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Sporophytic apomixis in polyploid *Anemopaegma* species (Bignoniaceae) from central Brazil

DIANA SALLES SAMPAIO[1], NELSON SABINO BITTENCOURT JÚNIOR[2] and PAULO EUGÊNIO OLIVEIRA[1]*

[1]*Instituto de Biologia, Universidade Federal de Uberlândia, Campus Umuarama, Uberlândia, 38400-902 Minas Gerais, Brazil*
[2]*Departamento de Zoologia e Botânica, Universidade Estadual Paulista 'Júlio de Mesquita Filho', Campus de São José do Rio Preto, Rua Cristóvão Colombo, 2265, Jardim Nazareth, São José do Rio Preto, 15054-000 São Paulo, Brazil*

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Apomixis and polyploidy have been important in the evolution of the angiosperms, and sporophytic apomixis has been associated with polyembryony and polyploidy in tropical floras. We studied the occurrence of polyembryony in populations of tetraploid *Anemopaegma acutifolium*, *A. arvense* and *A. glaucum* from the Brazilian cerrados, and histological features of sexual and apomictic processes were investigated in *A. acutifolium*. All populations and species were polyembryonic (68.9–98.4% of seeds). Normal double fertilization occurred in most ovules, with exceptions being that 3% of ovules were penetrated but not fertilized and in 4% of ovules both synergids were penetrated. The penetration of both synergids suggests a continuous attraction of pollen tubes and polyspermy. Adventitious embryo precursor cells (AEPs) arose from nucellar and integumental cells of the ovule in pollinated and unpollinated *A. acutifolium*, indicating sporophytic apomixis. However, further embryo and endosperm development required pollination and fertilization. This pseudogamy also allows concurrent sexual embryo development. Similar polyembryony rates and polyploidy indicated that *A. arvense* and *A. glaucum* are also apomictic, forming an agamic complex similar to that observed for some species of confamilial, but not closely related *Handroanthus*. The co-occurrence of apomixis and polyploidy in different groups of Bignoniaceae indicates homoplasious origin of these agamic complexes. © 2013 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2013, **173**, 77–91.

ADDITIONAL KEYWORDS: catuaba – double fertilization – polyembryony – polyspermy – pseudogamy – synergids – tetraploid.

INTRODUCTION

Apomixis is a process in which asexual embryos develop inside the seed, and it can have important consequences for the ecology and evolution of apomictic species (Asker & Jerling, 1992; Koltunow, 1993; Hörandl, 2010). The mechanisms of apomictic expression have been extensively investigated because of its potential for crop breeding, and allopolyploidy seems to be a trigger for its expression in many angiosperms (Paun, Stuessy & Hörandl, 2006; Carman, 2007; Hörandl, 2010). Although this relationship has been described for mostly temperate gametophytic apomictic species (Asker & Jerling, 1992; Richards, 2003; Whitton *et al*., 2008), tropical sporophytic apomictic species also seem to be polyploid (Oliveira *et al*., 1992; Piazzano, 1998; Mendes-Rodrigues *et al*., 2005; Bittencourt Júnior & Moraes, 2010). Studies that detect apomictic species and describe details of apomictic processes are important to understand evolution and diversification of tropical plants and ecological relationships in tropical communities (Asker & Jerling, 1992; Batygina, 1999a, b).

In gametophytic apomixis, apospory involves the concomitant development of reduced and unreduced embryo sacs, whereas, in diplospory, only one unre-

*Corresponding author. E-mail: poliveira@ufu.br

duced embryo sac is produced; in both cases, asexual embryos develop from the unreduced egg cell (Koltunow, 1993). In sporophytic apomixis, adventitious embryos arise from somatic tissues of the ovule, more or less in parallel with sexual embryogeny (Koltunow, 1993). Apospory and sporophytic apomixis often induce the production of more than one embryo per seed, resulting in polyembryonic seeds (Asker & Jerling, 1992; Koltunow, 1993). However, studies in Bignoniaceae and Malvaceae indicate that sporophytic apomixis results in much higher percentages of polyembryonic seeds than in aposporic gametophytic apomicts (Mendes-Rodrigues *et al.*, 2005, 2012; Mendes-Rodrigues & Oliveira, 2012). Although polyembryony is a good indicator of apomixis, it can also be a result of sexual processes by the development and fertilization of more than one reduced embryo sac, or by zygote cleavage (Maheshwari, 1950), and histological studies are thus necessary to confirm an apomictic origin.

Although autonomous endosperm formation occurs in some gametophytic apomictic species, in most apomicts pollination and formation of a bisexual endosperm are necessary for asexual embryos to develop (Asker & Jerling, 1992; Koltunow & Grossniklaus, 2003; Hörandl, 2010). Pollination followed by fertilization can also provide nourishment for a sexual embryo to reach maturity in the same seed (Batygina, 1999a, b ; Batygina & Vinogradova, 2007). Sexual embryos, if they survive, increase genetic variability in apomictic plant populations, but such populations can also maintain well-established genotypes through clonal adventitious embryo formation (Batygina, 1999a, b; Bayer & Chandler, 2007; Hörandl & Paun, 2007; Talent & Dickinson, 2007).

Polyploid agamic complexes with sporophytic apomixis have recently been described for some tropical woody Bignoniaceae and Malvaceae (Oliveira *et al.*, 1992; Costa *et al.*, 2004; Mendes-Rodrigues *et al.*, 2005; Bittencourt Júnior & Moraes, 2010). Most Bignoniaceae have diploid chromosome number of $2n = 40$ (Goldblatt & Gentry, 1979) and polyploidy is often, but not exclusively, found in apomictic species (Goldblatt & Gentry, 1979; Piazzano, 1998; Bittencourt Júnior & Moraes, 2010). The cases in which polyploidy is related to apomixis are described for some, possibly hybrids of *Handroanthus* Mattos (Gentry, 1992), that form polyploid agamic complexes with variations in ploidy and breeding system between populations of the same species (Gibbs & Bianchi, 1993; Piazzano, 1998; Costa *et al.*, 2004; Guerra & Natera, 2007; Bittencourt Júnior & Moraes, 2010; D. S. Sampaio unpubl. data).

