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Structure and development of male gametophyte in *Carya illinoensis* (Wangenh.) K. Koch

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Abstract. In order to understand the differentiation of staminate flowers of pecans (*Carya illinoensis* (Wangenh.) K. Koch), we carried out an integrated study of staminate flower development in a protogynous cultivar, Mahan, by assessing changes in external morphology and microstructure at multiple levels. Results showed that the staminate inflorescence differentiation cycle for pecans was 1 year. Staminate inflorescence development was acropetal. When inflorescences developed to 5–8 cm, the microspore mother cells in the base florets of the inflorescences entered into meiosis prophase and the middle layer started to degrade. When inflorescences grew to 8–10 cm, the microspore mother cells in the based florets of the inflorescences were at the peak of meiosis and cytokinesis was synchronous. When bracts have opened to 15°, the microspore mother cells of the basal florets had undergone two divisions to form tetrads. When bracts have opened to 45°, the basal florets entered the mid-late uninucleate stage and the tapetum underwent degradation and autolysis. When bracts opened to >90°, mature pollen grains were 2-celled, with three germ pores and the middle layer tapetum completely degraded. Anther wall development followed the basic type, which was composed of an epidermal layer, an endothecial layer, middle layer (1–3 layers, fibrous thickening absent) and the tapetal layer (cell division was from uninucleate to an octonucleate cell). In summary, external morphology and gametophyte development in pecan staminate flowers were consistent to related; thus, the internal gamete development status can be determined from external morphological characteristics of the flower. This provided a sampling basis and theoretical foundation for *in vitro* culture of pollen grains and elucidation of flowering mechanisms.

Keywords. Microspores, male gametophytes, tapetum, pollen morphology.

The pecan (*Carya illinoinensis* anth; *Juglandaceae*) is a deciduous tree native to North America with pleasant flleasa and medicinal & nutritional properties, popular with Chinese consumers (Thompson and Conner 2012). The pecan was introduced to China more than 100 years ago and is now extensively cultivated in Jiangsu, Zhejiang, and Yunnan Provinces (Zhang Ret al. 2015). Among the pecan cultivars, the protogynous variety "Mahan"

has the best combined traits of any pecan grown in eastern China (Zhang R et al. 2013). The pecan industry is rapidly developing but progress on related basic research on flower and fruit development has been slow. Monoecious pecans are cross-pollinated and dichogamous. Pecans are classified as protoandrous and protogynous according to whether the male or female reproductive parts first to develop and mature. A combination of protogynous and protoandrous varieties is a prerequisitie for fruit setting in a pecan plantation (Zhang R etal. 2015). To date, local studies on flower development have mostly dealt with external morphology and flowering phenology (Xie J 2013; Li C 2012; Xie J. 2011.). This information is used to design varietal combination verities for pecan plantations.

As a wind-pollinated plant, pecan trees produce large amounts of mature pollen to guarantee pollination. Research on staminate flower development in pecans can provide a theoretical basis for controlling the amount of flowers, improving flower quality, promoting normal development of pollen, and decreasing pollen abortion in staminate flowers. A large number of studies on staminate flower development of pecans appeared at the end of the 20th century. Woodroof (1924) was the first person to use hand-drawn figures to describe flower development. Yates and Sparks (1992) used the angle between the bract and the inflorescence axis to divide staminate flower development into 5 stages. On this foundation, Yates described the external characteristics of staminate flower development in the protogynous "Stuart" variety and the protoandrous "Desirable" variety. He further demonstrated the internal development map of these varieties, such as microspore tetrads, free microspores, and binucleate pollen grains (Yates and Sparks 1992; Shuhart 1932). However, he did not carry out cytological validation of the detailed process of development of gametes. In China, only Yang (2014) described some of the anatomical structures involved in staminate flower development, specifically tetrads and bunucleate pollen grains, in fruit abscission research in pecans. However, the stage during which abortion occurs in staminate flowers (such as microspore mother cell meiosis) has not been fully described. There is also no systematic description of changes in the tapetum or middle layer that could ensure pollen maturation or provide large amounts of nutrients for pollen development (Yates and Sparks 1992). There was no discussion of the evolution of pollen morphology.

We used observations of external morphology and internal anatomy during staminate inflorescence development to determine stages of differentiation at the microscopic level to establish the relationship between internal and external development. This may help observers determine the internal cellular development status from external morphological characteristics of the flower and provide a sampling basis and theoretical foundation for *in vitro* pollen culture and elucidation of flowering mechanisms. Additionally, timely measures can be employed according to the development status of pecan staminate flowers in order to provide guidance for practical production, such as variety collocation, prediction of flowering period, and performance of artificial pollination and removal of staminate flowers at appropriate times and in appropriate quantities.

1. MATERIALS AND METHODS:

1.1 Experiment materials and study site

Materials were obtained from the pecan cultivation base (32°19'59.48"N, 118°52'22.37"E) at Shanbei Village, Xiongzhou Street, Luhe District, Nanjing City, Jiangsu Province. This site has a humid subtropical climate, with an average annual temperature of 20°C , annual precipitation of 800–1000 mm, thick soil with a pH of 6.5–7.5. This region is rich in pecan resources, with good population and individual phenotypes. The sampling points for this study were on the northern side of mountain at an altitude of 50–200 m. Five mature trees with strong tree vigor, free from diseases and pests, were randomly selected from the Mahan variety (currently, the only known dominant homozygote (pp or PP) protogynous variety, with protogynous progeny) (Thompson and Romberg 1985).

1.2 Experimental methods

1.2.1 External morphological observations of pecan staminate floral bud and staminate inflorescence development

Experimental observations and sampling were carried out from February 2014 to June 2015. For each sample tree, five measurable branches with terminal buds were selected and labeled. Observation, recording, and photography were carried out every morning and the morphological characteristics of floral buds were recorded. The observation period started from when brown scales of staminate floral buds fell off during spring until staminate inflorescences matured.

1.2.2 Collection of microspore samples and male gametophytes of pecans

Three trees with normal growth and free from diseases and pests were randomly selected for sampling.

