivos foram realizados em acessos brasileiros de poaia, *Cephaelis ipecacuanha*. O comportamento meiótico foi estudado usando a técnica de esmagamento. Foi observada segregação irregular de cromossomos durante meiose I e II, muitos grupos de cromossomos em telófase II, micronúcleos, divisão incorreta do citoplasma, citocinese incompleta e produtos pós-meióticos anômalos, principalmente políades. A média do índice meiótico foi inferior a 72%. A viabilidade polínica foi analisada utilizando-se solução de Alexander e a percentagem de pólen viável variou entre as formas florais, brevistila e longistila (85,3% a 93,1%), e entre as diferentes localidades (82,5% a 92,6%) analisadas. O tamanho do pólen variou entre viáveis e inviáveis, e entre os inviáveis vazios e contraídos. Em seu habitat natural, a poaia apresenta propagação por multiplicação vegetativa, mas a reprodução sexuada parece ser tão importante para essa espécie quanto a propagação vegetativa.

Palavras-chave: poaia, heterostilia, meiose, microsporogênese, microgametogênese, viabilidade polínica.

INTRODUCTION

Poaia, ipeca or ipecacuanha ($n = 11$; Assis, 1992) are terms used in Brazil to designate the medicinal species *Cephaelis ipecacuanha*, known as ipecac in English language countries and raicilla in Central American countries (Torres, 1972). Its center of origin is located in the Americas. Native populations are restricted to three regions: Central America, Brazil's southwestern Amazonia (states of Mato Grosso and Rondonia) and the Atlantic forest, mainly the states of Minas Gerais, Espírito Santo, Rio de Janeiro and Bahia (Assis, 1992). The plant's natural propagation occurs by both vegetative multiplication and transportation of seeds by birds (Sick, 1993). This species presents heterostyly (Martins, 2000) like many other species belonging to the Rubiaceae family (for a review, see Vuilleumier, 1967; Bir Bahadur, 1968).

According to Akerele *et al.* (1991), *C. ipecacuanha* is considered one of the world's most important medicinal plants and Brazilian ipecac is considered the most valuable because it shows the highest emetine content (Assis, 1992). However, like other Brazilian native species that are disappearing due to the reduction of their habitats in areas of natural vegetation, ipecac is now considered a threatened species, (Oliveira & Martins, 1998). Many wild species have undergone a narrowing of their genetic base due to predatory collection or destruction of vegetation cover (McKeown, 1996), possibly endangering them through the action of natural selection (Brown, 1988).

Vegetative propagation is considered the main mode of reproduction for *C. ipecacuanha* (Sick, 1993), but sexual reproduction which depends on meiosis to produce gametes usually plays an important role in heterostylous species. Sexual reproduction involves two important events that lead to genetic diversity: fusion of gametes with different gene information and exchange between genetic materials (crossing over).

Meiosis is a highly dynamic process and the main cell mechanism enabling the occurrence of sexual reproduction in angiosperms. The complex orchestration of a normal cell division includes chromosome pairing in zygotene, genetic exchange in pachytene, chiasmata formation in diplotene and chromosome segregation in anaphase I and II (Franklin *et al*., 1999). Meiosis is genetically controlled (Gottschalk & Kaul, 1974; Golubovskaya, 1979). Although the meiocyte is a highly specialized cell capable of producing four haploid cells, mutations, hybridizations, environmental stress, endogamy and other factors may alter the constitution or the expression of genes that act during meiosis (Utsunomiya *et al*., 2002). More than 20 genes that affect meiosis have been described for maize, most of which are known to cause sterility (Golubovskaya, 1989, quoted by Defani-Scoarize *et al*., 1995).

Micropropagation has served as a tool for rescuing endangered plants (Palomino *et al*., 1999). Studies on *C. ipecacuanha* to obtain whole plants *in vitro* have been successful (Jha & Jha, 1989; Ideda *et al*., 1989), but *in situ* germplasm preservation can also be an important strategy to prevent narrowing of the genetic base. However, studies to ascertain the importance of sexual reproduction of this species have not been conducted. Therefore, this study aimed to document and report valuable information on meiotic behavior and pollen viability, thereby contributing toward a better understanding of the reproductive aspects and the importance of sexual reproduction of this species, in view of its considerable medicinal importance.

MATERIAL AND METHODS

Brazilian accessions of *C. ipecacuanha* collected in the municipalities of Caratinga and Carangolas (Minas Gerais) and Itaperuna (Rio de Janeiro) were analyzed. The species were kept in pots in a greenhouse in the Research Support Unit at UENF.

