Reproductive Studies in Ipecac (*Cephaelis ipecacuanha* (Brot.) A. Rich; Rubiaceae): Flower Bud and Anther Size Associated to Male Gamete Development Stages

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Summary Reproductive studies were carried out on Brazilian accessions of *Cephaelis ipecacuanha*, which is considered a threatened species. Meiotic behavior was studied using squashing technique with 1% acetic carmine and floral buds and anther were measured for the following male gamete development stages: meiosis I, meiosis II, tetrads, microspores and pollen. Bud and anther length showed significant differences ($P < 0.01$) among the stages of pollen development. There was high significant correlation between bud and anther length by Pearson correlation. Tukey test results indicated that each analyzed stage could be associated to bud length mean but it was not found the same association for anther length.

Key words *Cephaelis ipecacuanha*, Poaia, Microsporogenesis, Microgametogenesis, Flower bud and anther size.

Ipeca, poaia or ipecacuanha ($n=11$; Assis 1992) is the term used in Brazil to designate the medicinal species *Cephaelis ipecacuanha*, known as ipecac in English language countries and raicilla in Central America countries (Torres 1972). Its center of origin is located in American continent. Native populations are restricted to three regions: Central America, Southwest of the Brazilian Amazon region (Mato Grosso and Rondonia States) and Atlantic forest, principally in Minas Gerais, Espírito Santo, Rio de Janeiro and Bahia States (Assis 1992).

According to Akerele *et al.* (1991), ipecac was considered one of the more important medicinal plants of the world. The more remarkable medicinal characteristics are found in the roots with its flavor bitter taste and nauseating smell (Gattoni 1960, Oliveira and Martins 1998). The main compounds responsible for its therapeutic applications are the alkaloids emetine, cephaeline and psychothrine (Sousa *et al.* 1991). Brazilian ipecac is considered the most valuable because it shows highest emetine content (Assis 1992). These composites are effective to provoke vomits (Sousa *et al.* 1991), act as expectorant and treat against amoeba *Entamoeba histolytica* (Bruneton 1995), among other applications (Trease and Evans 1989, Sousa *et al.* 1991, Carrinconde *et al.* 1995).

Ipecac is considered a threatened species as others Brazilian native species that are disappearing due to diminution of their habitats in natural vegetation areas (Oliveira and Martins 1998, Costa

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et al. 2000). Many wild species have suffered narrowing of their genetic base due to predatory collection or vegetation cover destruction (McKeown 1996), and this fact can lead them to be endangered by natural selection action (Brown 1988). On this bases, the aim of this study was to document and report information on identification of flower bud and anther size associated to main stages of male gamete development to help providing a better understanding of reproductive aspects in this species in despite of its considerable medicinal importance, in order to give support to preservation and breeding strategy.

Material and methods

We analyzed *C. ipecacuanha* accessions from Brazil, collected in Caratinga and Carangolas (Mato Grosso), and Itaperuna (Rio de Janeiro) cities. The species were kept in pots in a greenhouse at 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. Before squashing, buds and anthers were measured and the values were associated to microsporogenesis and microgametogenesis stages. Data was submitted to analysis of variance (ANOVA), means were tested by the procedure of Tukey at 5% probability and Pearson correlation was performed between bud and anther sizes. Statistical analysis was performed considering a randomized experimental design by the program Genes (Cruz 2001).

Results

Mean values of flower bud and anther are shown on Table 1. Bud and anther length showed significant differences ($P < 0.01$) among the stages of male gamete development by F test (Table 2), and microsporogenesis (Fig. 1a–e) and microgametogenesis (Fig. 1f) were associated to flower bud and anther length (Fig. 2). Meiosis I and II stages could not be differentiated in relation to anther length due to asynchronism into the same anther in relation to the division phases: anthers showing the same size displayed different meiotic phases; therefore, values found for one stage overlapped the values found for other one.

For studies in ipecac involving microspore (uninucleate, haploid cell) in vitro culture, it is rather indicated to use flower buds length from 1.8 mm and anther from 0.9 mm. For obtaining pollen grains, that is binucleate after its liberation from anther, it is indicated collecting from 2.5 mm for flower bud length and from 1.2 for anther. In relation to meiosis I, flower buds between 0.9–1.4 mm length, and to meiosis II, 1.5–1.6 mm length may be collected. For both meiosis I and II, anther length may be estimated between 0.5–0.7 mm.

Table 1. Pollen development stages associated to minimum, maximum and mean values of flower bud and anther length (mm) in *Cephaelis ipecacuanha*

Stages	Bud size			Anther size		
	Values	Mean ($\pm$ sd)	C.V. (%)	Values	Mean ($\pm$ sd)	C.V. (%)
Meiosis I	0.9–1.5	1.18 $\pm$ 0.17	14.54	0.5–0.7	0.56 $\pm$ 0.07	13.23
Meiosis II	1.5–1.8	1.58 $\pm$ 0.11	7.09	0.5–0.7	0.62 $\pm$ 0.07	11.31
Tetrad	1.6–2.0	1.83 $\pm$ 0.13	7.08	0.7–0.8	0.75 $\pm$ 0.05	7.12
Microspore	1.8–2.4	2.12 $\pm$ 0.23	11.17	0.7–1.2	0.93 $\pm$ 0.19	20.50
Pollen grain	2.5–2.7	2.58 $\pm$ 0.06	2.47	1.2–1.6	1.35 $\pm$ 0.13	9.69

sd=standard deviation; C.V.=coefficient of variation.