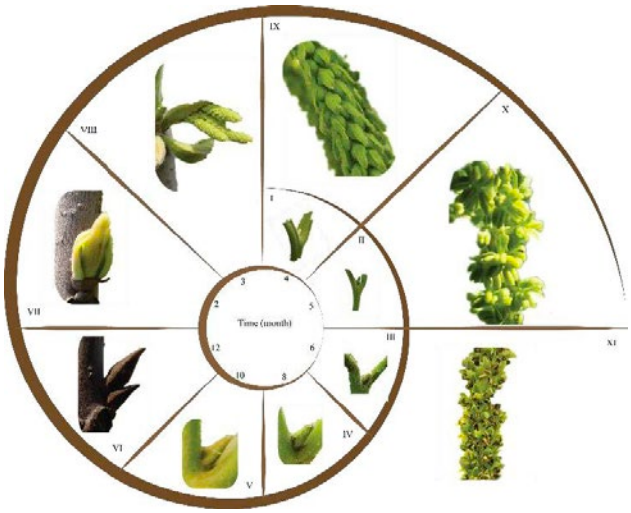

Figure 1. Cycle of pecan staminate flower differentiation over the course of one year.

The sampling time was determined according to the staminate flowering phenology of pecans (Figure 1, VII–XI). During late March of the second year, the leaves start to sprout and the brown scales on staminate floral buds abscise, taking on a broad ovate to triangular-ovate appearance, which is morphologically distinct from leaf buds. The first staminate inflorescence buds at the lower end of the main bearing branches were collected and sampling was carried out at 3-day intervals. Fifteen to twenty buds were collected during each sampling. Basal florets of staminate inflorescences from the sunward side of top and middle canopy layers were collected at 09:00–10:00 during the period from late March (when staminate flowers can be seen) to early May (when staminate flowers shed pollen). Collection was carried out according to different developmental stages (i.e. based on bract opening angle) since the flowering period of staminate flowers. The collected samples were immediately fixed and stored using FAA solution, then made into paraffin sections for microscopic observations and photography using an Olympus BX 60 microscope.

1.2.3 Sample preparation

Preparation of paraffin sections for optical microscopy: The sectioning technique was modified from Li (Li 1987). Flora buds were removed from fixation fluid and washed with distilled water. After cutting the buds in half along the middle axis, we used 10% ethylenediamine for 3–5 days of softening before dehydration using an alcohol gradient. Then, xylene was used for clearing and the plant tissue was embedded in paraffin for sectioning. The sections were 4–8 μm thick and were stained with safranin-Fast Green FCF and sealed in neutral resin. A LeicaDM-5000B microscope was used for observations and photography.

Sample preparation for scanning electron microscopy: One bunch of anthers which is goingto be shedding pollen were collected, fixed with glutaraldehyde, and washed 3–5 times with distilled water. A single pollen sac was cut transversely before dehydration using an ethanol gradient. Then it was dried to a critical point, placed on a platform, and sprayed with gold powder through ion spluttering. A FEI Quanta-200 scanning electron microscope was used for observations and photography.

DAPI fluorescence staining: Mature pollen grains were placed on glass slides and direct DAPI staining was carried out before the slides were sealed. Filter paper was used to absorb excess stain, nail polish was used to seal the sides, and the slides were stored at –20°C . The Olympus BH-2 epifluorescence microscope was used for observations, using a UGI (425 nm) excitation filter and an L420 (420 nm) emission filter.

1.2.4 Statistical analysis of pecan pollen morphology

By scanning electron microscopy, pole axis length (μm)/equator axis length (μm) and the number of particles per unit area (1 μm2) of 50 grains of pollen were measured using image processing software (Image J).

1.2.5 Image processing and data processing

All images were processed using Photoshop CS3 and Adobe Illustrator CS6.

2 RESULTS AND ANALYSIS

2.1 External morphology of pecan staminate flower development

As the bud scale abscise in late March, they expose the inner densely tomentose staminate floral buds and leaf primordia (Figure 2-1). The two lateral buds contain staminate inflorescences, which were tightly enclosed by large bracts and continue to differentiate. The floral axis continued to grow inside the bud and the bracts also continued to grow through early April(Figure 2-2). The middle bud contained unfolded new leaves that continued to grow. Inside, the new leaves started to uncurl (Figure 2-3). In mid-April, the staminate inflorescence extended and large bracts opened up (Figure 2-4). After large bracts had detached, the staminate inflorescence started to grow and

swell, taking on a slight curvature (Figure 2-9). The curved bracts were bound to the floral axis (Figure 2-10) and the pollen sacs were enlarged. The inflorescences continued to extend and swell, growing to around 5 cm. Slight separation of bracts and floral axis occurred (Figure 2-11).

In late April, the leaves had fully unfolded and the growth rate slowed down. The new shoot started to emerge slowly. The staminate inflorescence continued to differentiate and width was 2 cm. The perianth and anthers could be seen from outside the bud. Bracts started to straighten