For meiotic studies, flower buds were fixed in ethanol – acetic acid (3:1) at room temperature for 24 h, transferred to 70% alcohol and stored under refrigeration until use. Temporary slides were prepared by the squashing technique and the cells were stained with 1% acetic carmine. Although the flowers are very small and difficult to analyze, at least 25 cells were examined at each meiotic phase. The numbers of monads, dyads, triads, tetrads and polyads were recorded for calculation of the meiotic index (% IM = [number of normal tetrads x 100] ÷ total of post-meiotic products counted), according to Love (1951). The meiotic index considered in this study was the mean of four randomly sampled slides.

To ascertain viability, only the heterostyly of plants collected in Itaperuna and Caratinga was taken into account. Fixed pollen grains of flowers in anthesis were stained with Alexander solution (Alexander, 1969) for wall/cytoplasm reactivity, and were considered viable when their cytoplasm became stained and remained intact. The anthers were squashed in a drop of stain and, after two minutes at room temperature, the pollen grains were checked, counted and measured with the aid of an eyepiece micrometer. Mechanically damaged grains were easily distinguishable by their normal size and traces of cytoplasm inside and outside the pollen wall. The number of viable and sterile pollen grains considered in this study was the mean of the count of these cells on five randomly sampled slides (repetitions) for both brevistylous and longistylous morph and collection site.

An optical microscope was used for the observations and photographs. The statistical analysis was performed using the GENES program (Cruz, 2001).

RESULTS

Meiosis

Due to the small size of buds and chromosomes, the prophase I stages could not be clearly analyzed, so an accurate cytological analysis was impossible. A conventional cytological analysis was employed to verify the meiotic behavior, confirming that chromosomes of ipecac paired as 11 bivalents in metaphase I. Several meiotic irregularities were observed, whose type and frequency are presented in Table 1. Chromosomes showing irregular chromosome segregation (ascending and laggard; Fig. 1a-c) were the main problems found in meiosis I. Meiosis II showed the same type of irregularity, but telophase II displayed different numbers of chromosome sets (Fig. 1d-f) due to non-oriented bivalents on the equatorial plate. This fact was corroborated by the formation of abnormal post-meiotic products, mainly polyads, which showed irregularly shared cytoplasm and incomplete cytokinesis (Fig. 1g-h). The imbalanced distribution of chromosomes in anaphase II persisted up to telophase II. Linear tetrads were observed. As indicated by the data in Table 2, the meiotic index mean was 71.26%, with the quantity of polyads on average 72% greater than other irregularities. Micronuclei were observed (Fig. 1i) as a consequence of irregular chromosome segregation, and incomplete cytokinesis was also found in mature pollen grains (Fig. 2a).

Pollen size and viability

Pollen viability was found at three sites, ranging on average from 82.5 to 92.6% (Table 3). Pollen from two sites was analyzed in terms of heterostyly, with longistylous populations showing lower pollen viability than brevistylous populations (Table 4). Application of the F test (Table 5) revealed that: 1) the three sites analyzed showed significant differences in the percentage of pollen viability ($P < 0.01$); 2) Caratinga and Itaperuna showed no significant difference in the percentage of sterile pollen for the same floral morph; and 3) brevistylous and longistylous morphs from both Caratinga and Itaperuna showed significant differences with respect to their percentage of sterile pollen ($P < 0.01$). Although pollen from the Carangolas (MG) population was not analyzed

TABLE 1
Meiotic irregularities observed in *Cephaelis ipecacuanha*.

Meiotic phases	% Cells with			Total cells analyzed
	AC	LAG	DSC	
Metaphase I	1.9	9.3	-	39
Anaphase I	1.8	8.2	-	25
Telophase I	2.2	-	9.7	48
Metaphase II	1.8	7.1	-	35
Anaphase II	2.6	6.7	-	25
Telophase II	2.9	-	10.2	41

AC – ascending chromosomes; LAG – laggard chromosomes; and DSC – different sets of chromosomes.

