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MALE AND FEMALE GAMETOPHYTE DEVELOPMENT OF FLUE-CURED TOBACCO NANJIANG 3

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Abstract

Flue-cured Tobacco Nanjiang 3 was a kind of main cultivated varieties in Guizhou province. Its anatomical structure of microsporogenesis, megasporogenesis and development of male and female gametophyte were observed by paraffin section method. The results showed that, the anther wall is defined as basic development type with glandular tapetum. Microspore mother cell undergoes simultaneous division with two kinds of tetrad arrangements: tetrahedral or decussate type. The mature pollens belong to 2 cells type and have three or four apertures. The ovule of Flue-cured Tobacco Nanjiang 3 is anatropous and thin nucellus. The archesporial cell develops to megaspore mother cell directly and has larger volume. Four megaspore cells form a linear tetrad. The development pattern of embryo sac is Polygonum type, finally forms 7-celled and 8-nucleate mature embryo sac. Stamens develop earlier than gynoecium and the period of microsporogenesis, megasporogenesis and development of male and female gametophyte can be judged by appearance of the flower bud.

Key words: Flue-cured Tobacco; Microsporogenesis; Megasporogenesis; Male and female gametophytes.

Introduction

Flue-cured Tobacco Nanjiang 3 is an endemic variety in southwest and central of China, which was developed from a mutant of Honghua Dajinyuan using the systematic breeding method from 1997 to 2008, and now it is the most widely planted *Nicotiana tabacum* compared with other varieties in Guizhou province (Li *et al*., 2009). The *Nicotiana tabacum* occupies an important position in the family Solanaceae (Waltraud *et al*., 1991; Bokvaj *et al*., 2014). A great deal of information concerning about megasporogenesis, microsporogenesis and the development of male and female gametophytes of Solanaceae were found in the literature (Karihaloo & Malik, 1996; Villari& Messina, 1996; Carolina, 2003; Perveen & Qaiser, 2007). Ghimire & Heo (2012) made a detail described in detail the embryology of *Withania somnifera* and compared the results with embryological data on other members of the family Solanaceae. Carrizo Garcia (2002) expatiate the different anther wall formation in a total of 32 species belonging to 27 genera of Solanaceae. In contrast, the research on Nanjiang 3 which mainly focused on the ecological environment adaptability, cultivation and baking technology, the analyzing of nicotine content and other chemical composition (Gao *et al*., 2011; Zhou *et al*., 2011; Ai *et al*., 2012; He, 2012; Jin *et al*., 2012), however the investigations on the embryology and floral morphogenesis characters in Nanjiang 3 have not been conducted.

Reproductive growth plays an important role in the individual development of the whole flowering plants, bearing not only the vegetative growth of the contemporary plants, but also the life beginning of their offspring (Ma *et al*., 2012). Embryology is one of the most important parts in the process of sexual reproduction of the angiosperm (Carolina, 2003; Neal & Anderson, 2005). The growth progress of Nanjiang 3 can be divided to vegetative growth and reproductive growth. When tobacco plants start flower bud differentiation, the lower leaves get senescent first then the leaves from bottom to top stop growing gradually, and turn to reproductive growth from vegetative growth. A large amount of nutrients transfer to the top of inflorescence while tobacco growing into the reproductive growth period, which prejudice the accumulation of dry matter and reduce the yield and the quality of tobacco leaves(Erika *et al*., 2010; Farrokh *et al*., 2012).

The objective of this study was to find out the role of embryology of Nanjiang 3, which would provide the theoretical basis for preventing tobacco going into reproductive growth untimely, and avoiding the occurrence of large number of flowers and buds that would inhibit its nutrition growth.

Materials and Methods

Plant material: Flower buds of Nanjiang 3 at various developmental stages were continuously collected from fields in Ludi village, Qingzhen, Guizhou province, China, in 2014 and 2015.

Methods: From May to September in 2014, the different sizes buds were taken, and the length of buds were measured, then fixed with 70% FAA(formalin: acetic acid: 70% alcohol=5:5:90) for at least 24 hours, then dehydrated in ethanol, cleared by xylene and embedded with paraffin. Sample slice was 7-9 μm, and then dyed by safranin-fast green and slice was sealed by Canada balsam. Figures of cross-sections and longitudinal-sections were drawn using Olympus BX51 (Li *et al*., 2016).