Table 2. ANOVA summary for the characteristics bud length and anther length in relation to stages of male gamete development in *Cephaelis ipecacuanha*

Source of variation	DF	Mean squares	
		Bud length	Anther length
Stages	4	2.25275**	0.801625**
Error	35	0.024	0.135
CV		8.30%	13.75%

** Significance at the 1% ($P < 0.01$) probability level by F test, CV: Coefficient of variation, DF: Degree of freedom
 * The results are means of eight repetitions.

Fig. 1. Microsporogenesis and microgametogenesis in *Cephaelis ipecacuanha*. a) Metaphase I, b) Early telophase I, c) Telophase II, d) Tetrads, e) Late microspore, f) Binucleate pollen.

Table 3. Pearson correlation between the variables bud length and anther length in *Cephaelis ipecacuanha*

Variables	Cov (X, Y)	Observations	Total number of Correlation	α (%)
Bud length $\times$ anther length	1.1201	80	0.3753	0.0719**

** Significance at the 1% probability level.

Fig. 2. Mean values of bud and anther length in function of male gamete development stages in *Cephaelis ipecacuanha*. Means followed by the same letter in the same line do not differ by the Tukey test at 5% probability.

Means of bud length were grouped in five levels (Fig. 2a–e) while for length anther it was observed four levels (Fig. 2a–d) by Tukey test, indicating that each analyzed stage could be associated to bud length mean but it was not found the same association for anther length, that is indicates only to differentiation between the microgametogenesis stages. There was high significant correlation between bud and anther length by Pearson correlation (Table 3).

Discussion

Pollen development in higher plants is a cooperative process requiring multiple interactions between the sporophytic plant and the developing male gametophyte (Chaubal *et al.* 2000). Anther development can be divided into 10 stages. They are based primarily on the state of the male cells and the tapetum, and the surrounding anther wall tissues: 1) mass stages; 2) meiocyte stage; 3) dyad stage; 4) tetrad stage; 5) early microspore stage; 6) midmicrospore stage; 7) late microspore stage (uninucleate); 8) early pollen stage (binucleate); 9) midpollen stage; 10) late pollen stage (bi or trinucleate), so that stages 2–7 represent microsporogenesis and stages 8–10 represent microgametogenesis (Horner and Palmer 1995, Palmer and Horner 2000).

Due to embryos arising from vegetative cell, generative cell, or symmetrical mitotic division of haploid microspore, many authors try for monitoring the meiotic division process to associate the flower bud size to different meiotic phases or to pollen development stages. Souza *et al.* (2002) could associate only the microgametogenesis stages to anther and bud length in yellow passion fruit. Andrade *et al.* (1996) made cytological analysis in four coffee cultivars (*Coffea arabica* L.), and in all the genotypes flower bud and anther size were associated only to microspore development stages.

Other characteristics beyond flower bud size could be associated to microgametogenesis stages. Lauxen *et al.* (1995) related microsporogenesis stages to four pre-established intervals of

flower bud length in three Brazilian cultivars of soybean (*Glycine max* (L.) Merrill), and observed the lack of synchronism among anthers from the same flower bud in relation to meiosis I and II. In sweet pepper (*Capsicum annuum* L.), the morphological characteristics could be associated to microspore stages in all the genotypes that showed flower buds with the same-sized petals or petals a little larger than sepals, and anthers with anthocyanin traces in its extremity (Silva *et al.* 1995). Luz *et al.* (2000) collected sweet pepper bud flowers when the petals were about the same size of sepals, which correspond to anthers containing unvacuolated microspores, with a central nucleus. Willcox *et al.* (1990), studying peanut (*Arachis hypogaea* L.), observed that not the size but the flower bud shape might be correlated with the microspore development, even though this difference could occur due to environmental conditions and not only as a result of genotype differences.

That type of information is important for the success of research that involves anther culture technique, and the non-acquaintance in relation to the flower development period that the microsporogenesis and microgametogenesis stages are occurring is an obstacle. Anthers that show from tetrads to binucleate pollen stages are the best to the *in vitro* culture, but for most species, it is necessary the manipulation of the uninucleate, gametic cell (Zhang and Lespinasse 1992). According to Mascarenhas (1990), rRNA genes of pollen grains seem to be switched off 24 h after the first mitosis being impossible to revert the cell development to form a plant and not a pollen grain.

Micropropagation has been a tool to rescue endangered plants (Moura-Costa *et al.* 1993, Palomino *et al.* 1999). In *C. ipecacuanha* callus culture were performed to produce alkaloids (Teshima *et al.* 1988), and studies *in vitro* to obtain the whole plants was successful (Jha and Jha 1989, Ideda *et al.* 1989). Costa *et al.* (2000) obtained an excellent emetine production using twelve-year-old plants from tissue culture. As observed in this study, the flower bud size might be used as an indicator to main stages of pollen grain development, while the same association does not occur in many other species (Willcox *et al.* 1990, Souza *et al.* 2002). These stages of development of gamete at the time of culture has been shown to be an important factor in the production of haploids via anther culture (Maheshwari *et al.* 1980), which could be an interesting option regarding future breeding programs involving *C. ipecacuanha*, for achieving homozygosity in relation to composites production once the alkaloid contents range among plants (Cruz 1935, Addor 1945). Making feasible the *in vitro* propagation of ipecac could be an important strategy to obtain clones that show high yield beyond constancy in the production and accumulation of alkaloids (Oliveira and Martins 1998).

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