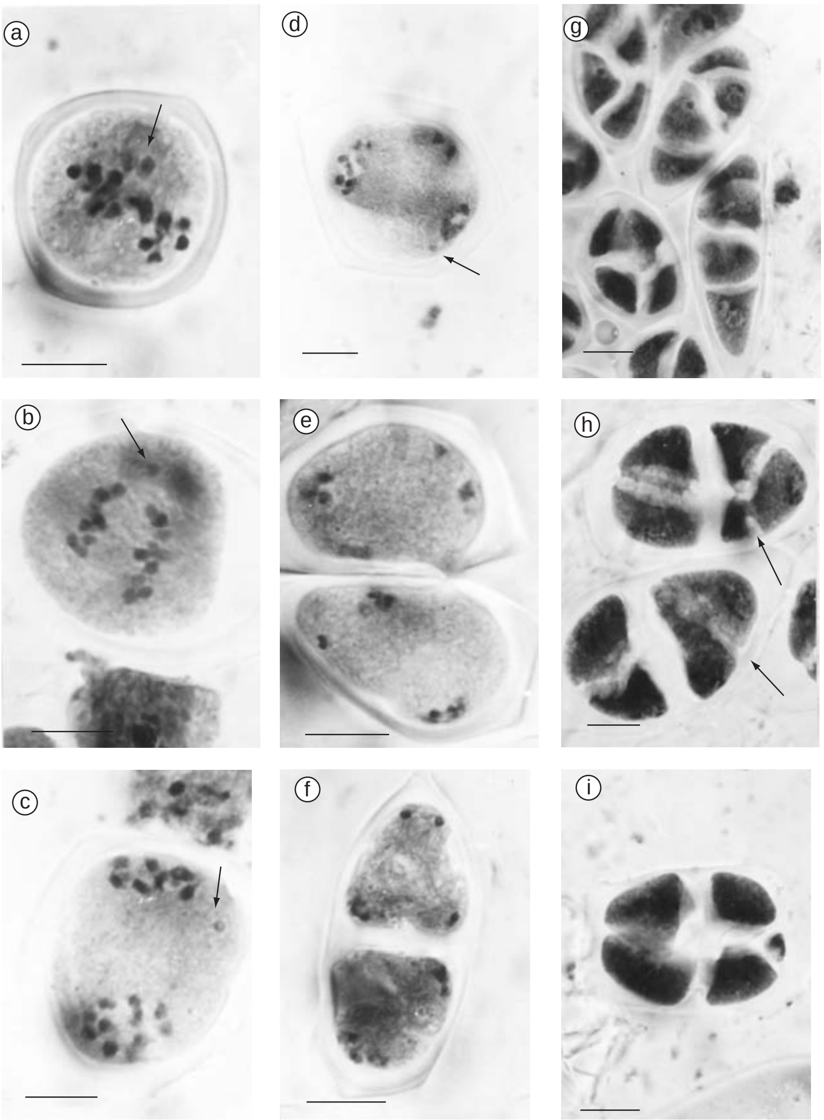

Fig. 1 — Meiosis in *Cephaelis ipecacuanha*. a) Metaphase I with ascending chromosomes (arrow); b) Anaphase I with laggard chromosome (arrow); c) Telophase I with laggard chromosome (arrow); d) Telophase II with three sets of chromosomes and laggard chromosome (arrow); e) Cells in telophase II with different sets of chromosomes; f) After cytokinesis, irregular distribution of chromosomes and cytoplasm in cell; g) Linear tetrads; h) tetrads showing incomplete cytokinesis (arrows); and i) Tetrad with micronucleus. Bars = 20 µm.

in terms of heterostyly, its values were similar to those of the longistylous populations.

Two types of sterile pollen grains were observed: a) empty (Fig. 2b), measuring from 8 to 40 µm (27.92 ± 7.68 µm), with only the wall reacting to the stain and indicating absence or only traces of cytoplasm; and b) shrunken (Fig. 2c-d), measuring from 24 to 56 µm (38.95 ± 8.46 µm), with both the wall and the cytoplasm reacting to the stain, although a space was found between the plasmatic membrane and the cell wall, accompanied by cytoplasm contraction. The presence of micronuclei led to the formation of microcytes and hence, anomalous pollen grains (Fig. 2e). The size of the strongly stained pollen grains varied from 36 to 80 µm (55.56 + 8.71 µm), and some gametes