Results

The development of microsporangium: 3-5 stamens are found in each bisexual flower and the anthers are tetralocular. The crosscutting section of anthers was butterfly-shaped and the pollen sac was small and characterized by half-moon in its initial stage and enlarged and rounded in microsporocyte meiosis anaphase. The anther wall development followed the basic type.

When stamen primordium developed into anther primordia, the cells divided rapidly at four corners, showing a four-sided structure. Also, there appeared one or several archesporial cell of big nucleus and dense cytoplasm below the epidermis. Nanjiang 3 had not been budding in this period while the top floral buds were dark green and 0.30 ~ 0.50 cm long. By the periclinal division, the archesporial cell formed two cell layers which were referred to the primary parietal cell outer and the sporogenous cell inner (Fig. I-1). By several periclinal divisions and anticlinal divisions, the primary parietal cells generated 3-5 cell layers that together with epidermis, the anther wall from the outer to the inner: an epidermis, an endothecium, 2-3 middle layers and a glandular tapetum (Table 1; Fig. I-3).

Anther wall development: In the development of anther wall, epidermis was produced at the first with its cells closely arranged for a protective effect. Later, with the growth of anthers, it gradually extended into a prolate shape and at the time of dehiscence, the anther wall withered. The endothecium formed of regular and well-organized cells and was completely differentiated at the stage of sporogenous cell division (Fig. I-1) and radially elongated at early meiosis stage (Fig. I-4 to 7). Cell with vacuolation reached the largest volume at the stage of mononuclear pollen and its tangential section and radial section appeared to be the thickened fibroid while cytoplasm and nucleolus disappeared before the dehiscence of anthers (Fig. I-12).

During the microsporocyte meiosis, the cytoplasm in the middle layer was reduced and the cells became flat (Fig. I-4). In the late stage of the binucleated pollen, the middle cells were gradually turned into cytoclasis and absorbed and disappeared (Fig. I-10, 11).

The tapetum cells were uninuclear with dense cytoplasm and few vacuoles at the beginning. With the microspore mother cell proceed meiosis, the tapetum cells appeared binucleate and even multinucleate phenomenon (Fig. I-5). When the meiosis was nearly completed, the tapetum cells degenerated and there were only residual tapetumor even nothing in the late period of the microspore development. In the development of the pollen most of the tapetum cells degraded in situ and the tapetum belonging to the glandular tapetum type, various substances secreted from tapetum into the pollen sac for the formation of microspore until the pollen matured (Fig. I-12).

Microsporogenesis and microgametogenesis: The primary sporogenous cell is the source of the pollen mother cell and undergoes mitosis to form the secondary sporogenous cells, also known as microsporocyte (Fig. I-2). The shape and structure of microsporocyte is significantly different from the surrounding cells which were arranged closely with the characteristics of big nucleus, dense cytoplasm, no eminent vacuole, filamentous chromosome and large amount of callose deposited around plasma membrane (Fig. I-3). During this period, the top flower buds are dark green and the length of buds ranges from 0.5 to 0.7 cm. In the progress of meiosis I, the chromosomes are clearly arranged on both side of cell equatorial plate at metaphase of meiosis I, and the chromosomes are also clearly located in two poles of cell during anaphase of meiosis I (Fig. I-4). The cytoplasm does not fissure after the meiosis I of microspore mother cell and 4-nucleate surrounding by common callose after the meiosis II (Fig. I-5). Then the cytoplasm fissure is and become tetrad (Fig. I-6). The meiosis of Nanjiang3 belongs to simultaneous type and the arrangements of tetrad are tetrahedral tetrad or cross type (Fig. I-7). At beginning of meiosis, the top flower buds are dark green and the length of buds ranges from 0.7 to 0.95 cm, then becoming to tetrad dark green buds stretching to 0.95～1.10cm (Tab I).

Table 1. Relationship between floral bud and developmental stage of Flue-cured Tobacco Nanjiang 3 hybrid stamen and pistil.

