

Polyploidy and polyembryony in *Anemopaegma* (Bignoniaceae, Bignoniaceae)

Fabiana Firetti-Leggieri · Lúcia G. Lohmann ·
Suzana Alcantara · Itayguara Ribeiro da Costa ·
João Semir

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Abstract Polyploidy is a key process in plant evolution, with the asexual formation of embryos representing a way through which polyploids can escape sterility. The association between polyploidy and polyembryony is known to occur in Bignoniaceae. In this study, we investigate polyembryony in four polyploid species of *Anemopaegma*: *A. acutifolium*, *A. arvense*, *A. glaucum* and *A. scabriusculum* as well as in one diploid species, *A. album*. Polyembryony was observed only in polyploid species. We used seed dissection and germination tests to compare the number of polyembryonic seeds. We tested how the pollen source influences the number of polyembryonic seeds and the number of embryos per seed and tested the correlation between the number of viable seeds per fruit and mean

number of embryos per seed. The number of polyembryonic seeds observed by seed dissection was higher than the number of polyembryonic seeds determined by the germination test, with the number of embryos produced per seed being higher than the number of seedlings. The dissection of seeds of *A. glaucum* indicated that a higher number of polyembryonic seeds and a higher number of embryos were present in seeds from cross-pollination than in seeds from self-pollination. On the other hand, germination tests indicated that a higher number of polyembryonic seeds were present in fruits from self-pollination than from cross-pollination. The mean number of embryos per seed was not influenced by the number of viable seeds per fruit in fruits from open pollination. These results indicate a positive relationship between polyembryony and polyploidy in *Anemopaegma*.

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F. Firetti-Leggieri (✉) · L. G. Lohmann (✉) · S. Alcantara
Departamento de Botânica, Instituto de Biociências,
Universidade de São Paulo, Rua do Matão 277,
São Paulo, SP CEP 05508-090, Brazil
e-mail: ffiretti@gmail.com

L. G. Lohmann
e-mail: llohmann@usp.br

S. Alcantara
e-mail: suzanaalcantara@gmail.com

F. Firetti-Leggieri · J. Semir
Departamento de Biologia Vegetal, Instituto de Biologia,
Universidade Estadual de Campinas, Caixa Postal 6109,
Campinas, SP CEP 13083-970, Brazil
e-mail: semir@unicamp.br

I. R. da Costa
Departamento de Biologia, Universidade Federal do Ceará,
Campus do Pici, Bloco 906, Fortaleza,
CE CEP 60451-970, Brazil
e-mail: itayguara@gmail.com

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Introduction

Polyploidy is the presence of three or more chromosome sets in the same nucleus (Soltis and Soltis 2000). It is considered a common phenomenon in plants and thought to represent a key process for the generation of genetic diversity and sympatric speciation in plants (Grant 1981; Rieseberg 1997; Otto and Whitton 2000; Adams and Wendel 2005; Soltis et al. 2009). The occurrence of polyploidy varies among different plant groups, being rare in gymnosperms, but very common in Pteridopsida (de Wet 1971; Soltis and Soltis 2000). In angiosperms, the frequency of polyploidy ranges from 30–35 (Stebbins 1971) to 70 % (Masterson 1994). A polyploidization event seems

to have happened before the radiation of the angiosperms, suggesting that most (or all) lineages are paleopolyploids (Bowers et al. 2003; Soltis et al. 2009).

Polyploids generally arise from unreduced gametes that are produced from failures in meiosis during sporogenesis (de Wet 1971; Levin 2002). However, polyploidy can also arise from somatic cell chromosome doubling (Levin 2002). Independent of their origin, polyploids are classified as autopolyploids when derived from self-pollination and crosses between individuals of the same species, or as allopolyploids when derived from interspecific hybridization (Soltis 2005; Otto 2007). Recent studies have shown that the process of polyploidization is recurrent and predominant in hybrids (Soltis and Soltis 1999; Bowers et al. 2003; Schifino-Wittmann 2004) and those presenting increased amounts of genetic diversity subjected to the action of selective pressures (Soltis and Soltis 1999; Schifino-Wittmann 2004; Adams and Wendel 2005; Soltis 2005).

The evolutionary success of polyploids depends, in large part, on the ability of individuals to reproduce and pass their genes to subsequent generations. Therefore, the sexual system plays a key role in the establishment of polyploids (Pannell et al. 2004). In allopolyploids, asexual reproduction is essential for the initial survival of hybrids since interspecific hybrids are often sterile because of problems with chromosome pairing (Stebbins 1971). In triploids, which result from the crossing between hybrid polyploids and their parental diploids, low fertility is common (Otto and Whitton 2000). This barrier is often overcome through asexual reproduction and the increased rate of self-fertilization promoted by the reduction in inbreeding depression in these taxa (Soltis and Soltis 2000; Barringer 2007; Otto 2007). In this sense, apomixis represents the most common mode of asexual reproduction in polyploids (Asker and Jerling 1992). It prevents meiosis and fertilization leading to a clonal progeny (Nogler 1984; Spillane et al. 2001) and presents a sexual development that is deregulated at several stages where (1) meiosis is impaired or absent resulting in an unreduced female gametophyte that is genetically identical to the parental plant; (2) fertilization is avoided, leading to the production of an autonomous embryo (parthenogenesis); and (3) the development of the endosperm is autonomous or sexual, that is, requiring fertilization of the central cell (Koltunow 1993; Spillane et al. 2001; Koltunow and Grossniklaus 2003; Tucker and Koltunow 2009). Apomixis is often divided into three different types, depending on the origin and location of the initial cells during apomictic development in angiosperms (Koltunow 1993): apospory and diplospory (gametophytic apomixis) and adventitious embryony (sporophytic apomixis; see Nogler 1984; Koltunow 1993;