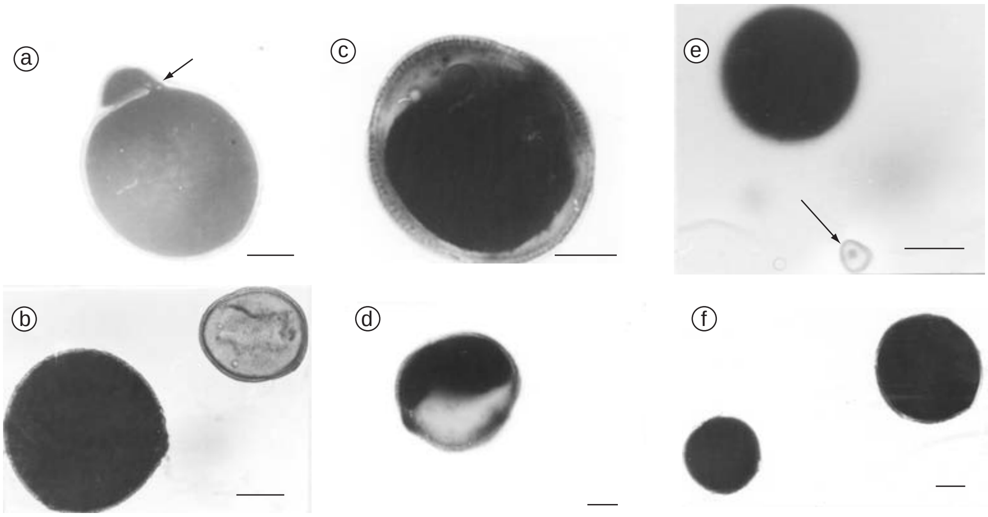

Fig. 2 — Pollen viability in *Cephaelis ipecacuanha*. a) Anomalous pollen grain showing incomplete cytokinesis (arrow); b) Sterile empty and well-stained viable polle; c) Shrunken sterile pollen grain in the initial stage; d) Shrunken sterile pollen grain in advanced stage; e) Very small, empty sterile cell (arrow) and viable pollen grain; and f) Giant cell and normal pollen. Bars = 20 µm.

TABLE 2
Post-meiotic products and meiotic index (MI) in *Cephaelis ipecacuanha*.

Repetitions	Number of products resulting from meiosis II					Total cells analyzed	MI (%)
	Monads	Dyads	Triads	Tetrads	Polyads		
01	01	03	11	174	58	247	70.44
02	-	01	10	102	28	141	72.34
03	01	02	13	114	29	159	71.69
04	-	01	19	156	45	221	70.58
Total	02	07	53	546	160	768	Mean: 71.26

TABLE 3
Pollen viability in *Cephaelis ipecacuanha*: collection without considering heterostyly.

Locality	Sterile pollen		Total PG scored*	% PV
	Total observed*	(%)		
Carangolas (MG)	233	17.42	1337	82.58
Caratinga (MG)	155	9.98	1552	90.02
Itaperuna (RJ)	172	7.39	2326	92.61

PG, pollen grains; PV, pollen viability. * The results are the sum of five repetitions.

TABLE 4
Pollen viability in brevistylous and longistylous flowers of *Cephaelis ipecacuanha*.

Locality	Brevistylous*			Longistylous*		
	SPGT	OPGT	% PV	SPGT	OPGT	% PV
Caratinga (MG)	111 (6.90%)	1608	93.10	220 (14.71%)	1495	85.29
Itaperuna (RJ)	240 (7.67%)	3127	92.33	275 (12.97%)	2120	87.03

SPGT, sterile pollen grain total; OPGT, observed pollen grain total; PV, pollen viability. * The results are the sum of five repetitions.

TABLE 5
ANOVA summary for the characteristics of sterile pollen grains (SPG), brevistylous sterile pollen grains (BSPG), longistylous sterile pollen grains (LSPG), sterile pollen grains in the different flower morphs from Caratinga (FMC), and sterile pollen grains in the different flower morphs from Itaperuna (FMI) in *Cephaelis ipecacuanha*.

Source of variation	Mean squares[E]					
	DF	SPG[&]	BSPG	LSPG	FMC	FMI
Locality[1]	2	193.17**	-	-	-	-
Error	12	26.22	-	-	-	-
CV (%)	-	41.91	-	-	-	-
Locality	1	-	1.59[NS]	15.92[NS]	-	-
Error	8	-	0.56	9.15	-	-
CV (%)	-	-	10.37	21.47	-	-
Flower morph	1	-	-	-	179.86**	66.56**
Error	8	-	-	-	8.00	1.71
CV (%)	-	-	-	-	25.46	12.79

** significance at the 1% ($P < 0.01$) probability level by F test; CV, coefficient of variation; [NS] Not significant; DF, Degrees of Freedom; [E]The results correspond to the mean of five repetitions; [&] Collection without considering heterostyly; and [1]Analysis without considering heterostyly.

