MICROSPORE DEVELOPMENT IN *ANNONA* (ANNONACEAE): DIFFERENCES BETWEEN MONAD AND TETRAD POLLEN

JORGE LORA [2,4] , MARIA HERRERO [2] , AND JOSE I. HORMAZA [3]

[2] Department of Pomology, Estación Experimental 'Aula Dei', CSIC, Apdo. 13034 50080 Zaragoza, Spain; and [3] Department of Subtropical Fruits, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" (IHSM-UMA-CSIC) 29750 Algarrobo-Costa, Málaga, Spain

Author for correspondence (e-mail: jlora@eead.csic.es)

• *Premise of the study:* Permanent tetrads are the most common form of pollen aggregation in fl owering plants. The production of pollen in monads is plesiomorphic in angiosperms, but the aggregation into tetrads has arisen independently different times during the evolution of fl owering plants. The causes behind the recurrent evolution of pollen aggregation from monads remain elusive. Permanent tetrad pollen is quite common in the Annonaceae, the largest family in the early-divergent order Magnoliales. In some genera, such as *Annona* , both tetrad- and monad-producing species can be found.
• *Methods:* In this comparative study of pollen development, we use immunolocalization, cytological characterization, and enzymatic assays of four species in the genus *Annona* and one species in its closely related genus *Asimina* that release pollen in tetrads and two species in the genus *Annona* that release pollen in monads.
• *Key results:* The main difference between species with tetrad and monad pollen is a delayed digestion of callose and cellulose at the pollen aperture sites that resulted in nonlayering of the exine in these areas, followed by a rotation and binding of the young microspores at the aperture sites.
• *Conclusions:* Small changes in development resulted in clear morphological changes on pollen dispersal time and open a window on the possible selective advantage of the production of aggregated pollen.

Key words: *Annona* ; Annonaceae; aperture; callose; monad; pollen development; tetrad.

This work was supported by Ministerio de Economía y Competitividad–European Regional Development Fund, European Union (AGL2010-15140, AGL2012-40239, and AGL2013-43732-R), the BBVA Foundation (BIOCON 08-184/09), INIA (RF2012-00010 and RFP2012-00016), and Gob. Aragón A-43. J.L. was supported by a Juan de la Cierva postdoctoral grant of the Spanish Ministry of Science and Innovation.

Most angiosperms release pollen as monads at anther dehiscence (Pacini and Franchi, 1999 ; Harder and Johnson, 2008), but various forms of aggregated pollen have arisen independently several times during the evolution of fl owering plants. Pollen can aggregate as dyads, triads, tetrads, or polyads, but tetrads are the most common form of aggregation in angiosperms (Willson, 1979). While pollen development is a wellcharacterized and highly conserved process in angiosperms (McCormick, 2004 ; Scott et al., 2006 ; Blackmore et al., 2007), the ultimate causes behind the recurrent evolution of aggregated pollen remain elusive. These aggregations are quite common in the Annonaceae (Doyle and Le Thomas, 1994), the largest family in the early-divergent order Magnoliales (APG II, 2003 ; APG III, 2009).

Aggregated pollen is also common in other Magnoliids such as Lactoridaceae in the Piperales and Winteraceae in the Canellales (Walker and Doyle, 1975). Aggregated pollen has been reported in mutants from the model plant *Arabidopsis thaliana* , a core eudicot that releases pollen in monads. Genetic studies showed that a mutation in the *QUARTET* locus results in the release of tetrad pollen due to failure of microspore separation during pollen development (Preuss et al., 1994). The characterization of these mutants revealed a defect in degradation of the wall of the pollen mother cell (PMC) that is associated with tetrad pollen formation in the *quartet* mutants (Rhee and Somerville, 1998). Later studies showed that a similar phenotype could result from mutations of different loci, such as *QUARTET2* (Ogawa et al., 2009) and *QUARTET3* that code for polygalacturonases (Rhee et al., 2003) and *QUARTET1* that codes for a pectin methylesterase (Francis et al., 2006). However, studies similar to these with mutants in *Arabidopsis* are more diffi cult for other species where similar tools are scarce. This is the case for most earlydivergent angiosperms, which could contribute signifi cantly to our understanding of how aggregated pollen has evolved in flowering plants.

In a previous study in *Annona cherimola* , a species in the Annonaceae that releases pollen in tetrads, we showed that the delayed digestion of the PMC wall and the tapetal chamber helped to hold together the four microspores, that rotate and then bind through the aperture sites with small pectin bridges, followed by joint sporopollenin deposition (Lora et al., 2009). Similar studies with different genera and species of the Annonaceae such as *Pseuduvaria* (Su and Saunders, 2004), *Annona glabra* , *A. montana* , and *Cymbopetalum baillonii* (Tsou and Fu, 2002 , 2007) have shown differences in the cohesion mechanism among the pollen grains. Thus, pollen grains in *Pseuduvaria* have cross-wall cohesion with wall bridges involving both the exine and intine, while pollen grains of *A. glabra*, *A. montana* , and *Cymbopetalum baillonii* are connected by a mass of callose and cellulose. These differences among closely related species are puzzling and refl ect the need for additional ontogenetic studies in this family that show a widely diverse pollen morphology (Doyle and Le Thomas, 2012). The fact that species with tetrad and species with monad pollen can be found in the same genus (*Annona*) provides an excellent opportunity for comparative studies to investigate the causes behind the formation of tetrad pollen and its evolutionary implications.

In this study, we compare pollen development in four species of *Annona* and one of *Asimina* that release pollen in tetrads and in two species of *Annona* that release pollen in monads. We paid special attention to the tetrad stage during microspore development, where clear developmental differences were observed between species that release pollen as tetrads or monads. These results show that small changes in development can result in clear morphological differences in pollen at dispersal time.

MATERIALS AND METHODS

Plant material **—** Two species with monad pollen [*Annona emarginata* (Schltdl.) H. Rainer and *Annona neosalicifolia* H. Rainer] and fi ve species with tetrad pollen [*Annona cherimola* Mill., *Annona squamosa* L., the hybrid (*A. squamosa* × *A. cherimola*) 'atemoya' (*Annona* × *atemoya* Mabb.), *Annona senegalensis* Pers. and *Asimina triloba* (L.) Dunal] were used in this study. *Asimina* is a closely related genus to *Annona* (Richardson et al., 2004). Adult trees were located in a fi eld germplasm collection at the IHSM La Mayora-CSIC, Málaga, Spain.

Light microscopy **—** To follow pollen development, we collected anthers from fl ower buds at a range of developmental stages, from differentiation up to anther dehiscence, which usually takes 30 d under the environmental conditions of the experiments. The anthers were fi xed in 2.5% v/v glutaraldehyde in 0.03 M phosphate buffer (Sabatini et al., 1963), dehydrated in an ethanol series, embedded in Technovit 7100 (Kulzer & Co, Wehrheim, Germany), and sectioned at 2 μm.

For general histological observations, sections were stained with 0.5% (w/v) periodic acid for 2 h, washed three times with water and held in Schiff's reagent in the dark for 1.5 h (Feder and O'Brien, 1968). After three washes with water, the sections were stained with aqueous 0.2% (w/v) toluidine blue. Intine and exine were observed with a 3 : 1 aqueous mixture of 0.01% (w/v) auramine and 0.007% (w/v) calcofl uor (Lora et al., 2009).

Additional fl ower buds were fi xed in FAA (70% ethanol-glacial acetic acidformalin [18 : 1 : 1; v/v/v]), dehydrated in an ethanol series, and then embedded in paraffi n wax, sectioned at 10 μm and stained with 0.1% (w/v) aniline blue in 0.1 N PO_4K_3 (Currier, 1957) to observe callose. Preparations were observed with a Leica DM LB2 epifl uorescence microscope with a 340–380 excitation fi lter and an LP 425 barrier filter for auramine and calcofl uor, and with a 515–560 excitation fi lter and an LP 590 barrier filter for aniline blue.

For the study of pollen morphology and pollen size, dehisced anthers were sieved through a 0.26 mm mesh sieve, and pollen was placed in glacial acetic acid and stored at room temperature until acetolysis. For acetolysis, following a modification of the method by Erdtman (1960) , pollen grains were placed into a mixture of 9 : 1 acetic anhydride–concentrated sulphuric acid at 65ºC for10 min, then washed with glacial acetic acid and washed again three times with water.