Anemopaegma Mart. ex Meisn., with species that are mostly lianas, includes several shrubby species in the cerrados, the Neotropical savanna areas in central Brazil (Gottsberger & Silberbauer-Gottsberger, 2006). Three of these cerrado *Anemopaegma* shrubs, *A. acutifolium* DC., *A. arvense* (Vell.) Stellf. ex de Souza and *A. glaucum* Mart. ex DC., (the *A. arvense s.l.* species complex), are tetraploids with a putative hybrid origin (Firetti-Leggieri *et al.*, 2011). If these species show the same relationship between polyploidy (probable allopolyploidy) and apomixis, as reported for some *Handroanthus* spp., the elucidation of the apomictic mechanism in *Anemopaegma* could potentially contribute to our understanding of the evolution of polyploid species in the genus, and also aid ecological studies of cerrado species. Moreover, as polyploid *Anemopaegma* spp., locally known as catuaba, have potential pharmacological applications (Uchino *et al.*, 2004; De Andrade *et al.*, 2008) and are currently collected from natural populations (Pereira *et al.*, 2003), the stability of apomictic populations with clonal embryos would be of great interest for plant breeding programmes (Koltunow & Grossniklaus, 2003), which may be an alternative to tissue culture (Pereira *et al.*, 2003).

Polyploidy has been associated with sporophytic apomixis and high polyembryony rates in other Bignoniaceae of putative hybrid origin (Costa *et al.*, 2004; Bittencourt Júnior & Moraes, 2010). Because such species also have putative hybrid origin and are described as tetraploids (Firetti-Leggieri *et al.*, 2011), we hypothesized that similar associations would be found in tetraploid populations of *A. acutifolium*, *A. arvense* and *A. glaucum*. In this study, we show that *A. acutifolium* is a species with sporophytic apomixis and high percentages of polyembryonic seeds. Sexually produced embryos are also formed concurrently with apomictic embryos in the same seed. We also document tetraploidy and polyembryony in *A. arvense* and *A. glaucum* and discuss the consequences of sporophytic apomixis for the ecology and evolution of the group.

MATERIAL AND METHODS

STUDY SPECIES AND SITES

The three species *A. acutifolium*, *A. arvense* and *A. glaucum* (Fig. 1A–C), are subshrubs with well-developed underground systems. *Anemopaegma* spp. characteristically have trifoliolate compound leaves and white tubular flowers with a yellow throat (Fig. 1B, C). The fruit is a stipitate capsule that reaches maturity in approximately 1 year. The wind-dispersed seeds are disc like and winged (Fig. 1D).

The study was conducted from 2006 to 2008 in different cerrado areas in Goiás (GO), Minas Gerais (MG) and São Paulo (SP) states, in Brazil. The presence and frequency of polyembryonic seeds and the ploidy of seedlings were investigated in three popu-

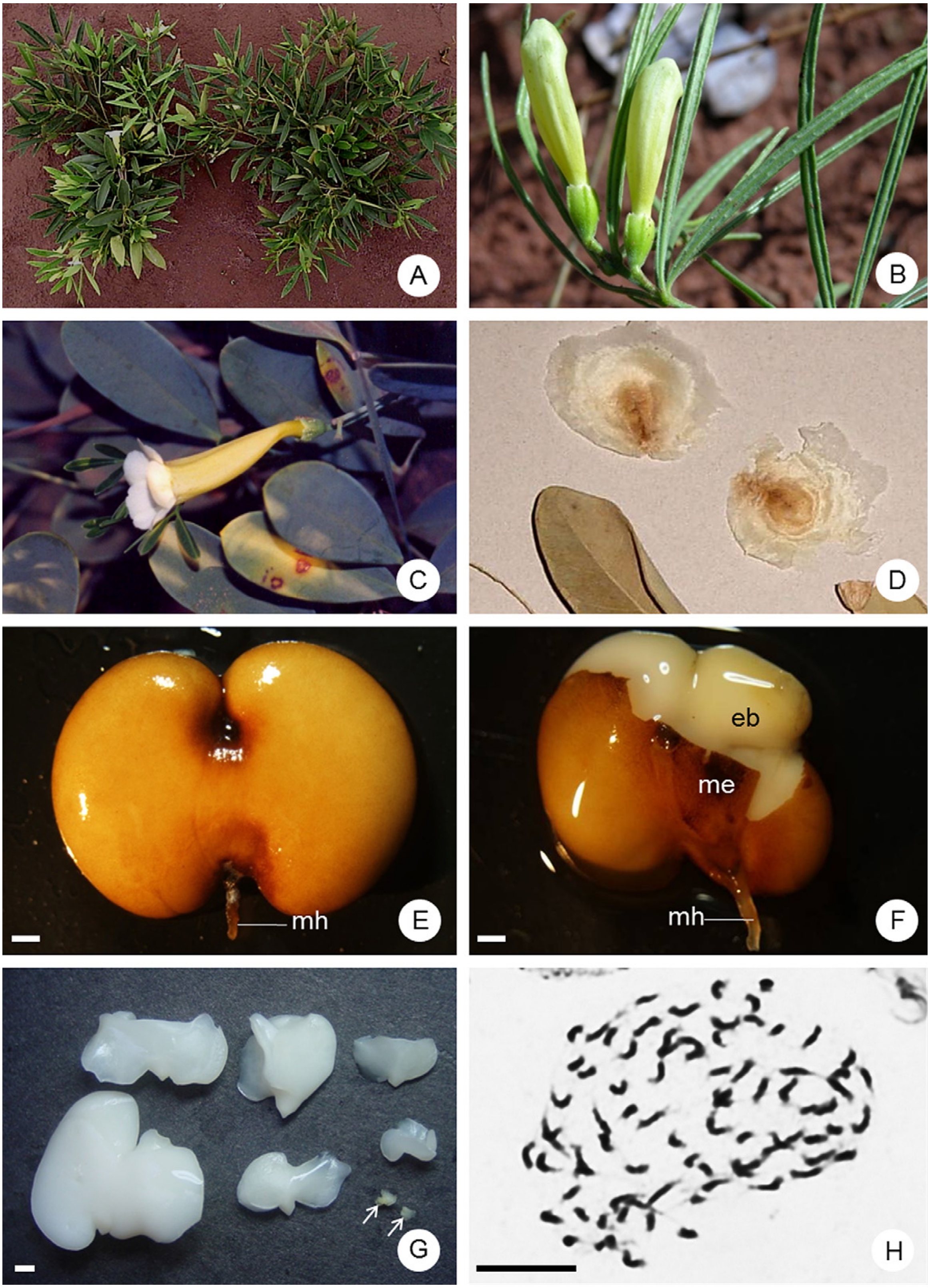

Figure 1. See caption on next page.