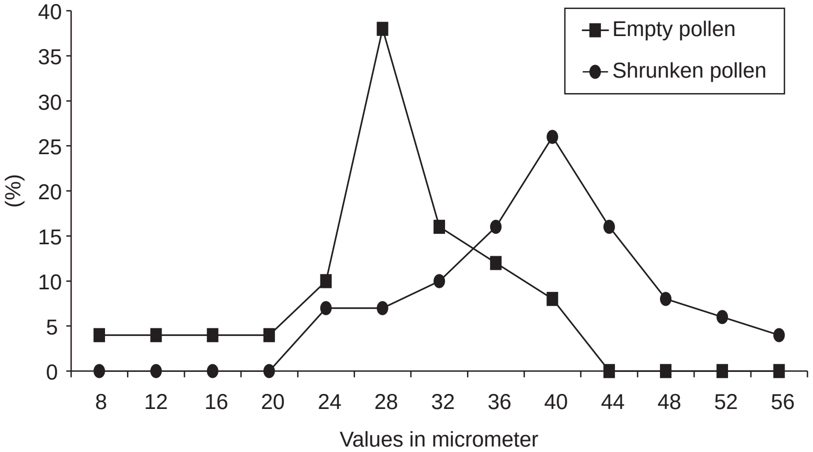

Fig. 3 — Frequency of sterile pollen grain in relation to pollen size in *Cephaelis ipecacuanha*.

were considered giant cells (Fig. 2f), being up to 43.98% larger than the mean viable pollen grain size. Fig. 3 shows the sterile pollen grain frequencies in relation to their size.

DISCUSSION

C. ipecacuanha is a diploid species with 11 bivalents. The basic number of the genus *Cephaelis* (= *Psychotria* L.) is x = 11 (Kiehn, 1986). According to Assis (1992), the karyotype of *C. ipecacuanha* is symmetric, displaying metacentric chromosomes, and is included among the more primitive types. However, a meiotic analysis revealed abnormal behavior. Irregular chromosome segregation in meiosis I and II could be the result of the non-oriented chromosomes, which show an inability to congregate on the equatorial plate, resulting in the grouping of many sets of chromosomes in telophase II, as well as micronuclei, incorrect cytoplasm division, incomplete cytokinesis and polyads.

According to Nicklas & Ward (1994), non-oriented bivalents may be related to impaired attachment of kinetochores to the spindle fibers. Pagliarini (1990) reported that laggards may result from late chiasma terminalization. Ascending chromosomes are the result of precocious migration and, according to Utsunomiya *et al.* (2002), generally consist of univalent chromosomes formed during late prophase I stages by precocious chiasma terminalization in early metaphase I, or may even result from low chiasma frequency or from the presence of asynaptic or desynaptic genes (Pagliarini, 2000). Laggards and non-oriented chromosomes may produce micronuclei if they fail to reach the poles in time to be included in the main telophase nucleus (Koduru & Rao, 1981; Utsunomiya *et al.*, 2002), leading to the formation of micro-pollen and, probably, to gametes with unbalanced chromosome numbers (Mansuelli *et al.*, 1995), such as aneuploids (Defani-Scoarize *et al.*, 1995). Despite meiotic irregularities, Kenton *et al.* (1988) stated that a period of chromosome instability can lead to the establishment of a new equilibrium and may represent an important source of variability in species with vegetative reproduction.

Abnormal cytokinesis was observed in *C. ipecacuanha*, resulting in the formation of polyads. Two types of microsporogenesis are generally recognized, *i.e.*, successive or simultaneous. In the same anther of *C. ipecacuanha*, we found linear and T-shaped tetrads that are characteristic of successive micropsporogenesis, and tetrahedral tetrads that are the result of simultaneous microsporogenesis (see Furness & Rudall, 1999). However, an intermediate type called "modified simultaneous" may have occurred in *C. ipecacuanha*, whereby an ephemeral cell plate is laid down after the first meiotic division, which then disappears and cytokinesis occurs simultaneously; sometimes the second division rapidly follows the first, before the cell plate is completely formed (Murty, 1964; Furnes & Rudall, 1999). This may explain why some meiocytes presented cells with partial cytokinesis. In mutants, some genes have been identified as responsible for abnormal cytokinesis. In maize, the *va* (*variable sterile*; *va1* and *va2* alleles; Beadle, 1932) and *el* (Rhoades & Dempsey, 1966) genes were responsible for the absence or partial formation of the cell plate, forming irregular post-meiotic products. Not only genetic factors but also several environmental factors can account for the frequency of anomalous post-meiotic products due to differences in the genetic background, as in the case of *T. latifolia* (Berdnikov *et al.*, 2002).