Naumova 1993, 1997; Bicknell and Koltunow 2004). Polyembryony is characterized by the presence of two or more embryos in the same seed (Naumova 1993). Supernumerary embryos of polyembryonic seeds may be sporophytic or gametophytic and can be generated by sexual reproduction or apomixis (Lakshmanan and Ambegaokar 1984). The sexual production of these embryos can result either from the cleavage of cells derived from zygotic embryo or from the fertilization of the egg cell and one of the synergids. The asexual production of sporophytic embryos, on the other hand, usually occurs from the cells of nucellus or from the integuments (Naumova 1993). Despite the multitude of origins, the high incidence of polyembryonic seeds per fruit, the presence of seeds with more than three embryos and embryos at multiple stages of development within the same seed represent typical features of adventitious embryony (Naumova 1993). Adventitious embryony, the most widespread form of apomixis, is thought to represent a strategy of the parental plant to counteract the reduction in offspring caused by the abortion of seeds derived from a single fruit which could, in turn, result from the competition among seeds for maternal resources (Ganeshaiyah et al. 1991).

In Bignoniaceae, polyembryony was first described in *Handroanthus chrysotrichus* (Piazzano 1998) and subsequently documented in *H. ochraceus* (Salomão and Allen 2001; Costa et al. 2004), *Anemopaegma chamberlaynii* (Correia et al. 2005) and *A. arvense* (Pereira et al. 2007). All of these taxa are polyploids, except for *A. chamberlaynii*, which is diploid. Histological studies performed in *A. acutifolium* (Sampaio 2010), *H. chrysotrichus* (Souza et al. 2005; Bittencourt and Moraes 2010) and *H. ochraceus* (Costa et al. 2004) further documented the occurrence of adventitious embryony with the formation of supernumerary embryos from the hypostase (the differentiated region of the chalaza, located between the funicular bundle and embryo sac, whose cells have thick walls and dense cytoplasm) and endothelial cells.

Here, we conducted further studies with representatives of *Anemopaegma* in order to investigate potential associations between polyploidy and polyembryony in the genus. We further address (1) whether polyploid species produce polyembryonic seeds and, if so, whether the number of polyembryonic seeds observed in the fruit is influenced by the method of observation, that is, seed dissection and germination testing; (2) whether the source of pollen (self- and cross-pollination) influences the production of polyembryonic seeds in the fruits and the production of embryos in the seeds; and (3) whether a correlation exists between the number of viable seeds per fruit and mean number of embryos produced per seed in each fruit, as evidenced by Ganeshaiyah et al. (1991) for species of *Citrus*.

Materials and methods

Focal taxa

This study focuses on four species of *Anemopaegma* (*A. acutifolium* DC., *A. arvense* (Vell.) Steff. ex de Souza, *A. glaucum* Mart. ex DC. and *A. scabriusculum* Mart. ex DC.), all of which have been previously shown to be polyploids ($2n = 80$; Firetti-Leggieri et al. 2011), plus *Anemopaegma album* Mart. ex DC., a diploid species ($2n = 40$; Firetti-Leggieri et al. 2011; Table 1). These species produce showy yellow flowers in axillary racemes, pollinated by *Apis*, *Centris* and *Euglossa* bees. Fruits are dehiscent capsules that release wind-dispersed seeds surrounded by hyaline-membranaceous wings. *A. acutifolium*, *A. arvense* and *A. glaucum* are shrubs distributed throughout the cerrado, Brazil, Bolivia and Paraguay (Firetti-Leggieri 2009; Lohmann and Taylor 2013). These species occur sympatrically and flower synchronously in the late dry season and early wet season (August to November) and release mature fruits and seeds during the dry season of the following year. They are interfertile, and intermediate morphotypes are thought to represent hybrids (Firetti-Leggieri 2009). Therefore, all of these taxa are collectively included in the “*Anemopaegma arvense* species complex.” *Anemopaegma scabriusculum*, previously considered a member of the *A. arvense* complex, is a 1–2-m-tall shrub occurring in “cerrados” and “campos rupestres” (rocky fields) areas of eastern Brazil (Bahia, Minas Gerais, Goiás, Pará and Piauí States) (Lohmann and Taylor 2013). This species flowers and produces mature fruits two to three times throughout the year (Silva and Queiroz 2003). *Anemopaegma album* is a 2–3-m-tall shrub or small tree distributed in “cerrados” from Bahia to the north of Minas Gerais (Brazil) (Lohmann and Taylor 2013) and is phylogenetically distant from the species of the *A. arvense* complex.

Field experiments and seed collection

From 2004 to 2008, ripe fruits of natural pollination were obtained from populations of *A. acutifolium*, *A. arvense* and *A. glaucum* located in five “cerrado” areas in Brazil: (1) Reserva Ecológica do IBGE— $15^{\circ}56'41''\text{S}$ $47^{\circ}53'07''\text{W}$, Brasília, Distrito Federal; (2) Universidade de Brasília, $15^{\circ}45'57''\text{S}$ $47^{\circ}57'22''\text{W}$, Brasília, Distrito Federal; (3) Prata, $19^{\circ}30'26''\text{S}$ $48^{\circ}50'43''\text{W}$, Minas Gerais State; (4) Assis, $22^{\circ}39'40''\text{S}$ $50^{\circ}23'58''\text{W}$, São Paulo State; and (5) Fazenda Campininha, $22^{\circ}18'\text{S}$ $47^{\circ}11'\text{W}$, Mogi-Guaçu, São Paulo State. In 2006 and 2007, fruits derived from natural pollination were obtained for *A. album* and *A. scabriusculum* from populations located in “cerrado” areas of Mucugê ($13^{\circ}07'20''\text{S}$ $41^{\circ}31'64''\text{W}$, Bahia State). Specimens representing each population were deposited in the herbarium of the Universidade Estadual de Campinas (UEC-UNICAMP) (Table 1).