No.	Morphological feature	Development stage of stamen	Development stage of pistil
I	Un-squaring; Top flower bud with length from 0.30 to 0.50 cm; Dark green	Archesporium cell	/
II	Un-squaring; Top flower bud with length from 0.50 to 0.70 cm; Dark green	Archesporium cell; Microspore mother cell	/
III	Un-squaring; Top flower bud with length from 0.70 to 0.95 cm; Dark green	Microspore mother cell; Meiosis stage	Archesporium cell
IV	Un-squaring; Top flower bud with length from 0.95 to 1.10 cm; Dark green	Tetrad stage	Archesporium cell
V	Squaring stage; corolla with faint yellow and extents out; Top flower bud with length from 1.10 to 1.40 cm	Monokaryotic microspore stage	Archesporium cell
VI	Corolla with faint yellow; Top flower bud with length from 1.40 to 1.70 cm; Ovary with length from 0.35 to 0.58 cm	2-celled pollen stage	Archesporium cell; Megaspore mother cell
VII	Top flower bud with length from 1.70 to 4.0 cm; Corolla with faint yellow; Ovary with length from 0.58 to 0.70 cm	2-celled pollen stage; Pollen tube elongation	Meiosis stage; Tetrad stage
VIII	Top flower bud swelling partly with length from 4.0 to 5.0 cm; Faint yellow; Ovary with length from 0.70 to 0.80 cm	2-celled pollen stage; Pollen tube elongation	Mononucleate embryo sac; Binucleate embryo sac
IX	Top flower bud dehiscing into 5 petals with length from 5.00 to 6.80 cm; Corolla with pink; Ovary with length from 0.80 to 0.90 cm	2-celled pollen stage; Pollen tube elongation	Bi- or Four- nucleate embryo sac
X	Top flower bud with length from 5.00 to 6.80 cm; Corolla with pink; Ovary with length from 0.90 to 1.10 cm	2-celled pollen stage; Pollen tube elongation	Four-nucleate embryo sac; Mature embryo sac

Pollen development in the anthers: Microspore released from tetrad is small with dense protoplasm and nuclear located in the centre of microspores. The microspores obtain nutrition from tapetum and volume increased gradually. Nucleus move from central of cell to side and the outer wall of the cell become thickened and form germ pore. Spherical-like pollens have commonly three germ pores and some square pollen have four germ pores (Fig. I-8, 9). At this time, buds and the top flower with faint yellow corolla appear. Calyx turn light green and the length of buds ranged from 1.10 to 1.40cm, then form two-cell pollen grain through the asymmetrical mitosis (Fig. I-10). The big cell in pollen grain is vegetative cell with large amount of starch, fat and other substances. The small cell in pollen grain is generative cell and becomes spindle-like, then the generative cell deviates from pollen wall into the cytoplasm of vegetative cell (Fig. I-11). During the period of two-cell pollen grain, corolla of top flowers are faint yellow, the length of buds ranged from1.40to 1.70cm and the length of ovary ranged from 0.35 to 0.58 cm. Vegetative cell is related to the form and growth of pollen tube and generative cell develop two spermatids, which are directly involved in reproductive process (Fig. I-12). At this time, the length of top flower buds is more than 1.70cm, pollen grain is mature and pollen tubes can be clearly found. No abnormality was observed in microsporogenesis and development of male gametophyte.

The development of ovule: The ovary of Nanjiang3 includes 2 carpels combining to 2anther chambers, axile placenta, anatropous ovule, many ovules and development of the embryo sac is Polygonum type.

Ovule grows on placenta and the nucellusis consisting of parenchyma cell at first. The cell under nucellus epidermis cell differentiates an archesporial cell (Fig. II-1), which has big volume, dense cytoplasm and clear nucleus. The archesporial cell differentiates from the megaspore mother cell directly and the volume of nucellus cell is increased to surround megasporocyte through the anticlinal division (Fig. II-2). At this period, the corolla of top flowers is faint yellow, the length of buds ranged from 1.40 to 1.70cm and ovary is 0.35 to 0.58 cm. Then both inner and outer layer integument gradually wrap the nucellus, outer integument forming micropyle.