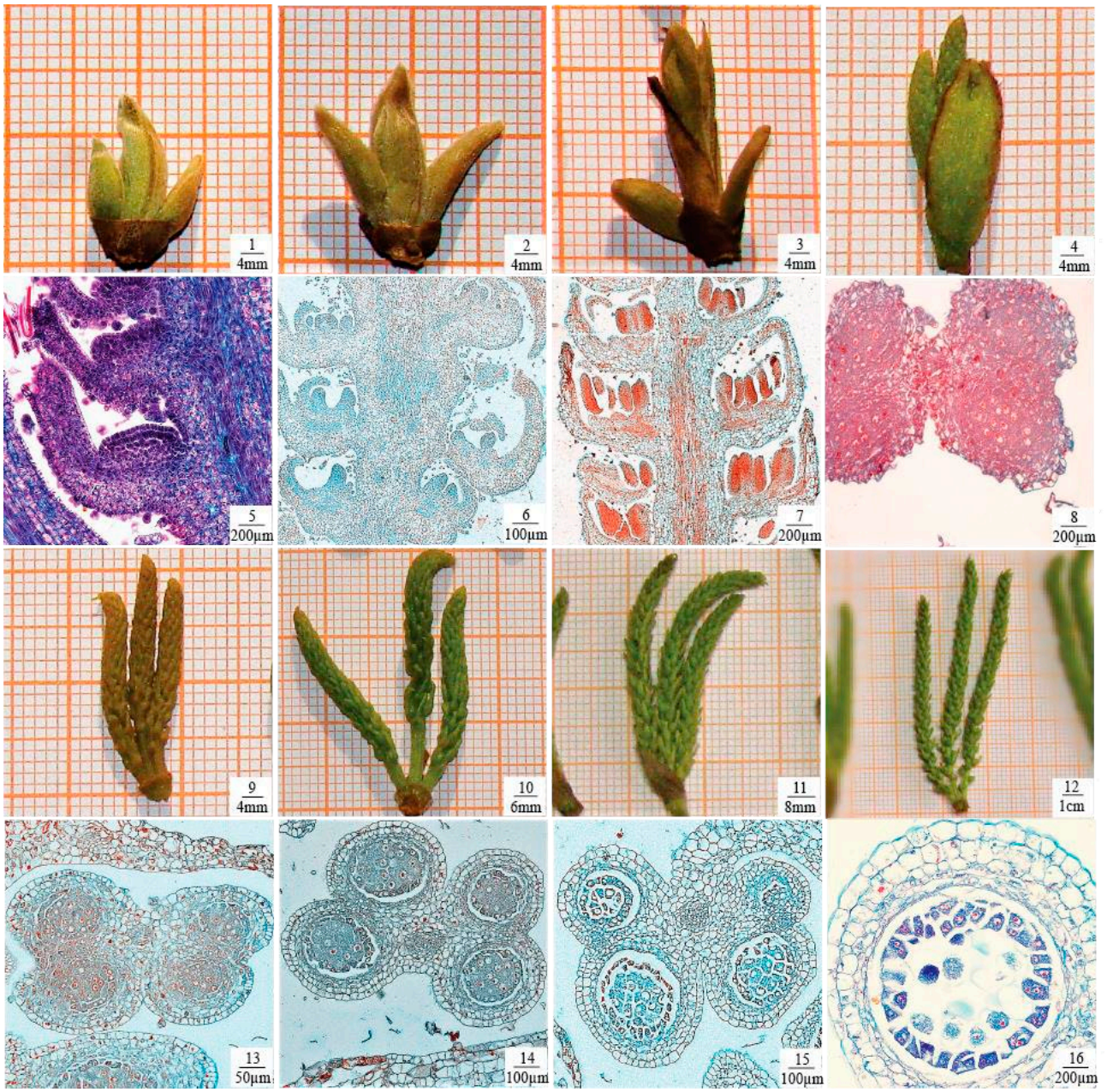

Figure 2. Internal and external structure of bud and staminate flower differentiation in pecan. 1) Staminate flower buds elongated and swollen. 2) Staminate flower buds elongating in bracts. 3) Leaves separation. 4) Side bracts cracking and staminate inflorescences extending. 5) Floret primordium expansion. 6) Bracteole differentiation. 7) Pollen sac elongation. 8) Secondary sporogenous cell differentiation. 9) Emergence of large deciduous bracts. 10) Inflorescence elongation and swelling. 11) Swelling of pollen sacs. 12) Visible pollen sacs. 13) Microspore mother cell differentiation. 14) Pollen sac: 4 chamber. 15) Microspore mother cells entering prophase. 16) Tapetum cell proliferation, microspore mother cell mitosis to dyad.

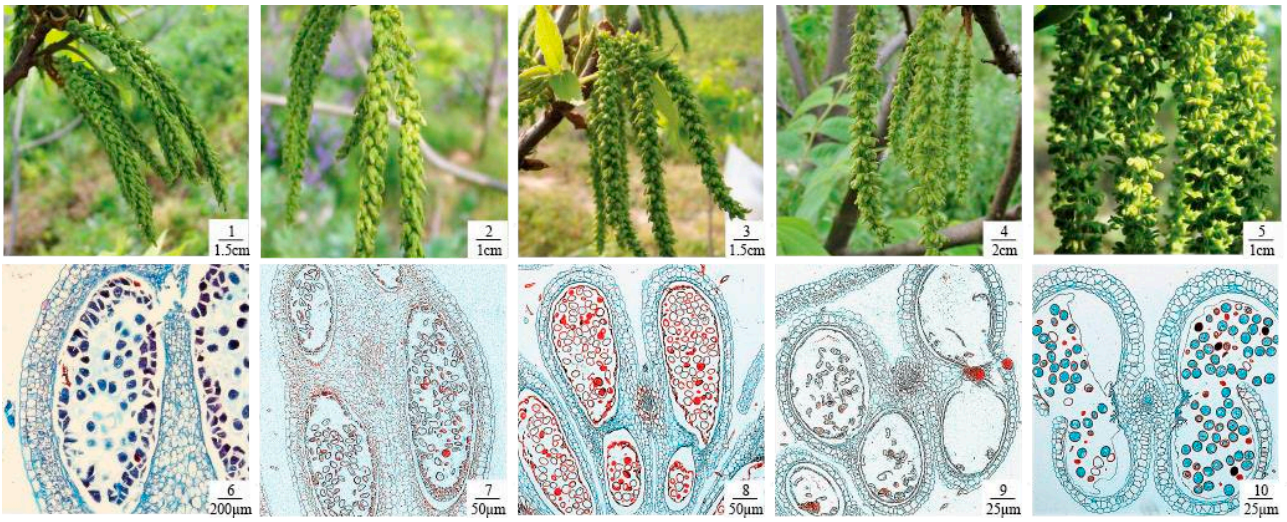

Figure 3. Internal and external structure of staminate flower differentiation in pecan. 1) Inflorescence elongated, angle between bracts and inflorescence axis around 5°. 2) Anthers dilated, bracts open, angle between bracts and inflorescence axis 10°. 3) Anther differentiation, angle between bracts and inflorescence axis 45°. 4) 4–6 anthers visible, angle between bracts and inflorescence axis 60°. 5) Anthers yellow-green, fully visible, turned outward, bracts open and inflorescence axis angle greater than 90°. 6) Microspore mother cells in metaphase nuclei and the nucleus polarized. 7-8) mononuclear microspores moved aside, and the tapetum degraded. 9) Microspore mother cells aborted, pollen wall degradation. 10) With the disintegration of the anther wall in each chamber, the pollen sac cracks and mature pollen was dispersed.

from a curved shape through extension and growth (Figure 2-10~Figure 2-12). The inflorescence grew until it reached a length of 9 cm, and bracts started to open (Figure 2-12). Bractlets gradually unfolded and their angle with the floral axis started to increase (Figure 3-1~Figure3–5). During the growth phase of the new shoots, leaves started to unfold and the tips of the leaves were reddish in color. In this period, staminate inflorescence length was around 5 cm and exhibited a cone shape. The anthers were enlarged and gradually changed from light green to emerald green before undergoing rapid enlargement. The four pollen sacs could be gradually seen and changed from green to yellow-green, and the texture of the pollen sacs changed from soft to hard, leathery, and reflective (Figure 3-5). In early May, when the staminate inflorescence stopped extending and bracts unfolded at an angle greater than 90°, unicellular pollen grains further developed into bicellular pollen grains until maturity, when anther dehiscence expose the yellow pollen. At the end of the pollen shedding period, the anthers shriveled, turned dark green, and gradually withered and fell off.