In order to determine whether the source of pollen influences the level of polyembryony (amount of seeds and embryos produced), controlled hand-pollination experiments were performed from 2005 to 2007 in populations of *A. acutifolium*, *A. arvense* and *A. glaucum* from Brasília (DF, Brazil). Inflorescences containing flower buds were bagged with nylon bags. On subsequent days, flowers of first-day anthesis were self- or cross-pollinated before being rebagged. Some inflorescences with flower buds were only bagged, and flowers, emasculated or not, were not pollinated to check for spontaneous fruit development. Fruits derived from natural pollination and hand-pollination were stored in the greenhouse of the Department of Plant Biology at UNICAMP (Campinas, São Paulo, Brazil) and exposed to sunlight until full dehiscence. After fruit dehiscence, aborted seeds (without embryos) were discarded, while viable seeds (with embryos) were used for both tests to detect polyembryony.

Table 1 Studied populations of *Anemopaegma* and respective chromosome numbers (from Firetti-Leggieri et al. 2011)

Species	Locality	Ploidy	Voucher (population)
<i>A. acutifolium</i> DC.	DF-Brasília, Reserva Ecológica do IBGE	$2n = 80$	F. Firetti and C.P. Firetti 194
	MG-Prata	$2n = 80$	F. Firetti et al. 43
<i>A. album</i> Mart. ex DC.	BA-Mucugê	$2n = 40$	F. Firetti et al. 39
<i>A. arvense</i> (Vell.) Steff. ex de Souza	SP-Mogi-Guaçu, Fazenda Campininha	$2n = 80$	F. Firetti et al. 129
	DF-Brasília, Reserva Ecológica do IBGE	$2n = 80$	F. Firetti and C.P. Firetti 49
	DF-Brasília, Universidade de Brasília	$2n = 80$	F. Firetti and C.P. Firetti 78
<i>A. glaucum</i> Mart. ex DC.	SP-Assis	$2n = 80$	F. Firetti et al. 19
	DF-Brasília, Reserva Ecológica do IBGE	$2n = 80$	F. Firetti et al. 57
	DF-Brasília, Universidade de Brasília	$2n = 80$	F. Firetti et al. 76
<i>A. scabriusculum</i> Mart. ex DC.	BA-Mucugê	$2n = 80$	F. Firetti et al. 91

Polyembryony determination

The occurrence of polyembryony in species of *Anemopaegma* was verified through the dissection and germination of seeds. For the seed, we analyzed viable seeds from fruits derived from natural and hand-pollination. Seeds were kept in distilled water for 24 h and subsequently dissected under an Olympus stereomicroscope in order to verify the number of embryos by seed and to characterize the embryo morphology, following Naumova (1993). A further morphological representation of the embryos was conducted through photographs taken with a digital camera attached to a Fieldmann Wild Leitz (FWL-SM 7.5) stereomicroscope. From fruits obtained from natural pollination, 51–312 seeds were analyzed per species, as follows: *A. acutifolium*: 149 seeds ($n = 10$ fruits); *A. album*: 75 seeds ($n = 5$ fruits); *A. arvense*: 163 seeds ($n = 10$ fruits); *A. glaucum*: 312 seeds ($n = 14$ fruits); and *A. scabrusculum*: 51 seeds ($n = 5$ fruits). Viable seeds were derived from self- and cross-pollination of *A. acutifolium* (118 seeds from 10 fruits obtained by self-pollination; 19 seeds from 2 fruits obtained by cross-pollination), *A. arvense* (32 seeds from 2 fruits obtained by self-pollination) and *A. glaucum* (177 seeds from 9 fruits obtained by self-pollination; 126 seeds from 6 fruits obtained by cross-pollination) (Table 2).

In order to verify the relationship between the number of viable seeds per fruit and mean number of embryos per seed of the same fruit, all seeds of each fruit from natural pollination that showed no sign of predation were counted and separated into aborted or viable seeds. A total of 10 fruits (149 seeds) of *A. acutifolium*, 10 fruits (163 seeds) of *A. arvense* and 14 fruits (312 seeds) of *A. glaucum* were dissected using viable seeds.

A germination test was conducted to verify the number of seedlings produced per seed and to compare the number of seedlings with the number of embryos observed in the dissection test. Viable seeds were initially soaked in a solution of 10 % sodium hypochlorite for ca. 5 min and washed with distilled water. We used seeds with intact wings and seeds with wings partially removed. Seeds were placed on Gerbox germination boxes with germination paper moistened with distilled water. In each Gerbox we placed seeds from a single fruit from a particular pollination treatment. Each Gerbox was then kept in a germination chamber (Tecna TEC-401) with temperatures between 25 and 27 °C and constant light for 45 days. After 45 days, we recorded the number of (1) germinated seeds; (2) seeds that did not germinate; and (3) seedlings produced by seed. Polyembryony was considered positive when two or more seedlings emerged from each seed. For this test, we used a variable number of viable seeds derived from natural pollination for all five species of *Anemopaegma* as follows: *A. acutifolium*: 155 seeds ($n = 11$ fruits); *A. album*: 218 seeds ($n = 7$ fruits); *A. arvense*: 356