Megasporogenesis and megagametogenesis: At the beginning of meiosis of megaspore mother cells, chromation condensation and megaspore enlarged the volume and callose wrap the whole of cells. The megaspore mother cells become dyads undergo the meiosis I (Fig. II-3), and then further divides into linear tetrad (Fig. II-4, 5). In this period, the length of buds ranged 1.70 to 4.0 cm, the corolla of top flower is faintly yellow and the length of the ovary ranged from 0.58 to 0.70 cm. The chalazal end of the megaspore increases its volume and develop into functional macrospore further, the rest of megaspore is degenerated (Fig. II-6).

The functional megaspore continues to grow along longitudinal axis of the nucellus, with being vacuolization and mononuclear hanging in the center of cell, and then develop into mononuclear embryo sac (Fig. II-7). In the period of mononuclear embryo sac, the length of top flower bud ranged from 4.0 to 5.0 cm and ovary ranged from 0.70 to 0.80 cm with the faint yellow slightly opening buds. As the functional megaspore grows for several days, it forms binucleate embryo sac (Fig. II-8) and four-nucleate embryo sac (Fig. II-9) successively and 8-free nucleate embryo sac (Fig. II-10) through 3 times mitosis. Finally there are 4 nuclei at both ends of the original embryo sac, and then3 of the 4 nuclei at the micropylar end constitute into the egg apparatus, including 1 egg cell and 2 synergids and the forth nucleus develops into upper polar nucleus. 3 of the 4 nuclei at the chalazal end develop into antipodal cells (Fig. II-11, 12). The forth nucleus as the lower polar nucleus usually moves upward to the side of upper polar nucleus to form the central cell which contain 2 nuclei, then fuse to a double nuclear named secondary nucleus. Finally, the embryo sac is consisted of 7 cells. In the binucleate and four-nucleate period of embryo sac, the length of top flower bud ranged from 5.0 to 6.8 cm and ovary ranged from 0.80 to 0.90 cm, the corolla was gradually cracked having 5 pink lobes. As the embryo sac becomes mature, the length of top flower bud ranged from 5.0 to 6.8 cm and ovary ranged from 0.90 to 1.0 cm with pink petals. No abnormal phenomenon was observed in the progress of megasporogenesis and the development of female gametophyte.

Discussion

The main characters of embryology of Nanjiang 3 are that the type of anther wall was basic development type with glandular tapetum. The cell meiosis of microspore mother cell is simultaneous division, the mature pollen belongs to 2cell type, arrangements of tetrad are tetrahedral or decussate tetrad and the pollen has 3 or 4 apertures. The ovule is anatropous and thin nucellus. The archesporial cell develops to megaspore mother cell directly and has larger volume then develop four macrospore cells through cell meiosis and produces formed a linear tetrad. The development pattern of embryo sac is Polygonum type and finally forms 7-cellsand 8- nuclei.

The anther of higher plants is a complex and heterogenous structure in which different tissues like sporogenous, tapetum and wall layers interact with each other during microsporogenesis, culminating in pollen formation (Manoharan & Rudramuniyappa, 1998). The anther wall layers have been defined into four different wall formation types: basic, dicotyledonous, monocotyledonous and reduced (Davis, 1966). For now, the formation of anther wall we have observed in Solanaceae specises only basic and dicotyledonous type (Carrizo Garcia, 2002; Carrizo Garcia, 2003.).Our results about anther development pattern are similar to that observed in different genera and other species of Solanaceae, while basic type was present in flowers of Nanjiang 3 and has a glandular tapetum. Some previous studies on *Withania somnifera* indicated that the tapetum was glandular but anther wall formation was dicotyledonous-type (Ghimire & Heo, 2012).