2.2 Primordium development and occurrence of microspores in staminate flowers

In late March, floret primordia protrusions appeared at the base of bract tissue and the top of the primordia became flatter and wider (Figure 2-5). Staminate inflorescences elongated and the number of bracts increasing. The bracts at the base of the inflorescence were relatively large while bracts at the top were smaller. The staminate flower primordia grow gradually and continue to differentiate into anthers. Column-shaped anthers became elongated and were arranged side by side within bracts (Figure 2-6).

In early April, archesporial cells appeared and underwent further periclinal division to form primary sporogenous cells. Then, the sporogenous cells differentiated into secondary sporogenous cells, forming young anthers that had a near-rectangular shape in longitudinal section (Figure 2- 7). Primary peripheral cells were formed by outward division, which then further differentiated into butterfly-shaped pollen sacs (Figure 2-8). Primary sporogenous cells continued to undergo mitosis to form several secondary sporogenous cells. These cells had a tight arrangement, thick cytoplasm, large nuclei, and polygonal shapes (Figure 2-8). Secondary sporogenous cells continued to undergo mitosis to form even more secondary sporogenous cells, which were arranged tightly inside the anther locule. The volume of the anther locule also increased. At the late stage of division of secondary sporogenous cells, cell-cell connections became weaker and large gaps appear. The cytoplasm became thinner while the nucleolus became apparent and was stained deeply (Figure 2-14). The secondary

sporogenous cell phase lasted 1 week, after which the nucleoplasm became thick again, the nucleolus ceased to be visible, microspore mother cells formed (Figure 2-15), and callose deposition began. Primary peripheral cells underwent periclinal division and were differentiated into an inner layer and outer layer of secondary peripheral cells (Figure 2-16). Cells actively differentiated inside the pollen sacs and both microspore mother cells and tapetal cells underwent vigorous division (Figure 2-16).

2.3 Microspore meiosis

After microspore mother cells became surrounded by callose they undergoes meiosis, the nuclear membrane and nucleolus disintegrated and microspore dyads formed (Figure 4-10). These dyads continued to divide into tetrads (Figure 4-12), which eventually formed pollen grains. Changes in chromosome behavior during meiosis of microspore mother cells were described as follows: (1) Prophase I: Chromosomes were extracted from the nucleolus (Figure 4-1~Figure 4- 2); the nucleolus became smaller (Figure 4-3) and gradually disintegrated (Figure 4-4) and disappeared. The chromosomes became short and thick (Figure 4-5). (2) Metaphase I: The spindle fibers were attached to the centromeres (Figure 4-6) and homologous chromosomes were pulled towards the two poles. Bivalent pairing could be observed at the polar view (Figure 4-7). From the lateral view, it can be seen that chromosomes were arranged on the equatorial plate (Figure 4-8). (3) Anaphase I: The nucleolus and nuclear membrane disappeared, homologous chromosomes that formed bivalents separated and continued to move towards the two poles (Figure 4-9). (4) Telophase I: The chromosomes that migrated to the poles disappeared and aggregated to form an irregular mass. Cytoplasm cleavage occurred and a binucleated cell was formed (Figure 4-10). Subsequently, the cell directly entered prophase II and stratification of anther wall cells was apparent (Figure 2-13). (5) Metaphase II: The nuclear membrane disappeared and chromosomes were arranged on two sides of the equatorial plate in the mother cell. The same anther locule exhibited synchronous progression (Figure 4-11). (6) Telophase II: cytoplasm cleavage occurred again and four cells surrounded by callose were formed, while the cell wall of each cell also took shape (Figure 4-12). Finally, the tetrad was formed.

2.4 Development of male gametophytes

At the end of April to early May, the staminate inflorescence grew rapidly. The four cells in the tetrad separated to form free microspores that were uninucleate. These microspores had thin walls and thick cytoplasm, and the nucleus was located in the center of the cell (Figure 5-1). Figure 5-2 shows a free microspore by fluorescent staining. The cells were red and slightly swollen. The uninucleate pollen grain absorbed nutrients from tapetal secretions or its degradation products and its volume increased. Cytoplasmic vacuolation was significant (Figure 5-3), forming a large central vacuole. The nucleus was compressed by the large vacuole and move close to the pollen wall (Figure 5-4). The free microspore entered the mid-late uninucleate stage (Figure 5-5) while cells became transparent (Figure 5-6). The nucleus underwent unequal division near the wall (Figure 5- 7) to form binucleate cells of different sizes. The vegetative cell near the vacuole was larger and the genital cell that was near the pollen wall was smaller. The cell plate disappeared and the large and small cells moved freely between the vacuole and the cell wall (Figure 5-8). With further development of the male gametophytes, the germ cells left the pollen wall and vacuolation decreased (Figure 5-9). They moved towards the center of the pollen grain and became separated from the vegetative cells (Figure 5-10). Figure 5-11 shows developing pollen grains. The genital cell gradually elongated and took on a crescent shape (Figure 5-12), then a spindle-shape (Figure 5-13) and the liquid-pattern nucleus (Figure 5-14) flew through the pollen tube through the germ pore (Figure 5-15).

2.5 Anther wall development

After one periclinal division and multiple anticlinal divisions, the primary peripheral cells differentiated into secondary peripheral cells (Figure 6-1). The two layers of cells divided, with the outer cells differentiating into the endothecium and middle layer while the inner cells developed into the middle layer and the tapetum (Figure 6-2). Cells in the middle layer of the anther locule and tapetal cells divided further, forming 1–3 layers of cells. Anther wall development was simulations with the occurrence of microspores and development of the male gametophytes. During meiosis of microspore mother cells, anther wall differentiated into an epidermal layer, endothecium, middle layer, and tapetum in the end (Figure 6-3).