Giant cells were observed in *C. ipecacuanha*. These are imbalanced gametes probably formed by the absence of cytokinesis and/or irregular chromosome segregation. These imbalanced gametes exert a strong influence on sexual propagation, so that chromosomal instability can produce gametes with the addition or loss of chromosomes. Sometimes, such abnormal gametes are tolerant and participate in fertilization, but the union of these gametes can produce considerable variations in chromosome numbers in subsequent generations (Caetano-Pereira *et al.*, 1998). In maize, imbalanced gametes are unable to compete with normal gametes (Defani-Scoarize *et al.*, 1995). In *Typha latifolia*, nullisomy was lethal to a developing gametophyte while disomy was tolerable, resulting in non-disjunction in the second division (Berdnikov *et al.*, 2002).

C. ipecacuanha presented different pollen viability percentages in relation to floral morph and population locality. Assis (1992) studied *C. ipecacuanha* populations from the states of Minas Gerais (MG), Espírito Santo (ES), Mato Grosso (MT) and Rondonia (RO) Brazilian without taking into consideration the heterostyly. Only the MG population showed a very low pollen viability of 47.74%, while plants in ES showed 81.46% and indexes exceeding 91% were found in MG. The latter findings are congruent with those found in this study. The populations under study showed higher pollen viability when the floral morph was brevistylous, *i.e.*, above 90%, while longistylous presented values between 85.2 to 87%. Carangolas (MG) presented 82.58% pollen viability, and was probably a longistylous population. The meiotic index was 71.26%, probably indicating that the many types of irregularities found during meiosis affected the percentage of sterile pollen. According to Love (1951), plants with a meiotic index of 90% to 100% may be considered quite cytologically stable, but it is actually impossible to ensure stability in plants with indices of 88% to 92%, since plants with a meiotic index of less than 90% are likely to encounter difficulties in outbreeding.

Two types of sterile pollen were observed: empty and shrunken. *C. ipecacuanha* showed smaller empty pollen grains, indicating that the malformation probably occurred during chromosome segregation (microsporogenesis), while the shrunken pollen grains were larger and practically the same size as the viable pollen grains, which may have suffered genetic action during microgametogenesis (see Twell, 1995). Gamete development can be divided into three sequential and distinct stages: pre-meiosis, meiosis and post-meiosis (Caetano-Pereira *et al*., 1997). The normal and harmonious course of meiosis (microsporogenesis) would ensure viability of the gamete, but the post-meiotic genes, *i.e*., genes that transcribe in microgametogenesis, act in the interaction between the generative and vegetative cell. A break in cell-to-cell communication can lead to gamete malformation (Twell, 1995), making it equally unviable. Also according to Mascarenhas (1990), genetic expression during pollen grain development is divided into two different stages, with transcripts that correspond to the expression of the early genes in the development of the microspores, but a decline after anthesis and late gene transcripts appearing shortly after mitosis of the microspores, accumulating until anthesis.

In some species, abnormal meiotic behavior is considered responsible for pollen sterility and low seed production (Golubovskaya, 1979; Koul, 1990; Pagliarini *et al*., 1992, 1993; Consolaro *et al*., 1996). The presence of meiotic irregularities has significantly affected the meiotic index and pollen viability of *C. ipecacuanha* and may imply that the process of sexual reproduction is not totally effective. However, in this species, sexual reproduction seems to be as important as vegetative propagation. The existence of differences in the breeding mechanism (heterostyly) among populations strengthens this idea. Heterostyly is thought to have evolved because it renders cross-pollination efficient (Björkman, 1995) and favors allogamy (Robbrecht, 1988). According to Vuilleumier (1967), heterostylous species can maintain a balance between inbreeding and outbreeding; this flexibility makes it a highly adaptable reproductive system, which can change under different environmental pressures to divergent breeding systems. In *Salvia brandegeei*, the evolution of distyly may have been associated with an ecological shift to a new environment in which protandry failed to prevent increased levels of geitonogamy; heterostyly was then selected because it increased the efficiency of cross-pollination (Barrett *et al*., 2000).

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