Figure 1. *Anemopaegma* species studied, polyembryony and chromosome counting. A, *Anemopaegma acutifolium* habit. Photo: Marcelo Rodrigo Pace. B, leaves and flower buds of *A. arvense*. C, leaves and flower of *A. glaucum*. D, seeds of *A. glaucum*. E–G, pictures taken with a stereomicroscope. E–F, *A. glaucum*. E, embryo(s) surrounded by membranaceous tissue. (mh) micropylar haustorium of the endosperm. Scale bar, 1 mm. F, Membranaceous tissue (me) being removed to count embryo number per seed. (eb) embryo. Scale bar, 1 mm. G, eight embryos from the same seed of *A. acutifolium*. Two smaller embryos are indicated by arrows. Scale bar, 1 mm. H, photomicrograph of a mitotic prometaphase from the root apical meristem of *A. arvense*, $2n = 80$. Scale bar, 10 μm.

lations of *A. acutifolium*, three of *A. arvense* and one of *A. glaucum* (Table 1). Seed collections were made from three to 15 individuals in each population. For *A. acutifolium*, we collected three fruits at Fazenda Água Limpa, MG and seven fruits in each of the other populations. For *A. arvense*, we collected nine fruits in Caldas Novas, GO and five fruits in each of the other populations. Finally, for *A. glaucum*, we collected 27 fruits at Santana do Riacho, MG. The number of seeds analysed varied for each population (Table 1). The material for histological analyses was collected from 17 individuals of the population of *A. acutifolium* located in a private natural reserve of the Clube de Caça e Pesca Itororó de Uberlândia (CCPIU), Uberlândia, MG. Vouchers were deposited in Herbarium Uberlandense (HUFU), Uberlândia, MG, under numbers HUFU 44909, 47295, 48834, 50820, 50821 and 50823.

Chromosome number analysis

Chromosome counts were made to check any association between polyploidy, apomixis and polyembryony. Although the three species studied have been already reported as tetraploid (Firetti-Leggieri *et al*., 2011), variations in ploidy are common among agamic complexes (Talent & Dickinson, 2007). Root apical meristems were obtained by germination of seeds sowed in Gerbox plastic boxes at room temperature (25–28 °C) and under natural light. The root tips were pretreated in para-dichlorobenzene (Aldrich) saturated solution for 4 h, between 16 and 18 °C. They were then fixed in 3:1 Carnoy solution (three parts of ethanol:1 part of glacial acetic acid) for 24 h and stored in 70% ethanol in a freezer below 0 °C. The root tips were digested in a solution of 5 M hydrogen chloride (HCl) for 20 min at room temperature and subjected to a standard squash technique. Coverslips were removed after immersion in liquid nitrogen. Staining was performed with 2% Giemsa solution. After drying, the slides were sealed with Entellan. We analysed from two to five slides (each slide corresponded to a seedling) of each population, and at least 20 cells, with clear chromosome morphology and separation. Chromosome counting was performed under an optical microscope (BX51TF; Olympus, Tokyo, Japan) and good metaphase plates were photographed with a digital camera (DP70; Olympus, Tokyo, Japan).

Embryo number per seed

To define the frequency of polyembryonic seeds and number of embryos per seed, mature seeds from all populations were fixed and stored in formaldehyde–acetic acid-alcohol (FAA)70 (Johansen, 1940). The seeds were dissected with fine-tipped tweezers and scalpels under a stereo microscope (SZ40; Olympus, Tokyo, Japan) to verify the number of embryos per seed. As many individuals with mature fruits were found only for the *A. glaucum* population, we used this species to compare the differences in mean embryo number per seed among individuals. Fifteen individuals were studied, and from five to 43 seeds were analysed per individual (in total, 292 seeds were dissected; Table 1). The data were analysed using a Kruskal–Wallis test in BioEstat (Ayres *et al*., 2007).

Histological analysis

Histological analysis of pistils and young fruits of *A. acutifolium* were performed to define the occurrence of apomixis, its mechanism and concurrent sexual process. Floral buds were bagged with nylon mesh to exclude pollinators and other floral visitors. Pistils of *A. acutifolium* were submitted to hand self- and cross-pollinations of previously emasculated first-day flowers, and two to seven pistils of each treatment were collected from 24 to 120 h after pollination (45 pistils were analysed). Five unpollinated pistils from first-day emasculated flowers were also collected 120 h after the onset of anthesis. After emasculation or controlled pollination treatments, flowers were bagged again until they were collected. All collected pistils resulting from pollination treatments had ovaries/young fruits shorter than 3.5 cm. Other fruits, > 5.0 cm long, resulting from untreated, naturally pollinated pistils, were used to observe advanced developmental stages. Entire pistils from pollination treatments and seeds extracted from larger fruits were fixed in a 1% glutaraldehyde and 4% formaldehyde solution (McDowell & Trump, 1976) in sodium

Table 1. Chromosome number (number of seedlings), mean number of embryos per seed, percentage of polyembryonic seeds and frequency of seeds with different number of embryos in distinct populations and collection years for *Anemopaegma acutifolium*, *A. arvense* and *A. glaucum*

Species	Population/ geographical location	Chromosome number (*n*)	Collection year	Mean number of embryos per seed ± SD (*n*)	Polyembryonic seeds %	Frequency of seeds with different number of embryos (%)													
						1	2	3	4	5	6	7	8	9	10	11	12	13	14
A. acutifolium	Botucatu, SP 22°57′07.1″S, 48°29′10.6″W	$2n = 80$ (3)	2008	2.52 ± 1.01 (96)	87.5	12.5	42.6	29.4	12.4	2.1	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. acutifolium	CCPIU, Uberlândia, MG 18°58′48.5″S, 48°17′45.8″W	$2n = 80$ (3)	2007	5.21 ± 2.59 (143)	97.2	2.8	11.2	14.7	13.3	18.9	13.3	7.0	8.4	2.8	0.7	4.2	1.4	0.0	0.7
			2008	3.48 ± 1.88 (97)	86.6	13.4	21.7	20.6	19.6	6.2	15.5	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0
A. acutifolium	Faz. Água Limpa, Uberlândia, MG 19°05′22″S, 48°21′15″W	$2n = 80$ (5)	2006	2.59 ± 1.50 (27)	74.1	25.9	29.6	22.2	11.1	7.4	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. arvense	Bauru, SP 22°19′32.7″S, 49°00′34.3″W	$2n = 80$ (2)	2008	2.07 ± 0.89 (45)	68.9	31.1	35.6	28.9	4.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. arvense	Botucatu, SP 22°57′07.1″S, 48°29′10.6″W	$2n = 80$ (2)	2008	2.22 ± 0.91 (50)	78.0	22.0	44.0	24.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. arvense	Caldas Novas, GO 17°46′45″S, 48°40′26″W	$2n = 80$ (3)	2007	2.44 ± 0.98 (18)	88.9	11.1	50.0	27.8	5.6	5.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. glaucum	Santana do Riacho, MG 19°18′19.3″S, 43°36′17.5″W	$2n = 80$ (2)	2006	3.90 ± 1.52 (308)	98.4	1.6	16.2	27.0	22.1	20.5	7.1	3.9	0.7	0.3	0.7	0.0	0.0	0.0	0.0

phosphate buffer 0.1 M, pH 7.2. After dehydration in an ethanol series, an ethanol:chloroform series (3:1, 1:1, 3:1) was used to remove epicuticular waxes. Embedding was carried out with hydroxyethylmethacrylate (Leica Microsystems Inc., Heidelberger, Germany) (Gerrits & Smid, 1983) and serial sections from 2 to 4 μm thick were obtained using a rotary microtome (RM2135; Leica, Wetzlar, Germany) with a 8-mm wide Leica glass knife. The sections were stained with Toluidine Blue O 0.05%, in benzoate buffer, pH 4.4 (Feder & O'Brien, 1968), and the slides were sealed with Permount. Analyses and photomicrographs were made using a light microscope (BX51TF; Olympus, Tokyo, Japan) equipped with a digital camera (DP70; Olympus, Tokyo, Japan).