Tapetal cells: The tapetal cells of the pecan anther wall belong to glandular tapetum type. During the initial phase of secondary sporogenous, it can be seen that the morphology of early tapetal cellswas similar to that of anther wall cells, with thick cytoplasm (Figure 6-4). Coincident with meiotic prophase in the microspore

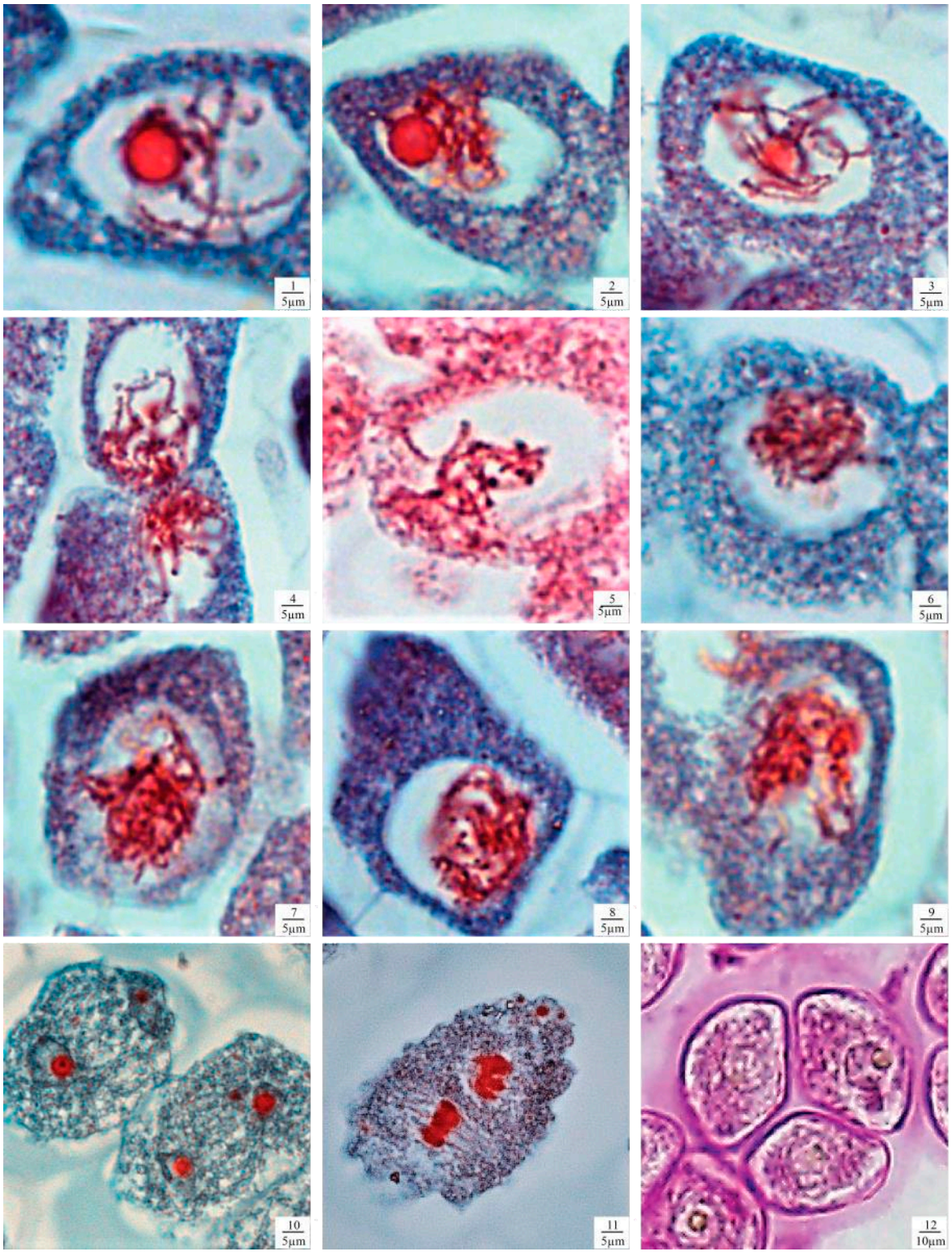

Figure 4. Male gametophyte development of pecan. 1) Microspore mother cells separating and detaching from each other. 2) Pre-prophase, leptonema I, chromosome extraction. 3) Zygotene I, nucleolus gradually disappearing. 4) Pachytene I, chromosomes shorter and thicker, relatively concentrated. 5) Diplotene I, chromosome pairing; Figure 4-6. Metaphase I (polar view). 7) Metaphase I (side view). 8-9) Anaphase I, chromosomes at poles. 10) Dyad, visible binucleated cells. 11) Metaphase II, spindle apparatus. 12) Tetrad stage of microspore development.