Scanning electron microscopy (SEM) — Pollen was fresh dried with silica gel and directly attached to SEM stubs using adhesive carbon tabs and observed with a JSM-840 scanning electron microscope (JEOL) operated at 10 kV.

Immunocytochemistry — Anthers from three flowers per developmental stage were fixed in 4% v/v paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.3, left overnight at 4 ºC, dehydrated in an acetone series, embedded in Technovit 8100 (Kulzer), polymerized at 4 ºC, and sectioned at 2 µm. Sections were placed in a drop of water on a slide covered with 2% v/v 3-aminopropyltrietoxy- silane (Sigma, St. Louis, Missouri, USA) and dried at room temperature (Satpute et al., 2005 ; Solís et al., 2008).

Different antibodies were used to localize specifi c cell components: JIM7 and JIM5 rat monoclonal antibodies (Carbosource Service, University of Georgia, Athens, Georgia, USA), which recognize methyl-esterifi ed and unesterified pectins, respectively (Knox, 1997), and an anticallose mouse monoclonal antibody (Biosupplies, Parkville, Australia) for callose.

Following the protocol of Lora et al. (2009) , sections were incubated with PBS for 5 min and later with 5% w/v bovine serum albumin (BSA) in PBS for 5 min. Then, different sections were incubated for 1 h with the primary antibodies: JIM5 and JIM7 undiluted and anticallose diluted 1/20 in PBS. After three washes in PBS, the sections were incubated for 45 min in the dark with the corresponding secondary antibodies (antirat for JIM5 and JIM7, and antimouse for anticallose) conjugated with Alexa 488 fl uorochrome (Molecular Probes, Eugene, Oregon, USA) and diluted 1/25 in PBS. After three washes in PBS and water, the sections were mounted in Mowiol 4-88 (Sigma) or ProLong Gold Antifade Reagent (Invitrogen), examined with a Leica TCS SP5 II confocal microscope and with a Leica DM2500 epifl uorescence microscope equipped with a Leica DFC310 FX camera. Filters were 470/525 nm for the Alexa488 fluorescein label of the antibodies. Overlapping photographs were obtained with the Leica Acquisition Station AF6000 E.

Fluorescence measurement — Callose from microspore tetrads was stained with 0.8 µg/µL sirofl uor in water (Biosupplies), and the fl uorescence intensity signal was measured with the program Image J (National Institutes of Health, Bethesda, Maryland, USA) and evaluated with an ANOVA. Duncan's multiple range test was used to separate means ($P \leq 0.01$). Statistical analyses were performed with SPSS 12.0 statistical software. The photographs were obtained in a Leica TCS SP5 II confocal microscope using an LP 590 barrier fi lter for sirofluor.

Digestion assays — Callose was digested with 1.3 µg/µL lyticase (2000 U/mg protein, Sigma); 8×10^{-4} µg/µL sirofluor in water was added to detect callose. This protocol was used for anthers previously screened with the microscope to confi rm the presence of young microspores in the tetrad stage. Such detection was done every hour for 7 h. At the same developmental stage of the anthers, cellulose was digested using 13 µg/µL of cellulase with 2.3×10^{-7} µg/µL of calcofluor in water to detect cellulose. The young microspores were then observed after 30 min, 60 min, 90 min, 120 min, and 180 min. For simultaneous digestion of cellulose and callose, 8 µg/µL of cellulase (Macerozyme R-10, Duchefa, Haarlem, Netherlands) and 0.8 µg/µL of lyticase were jointly used. For callose detection, 5×10^{-4} µg/µL of sirofl uor in water was also added. Samples were observed for callose digestion every hour for 7 h. Microspore tetrads that showed signal close to the microspore wall were considered with signal for callose and cellulose detection.

RESULTS

Pollen had a globose shape in all the species examined (Walker and Doyle, 1975). Tetrads were tetragonal in *Annona squamosa* (Fig. 1A) and rhomboidal in atemoya, *A. senegalensis*, and *Asimina triloba* (Fig. 1C, E, G). Punctuated exine cohesion (Fig. 1B, D, F, H) could be observed in all examined species with tetrad pollen. *Annona emarginata* and *A. neosalicifolia* showed inaperturate monad pollen (Fig. 1I, J).

Microsporogenesis and microspore tetrad release — Microsporogenesis in atemoya, a species with tetrad pollen (Fig. 2A, B), was similar to that of *A. emarginata* , a species with monad pollen (Fig. 2E, F). These similarities remained when compared with three other species with tetrad pollen (*A. senegalensis* , *A. squamosa* , and *Asimina triloba*) and an additional species with monad pollen (*Annona neosalicifolia*) (Appendix S1, see Supplemental Data with the online version of this article). In all the species studied, the PMCs increased in size (Fig. 2A, E), then successive cytokinesis was observed, and starch grains appeared in the young microspore just after meiosis (Fig. 2F ; Appendix S1). The main developmental difference between microspores that form permanent tetrads and monads was that, just after meiosis, the microspores that will be released as tetrads showed a wide aperture site devoid of exine (Fig. 2C, D, H ; Appendix S1C). In contrast, in microspores that will be released as monads, the exine was generally uniform all around the microspore (Fig. 2G ; Appendix S1F). Upon completion of meiosis, in the pollen that will be released as tetrads, the aperture site was initially located distally in the tetrad (Fig. 2C, H ; Appendix S1C). The young microspores then rotated 180 º, so that the aperture site faced the center of the tetrad (Fig. 1D) (Lora et al., 2009). In the species with monad pollen, since the aperture site is not apparent, the existence of a similar rotation could not be determined.

No apparent differences were detected in the timing of pollen development relative to the timing of tapetum degeneration between species that release pollen in tetrads or monads. All the species studied showed a secretory tapetum with tapetal septa, as previously observed in *A. squamosa* (Periasamy and Kandasamy, 1981) and *A. cherimola* (Lora et al., 2009) (Fig. 2C, D, G, H ; Appendix S1).

Microspore wall — Remnants of cellulose stained with calcofluor were visible before and during the rotation phase in two species with tetrad pollen, atemoya and *A. squamosa* (Fig. 3A, B) whereas they were missing in the two species with monad pollen (Fig. 3C, D). Similarly, antibodies against callose also showed remnants of callose in the aperture sites in species with tetrad pollen (Fig. 3E, F), while no remnants were observed at this stage in species with monad pollen (Fig. 3G, H). Remnants of callose and cellulose were also observed in the other species with tetrad pollen, *A. cherimola* (Lora et al., 2009), *A. senegalensis*, and *Asimina triloba* (Appendix S2, see online Supplemental Data).

To study with more detail the differences observed between tetrad and monad pollen, enzymatic assays were also performed in tetrad pollen of atemoya and *Annona cherimola* and monad pollen of *A. emarginata* and *A. neosalicifolia* . Upon completion of meiosis, and before digestion, cellulose was observed at the tetrad stage surrounding all the microspores analyzed (Fig. 4A–D). The tetrad microspores of *A. cherimola* and atemoya still showed cellulose remnants 1 h after cellulose digestion (Fig. 4E, F), and digestion was not completed until 3 h after the treatment (Fig. 4I). However, monads did not show remnants of cellulose 1 h after the treatment (Fig. 4G, H).

Similarly, upon completion of meiosis, callose was also present in the tetrad stage of all species examined with a similar intensity signal measured as relative fl uorescence units (RFU) (Fig. 5A–D). There was a signifi cant difference in fl uorescence intensity among species ($F_{2,34} = 3.67$, $P = 0.036$); however, when species means were analyzed with a post hoc Duncan's multiple range test at $P < 0.01$, no differences were found between any two species, whether they had tetrad pollen (93 □} 9 RFU, $N = 14$ for *A. cherimola* ; 89 □} 12 RFU, $N = 7$ for atemoya or monad pollen; 83 □} 10 RFU, $N = 16$ for *A. neosalicifolia*). The digestion of callose with lyticase also revealed that the initiation of digestion of callose was delayed by 3 h in atemoya and 6 h in *A. cherimola* (Fig. 5E, F, I) and at this time continued to be present at the aperture sites. However, monad pollen species *A. neosalicifolia* and *A. emarginata* showed a quicker digestion, with no callose 1 h after the treatment (Fig. 5G–I).