Table 2 Polyembryony in the species of *Anemopaegma* dissected

Species	Pollination treatment	Fruits	Viable seeds	Polyembryonic seeds total (%)	Embryos total (mean; sd)	Embryonic classes (seeds with variable number of embryos)						
						1	2	3	4	5	6	7
<i>A. acutifolium</i>	Self	10	118	49 (41.53)	198 (1.71 ± 0.38)	69 (58.47)	26 (22.03)	17 (14.4)	4 (3.39)	2 (1.69)	0	0
	Cross	2	19	6 (31.57)	30 (1.64 ± 0.15)	11 (57.89)	5 (26.31)	3 (15.79)	0	0	0	0
	Natural	10	149	77 (51.68)	280 (1.83 ± 0.47)	72 (48.32)	43 (28.85)	22 (14.86)	7 (4.69)	2 (1.34)	3 (2.01)	0
<i>A. album</i>	Natural	5	75	0	75	75 (100)	0	0	0	0	0	0
<i>A. arvense</i>	Self	2	32	29 (90.62)	109 (3.35 ± 0.6)	3 (9.37)	7 (21.87)	6 (18.75)	9 (28.12)	4 (12.5)	3 (9.37)	0
	Natural	10	163	134 (82.2)	452 (2.79 ± 0.69)	29 (17.79)	51 (31.28)	39 (23.92)	20 (12.27)	20 (12.27)	4 (2.45)	0
<i>A. glaucum</i>	Self	9	177	89 (50.28)	322 (1.82 ± 0.53)	88 (49.71)	50 (28.24)	27 (15.25)	8 (4.52)	3 (1.69)	1 (0.56)	0
	Cross	6	126	90 (71.42)	302 (2.31 ± 0.73)	36 (28.57)	39 (30.95)	26 (20.63)	15 (11.9)	10 (7.93)	0	0
	Natural	14	312	255 (81.73)	902 (2.92 ± 0.53)	57 (18.27)	76 (24.35)	78 (25)	56 (17.95)	38 (12.18)	4 (1.28)	3 (0.96)
<i>A. scabrusculum</i>	Natural	5	51	5 (9.8)	56 (1.06 ± 0.08)	46 (90.19)	5 (9.8)	0	0	0	0	0

seeds ($n = 20$ fruits); *A. glaucum*: 296 seeds ($n = 18$ fruits); and *A. scabriusculum*: 100 seeds ($n = 5$ fruits). Seeds derived from controlled hand-pollination experiments were obtained for *A. acutifolium* (30 seeds from 2 fruits obtained by self-pollination), *A. arvense* (135 seeds from 7 fruits obtained by self-pollination; 140 seeds from 8 fruits obtained by cross-pollination) and *A. glaucum* (92 seeds from 4 fruits obtained by self-pollination; 179 seeds from 8 fruits obtained by cross-pollination; Table 3).

In order to evaluate the germinating capacity of embryos of different sizes, a sample of 228 embryos of *A. glaucum* were removed from 76 seeds from natural pollination and were subjected to the germination procedure mentioned above.

Statistical analyses

A chi-square analysis was used to: (1) compare the number of polyembryonic seeds produced in fruits of *A. arvense* and *A. glaucum* from self- and cross-pollination and (2) to compare the number of polyembryonic seeds obtained by seed dissection and germination tests in fruits derived from natural pollination of *A. acutifolium*, *A. arvense*, *A. glaucum* and *A. scabriusculum*. The number of embryos produced per seed in fruits from self- and cross-pollination of *A. glaucum* was compared by *t* test to samples with equal variance ($F = 1.39$; $p = 0.062$). The relationship between the number of viable seeds per fruit and mean number of embryos produced by seeds of the same fruits from natural pollination of *A. acutifolium*, *A. arvense* and *A. glaucum* was evaluated with linear correlation. All statistical tests were performed in VassarStats: Website for Statistical Computation (<http://faculty.vassar.edu/lowry/VassarStats.html>).

Results

Occurrence of polyembryony

Polyembryony was recorded in seeds of all polyploid species analyzed, whereas the diploid species, *Anemopaegma*

album, had 100 % of monoembryonic seeds. Only flowers pollinated by controlled hand-pollination (self- and cross-pollination) or under natural pollination produced fruits and seeds. Non-pollinated flowers, emasculated or not, with no pollen grains actively deposited on stigma, detached from the inflorescence 3–5 days after the onset of anthesis.

Dissection test

Seed dissection revealed the presence of a variable number of embryos with distinct morphologies and at different stages of development. In monoembryonic seeds, embryos are white, ca. 1.02×1.63 cm (on average) and present a short radicle–hypocotyl axis and two bilobed cotyledons (Fig. 1a). In polyembryonic seeds, on the other hand, the embryos show variation in size and stage of development (Fig. 1b, c). When multiple embryos were encountered, they deformed and membranous cotyledons (Fig. 1c).

The number of polyembryonic seeds varied widely among fruits. The percentage of polyembryonic seeds resulting from natural pollination ranged from 20.00 to 76.47 % in *A. acutifolium* (mean = $49.2 \% \pm 18.09$), from 50 to 92.85 % in *A. arvense* (mean = $81.33 \% \pm 13.15$), from 60.00 to 96.29 % in *A. glaucum* (mean = $80.4 \% \pm 9.12$) and from 0 to 12.19 % in *A. scabriusculum* (mean = $6.09 \% \pm 8.61$).

In *A. glaucum*, the number of polyembryonic seeds derived from cross-pollination (90 from 126 seeds, 71.42 %) was significantly higher than the number of polyembryonic seeds in fruits from self-pollination (89 from 177 seeds, 50.28 %; $\chi^2 = 13.61$; $df = 1$; $p < 0.05$).