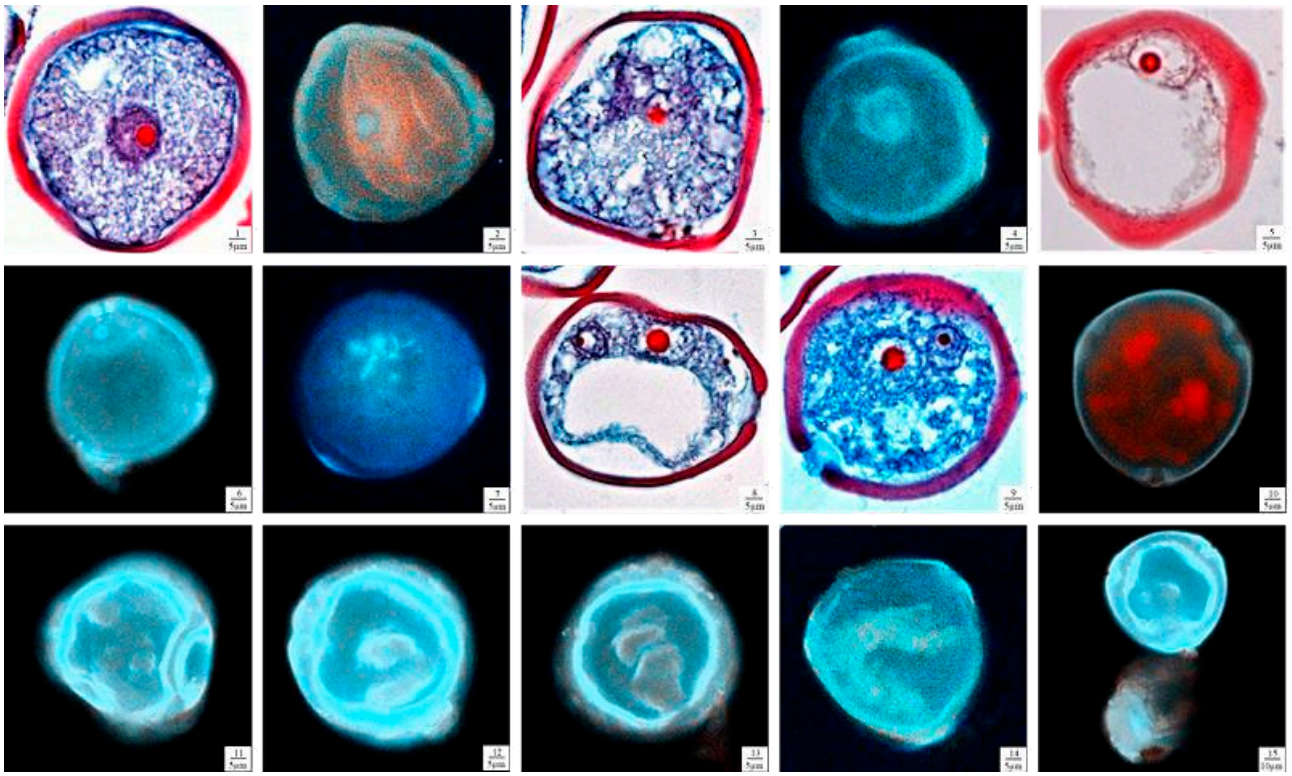

Figure 5. Observations of DAPI fluorescence staining of Microspore in *Carya illionensis*. 1-2) DAPI. Single isolated microspore. 3-4) DAPI. Central microspore. 5-6) DAPI. Uninucleate microspore in periphery stage. 7) Germ cell mitosis (DAPI). 8) Germ cells immersed in the cytoplasm of a vegetative cell and close to the cell wall. 9) Germ cells and vegetative cells free to the center of the nucleus. 10) Germ cells immersed in the cytoplasm of vegetative cells (DAPI). 11) Two-celled mature pollen (DAPI). 12) Differentiation of germ cells (DAPI). 13-14) Nucleus inclusions precipitated from the germination pore(DAPI). 15) Pollen germination(DAPI).

mother cells, the cytoplasm coincident with became thinner, and single nucleus could be seen. It then began to divide (Figure 6-5). Coincident with anaphase I of microspore mother cell meiosis, the tapetal cells were binucleated (Figure 6-6). The tapetal cells continued to divide and had a near-diamond shape, large nucleus, thick cytoplasm, small vacuole, and large volume. These cells are many times larger than other anther wall cells and had four, eight, or more nuclei (Figure 6-7). Tapetal cells divided into septal cells earlier than microspores (Figure 6-8). When microspore mother cells are at diakenesis I, the tapetum was formed (Figure 6-9). During the entire development process, the position of tapetal cells was unchanged and intracellular protoplastids provided nutrients and structural materials for microspore development through intracellular tangential surfaces. The tapetum underwent degradation and autolysis during the mid-late uninucleate stage of microsporogenesis. This was mainly manifested as cell wall degradation from the inner tangential wall towards the outer tangential wall (Figure 6-10). During the process of tapetum degradation, the tapetum provided nutrition for pollen grain elongation and structural materials (Figure 6-11~Figure 6-15). During the mid-late uninucleate stage, the tapetum underwent in situ disintegration and only a single layer of remnants was retained (Figure 6-16).

Middle layer: The 2–3 layers of cells were surrounded by the endothecium (Figure 6-6). During the formation of the microspore mother cell, the middle layer's second layer was compressed and degenerated to some degree (Figure 6-12~Figure 6-13). For example, three layers showed no sign of degeneration (Figure 6-14). The cells gradually atrophied and flattened, disintegrated, and were absorbed (Figure 6-17~Figure 6-18). When the pollen grain was mature and anthers underwent dehiscence, the middle layer of cells basically disintegrated and disappeared (Figure 6-18~Figure 6-19).

Endothecium: A layer of cells were near the epidermis (Figure 6-2). Cells were large and round during prophase (Figure 6-3). As the anther develops, the anther locule expanded, the diameter of inner wall cells