The digestion of callose was earlier when cellulase was also used with the species with permanent tetrad pollen, atemoya, and *A. cherimola* . The digestion was completed in monads 1–2 h after the treatment. However, in species with tetrad pollen, digestion was not completed until 5–7 h after treatment (Fig. 5J). In all cases, callose and cellulose remnants were located in the aperture zone of the microspores in species with tetrad pollen, but not in the microspores of species with monad pollen (Figs. 4, 5).

Pectin distribution — Since pectins are one of the main components of the microspore walls involved in the formation of the exine (Majewska-Sawka and Rodriguez-Garcia, 2006), we used immunocytochemistry to assay for the presence of methylesterified and unesterifi ed pectins in the cell wall during pollen development. We used the antibodies JIM7 and JIM5 that react to methyl-esterified and unesterified pectins, respectively.

In all the cases studied, the PMC walls had methyl-esterified and unesterified pectins upon completion of meiosis (Fig. 6A–D ; online Appendix S3, A–D). The young microspores at the tetrad stage reacted to these antibodies, but a weaker signal was present in the aperture sites of the species with tetrad pollen (Fig. 6E–H ; Appendix S3, E–H). Although the presence of a strong or weak signal in the aperture sites was the main difference between monad and tetrad pollen, respectively, additional differences in the pattern of pectin deposition were observed when the pollen became joined together. Pectins were conspicuous at this stage in all the analyzed species with tetrad pollen (Fig. 6I, J ; Appendix S3, I, J), excluding *A. senegalensis* , but were faintly detected in the young microspores of species with monad pollen (Fig. 6K, L ; Appendix S3, K, L). Later on pectin signal was strong in mature pollen in all tetrad and monad pollen species examined (Fig. 6M–P ; Appendix S3, M–P).

Annona senegalensis followed the same pattern as other tetrad pollen species examined, with pectin layering at the pollen mother cell (Fig. 7A–D), and a weak signal at the aperture site (Fig. 7E–H). But pectin labeling could not be detected after the 180 º rotation, when microspores began to increase in size and pollen joined together (Fig. 7I, J), unlike the species with tetrad pollen (Figs. 7K, 7L vs. 6I, 6J ; Appendix S3, I, J). In mature pollen, the pectin signal was again strong (Fig. 7M, N) as in all other monad and tetrad pollen species examined (Figs. 7O, 7P vs. 6M–P ; Appendix S3, M–P).

Pollen from the species with permanent tetrads showed the inner wall of microspores adjacent to each other, when the microspores started to increase in size and the main callose vanished. Inter-intine

connections of unesterifi ed pectins were observed in mature pollen of atemoya, *A. squamosa* , and *Asimina triloba* (Figs. 6N , 7P ; Appendix S3N). These connections were not observed in *Annona senegalensis* (Fig. 7N) or in the species with monad pollen (Fig. 6P ; Appendix S3P). However, all the species with permanent tetrad pollen resisted separation during acetolysis, revealing the permanence of joint sporopollenin.

The mature pollen of species with tetrads retained the differences in exine distribution observed in the young microspore stage. The intine covering was similar in species with tetrad and monad pollen. However, the exine covering was not completed, leaving a big aperture site in species with permanent tetrad pollen (Fig. 8A–C ; Appendix S4, A), while it fully covered the mature pollen grain in species with monad pollen (Fig. 8D, E). Following the 180 º rotation of the microspores, this large aperture site was the area where microspores joined together in the species with permanent tetrad pollen (Fig. 8F–H ; Appendix S4, B), whereas microspores remained isolated in species with monad pollen (Fig. 8I, J).

DISCUSSION

Microspore development in *Annona* species with monad and permanent tetrad pollen followed a similar sequence of events, but variations in the timing of the digestion of callose and cellulose was a starting point for the difference between further development of pollen as monads or as permanent tetrads. A longer retention of callose at the microspore wall aperture was associated with the absence of exine at the aperture site in permanent tetrad pollen. Microspore development was followed by rotation and binding of the young microspores at the aperture sites, resulting in subsequent joint sporopollenin deposition.

Different pollen apertures and callose remnants — Significant differences between tetrad and monad pollen were found in exine distribution. All species with permanent tetrad pollen had a large aperture devoid of exine, as compared with a homogenous exine all around the pollen grain in monad pollen.
The difference of exine distribution is related to the sequence of events near the time of callose digestion in the tetrad during microspore development.

During meiosis, the PMC is surrounded by a callose–cellulose material. Callose, deposited on the outer surface of the plasma membrane (Carpita and Gibeaut, 1993 ; Parre and Geitmann, 2005) acts as a permeability barrier and leak sealant (Parre and Geitmann, 2005). When meiosis is completed, callose is digested by an enzyme cocktail secreted from the tapetum (Scott et al., 2006). Interestingly, the pollen aperture location appears to be linked to the last point of callose deposition (Blackmore et al., 2007 ; Albert et al., 2010 ; Albert et al., 2011), and our results support this view, further showing that formation of the pollen aperture is related to delayed digestion of callose with no layering of exine at this site. A similar pattern has been described previously in other species of the Annonaceae with permanent tetrad pollen (Tsou and Fu, 2002 , 2007 ; Lora et al., 2009), supporting the idea that the pattern of exine layering is established at the tetrad developmental stage, when callose digestion is closely followed by the formation of the exine (Blackmore et al., 2007). Pectins are also involved in exine formation (Majewska-Sawka and Rodriguez-Garcia, 2006), and in species with permanent tetrad pollen, the aperture sites showed a weaker signal for methyl-esterifi ed and unesterifi ed pectins. Conversely, remnants of the callose–cellulose material were not observed in the developing microspores of the two species that produce pollen in monads, *A. neosalicifolia* and *A. emarginata* , where exine was layered all around the microspore and the signal against pectins was also homogeneous.

Callose and cellulose were digested earlier in species with monad pollen than in species with permanent tetrad pollen, in which the last remnants of callose and cellulose were always located at the aperture site, remaining even in mature pollen in species such as *A. squamosa* and atemoya. A delay in tapetum degeneration could explain this delay, but no differences in the timing of pollen development relative to the tapetum degeneration in the species studied were observed. Our results on callose remnants and exine layering are not unique to permanent tetrad pollen; they are shared by monoaperturate monad pollen (Toghranegar et al., 2013). However, the subsequent joining of the four microspores together, once they are free, is more intriguing.

Joining and binding of the microspores — Once the four microspores are shed free, they synchronize by rotating around themselves. In this way, after the 180 º rotation, the initially distal aperture sites face inward, and the thinner aperture sites of the four sibling microspores face each other. The rotation of the four microspores has been observed in other species in the Annonaceae, *A. glabra* , *A. montana* (Tsou and Fu, 2002), *A. cherimola* (Lora et al., 2009), and *Cymbopetalum baillonii* (Tsou and Fu, 2007). The reasons behind this rotation remain puzzling, but it has been suggested that the remnants of callose in the aperture sites pull the microspores for the 180 º rotation (Tsou and Fu, 2002 , 2007).

Once the microspores face each other, the question remains on how they adhere to each other. Results for three of the tetrad pollen species here studied show a similar pattern to that previously reported in *A. cherimola* (Lora et al., 2009). The thin microspore walls, at the aperture sites, adhere to each other through pectin bridges. Sporopollenin is then jointly deposited, further establishing a strong union resistant to acetolysis. Intine and exine cohesion was also reported for *Pseuduvaria* (Su and Saunders, 2004), also belonging to the Annonaceae. However, while sporopollenin bridging also occurs in *A. senegalensis* , intine cohesion was not observed in the mature permanent tetrad pollen of this species. Also a different pattern was reported for *Annona glabra* , *A. montana* (Tsou and Fu, 2002), and *Cymbopetalum baillonii* (Tsou and Fu, 2007) in which pollen grains are connected by a mass of callose and cellulose. Thus, the tetrad cohesion process could involve different steps.

The confi ned space where the microspores develop surely contributes to the establishment of these different cohesion mechanisms. Thus, a relationship between the locular space and the types of dispersal unit was previously suggested (Pacini, 2010). Studies with the quartet mutants of the model plant *Arabidopsis thaliana* show that a delay in the PMC wall dissolution is key to keeping the four pollen grains together (Rhee and Somerville, 1998). This conclusion obtained for a core eudicot was also observed in our previous study of *Annona cherimola* (Lora et al., 2009) and is supported by the results of the present study in which we also observed remnants of pectin in the PMC wall. Moreover, a degenerated septal tapetum still surrounds the four microspores, forming the tapetal chamber.