In polyembryonic seeds resulting from open pollination, the number of embryos per seed varied from two to six in *A. acutifolium* (2.7 ± 1.01) and *A. arvense* (3.16 ± 1.17), and from two to seven in *A. glaucum* (3.31 ± 1.16). On the other hand, the maximum number of embryos per seed was two in *A. scabriusculum*. For controlled hand-pollination experiments, the number of embryos per seed in fruits derived from self-pollination varied from two to five in

Table 3 Polyembryony in the species of *Anemopaegma* species germinated

Species	Pollination treatment	Fruits	Viable seeds	Germination total (%)	Polyembryonic seeds total (% ^a)
<i>A. acutifolium</i>	Natural	11	155	71 (45.8)	17 (23.94)
<i>A. album</i>	Natural	7	218	172 (78.89)	0
<i>A. arvense</i>	Self	7	135	65 (48.15)	39 (60)
	Cross	8	140	68 (48.57)	4 (2.85)
<i>A. glaucum</i>	Natural	20	356	213 (59.83)	73 (34.27)
	Self	4	92	56 (60.86)	20 (35.71)
	Cross	8	179	83 (46.37)	10 (12.05)
<i>A. scabriusculum</i>	Natural	18	296	141 (47.63)	60 (42.55)
	Natural	5	100	68 (68)	6 (8.82)

^a Percentage of polyembryonic seeds calculated from the germinated seeds

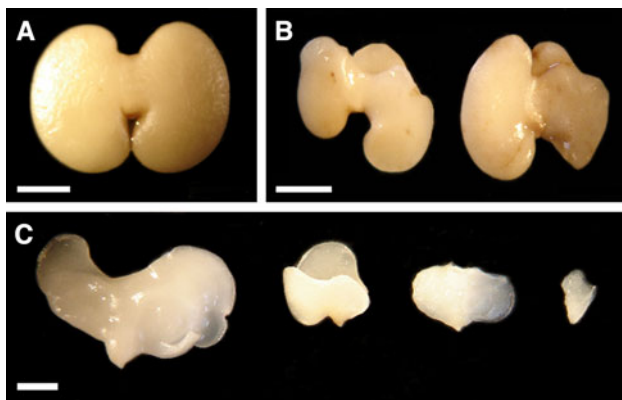


Fig. 1 Morphology of embryos of *Anemopaegma glaucum*. **a** Embryo of monoembryonic seed. **b** Abnormal embryos of polyembryonic seed with two embryos. **c** Abnormal embryos of polyembryonic seed with four embryos. Bars = 0.25 cm

A. acutifolium (2.63 ± 0.80) and from two to six in *A. glaucum* (2.63 ± 0.87). In fruits resulting from cross-pollination, the number varied from two to three in *A. acutifolium* (2.37 ± 0.51) and two to five in *A. glaucum* (2.96 ± 1.02 ; Table 2). The number of embryos per seed from different pollination treatments of *A. glaucum*, fruits resulting from cross-pollination produced significantly more embryos than those derived from self-pollination ($T = -2.29$; $df = 177$; $p < 0.05$).

There was no correlation between the number of viable seeds per fruit and the mean number of supernumerary embryos produced by seeds of the same fruits in *A. acutifolium* ($r^2 = 0.092$, $p = 0.1974$; Fig. 2a), *A. arvense* ($r^2 = 0.07$, $p = 0.2293$; Fig. 2b) or *A. glaucum* ($r^2 = 0.142$, $p = 0.0918$; Fig. 2c).

Germination test

Seed germination ranged from 12.5 to 100 % in fruits resulting from natural pollination. Despite this wide variation among fruits, the average germination was above 60 % in all species (*A. acutifolium* = 69.78 %; *A. album* = 78.21 %; *A. arvense* = 73.97 %; *A. glaucum* = 68.96 %; *A. scabriusculum* = 65.53 %). In fruits from controlled hand-pollination, the highest average percentage of germination was observed in seeds from self-pollination for both species, *A. arvense* (self = 62.75 %; cross = 46.67 %) and *A. glaucum* (self = 58.02 %; cross = 53.42 %). All seeds that germinated had wings partially removed; seeds with intact wings did not germinate.

Mono- and polyembryonic seeds were found in all polyploid species, independent of the pollination treatment (Table 3). The percentage of polyembryonic seeds observed in fruits from natural pollination ranged from 10.52 to 58.34 % (22.96 %) in *A. acutifolium*, from 0 to