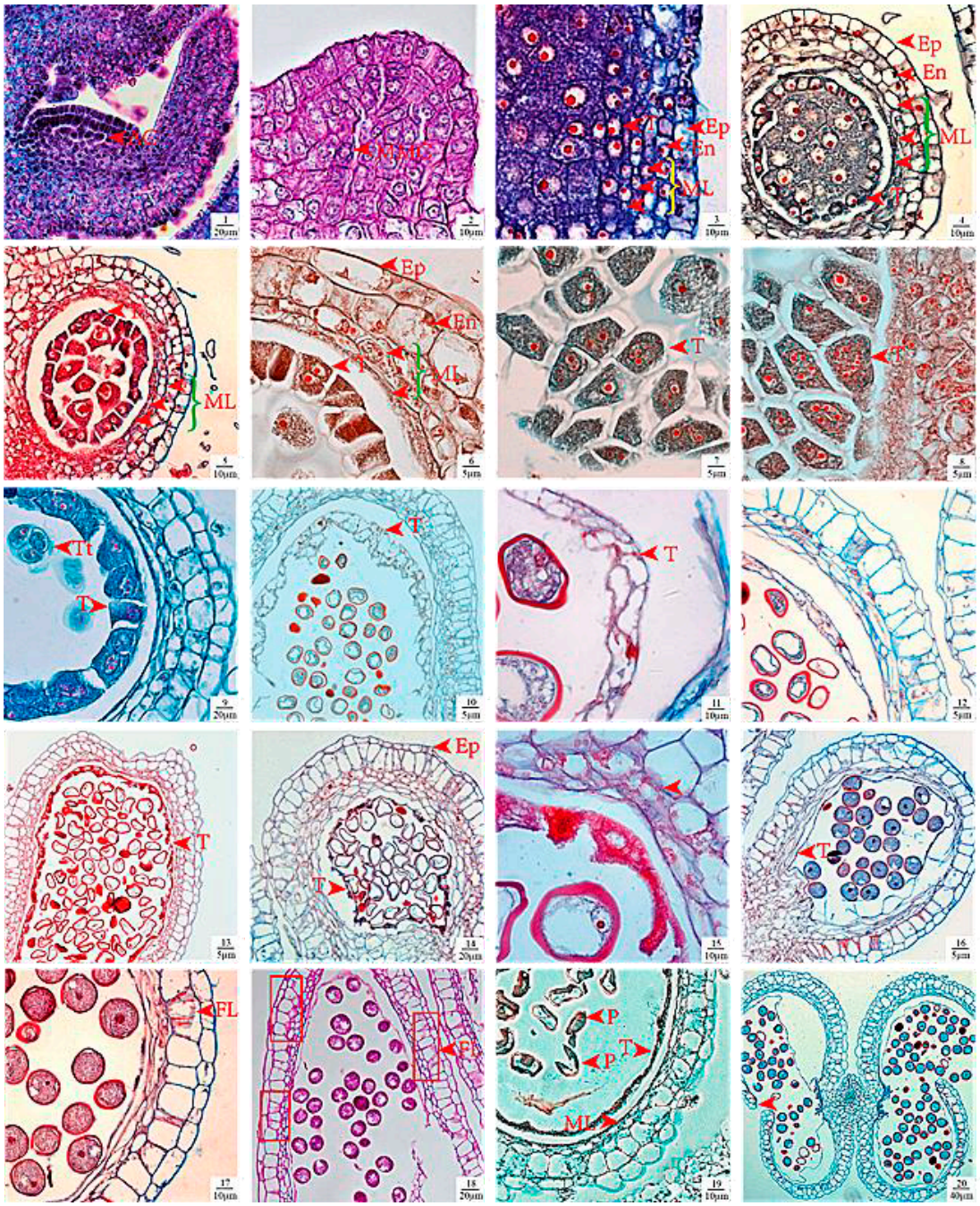

Figure 6. Development of male flower wall and tapetum of anther. Formation of the anther wall in *Carya illionensis*. AC, Archesporial cell; Ep, epidermis; En, endothecium; ML, middle layer, MMC. Microspore mother cell; Ta, Tapetum; FL, Fibrous layer, Tt, Tetrahedral tetrads; VC, vegetative cell; GC, generative cell. 1) Anther wall of primary sporogenous cell stage. 2) Anther wall of secondary sporogenous cells. 3) Anther wall of pollen mother cells: 6 layers, tapetum initial differentiation. 4) Pollen mother cells are separated from each other and differentiating tapetal cells are separated. 5) Microspore meiosis before the prophase, tapetum elongated and turn flattened. 6) Microspore meiosis I late, tapetal cell division. 7) Microspore meiosis I late transition period, heterotic tapetum division peak period. 8) Heterogeneous multicore tapetum; the middle containing starch granules and other nutrients. 9) Tetrad stage, epidermal expansion, the inner wall thickening, the middle 3 layers, glandular tapetum 2 to 3 layers. 10) Late uninucleate microspores: The tapetum wall beginning to disintegrate. 11) Tapetum showing disintegration and became thinning. 12) The inner layer of the tapetum was almost completely dissolved. 13) The tapetum had dissolved, leaving only a monolayer; the cell wall had dissolves, leaving the edge useless. 14) The tapetum was disintegrated outside the tangential wall and the middle layer was flattened. 15-16) The tapetum is almost completely dissolved. 17) The middle layer began to dissolve and banded; anther chamber wall showed fibrous thickening. 18) The cell walls of chamber showed fibrous thickening, and the middle layer had dissolved. 19) 2 nucleated stage, the middle layer is almost completely dissolved. 20) Interventricular rupture, pollen sac split.

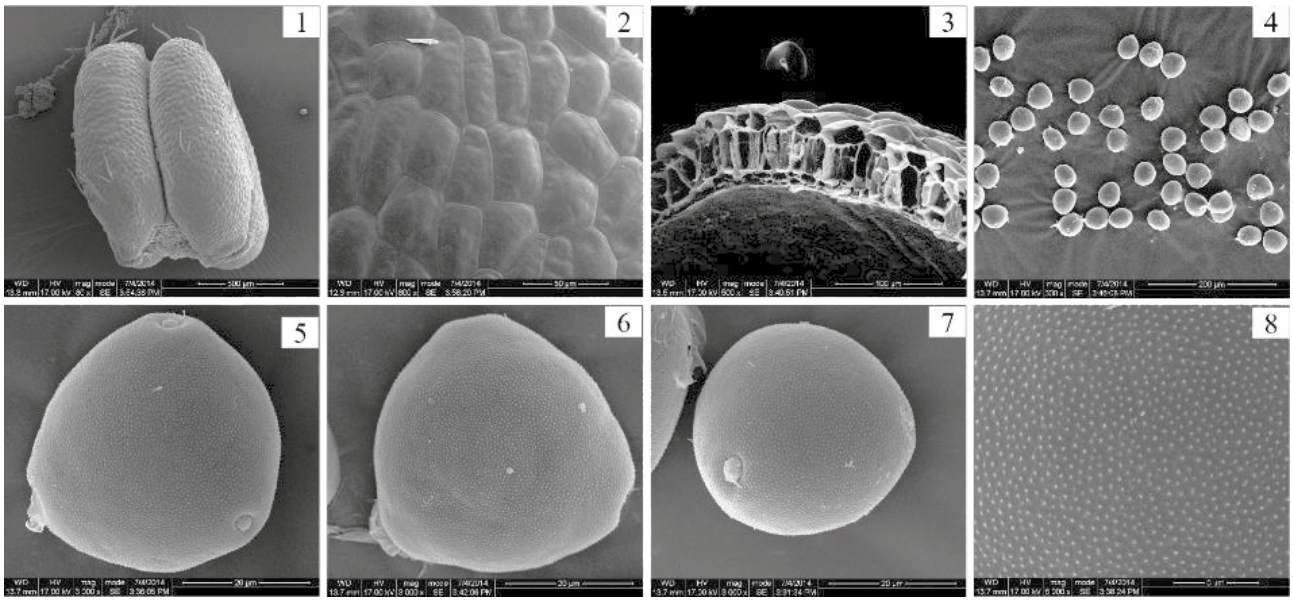

Figure 7. Scanning electron microscopic observation of pollen sac of pecan. 1) Overall appearance of anther. 2) Pollen sac surface. 3) Pollen sac wall anatomical structure when shedding pollen. 4) After pollen sac cracking, pollen group photo. 5) Pollen side view. 6) Pollen polar view. 7) Pollen equatorial plane view, showing micropyle. 8) Pollen surface ornamentation.

increased, and belt-like thickening took place outwards and upwards from the inner tangential wall (Figure 6-9). When the pollen grain was formed, the endothecium formed a fibrous bundle, also known as the fibrous layer (Figure 6-17~Figure 6-18). Secondary thickening did not occur in cells between two pollen sacs at one side of the butterfly-shaped pollen sac (Figure 6-14). During flowering, the entire pollen sac opened, shedding pollen (Figure 6-20), leaving the endothecium nearly empty (Figure 7-3).

Epidermis: Cross-sectional observations indicated that the epidermis exhibited a long rectangular shape, which then underwent anticlinal division in order to adapt to expansion caused by internal anther development. During meiosis of anther mother cells, the epidermis has a distinct cuticle that continued to thicken (Figure 6-6). When anthers were mature, the epidermis expanded and cells became flattened (Figure 6-10~Figure 6-13). Some of the cells disintegrated and only wavy residues were retained (Figure 6-18). The mature anthers contained hairs and the epidermal cells exhibited an irregular massive structure (Figure 7-1~Figure 7-2). Epidermal hairs decreased with anther development. Because anthers were exposed to the air, they underwent severe dehydration and the irregular magradually shrunk and protruded. Figure 7-3 shows the initiation of dehydration on the anther surface and severe dehydration caused the massive to shrink. The surface tension of the anther wall was increased, eventually causing dehiscence.