***Pollen development in Annonaceae* —** Results herein show that, even within the same genus, different origins may account for the presence of mature tetraspore pollen. However, all the species here examined, except for the fi rst cohesion mechanism, share a common developmental sequence of events. A strong reduction of exine at the pollen contact area is also found in other species of Annonaceae with aggregated pollen in the form of dyads, tetrads, or polyads (Le Thomas et al., 1986). In fact, although with some exceptions as the genus *Isolona* , where the presence of monads is probably a reversion from tetrads (Doyle and Le Thomas, 2012), a reduction of the exine in this area is correlated with compound pollen (Doyle and Le Thomas, 1994 , 2012).

Cladistic analyses (Doyle and Le Thomas, 1994 , 2012) showed that the monosulcate condition could represent the ancestral pollen type in Annonaceae as in other Magnoliales and that loss of the sulcus took place in the more evolutionarily derived species of the family including some groups (such as the Miliuseae in the subfamily Malmeoideae) with mostly monad pollen and other groups (such as the subfamily Annonoideae) with mostly aggregated pollen. Although tetrads have arisen independently in some lines of the Annonaceae, most taxa with tetrads belong to the subfamily Annonoideae (Doyle and Le Thomas, 2012).

A putative adaptive benefi t of permanent tetrad pollen with a big aperture could be a cooperative strategy between the four sibling pollen grains to prevent desiccation and the entrance of pathogens. In the case of monad pollen in *A. neosalicifolia* or *A. emarginata* , this strategy is not needed because the complete covering of the pollen wall does not leave any unprotected area. While such cooperative protection has not been previously suggested, the fact that pollen aggregation occurs mostly in insect-pollinated taxa (Hesse et al., 2000) has been related to an advantage in species where pollination visits are scarce (Harder and Johnson, 2008). This strategy could be the case in *Annona* where cantharophily (pollination by beetles) is the most common pollinating system (Gottsberger, 2012 ; Saunders, 2012), and usually these insects are less mobile than other pollinating insects such as flies or bees (Willmer, 2011). Moreover, pollen aggregation is often combined with floral thermogenesis and/or floral gigantism that increase pollination effi ciency (Davis et al., 2008 ; Endress, 2010).

***Conclusions* —** Results herein revealed the key events during pollen development behind this adaptive advantage of permanent tetrad pollen. The main difference between *Annona* species with tetrad and monad pollen is the delayed digestion of callose and cellulose at the pollen aperture sites, thus resulting in the lack of exine layering in these areas. The young microspores then rotate and bind at the aperture sites, fi rst via cohesion of the intine layers and later via cohesion of the adjacent exine layers at the margin of the apertures. These results could represent a widespread situation of the independent, recurrent appearance of aggregated pollen in fl owering plants and could be explained by relatively minor ontogenetic changes.

LITERATURE CITED

ALBERT , B. , S. NADOT , L. DREYER , AND A. RESSAYRE . 2010 . The influence of tetrad shape and intersporal callose wall formation on pollen aperture pattern ontogeny in two eudicot species. *Annals of Botany* 106 :557 – 564 .

ALBERT , B. , A. RESSAYRE , AND S. NADOT . 2011 . Correlation between pollen aperture pattern and callose deposition in late tetrad stage in three species producing atypical pollen grains. *American Journal of Botany* 98 : 189 – 196 .

APG II . 2003 . An update of the Angiosperm Phylogeny Group classifi cation for the orders and families of fl owering plants: APG II. *Botanical Journal of the Linnean Society* 141 : 399 – 436 .

APG III . 2009 . An update of the Angiosperm Phylogeny Group classifycation for the orders and families of fl owering plants: APG III. *Botanical Journal of the Linnean Society* 161 : 105 – 121 .

BLACKMORE , S. , A. H. WORTLEY , J. J. SKVARLA , AND J. R. ROWLEY . 2007 . Pollen wall development in fl owering plants. *New Phytologist* 174 : 483 – 498 .

CARPITA , N. C. , AND D. M. GIBEAUT . 1993 . Structural models of primary cell walls in fl owering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal* 3: 1– 30.

CURRIER , H. B. 1957 . Callose substance in plant cells . *American Journal of Botany* 44 : 478 – 488 .

DAVIS , C. C. , P. K. ENDRESS , AND D. A. BAUM . 2008 . The evolution of floral gigantism. *Current Opinion in Plant Biology* 11 : 49 – 57 .

DOYLE , J. A. , AND A. LE THOMAS . 1994 . Cladistic analysis and pollen evolution in *Annonaceae* . *Acta Botanica Gallica* 141 : 149 – 170 .

DOYLE , J. A. , AND A. LE THOMAS . 2012 . Evolution and phylogenetic significance of pollen in Annonaceae. *Botanical Journal of the Linnean Society* 169 : 190 – 221 .

ENDRESS , P. K. 2010 . The evolution of fl oral biology in basal angiosperms. *Philosophical Transactions of the Royal Society, B, Biological Sciences* 365 : 411 – 421 .

ERDTMAN , G. 1960 . The acetolysis method. *Svensk Botanicheskii Tidskrift* 54 : 561 – 564 .

FEDER , N. , AND T. P. O'BRIEN . 1968 . Plant microtechnique: Some principles and new methods. *American Journal of Botany* 55 : 123 – 142 .

FRANCIS , K. E. , S. Y. LAM , AND G. P. COPENHAVER . 2006 . Separation of Arabidopsis pollen tetrads is regulated by *QUARTET1* , a pectin methylesterase gene. *Plant Physiology* 142 : 1004 – 1013 .

GOTTSBERGER , G. 2012 . How diverse are Annonaceae with regard to pollination? *Botanical Journal of the Linnean Society* 169 : 245 – 261 .

HARDER , L. , AND S. JOHNSON . 2008 . Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Sciences* 169 : 59 – 78 .

HESSE , M. , S. VOGEL , AND H. HALBRITTER . 2000 . Thread-forming structures in angiosperm anthers: Their diverse role in pollination ecology. *Plant Systematics and Evolution* 222 : 281 – 292 .

KNOX , J. P. 1997 . The use of antibodies to study the architecture and developmental regulation of plant cell walls. *International Review of Cytology* 171 : 79 – 120 .

LE THOMAS , A. , W. MORAWETZ , AND M. WAHA . 1986 . Pollen of paleo- and neotropical Annonaceae: Defi nition of the aperture by morphological and functional characters . *In* S. Blackmore and I. K. Ferguson [eds.], Pollen and spores: Form and function, 375–388, Academic Press, London, UK.

LORA , J. , P. S. TESTILLANO , M. C. RISUENO , J. I. HORMAZA , AND M. HERRERO . 2009 . Pollen development in *Annona cherimola* Mill. (Annonaceae). Implications for the evolution of aggregated pollen. *BMC Plant Biology* 9 : 129 .

MAJEWSKA-SAWKA , A. , AND M. I. RODRIGUEZ-GARCIA . 2006 . Immunodetection of pectin and arabinogalactan protein epitopes during pollen exine formation of *Beta vulgaris* L. *Protoplasma* 228 : 41 – 47 .

MCCORMICK , S. 2004 . Control of male gametophyte development. *Plant Cell* 16 : S142 – S153 .

OGAWA , M. , P. KAY , S. WILSON , AND S. M. SWAIN . 2009 . ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in *Arabidopsis. Plant Cell* 21 : 216 – 233 .

PACINI , E. 2010 . Relationships between tapetum, loculus, and pollen during development. *International Journal of Plant Sciences* 171 : 1 – 11 .

PACINI , E. , AND G. G. FRANCHI . 1999 . Pollen grain sporoderm and types of dispersal units. *Acta Societatis Botanicorum Poloniae* 68 : 299 – 305 .

PARRE , E. , AND A. GEITMANN . 2005 . More than a leak sealant. The mechanical properties of callose in pollen tubes. *Plant Physiology* 137 : 274 – 286 .

PERIASAMY , K. , AND M. K. KANDASAMY . 1981 . Development of the anther of *Annona squamosa* L. *Annals of Botany* 48 : 885 – 893 .

PREUSS , D. , S. Y. RHEE , AND R. W. DAVIS . 1994 . Tetrad analysis possible in *Arabidopsis* with mutation of the *QUARTET* (*QRT*) genes. *Science* 264 : 1458 – 1460 .