RESULTS

Polyembryony and chromosome number

All individuals sampled had $2n = 4x = 80$ and polyembryonic seeds, with up to 14 embryos in a single seed (Table 1, Fig. 1E–H). During seed dissection, a membranaceous brownish tissue surrounded the embryos, maintaining them together as a unit (Fig. 1E, F). Embryos in distinct developmental stages were encountered in the mature seeds (Fig. 1G). The mean embryo number per seed ranged from 2.07 to 5.21 and the percentage of polyembryonic seeds varied from 68.89 to 98.38% for the different populations (Table 1).

In the studied population of *A. glaucum*, the percentages of polyembryonic seeds varied from 92.86% to 100%; and all seeds analysed were polyembryonic in 12 out of the 15 individuals studied. Significant differences in mean embryo number per seed were detected ($H = 40.111$, $P = 0 < 0.001$), varying from 3.09 ± 1.14 to 5.14 ± 0.69.

Ovule characterization

In *A. acutifolium*, the ovules were anatropous, unitegmic and tenuinucellate (Fig. 2A). The embryo sac was of the *Polygonum* type (Fig. 2B–D), showing two mature intact synergids in first-day unpollinated pistils (Fig. 2B). The synergids remained intact in unpollinated pistils over subsequent days. Only one out of the 1045 ovules showed two embryo sacs (Fig. 2E). During anthesis the nucellus consisted of a hypostase and remnants of the nucellar epidermis that was degenerated at the micropylar region (Fig. 2C). The endothelium surrounded the embryo sac at the chalazal region, but was degenerated at the micropylar region (Fig. 2C).

Fertilization

Ovule penetration was observed from 24 h after pollination (Fig. 2C). Ovules without mature embryo sacs were not penetrated (Fig. 2A). After ovule penetration, the pollen tube discharged its cytoplasmic contents into one of the synergids, increasing its volume and showing enhanced stainability (Fig. 2C). Subsequently, the formation of a cytoplasmic loop was seen between the chalazal face of the egg cell and the central cell (Fig. 2D). Ovules with a deeply stained penetrated synergid and cytoplasmic loop were considered fertilized (*sensu* Bittencourt Júnior, Gibbs & Semir, 2003; Bittencourt Júnior & Semir, 2005). Ovules from both self- and cross-pollinated pistils were penetrated and fertilized without any structural differences detected between them (Fig. 2C, D). Some penetrated but non-fertilized ovules were observed, in which pollen tubes continued growing inside the embryo sac (Fig. 2F). Sometimes two pollen tubes or tangled pollen tubes were found within a single non-fertilized ovule, in which both synergids had a degenerate aspect (Fig. 2G). Each of the two synergids was penetrated by a different pollen tube in 3.0% of the fertilized ovules from self-pollinated pistils and in 5.7% of the fertilized ovules from cross-pollinated pistils (Fig. 2H). The presence of two penetrated synergids per ovule was observed from 24 to 120 h after pollination in ovules with up to an eight-celled endosperm, so that this double penetration did not

Figure 2. Longitudinal sections of ovules and young seeds showing the embryo sac structure and fertilization process in *Anemopaegma acutifolium* (all with the chalazal end oriented to the top). A, ovule without embryo sac observed 24 h after self-pollination. The complete nucellus (nu) can be seen. h, hypostase; ne, nucelar epidermis. B, non-penetrated synergids (s) in first-day unpollinated flowers. cc, central cell. C, embryo sac with a penetrated synergid (ps) and an egg cell (ec) 24 h after hand cross-pollination. at, antipodes; et, endothelium; pn, polar nuclei. D, non-penetrated synergid and penetrated synergid with a cytoplasmic loop (cl) 48 h after manual self-pollination. m, micropyle. E, ovule with two embryo sacs 96 h after cross-pollination. F, embryo sac penetrated by a pollen tube (arrows) that did not discharge its cytoplasmic content, 48 h after self-pollination. G, synergids with a degenerated aspect and two pollen tubes (arrows) penetrating the embryo sac without discharge of their cytoplasmic content, 96 h after cross-pollination. H, both synergids were penetrated by pollen tubes, 48 h after self-pollination. The tip of an arriving pollen tube is indicated by arrows. I, both synergids penetrated and two-celled endosperm, 48 h after self-pollination. ccc, chalazal chamber cell; mcc, micropylar chamber cell. Scale bars, 50 μm.