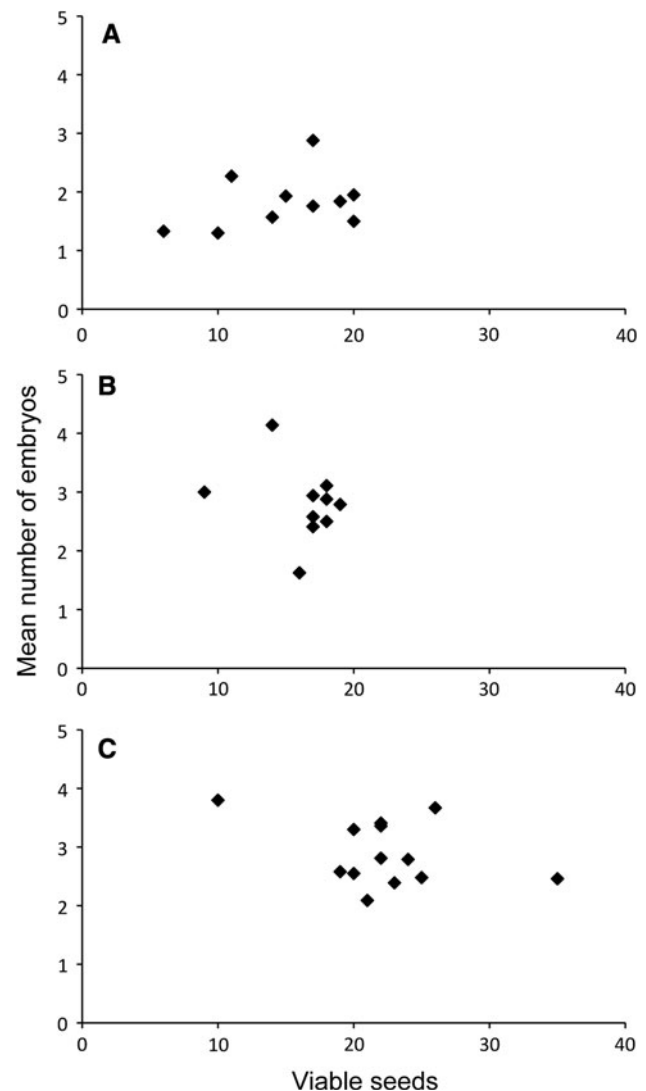


Fig. 2 Relationship between the number of viable seeds per fruit and mean number of embryos per seed in fruits from natural pollination. **a** *A. acutifolium* ($n = 10$ fruits; $n = 149$ seeds); **b** *A. arvense* ($n = 10$; $n = 163$ seeds). **c** *Anemopaegma glaucum* ($n = 14$ fruits; $n = 312$ seeds)

100 % (36.14 %) in *A. arvense*, from 16.67 to 100 % (47.28 %) in *A. glaucum* and from 5.26 to 20 % (10.98 %) in *A. scabriusculum*.

In *A. arvense* and *A. glaucum*, polyembryonic seeds in fruits from self-pollination (mean 35.07 and 29.58 %, respectively) were more common than in fruits from cross-pollination (mean 3.98 and 9.47 %, respectively). For *A. arvense* and *A. glaucum*, the chi-square test indicated a significant difference between self- versus cross-pollination for the number of polyembryonic seeds (*A. arvense*: $\chi^2 = 42.05$; $df = 1$; $p < 0.05$; *A. glaucum*: $\chi^2 = 9.71$; $df = 1$; $p < 0.05$).

In all the polyembryonic species of *Anemopaegma*, the number of seedlings produced per seed ranged from two to

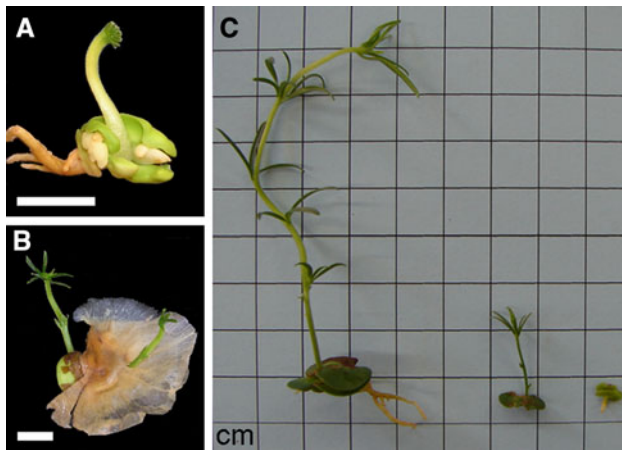


Fig. 3 Seedlings of *Anemopaegma glaucum*. **a** Polyembryonic seed with seedling and two embryos; 15 days after germination. **b** Polyembryonic seed with two seedlings. **c** Seedlings, from polyembryonic, 45 days after germination. Bars = 2.0 cm (**a**); 1.0 cm (**b**)

four, but predominantly two (Fig. 3a, b), except in *A. scabriusculum* whose polyembryonic seeds produced only two seedlings. Embryos of about 0.5 cm germinated, but seedlings did not develop (Fig. 3c), whereas embryos less than 0.5 cm and membranous cotyledons did not germinate.

Comparing the number of polyembryonic seeds obtained in both tests, dissection and germination, the chi-square test indicated a significant difference between tests for *A. acutifolium* ($\chi^2 = 15.11$; $df = 1$; $p = 0.0001$), *A. arvense* ($\chi^2 = 85.75$; $df = 1$; $p < 0.0001$) and *A. glaucum* ($\chi^2 = 41.34$; $df = 1$; $p < 0.0001$). In these species, the number of polyembryonic seeds observed by seed dissection was higher than by germination testing. In *A. scabriusculum* no significant difference was found ($\chi^2 = 0.03$; $df = 1$; $p = 0.8625$).

Discussion

Polyembryonic seeds were recorded in all polyploid species of *Anemopaegma* samples (*A. acutifolium*, *A. arvense*, *A. glaucum* and *A. scabriusculum*), but absent in *A. album*, the only diploid species included in this study. The patterns of polyembryony and polyploidy detected here have been previously reported in other species of Bignoniaceae and in other families, such as Malvaceae (Baker 1960; Mendes-Rodrigues et al. 2005) and Orchidaceae (Teppner 1996). In Bignoniaceae, the relationship between polyploidy and polyembryony was observed in *H. chrysotrichus* (Mart. ex DC) Mattos (Piazzano 1998; Ortolani 2007; Bittencourt and Moraes 2010) and *H. ochraceous* (Cham.) Mattos (Bittencourt Jr 2003; Bittencourt and Moraes 2010). Ortolani (2007) demonstrated the existence of diploid and tetraploid cytotypes in *H. chrysotrichus* and noticed the