RHEE , S. Y. , E. OSBORNE , P. D. POINDEXTER , AND C. R. SOMERVILLE . 2003 . Microspore separation in the *quartet 3* mutants of *Arabidopsis* is impaired by a defect in a developmentally regulated polygalacturonase required for pollen mother cell wall degradation. *Plant Physiology* 133 : 1170 – 1180 .

RHEE , S. Y. , AND C. R. SOMERVILLE . 1998 . Tetrad pollen formation in quartet mutants of *Arabidopsis thaliana* is associated with persistence of pectic polysaccharides of the pollen mother cell wall. *Plant Journal* 15 : 79 – 88 .

RICHARDSON , J. E. , R. H. J. ERKENS , L. W. CHATROU , J. B. MOLS , AND M. D. PIRIE . 2004 . Historical biogeography of two cosmopolitan families of flowering plants: Annonaceae and Rhamnaceae. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 359 : 1495 – 1508 .

SABATINI , D. D. , K. BENSCH , AND R. J. BARRNETT . 1963 . Cytochemistry and electron microscopy—Preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *Journal of Cell Biology* 17 : 19 – 58 .

SATPUTE , G. , H. LONG , J. SEGUÍ-SIMARRO , M. RISUEÑO , AND P. TESTILLANO . 2005 . Cell architecture during gametophytic and embryogenic microspore development in *Brassica napus* L. *Acta Physiologiae Plantarum* 27 : 665 – 674 .

SAUNDERS , R. M. K. 2012. The diversity and evolution of pollination systems in Annonaceae. *Botanical Journal of the Linnean Society* 169 : 222 – 244 .

SCOTT , R. J. , M. SPIELMAN , AND H. G. DICKINSON . 2006 . Stamen development: Primordium to pollen . *In* B. R. Jordan [ed.], The molecular biology and biotechnology of fl owering, 2nd ed., 298–331. CAB International, Wallingford, UK.

SOLÍS , M.-T. , B. PINTOS , M.-J. PRADO , M.-Á. BUENO , I. RASKA , M.-C. RISUEÑO , AND P. S. TESTILLANO . 2008 . Early markers of in vitro microspore reprogramming to embryogenesis in olive (*Olea europaea* L.). *Plant Science* 174 : 597 – 605 .

SU , Y. C. F. , AND R. M. K. SAUNDERS . 2004 . Pollen structure, tetrad cohesion and pollen-connecting threads in *Pseuduvaria* (Annonaceae). *Botanical Journal of the Linnean Society* 143 : 69 – 78 .

TOGHRANEGAR , Z. , S. NADOT , AND B. ALBERT . 2013 . Variation of microsporogenesis in monocots producing monosulcate pollen grains. *Annals of Botany* 112 : 135 – 139 .

TSOU , C. H. , AND Y. L. FU . 2002 . Tetrad pollen formation in *Annona* (Annonaceae): Proexine formation and binding mechanism. *American Journal of Botany* 89 : 734 – 747 .

TSOU , C. H. , AND Y. L. FU . 2007 . Octad pollen formation in *Cymbopetalum* (Annonaceae): The binding mechanism. *Plant Systematics and Evolution* 263 : 13 – 23 .

WALKER , J. W. , AND J. A. DOYLE . 1975 . The bases of angiosperm phylogeny— Palynology. *Annals of the Missouri Botanical Garden* 62 : 664 – 723 .

WILLMER , P. 2011 . Pollination and floral ecology . Princeton University Press, Princeton, New Jersey, USA.

WILLSON , M. F. 1979 . Sexual selection in plants. *American Naturalist* 113 : 777 – 790 .

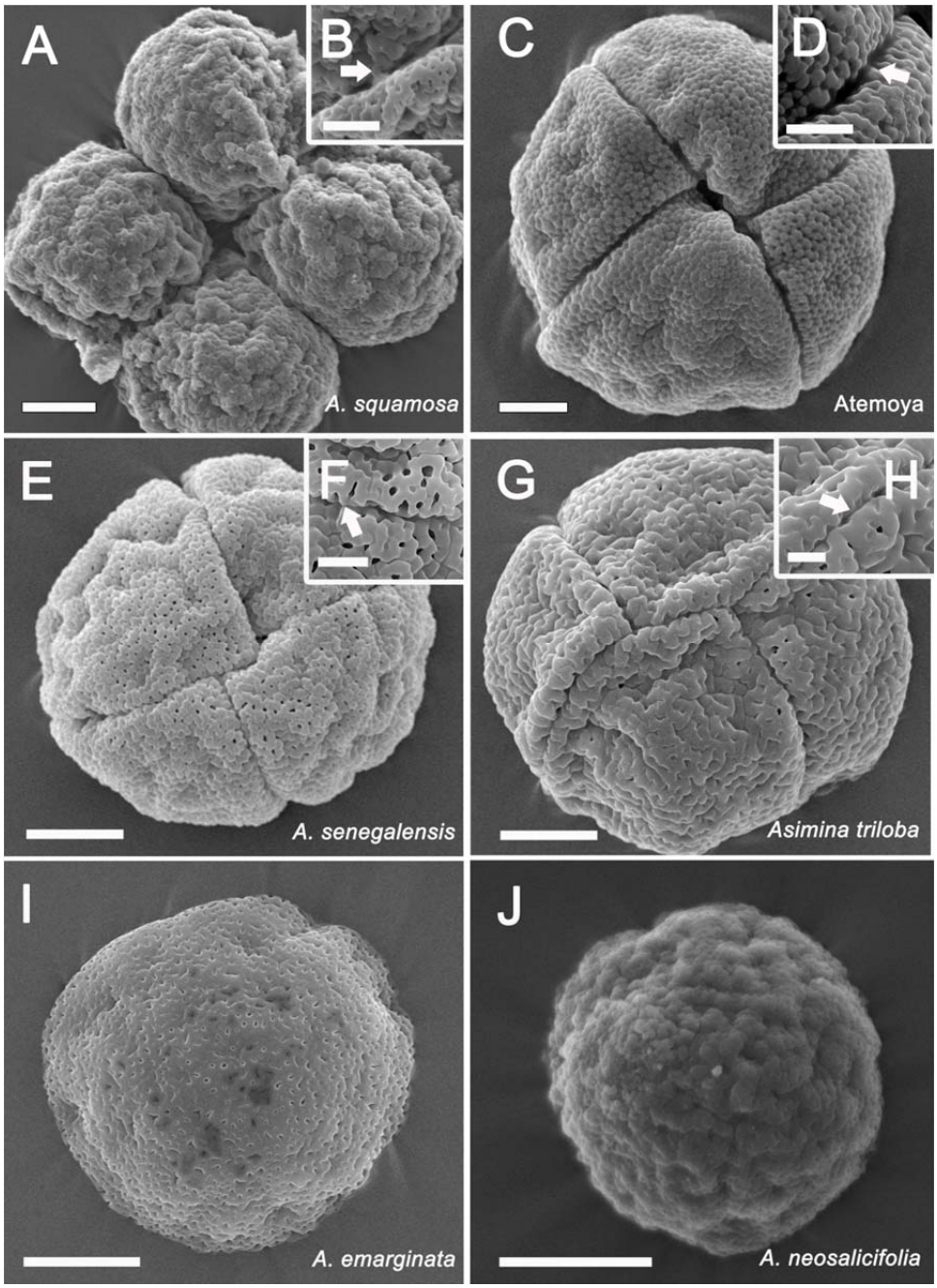

Fig. 1. Scanning electron microscopy of mature pollen. Mature pollen is globose in all species examined. Permanent tetrads are tetragonal in (A, B) *Annona squamosa* , whereas they are rhomboidal in (C, D) atemoya, (E, F) *A. senegalensis* , and (G, H) *Asimina triloba* . Exine cohesion (arrow) is shown in the upper square of species with tetrad pollen. (I) *Annona emarginata* and (J) *A. neosalicifolia* show inaperturate monad pollen. Scale bars: A, C, E, G = 10 μm; B, D, F, H = 5 μm; I, J = 20 μm.