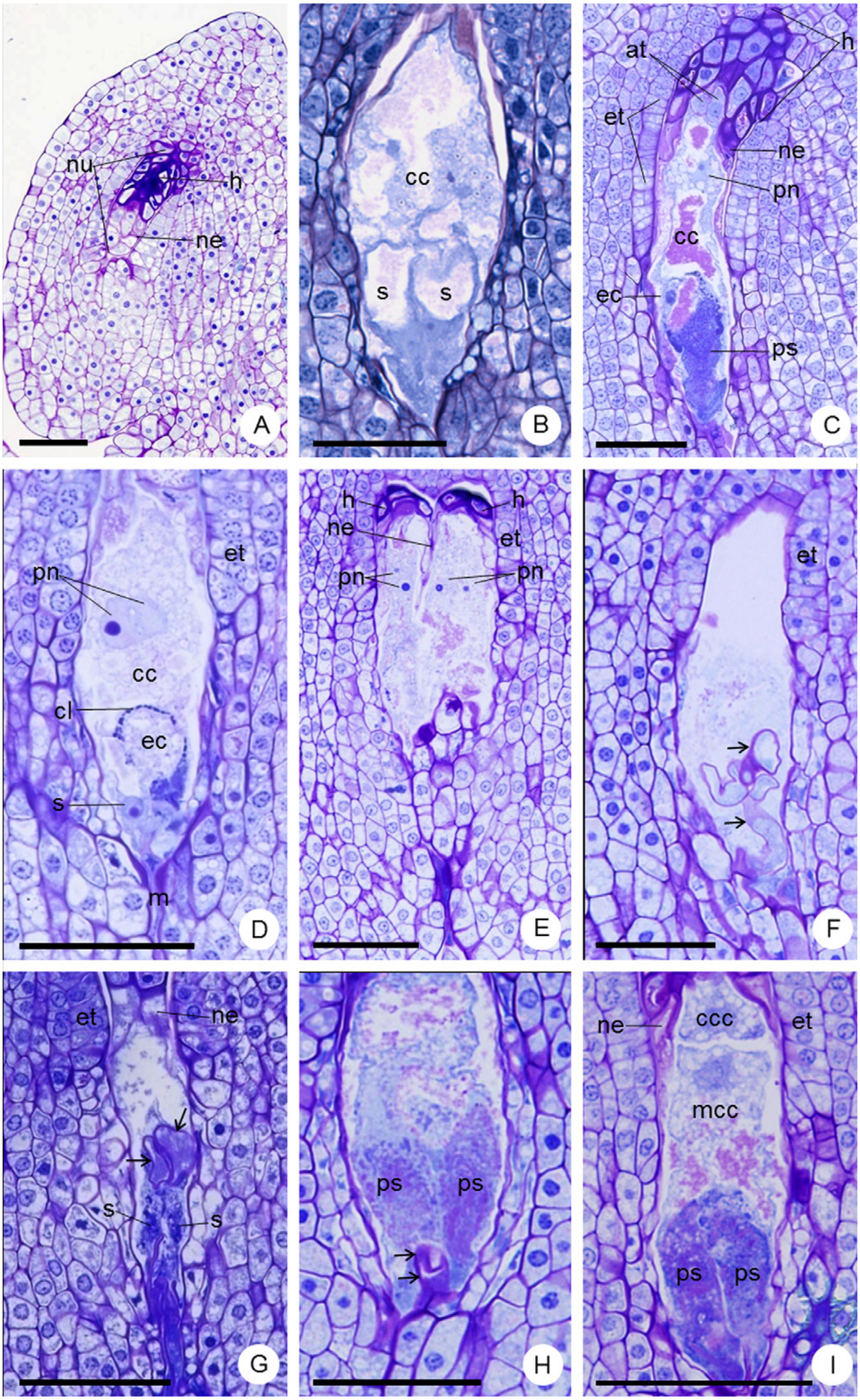

Figure 2. See caption on previous page.

seem to disturb the initial endosperm development (Fig. 2I).

Endosperm development

Polar nuclei did not fuse before the fertilization process (Fig. 2D). The endosperm was *ab initio* cellular, and a transverse primary endosperm cell division gave rise to a larger cell, the 'micropylar chamber' and a smaller one, the 'chalazal chamber' (Fig. 3A, B). The chalazal cell divided longitudinally, giving rise to two densely cytoplasmic cells that subsequently form a chalazal haustorium (Fig. 3C–F), while the micropylar cell divided longitudinally or transversely (Fig. 3C–E). Sometimes the second cycle of cell divisions was asynchronous in the micropylar and chalazal chambers, producing a transient three-celled endosperm. The formation of a six-celled endosperm occurred by a transverse or a longitudinal division of the two micropylar cells, depending on their former plane of division (Fig. 3D–F). A transient five-celled endosperm was formed whenever these divisions were asynchronous. Subsequently, the development of the endosperm proceeded by waves of transverse divisions of cells derived from the central tier of the six-celled endosperm. Some asynchrony between the two median cell divisions and the divisions of their derivatives allowed the occurrence of odd endosperm cell numbers. Up to 120 h after pollination, the pair of cells that originated from the chalazal chamber of the two-celled endosperm stage underwent a single simultaneous longitudinal division, giving a four-celled chalazal haustorium (Fig. 3G, H). Except for the chalazal haustorium, the endosperm remained with only two cell layers, with up to ten cells, at this stage after pollination (Fig. 3G).

In later stages, in naturally developed fruits 5.0 cm long or larger, the chalazal haustorium had degenerated and a circular group of large cells had differentiated at the chalazal end of the endosperm (Fig. 3I). Large and vacuolated endosperm micropylar cells also differentiated and constituted the micropylar haustorium (Fig. 4A, B). Periclinal and oblique divisions in the endosperm created a multilayered tissue, with the peripheral cells of the endosperm adjacent to the endothelium (Fig. 3I). The rupture of some mesotestal cell layers caused the embryos, endosperm, endothelium and a few inner layers of the mesotest to become isolated from the external layers of the integument (external layers of mesotest and the exotest), except at the chalazal region, where there was a connection with the vascular bundle (Fig. 4A). This group of peripheral endosperm and inner integument cell layers that surrounded the embryos corresponded to the membranaceous brownish tissue that enclosed the embryos at seed maturity (Fig. 1E, F). Endosperm did not develop in ovules of unpollinated pistils fixed up to 120 h after the onset of anthesis (Fig. 5A).

Supernumerary embryo origin

Hypostase cells were observed to protrude into the embryo sac, in ovules of unpollinated pistils collected 120 h after the onset of anthesis (Fig. 5A–C) and at the chalazal pole of the endosperm of young seeds of pollinated pistils collected 120 h after pollination (Fig. 5D–G). These hypostase cells were adventitious embryos precursor cells (AEPs). The AEPs that developed in pollinated pistils had a tube-like form with the nucleus in its distal portion (Fig. 5E–G), as observed for the zygote (Figs 3A, 5D), whereas those AEPs developed in unpollinated pistils did not have a regular shape and their nuclei were in the proximal portion (Fig. 5A–C). Up to 96 h after pollination, only the zygote was observed, whereas, at 120 h after pollination, this zygote was observed concomitantly with recently formed hypostase AEPs (Fig. 5D), at which stage no other AEPs coming from the micropylar region were observed.

In later stages, in naturally developed fruits larger than 5.0 cm, two elongated cells of the same length

Figure 3. Longitudinal sections of young *Anemopaegma acutifolium* seeds showing endosperm development (all with the chalazal end top oriented). A–C, 72 h after self-pollination. A, the arrow points to the first mitotic division of the primary endosperm nucleus. et, endothelium; h, hypostase; ne, nucelar epidermis; ps, penetrated synergid; z, zygote. Scale bar, 50 µm. B, two-celled endosperm. ccc, chalazal chamber cell; mcc, micropylar chamber cell. Scale bar, 50 µm. C, two-celled endosperm undergoing mitosis to form a four-celled endosperm (arrows). Scale bar, 50 µm. D, four-celled endosperm 48 h after self-pollination. ch, chalazal haustorium of the endosperm. Scale bar, 50 µm. E, four-celled endosperm 120 h after cross-pollination. The arrows point to the transversal cell wall between the two endosperm micropylar chamber cells. ed, endosperm. Scale bar, 50 µm. F, six-celled endosperm 72 h after cross pollination. Both synergids penetrated. Scale bar, 50 µm. G, ten-celled endosperm with four-celled chalazal haustorium 120 h after cross-pollination. The arrows point to the zygote. Scale bar, 50 µm. H, four-celled chalazal haustorium of the endosperm 120 h after self-pollination, only three cells can be seen in this section. at, antipodes. Scale bar, 20 µm. I, seed from a fruit that was more than 5.0 cm long. Multilayered endosperm with differentiated cells in the chalazal region and a degraded chalazal haustorium. Three globular proembryos can be seen. chc, chalazal haustorium chamber; dec, differentiated endosperm on the chalazal region; su, suspensor. Scale bar, 200 µm.