occurrence of polyembryony among polyploid individuals exclusively. In *Anemopaegma*, polyembryony was recorded in *A. acutifolium* (Sampaio 2010) and *A. arvense* (Pereira et al. 2007), both of which included in the present study, as well as in *A. chamberlaynii* (Correia et al. 2005). No relationship between polyploidy and polyembryony was observed in *Dolichandra unguis-cati* (L.) LG Lohmann (*Macfadyena unguis-cati* (L.) AH Gentry), a polyploid species (Bowden 1945; Venkatasubban 1944 *apud* Darlington and Wylie 1961; Jullier 1989; Piazzano 1998). According to the population level studies, the absence of polyembryony in polyploid species, or of polyploidy in polyembryonic species, could be attributed to a lack of investigations of such features in the same population. Therefore, future research should unite these two aspects so that the relationship between high ploidy levels and polyembryony can be corroborated or refuted for other species of Bignoniaceae. In the case of *A. chamberlaynii*, another possible explanation for the lack of relationship between polyploidy and polyembryony is the formation of supernumerary embryos in a few seeds (only two seeds in all samples; Correia et al. 2005) by processes unrelated to apomixis, such as cleavage of the zygotic embryo. On the other hand, the absence of polyembryonic seeds in fruits of *Dolichandra unguis-cati*, a polyploid species, can be explained by a recent polyploidization since polyembryony, generated by adventitious embryony, is more common in paleopolyploids (Carman 1997).

The absence of fruit production in flowers of *Anemopaegma* species that have not been through an active process of pollination indicates that the fertilization is required for the formation of the endosperm, a resource that is necessary for the development of adventitious embryos. This finding corroborates the results found for *Handroanthus chrysotrichus*, another apomictic species of Bignoniaceae. Histological studies performed in pistils of the *H. chrysotrichus* showed that the formation of the precursor cells of adventitious embryos does not depend on ovule fertilization and/or endosperm initiation, because these cells are formed in ovules of unpollinated pistils. However, the development of adventitious embryos requires nutritional supply from the endosperm (Bittencourt and Moraes 2010). In apomictic species that have no autonomous endosperm development, the adventitious embryos only grow to a certain stage by obtaining nutrients from degradation of the cells of the nucellus and inner integument. However, these embryos only reach the early stages of development and do not produce viable seeds (Wakana and Uemoto 1987; Naumova 1993; Koltunow and Grossniklaus 2003; Bittencourt and Moraes 2010).

The fruit and seed set by either self- or cross-pollination indicates that *A. acutifolium*, *A. arvense* and *A. glaucum* are self-fertile. Despite the widespread occurrence of

late-acting self-incompatibility (LSI) in the Bignoniaceae (Gibbs and Bianchi 1993, 1999; Bittencourt Jr 2003; Bittencourt et al. 2003; Bianchi et al. 2005; Bittencourt and Semir 2004, 2005, 2006; Gandolphi and Bittencourt 2010), polyploid and apomictic species of this family are self-compatible (Bittencourt and Moraes 2010; Sampaio 2010). Polyploid plants self-fertilize to a greater degree than their diploid relatives; therefore, an association between polyploidy and self-compatibility seems to exist (Barringer 2007; Hörandl 2010). However, Oliveira et al. (1992) and Bittencourt and Moraes (2010) suggested that self-fertility of polyploids and apomictic species of Bignoniaceae do not result from the breakdown of LSI, but from the emergence of pseudogamic apomixis favored by delay in the rejection of self-pollinated pistils. In this case, the adventitious embryos may sequester maternal resources that would prevent the abortion of these pistils. According to Gibbs and Bianchi (1993), adventitious embryos of selfed pistils would generate a correct hormonal feedback that is identical to that generated by developing crossed ovules, which would prevent the abortion of pistils after self-fertilization. In *H. chrysotrichus*, self-fertility seems to have resulted from the emergence of pseudogamic apomixis (Bittencourt and Moraes 2010).

Dissection tests performed on mature seeds of *A. acutifolium*, *A. arvense* and *A. glaucum* showed a high percentage of polyembryonic seeds per fruit, a highly variable number of embryos produced per seed and abnormal morphology of several embryos. Similar results were found by dissection tests in polyembryonic species of Bignoniaceae with adventitious embryony: *A. acutifolium* (Sampaio 2010), *H. chrysotrichus* (Bittencourt and Moraes 2010) and *H. ochraceus* (Costa et al. 2004). These findings suggest that adventitious embryony is also present in *A. arvense* and *A. glaucum*. Adventitious embryony, the most common form of apomixis for the formation of polyembryonic seeds, is characterized by a high number of polyembryonic seeds per fruit and seeds with multiple embryos at different stages of development (Naumova 1993). On the other hand, polyembryony in *A. scabriusculum* was found in only a small percentage of seeds, with each polyembryonic seed only producing two embryos. The sporadic occurrence of polyembryony in *A. scabriusculum* and the low production of embryos per seed may result from other factors that are unrelated to sporophytic apomixis (Sampaio 2010) or may result from the short period of fruit development, since this species has two to three reproductive cycles per year (Silva and Queiroz 2003). The other three polyembryonic species of *Anemopaegma* have only one flowering and fruiting event per year, with the fruits taking up to 10 or 12 months to reach full development (Firetti-Leggieri 2009). As observed in polyembryonic species of *Handroanthus* (Costa et al. 2004;

Bittencourt and Moraes 2010) and *Citrus* (Koltunow 1993), initiation of supernumerary embryos during the process of seed maturation and embryo competition for space and resources is responsible for the presence of embryos at different stages of development and different morphologies in polyembryonic seeds (Lakshmanan and Ambegaokar 1984).