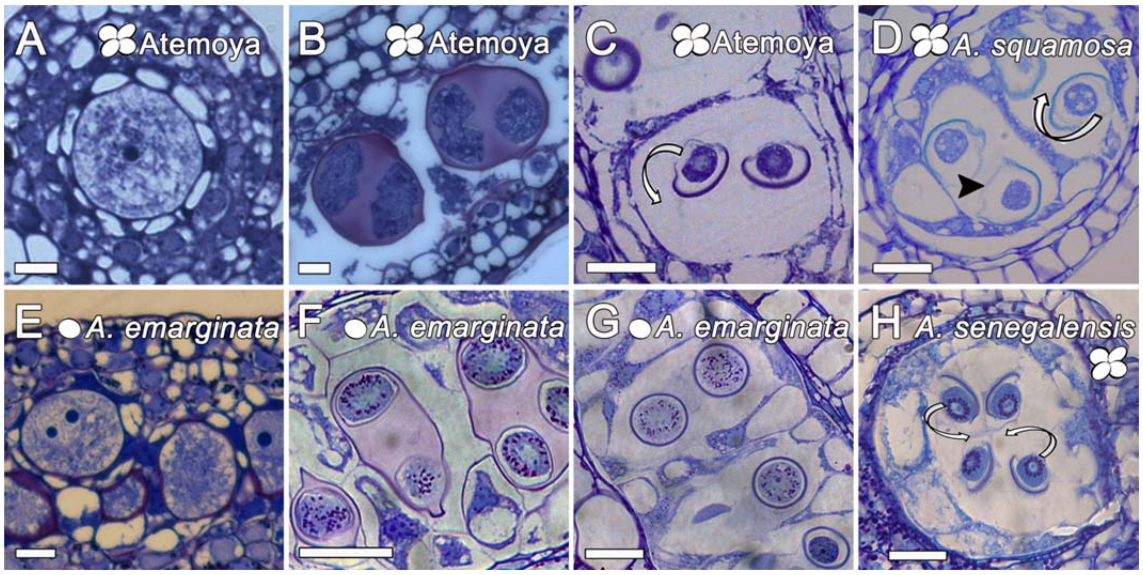

Fig. 2. Microsporogenesis in species with permanent tetrad (A–D, H), and monad (E–G) pollen. (A) Pollen mother cell (PMC) just before meiosis. (B) Successive cytokinesis. (C, D, H) Young microspores have rotated (white arrows), turning the distal thin wall aperture toward the center of the tetrad (black arrowhead in D). (E) PMC showing the fi rst meiosis. (F) Successive cytokinesis. (G) Young microspores showing starch grains and homogeneous thickness of the microspore wall. Anthers were stained with periodic acid–Schiff's reagent and toluidine blue. Scale bars = 20 μm.

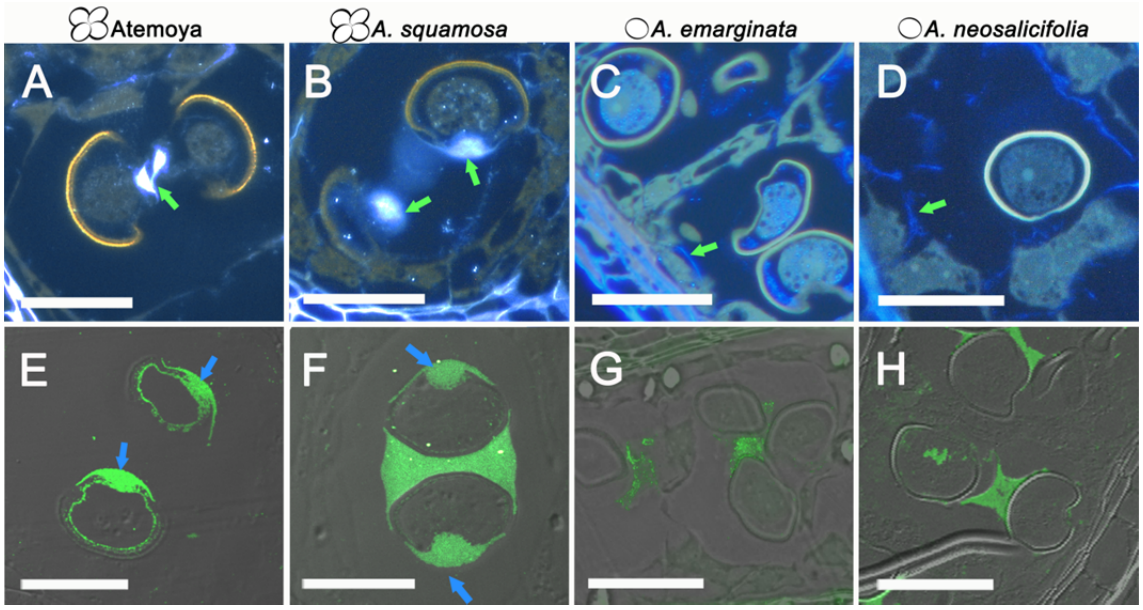

Fig. 3. Staining for cellulose (A–D) and callose (E–H) at the end of the tetrad developmental stage. Remnants of cellulose and callose were present in the aperture sites (arrows) of (A, E) atemoya and (B, F) *Annona squamosa* , but not in (C, G) *A. emarginata* or (D, H) *A. neosalicifolia* . Sections were stained with (A–D) a 3 : 1 mixture of auramine and calcofluor, and (E–H) callose was detected using an antibody against callose. Scale bars = 20 μm.

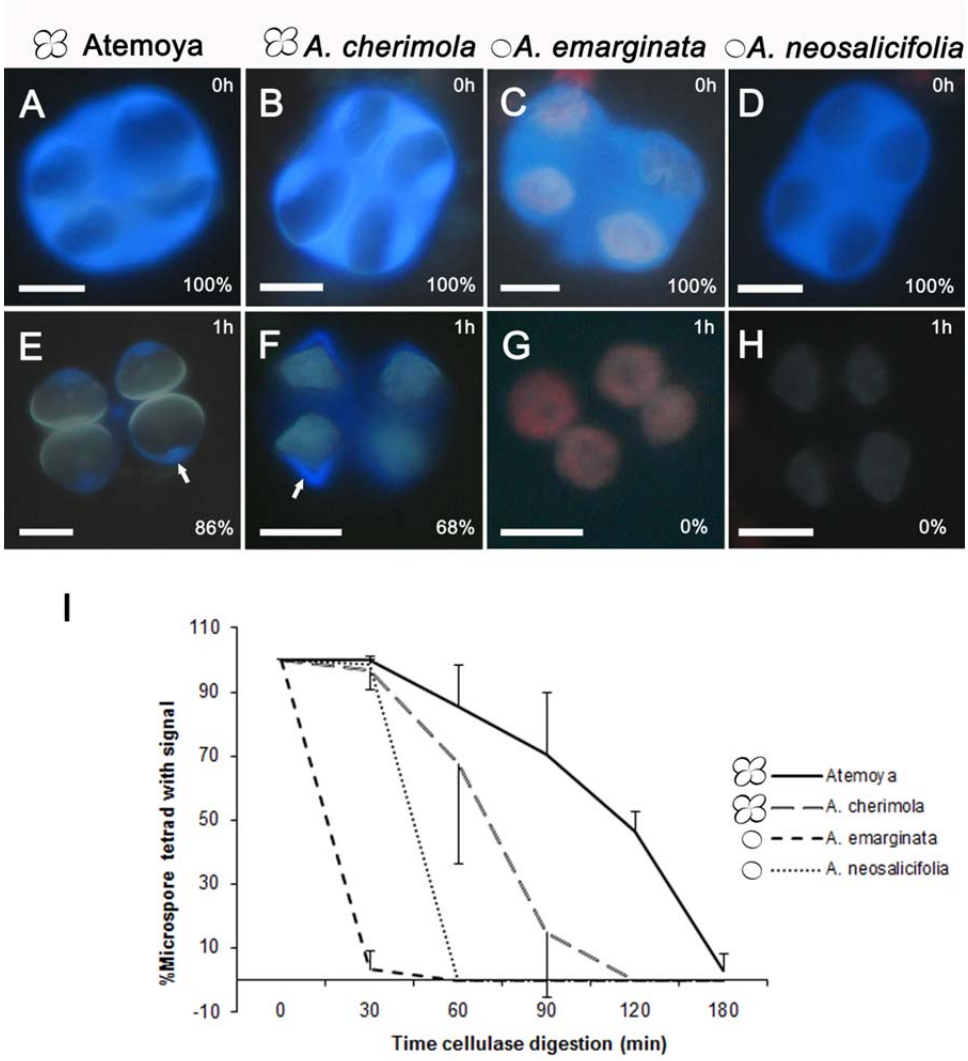

Fig. 4. Visualization of cellulose at the tetrad developmental stage. (A–D) Cellulose was detected in all examined species in the tetrad developmental stage. After 1 h of cellulose digestion, (E, F) remnants of cellulose were found in the aperture site (arrow) of the species with permanent tetrad pollen, whereas (G, H) cellulose digestion was completed in species with monad pollen. The percentage of microspores with cellulose is shown in the lower right corners. (I) Percentage of tetrads with cellulose staining, at different times of cellulose digestion, showing earlier digestion of cellulose in species with monad pollen than in species with tetrad pollen. Bars indicate SD. Cellulose was stained with calcofluor. Scale bars = 20 μm.