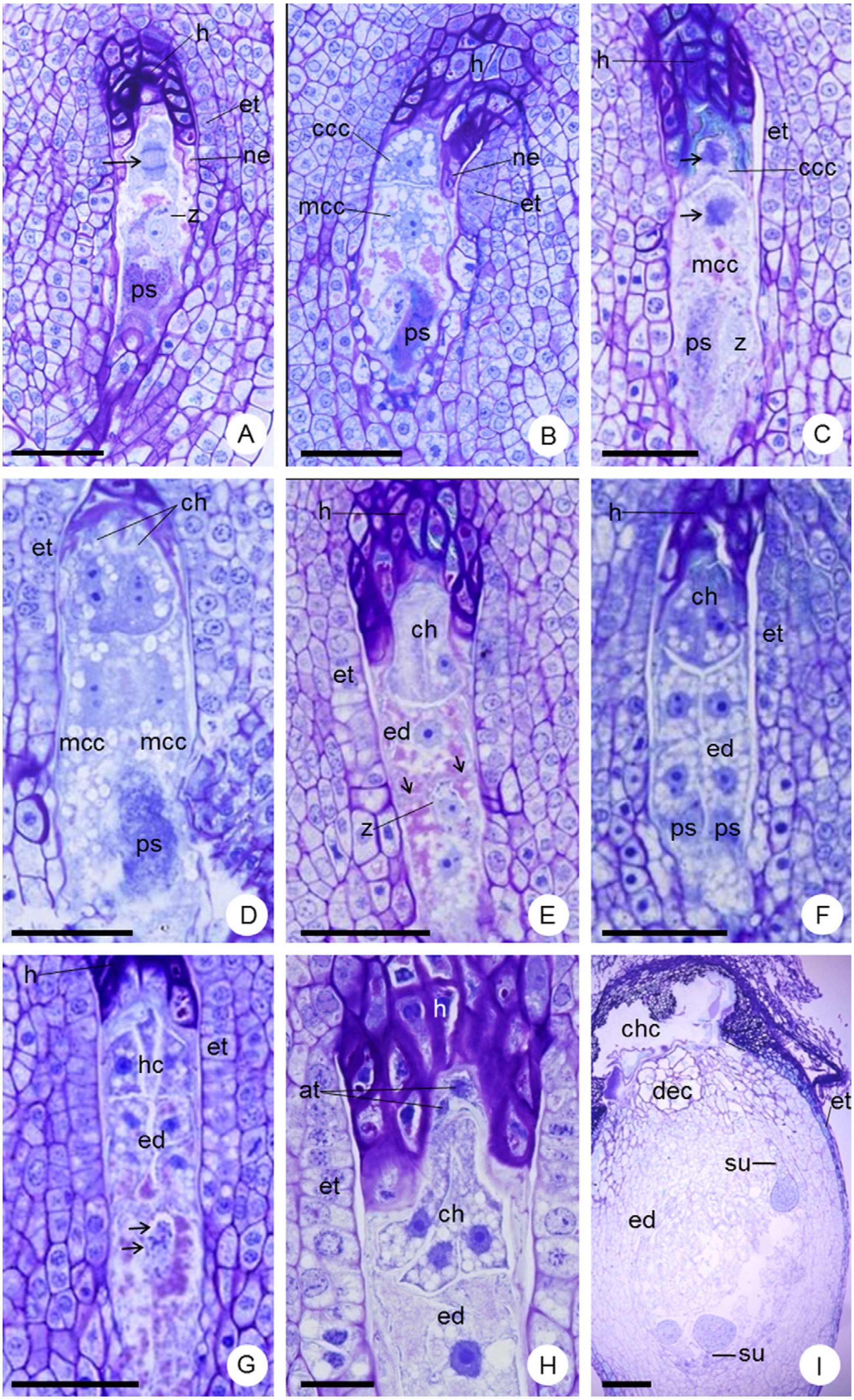

Figure 3. See caption on previous page.

Figure 4. Seeds from fruits of *Anemopaegma* spp., which were more than 5.0 cm long, dissected under a stereomicroscope. The external part of the integument was discarded. The internal integument layers, the endosperm and multiple embryos (arrows) can be seen. A, longitudinal section of a dissected young seed of *Anemopaegma acutifolium* (with the chalazal end left). ed, endosperm; et, endothelium; mh, micropylar haustorium of the endosperm; ms, mesotest. Scale bar, 500 μm. B, whole mount of a dissected young seed of *A. glaucum* (with chalazal end left). in, internal integument layers; me, membranaceous tissue. Scale bar, 1000 μm.

Figure 5. Longitudinal sections of ovules and young seeds of *Anemopaegma acutifolium* showing the adventitious embryos origin (all with the chalazal end top oriented). Adventitious embryo precursor cells (AEPs) are indicated by arrows. A–C, sequential sections of an ovule from an unpollinated pistil, 120 h after the anthesis beginning. Scale bar, 50 μm. A, embryo sac with a large AEP arising from the hypostase (h). ec, egg cell; et, endothelium; pn, polar nuclei; s, synergid. B, detail of AEP. C, two AEPs can be observed. D, eight-celled endosperm, zygote (z) and AEP 120 h after cross-pollination. ch, chalazal haustorium of the endosperm; ed, endosperm. Scale bar, 50 μm. E, 120 h after self-pollination. Scale bar, 20 μm. F-I, seeds from fruits more than 5 cm long. F–G, AEPs arising from hypostase cells. Scale bar, 20 μm. H, two AEPs arising on the micropylar region. Scale bar, 20 μm. I, three embryos on the micropyle. su, suspensor. Scale bar, 200 μm.

were observed coming from the inner face of the micropylar region (Fig. 5H). Those cells were also interpreted as AEPs because the zygote had developed much earlier. Although we did not observe differentiation of these micropilar AEPs, as there is no nucellus in this region, the only possible origin of these cells would be from integumentary cells. Embryos that originated from cells of the hypostase and micropylar region of the ovule were observed growing simultaneously in a single seed (Fig. 3I). Because more than one embryo originated in the micropylar region (Figs 3I, 5I), it is likely that the sexual embryo developed concurrently with asexual ones. As the zygotic proembryonic tube initiated its development earlier than the AEPs, the largest embryo observed in each developing seed was possibly of sexual origin (Figs 1G, 3I, 4B, 5I).