Our study has detected a difference in the number of polyembryonic seeds per fruit according to the method of analysis used. The number of polyembryonic seeds verified in fruits from natural pollination of *A. acutifolium*, *A. arvense* and *A. glaucum* revealed by dissection test was significantly higher than that recorded by germination tests. This pattern was also found in *Handroanthus ochraceus* (Salomão and Allen 2001; Costa et al. 2004) and *H. chrysotrichus* (Bittencourt and Moraes 2010). Using germination tests, Salomão and Allen (2001) reported 3 % polyembryonic seeds for *H. ochraceus*, while Costa et al. (2004) reported 81.37 % of seeds using seed dissection. Bittencourt and Moraes (2010) detected over 80 % of polyembryonic seeds histological, against 51–55 % of polyembryonic seeds by germination tests. Furthermore, the number of embryos per seed detected by dissection was higher than the number of seedlings emerging from polyembryonic seeds. The discrepancy between tests may result from the unviability of embryos with reduced dimensions and membranous cotyledons (i.e., with anomalous morphology), which do not produce seedlings and, hence, are detected only through seed dissection. The inability of embryos with reduced size and membranous cotyledons to generate seedlings was demonstrated through the germination of single embryos. The difference in the morphology of embryos and their ability to germinate and form seedlings might be explained by the continuous formation and maturation of adventitious embryos, such as those observed in *Citrus* (Koltunow 1993), by competition for resources (Uma Shaanker and Ganeshaiyah 1997; Mendes-Rodrigues et al. 2005; Bittencourt and Moraes 2010) or space for their complete maturation (Wakana and Uemoto 1987; Costa et al. 2004). As observed in *Eriotheca pubescens* (Mendes-Rodrigues et al. 2005, 2011), embryo development of *Anemopaegma* into seedlings seems to depend on their size and weight at the time of germination.

Source of pollen influenced the number of polyembryonic seeds per fruit and the number of embryos per seed in *A. glaucum*. The dissection test indicated a higher number of polyembryonic seeds and embryos per seed in fruits derived from cross-pollination than in fruits derived from self-pollination. On the other hand, the germination test indicated a higher number of polyembryonic seeds in fruits resulting from self-pollination, as opposed to polyembryonic seeds in fruits resulting from cross-pollination. Germination testing is similar to those found in *Opuntia*

(Cactaceae; Mondragon 2001), but differ from those obtained for *Eriotheca pubescens* (Malvaceae, Bombacoideae; Mendes-Rodrigues et al. 2005). In apomictic species belonging to self-incompatible groups, like *Anemopaegma*, seeds from cross-pollination seem to produce fewer adventitious embryos due to the greater vigor of the sexual embryo (Mendes-Rodrigues et al. 2005). The results obtained from germination tests for fruits from controlled hand-pollination of *A. glaucum* seem to corroborate this assumption. Although seeds from cross-pollination produce more supernumerary embryos, these embryos are less vigorous and are not converted into seedlings. In those cases, the offspring might compete intensely among themselves for maternal resources, leading to starvation or death of their siblings (Uma Shaanker and Ganeshaiiah 1997). The extent of sibling rivalry would be a function of the genetic relatedness among the offspring, being smaller among siblings that are more genetically related. In polyembryonic seeds, this competition would be more intense among less genetically related embryos generated by cross-pollination, which would explain the lower rate of embryo development into seedlings from cross-pollinated seeds.

In a study of several polyembryonic species of *Citrus*, the mean number of embryos produced by seeds was shown to be inversely related to the number of viable seeds in each fruit (Ganeshaiiah et al. 1991). Furthermore, species with adventitious embryony, such as in *Citrus*, were shown to produce supernumerary embryos to compensate for the loss of progeny by seed abortion, which was triggered by competition seeds of the same fruit (Ganeshaiiah et al. 1991; Uma Shaanker and Ganeshaiiah 1996, 1997). Therefore, the greatest number of embryos was found in fruits that had greater propensity to seed abortion. However, the correlation observed in *Citrus* species was not observed in the three studied species of *Anemopaegma*; instead, the number of embryos found in seeds of each fruit was unrelated to the number of viable seeds of each fruit. In *Anemopaegma* species, the production of several embryos in each seed is a strategy for colonization and survival in environments with periodic environmental disturbances, such as those found in the “cerrado,” rather than a way for the mother plant to minimize the loss of offspring through competition.

We highlight the importance of the results of *A. arvense* due to its wide use in folk medicine for its antitumor action (Lee et al. 2002; Uchino et al. 2004) and in the production of cosmetics (Kokou et al. 2000; Shimizu 2001). Because of its multiple properties, this species was included in a conservation program of the State of São Paulo, the Biodiversity Research Program of the State of São Paulo (BIOTA; Pereira et al. 2007). The identification of polyembryony might be useful for large-scale cultivation of this

species, especially since apomixes can lead to the formation of genetically uniform large populations and perpetuate hybrid vigor through successive generations of seeds (Calzada et al. 1996; Bicknell and Koltunow 2004).

The results reported here contribute to the knowledge of the reproductive behavior of *Anemopaegma* species and increase the number of records of polyembryony in polyploid species of Bignoniaceae. The occurrence of polyembryony, along with self-fertility, seems to be important for the maintenance of hybrids generated by interspecific crosses and for the colonization of open areas of “cerrado.” Even though we showed that adventitious embryony is in *Anemopaegma*, histological studies, chromosome counts and reproductive biology are needed to further understand the diversity of reproductive strategies encountered, as well as the processes that generate this diversity in representatives of the Bignoniaceae.

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