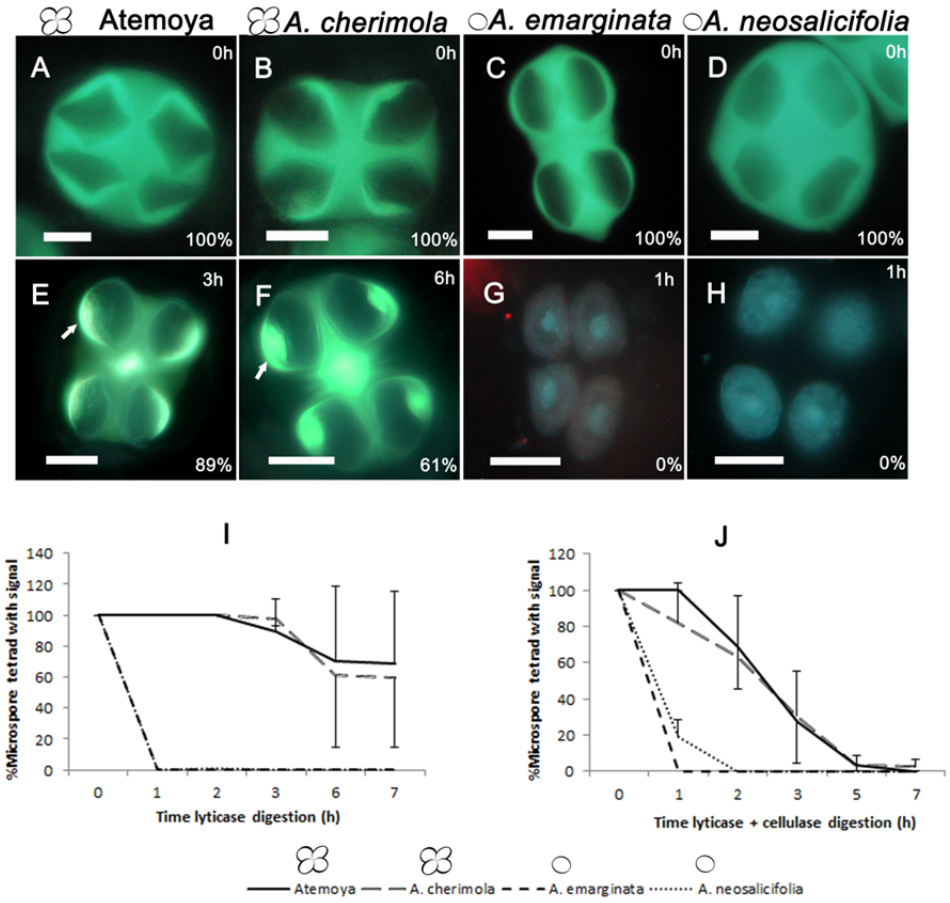

Fig. 5. Staining for callose at the tetrad developmental stage. (A–D) Callose was detected in all examined species in the tetrad developmental stage. After 1 h of callase digestion, (E, F) callose remnants were still present at the pollen aperture sites (arrows) in some of the tetrad pollen, whereas (G, H) callose was absent in species with monad pollen. The percentage of microspores with callose is shown in the lower right corners. (I, J) Percentage of tetrad microspores with (I) callose after digestion with callase or (J) with callase and cellulase together. Bars indicate SD. Callose was stained with sirofluor. Scale bars = 20 μm.

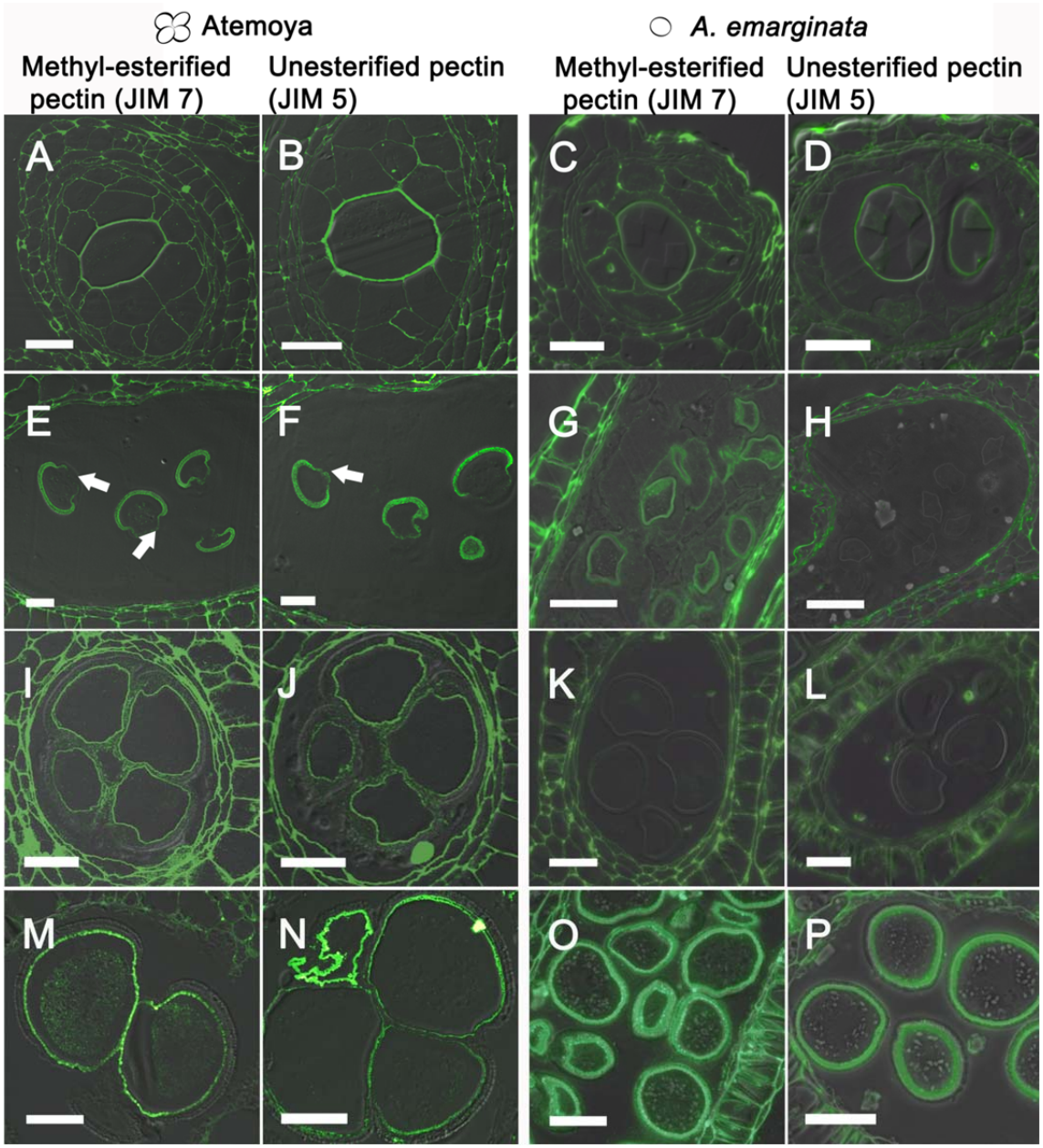

Fig. 6. Labeling with monoclonal antibody JIM 7 for methyl-esterifi ed and JIM 5 for unesterifi ed pectins, during pollen development, in tetrad (A, B, E, F, I, J, M, N) and in monad pollen (C, D, G, H, K, L, O, P). (A–D) Pollen mother cell walls showed both kinds of pectins. Tapetal cells showed (A, C) methyl-esterified pectins in both species, but (B, D) the signal was weaker for unesterified pectins. (E, F) Microspore wall of atemoya tetrad pollen showing (E) methyl-esterified and (F) unesterified pectins, but the signal is weaker in the aperture site (arrow). (G, H) Microspore pollen of Annona emarginata shows a homogeneous signal around the microspore wall for (G) methyl-esterifi ed and faintly detected for (H) unesterified pectins. (I–L) As microspores increased in size both pectins were labeled in the microspore wall of (I, J) atemoya tetrad pollen but not in (K, L) monad pollen of *A. emarginata.* (M–P) Mature pollen both pectins labeled in (M, N) tetrad and (O, P) monad pollen. Scale bars = 20 μm.