Pollination treatment did not seem to affect the development of AEPs from the hypostase in pistils collected 120 h after pollination, which also showed endosperms with up to ten cells and integument growth to form the seed testa. In contrast, although AEPs occurred in unpollinated pistils collected 120 h after the onset of anthesis, they were irregular in shape and there was no sign of endosperm development or integument cells proliferation in these ovules,

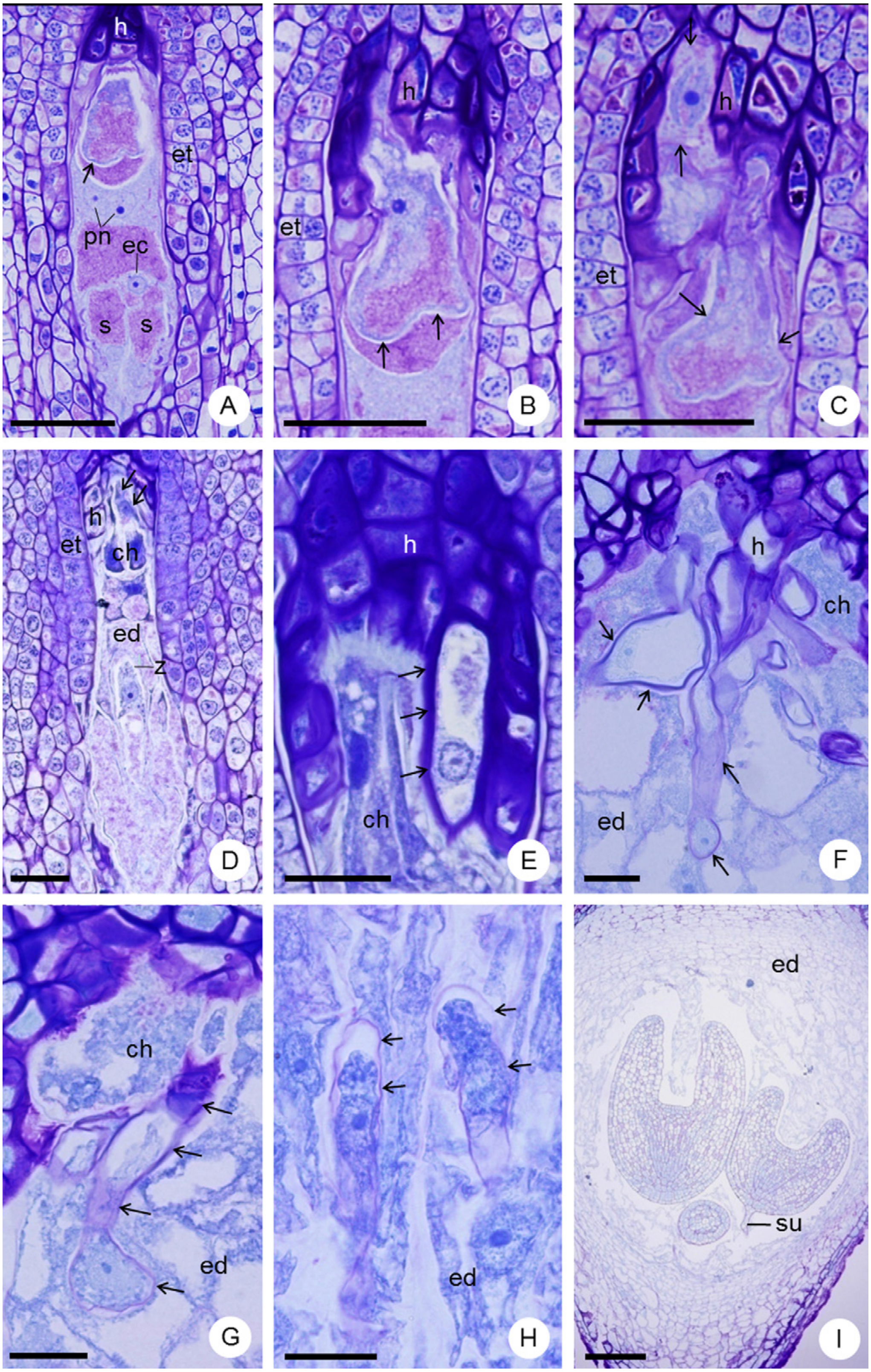

Figure 5. See caption on previous page.

indicating that pollination is essential for normal development of AEPs, endosperm and seed integument.

DISCUSSION

The origin of supernumerary embryos from somatic tissues of the ovule indicated sporophytic apomixis in *A. acutifolium* and explained the polyembryony found in this species. The regularity of tetraploidy and polyembryony in the species and populations of *Anemopaegma* studied indicates that there is a close association between the occurrence of polyploidy and apomixis in *Anemopaegma*. Double fertilization and endosperm development occurred normally in pollinated pistils, except for occasional ovules in which both synergids were penetrated. Sexual and adventitious embryos appear to grow together in a single seed. Although adventitious embryos were initiated in ovules of both pollinated and unpollinated pistils, there was no endosperm development in unpollinated ones, indicating pseudogamy.

The apomixis found in *A. acutifolium* is similar to that observed in *Handroanthus ochraceus* (Cham.) Mattos (Costa *et al*., 2004) and *H. chysotrichus* (Mart. ex DC.) Mattos (Bittencourt Júnior & Moraes, 2010). However, as *Anemopaegma* and *Handroanthus* are phylogenetically distant genera (i.e. Olmstead, 2013) in this largely diploid and sexually reproducing family (Olmstead *et al*., 2009), apomixis may have evolved independently at least twice in the Bignoniaceae. The similar pattern of apomixis expression in Bignoniaceae may be a result of a conservative embryology, as apomixis seems to result from asynchrony in the sexual process caused by hybridization and polyploidization (Koltunow & Grossniklaus, 2003; Carman, 2007).