2.6 Pollen morphology

The pollen wall has three germ pores, which were distributed along the equatorial axis. The polar reveals a near-triangular shape. The proximal polar and distal polar morphological structures were generally similar, and the pollen was isopolar. The surface of the pollen exhibited densely distributed granular ornamentation. Upon measurement, we found the ornamentation density to be 8.9 um and the coefficient of variation to be 8%. The average length of the polar axis of pollen from the Mahan pecan was 39.92 μm and the equatorial axis was 35.66 μm. P/E value of Mahan is 1.119 and belongs to the spheroidal type.

2.7 Consistency between external morphology and anatomical structures during staminate flower development in pecans

Pecan staminate flowers take approximately 1 year from development of the inflorescence primordia to pollen grain maturation. Through observations of staminate floral bud and staminate flower differentiation in pecans, we summarized the correlation between external morphological and tissue structure during differentiation (Table 1). Descriptions of different stages, such as the length of the staminate inflorescence, whether bracts, perianth, or anthers are visible, color changes in bracts

Table 1. Relationship between the external morphology and anatomical structure on staminate flora-bud development of *Carya illinonensis*.

2012/date	2013/date	External morphology	Anatomical structure
03-07~03-13	03-08~03-15	Inflorescence extending out of bract	Archesporium formed
03-14~03-20	03-16~03-22	A cone shaped inflorescence	Archesporium periclinal division
03-21~03-27	03-23~03-27	Inflorescence thickened, elongated, globose	Primary sporulation cells and primary parietal cells are formed
03-28~04-10	03-28~04-14	Inflorescence axis elongation, morphological differentiation completed	Primary sporulation and primary parietal cells continue to differentiate
04-11~04-23	04-15~04-25	Inflorescence elongation, pollen sac enlargement	Secondary sporulation forms, and anther wall begins to divide
04-24~04-26	04-26~04-30	Angle between rachis and bract increased to 30	Formation of microspore mother cells and obvious stratification of pollen wall
04-27~05-03	04-31~05-05	Angle between rachis and bract increased to 45	Microspore enters tetrad period
05-04~05-11	05-06~05-10	Angle between rachis and bract increased to 90	Single cell pollen formation and degeneration of tapetum cells
05-12~05-15	05-11~05-13	Anthers dehiscence to release yellow pollen grains	2-cell pollen, pollen wall rupture, tapetum disappeared

and anthers can be used as indicators of structural changes in tissues.

3. CONCLUSION AND DISCUSSION

3.1 Developmental characteristics of pecan staminate flowers

In pecans, the developmental progress of different parts of the same tree can be different: The periphery of the canopy develops early while the core develops later. The upper parts of the tree develop early and the lower parts develop later. Healthy branches develop early while thin and weak branches develop later. In the same inflorescence, microspore mother cells at the base of the florets develop slightly earlier than florets at the top. Staminate inflorescence development is acropetal and undergoes basifugal growth and development; i.e., development and maturation gradually occur at the base of the floral axis towards the top. This is consistent with the development of *Cyclocarya paliurus* (Juglandaceae) (Fu et al. 2010) and *Carya cathayensi*s Sarg (Huang et al. 2006). Through observation of staminate flower development status at basal of staminate flowers to determine the development status of staminate flower, we found that the developmental stages of staminate inflorescence are consistent with that described by Yates (1992). During development of the external morphology of pecans, the inflorescence elongates, florets enlarge, bracts dehisce, and anthers turn yellow. At the corresponding internal anatomical development stage, the anthers and anther wall, microspores, and male gametophytes develop. Most protoandrous varieties enter into dormancy at the year when flower primordia are form. The protogynous "Mahan" variety forms flower primordia in the spring of the following year, which gradually differentiates into staminate flowers. The external morphological characteristics during staminate flower development can be used to evaluate the maturation stages of reproductive cells in the anthers of pecans.

3.2 Developmental characteristics of microspores and male gametophytes of pecans

Through combination with field observations, anther microscopic examination showed that the "Mahan" pecan variety enters into meiosis prophase when staminate inflorescences reach 4–8 cm. Microspore mother cell meiosis in pecans is classified as synchronous meiosis and division presentation is generally consistent, which is different from *Catalpa bungei* (Fan et al. 2011) and *Atractylodes japonica* (Cao et al. 2004). The microspore mother cell undergoes differentiation and two mitotic divisions to form microspores. The cytokinesis mode used by this plant is synchronous, which is consistent with cucumbers (Cao et al. 2004) and broccoli (Wan et al. 2006). Staminate inflorescences containing anthers and bractlets that are enclosed by large bracts do not get contain reproductive cells (i.e. mature microspores to male gametophytes) and only flower primordia and sporogenous cells are present. Subsequently, free microspores can be observed with the naked eye in bractlets and. After undergoing one mitotic division, microspores gradually form mature pollen grains,

which are binucleated pollen grains. The mature pollenhas three germ pores and the surface ornamentation is granular. Walker (Walker and Lee 1976) carried out a classification of pollen external morphology and proposed that plants with many germ pores belong to relatively evolved clades, with ornamentation changing from absent to present. In addition, there is an evolutionary trend of aperture appearance,granular protrusions,elongated shapte (rod shape), stripes. From this, we can deduce that pecans should be relatively primitive. The development of microspores and male gametophytes of pecans is similar to that of *Carya cathayensi*s Sarg. (Xie 2006) and *Cyclocarya paliurus* (Feng 2006), and other plants from the family Juglandaceae, which are species with primitive development (Luza and Polito 1988). According to the palynology criteria reported by Wang *et al*. (1983) Mahan pollen grains are medium-sized pollen grains. According to the classification criteria for pollen shapes by Punt *et al*. (2007), pollen grains with a polar axis to equatorial diameter ratio (P/E) between 1.10 and 1.14 are classified as spheroidal.

3.3 Developmental characteristics of anther wall of pecans

According to Davis's (1996) classification criteria for anther walls, the development of the anther wall of the pecan can be classified as basic, which is composed of an epidermal layer, an endothecial layer, middle layer (1–3 layers) and the tapetal layer. The epidermis is maintained until anther maturation to support anther structure and ensure that anaphase development in microspores is completed. The number of epidermal hairs decreases with anther development and aids in drying and dehiscence of the epidermis (Yates and Sparks 1992). Fibrous thickening of the endothecium and slight lignification when mature can aid in pollen sac dehiscence. There are 3 cell layers in the middle layer of pecans, which provide abundant starch and nutrients to microspore mother cells undergoing mitosis. The tapetum of pecan is a glandular tapetum.

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