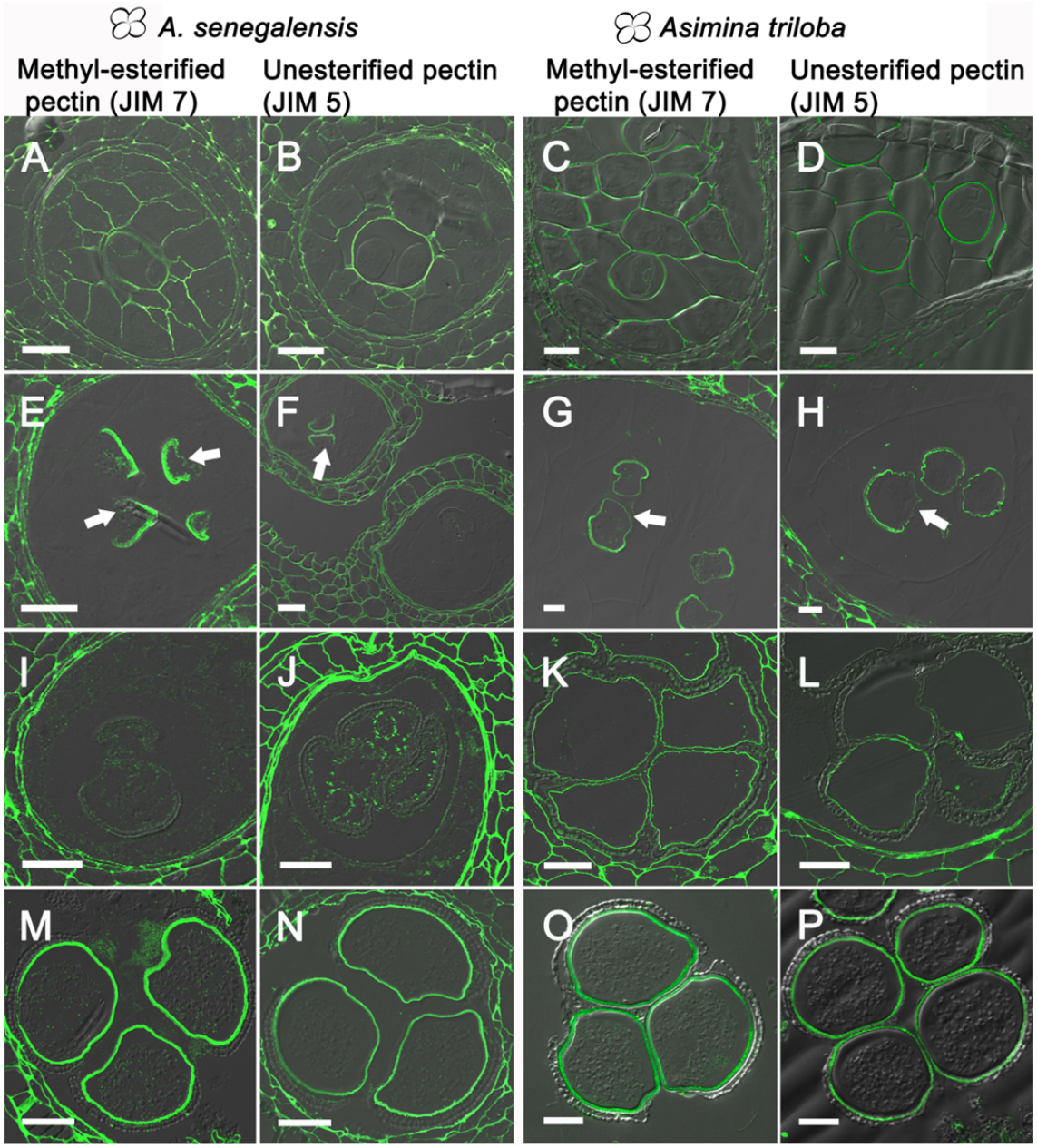

Fig. 7. Labeling with monoclonal antibody JIM 7 for methyl-esterifi ed and JIM 5 for unesterifi ed pectins, during pollen development in *Annona senegalensis* (A, B, E, F, I, J, M, N) and *Asimina triloba* (C, D, G, H, K, L, O, P). (A, C) Pollen mother cell (PMC) walls and tapetal cells showing methylesterified pectin in (A) *A. senegalensis* and (C) *Asimina triloba* . (B, D) Similarly, PMC walls also show unesterified pectins but weaker signal in the tapetal cells. (E, F) Microspore wall of *A. senegalensis* and *Asimina triloba* showing (E, G) methyl-esterifi ed and (F, H) unesterifi ed pectins but weaker signal in the aperture site (arrow). (I–L) Microspore has rotated 180º and increased in size; (K, L) methyl-esterified and unesterified pectins are detected only in the microspore wall of *A. triloba* . (M–P) Mature pollen showing methyl-esterifi ed and unesterifi ed pectins in (M, N) *Annona senegalensis* and (O, P) *Asimina triloba* in which cross-wall cohesion is present. Scale bars = 20 μm.

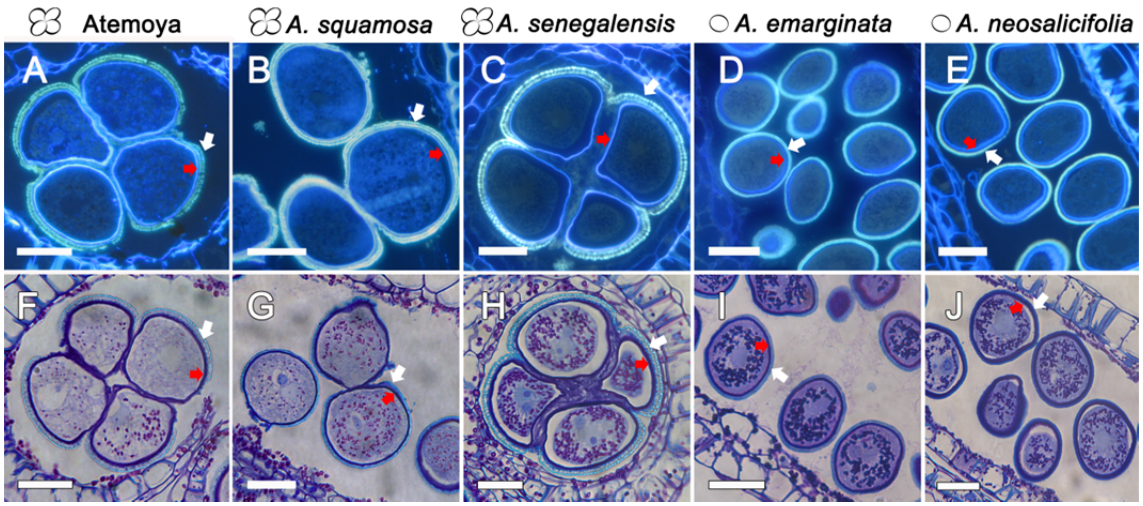

Fig. 8. Exine and intine distribution in mature pollen of species with tetrad (A–C, F–H) and monad (D, E, I, J) pollen. The intine (red arrow) covering is similar in all species, but the exine (white arrow) has a large, thinner aperture in species with tetrad pollen (A–C, F–H), whereas it is uniform around the pollen grain in species with monad pollen (D, E, I, J). (A–E) Exine was stained with auramine and intine with calcofluor after treatment with 3 : 1 auramine–calcofluor solution. (F–J) For general histological observations, the anthers were stained with periodic acid–Schiff's reagent and toluidine blue. Scale bars = 20 μm.