A correlation between high percentages of polyembryonic seeds and sporophytic apomixis in *A. acutifolium* is also found in species of *Handroanthus* and *Eriotheca* Schott & Endl. (Malvaceae–Bombacoideae) (Oliveira *et al*., 1992; Mendes-Rodrigues *et al*., 2005, 2012). The high incidence of polyembryonic seeds in these taxa contrasts with the low percentage of polyembryonic seeds (5.56%; Correia, Pinheiro & Lima, 2005) found in the diploid (Goldblatt & Gentry, 1979) and self-incompatible (Correia, Pinheiro & Lima, 2006) *Anemopaegma chamberlaynii* (Sims) Bureau & K.Schum., which does not seem to be related to apomixis. Adventitious embryos that arose in somatic tissues of the ovule caused the high polyembryony rates found in *A. acutifolium*, and this indicates that polyembryonic *A. arvense* and *A. glaucum* are also sporophytic apomictic species. Tetraploidy and close relationships among these three species (Firetti-Leggieri *et al*., 2011) also corroborate this view and indicate the existence of an agamic polyploid complex.

These species complexes in Bignoniaceae are of putative hybrid origin, based on morphology and breeding experiments (Gentry, 1992; Firetti-Leggieri *et al*., 2011). Thus, the sporophytic apomixis described here may be associated to allopolyploidy as observed for many gametophytic apomictic species (Asker & Jerling, 1992; Richards, 2003; Paun *et al*., 2006; Carman, 2007; Whitton *et al*., 2008; Hörandl, 2010). The coexistence of two genomes in a polyploid hybrid, from species that have different timing of reproductive tissue development, may cause asynchrony in the expression of genes related to meiosis, embryogenesis and endospermogenesis (Carman, 2007). In sporophytic apomictic species, the expression of genes that control embryogenesis would occur out of place (somatic tissue) and out of time, allowing somatic cells to develop in adventitious embryos.

The embryology of *A. acutifolium* is similar to that observed for other sexual Bignoniaceae. The *Polygonum*-type embryo sac and the cellular *Catalpa*-type endosperm (according to Mauritzon, 1935) with micropylar and chalazal haustoria are common for most Bignoniaceae (Govindu, 1950; Mehra & Kulkarni, 1985; Shivaramiah, 1998; Bittencourt Júnior *et al*. 2003; Sampaio, Costa & Paoli, 2007; Bittencourt Júnior & Moraes, 2010). However, the disc-like group of large cells that differentiated at a late stage in the chalazal region of the endosperm seems to be a novelty in the family and could have systematic value for *Anemopaegma*. The double origin of the membranaceous tissue that surrounds the embryos (derived from the integument and endosperm) observed in *A. acutifolium* has been reported for other Bignoniaceae and seems to be a constant in the family (Sampaio *et al*., 2007; Bittencourt Júnior & Moraes, 2010).

Ovule penetration and fertilization occurred irrespective of cross- or self-pollination treatment. Even in apomictic species, fertilization can be hindered by persisting self-incompatibility mechanisms (Hörandl, 2010). However, in *A. acutifolium* no marked differences were observed in fertilization and embryogeny following selfing and outcrossing (Sampaio, Bittencourt Júnior & Oliveira, 2013).

Some abnormal pollen tube growth and fertilization events were observed. Penetrated and non-fertilized ovules, in which the pollen tubes continue to grow inside the embryo sac, have been observed for other Bignoniaceae (Bittencourt Júnior *et al*. 2003; Bittencourt Júnior & Semir, 2005), and penetration of the embryo sac by more than one pollen tube was reported as polyspermy (Maheshwari, 1950; van Went & Willemse, 1984). These events were also reported in experiments with *feronia* mutants of *Arabidopsis thaliana* (L.) Heynh., in which other pollen tubes continue to be attracted into the embryo sac, probably because the fertilization does not occur and the β-glucuronidase (*GUS*) gene is continuously expressed, attracting pollen tubes (Huck *et al*., 2003). Moreover, in *A. acutifolium*, two pollen tubes sometimes penetrated a single ovule and discharged their cytoplasmatic contents into the two synergids. We have no idea of the effects of two penetrated synergids for seed development of *A. acutifolium*, but fertilization did occur and an apparently normal development of the endosperm and embryos ensued.

As observed for *A. acutifolium*, adventitious embryo initiation also occurs in unfertilized ovules of *Handroanthus chrysotrichus* (Bittencourt Júnior & Moraes, 2010) and *Citrus sinensis* (L.) Osbeck (Koltunow *et al*., 1995). However, in all these species, seeds reach maturity only in pollinated pistils, as the nourishment from the endosperm is essential to embryo maturation (Koltunow *et al*., 1995; Bittencourt Júnior & Moraes, 2010). Although we followed unpollinated pistils only up to 120 h after the onset of anthesis, no sign of endosperm development was observed, which indicates that, in this species, pseudogamy is obligate (Koltunow, 1993; Koltunow & Grossniklaus, 2003). Most apomictic species studied so far are pseudogamic and autonomous endosperm formation is infrequent in such taxa (Asker & Jerling, 1992; Koltunow, 1993; Richards, 2003; Whitton *et al*., 2008).

In all species of Bignoniaceae with pseudogamy, i.e. *A. acutifolium,* reported here, and also *H. chrysotrichus* and *H. ochraceus* (Costa *et al*., 2004; Bittencourt Júnior & Moraes, 2010), sexual and additional adventitious embryos develop concomitantly. This contrasts with *Eriotheca pubescens* (Mart. & Zucc.) Schott. & Endl. (Malvaceae–Bombacoideae), in which the sexual embryo developed late (Bombacoideae have a resting zygote) in comparison with the adventitious embryos. As a consequence, the sexual embryo was relatively small and was less capable of reaching maturity (Mendes-Rodrigues *et al*., 2005). This hazardous fate of the sexual embryo in *E. pubescens* would explain the low genetic variability and a mostly clonal population found in this species (Martins & Oliveira, 2003). In *A. arvense*, genetic variability is greater within populations than between them (Batistini *et al*., 2009) and this is probably attributable to the greater survival of viable sexual embryos, as described for other polyembryonic species (Batygina, 1999a, b; Batygina & Vinogradova, 2007; Hörandl & Paun, 2007).

All populations of *A. acutifolium*, *A. arvense* and *A. glaucum* studied so far appear to have sporophytic apomixis, and it is of interest that such apomixis has been reported for other species in the cerrado region (Mendes-Rodrigues *et al*., 2005, 2012). The ability to produce sexual (self- and outcross) and adventitious embryos in the same seed or plant would give a mixed-mating system, which may be advantageous as it maintains genetic variability, through sexual embryos and ‘frozen’, well-adapted genotypes (Bayer & Chandler, 2007; Majesky *et al*., 2012). Evolution of agamic polyploid complexes is intriguing and many lineages can be present in these complexes (Asker & Jerling, 1992). In Bignoniaceae, complexes comprising polyploid tropical woody species with sporophytic apomixis have evolved at least twice. This is an unexpected pattern for this kind of apomixis (sporophytic) that traditionally has been associated with diploid or palaeopolyploid species (Asker & Jerling, 1992; Richards, 2003; Whitton *et al*., 2008).

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