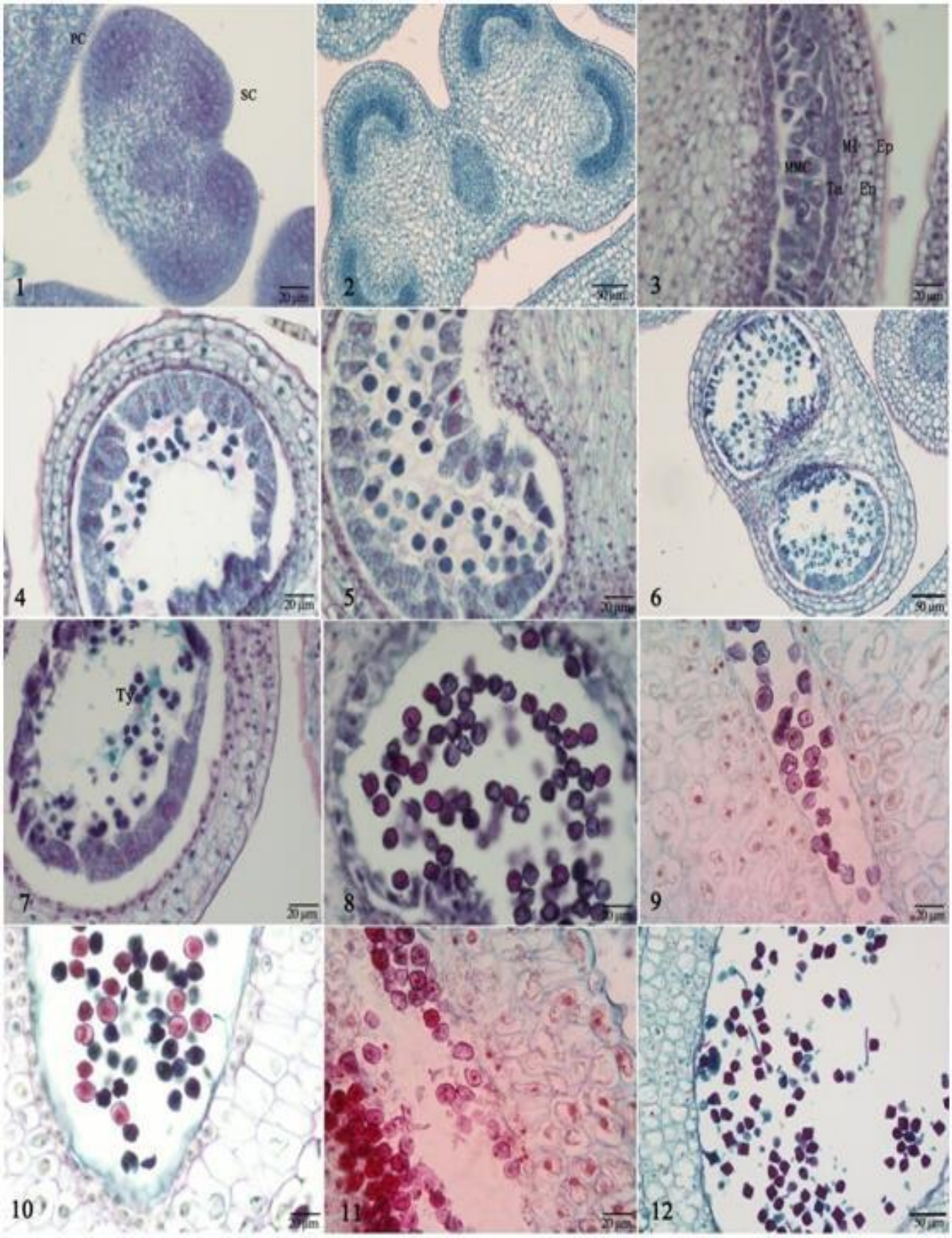

Fig. I. Microsporogenesis and development of male gametophyte of flue-cured tobacco Nanjiang 3.
1. The archesporiumis divided into a parietal cell and a sporogenous cell, by the periclinal division; 2. The microspore mother cell; 3. The microspore mother cell prepare meiosis with intense tapetal cytoplasm; 4. The first meiosis period; 5. The second meiosis period; 6. The tetrad stage; 7. The tetrad arrangement for tetrahedral or decussate tetrad; 8. The monokaryotic microspore; 9. Central and edge stage of the mononuclear pollen; 10-11. The 2-celled pollen stage; 12. The pollen tube elongation
PC. Peripheral cell; SC. Sporogenous cell; Ep. Epidermis; En. Endothecium; MI. Middle layer; Ta. Tapetum; MMC. Microspore mother cell; Ty. Tetrad

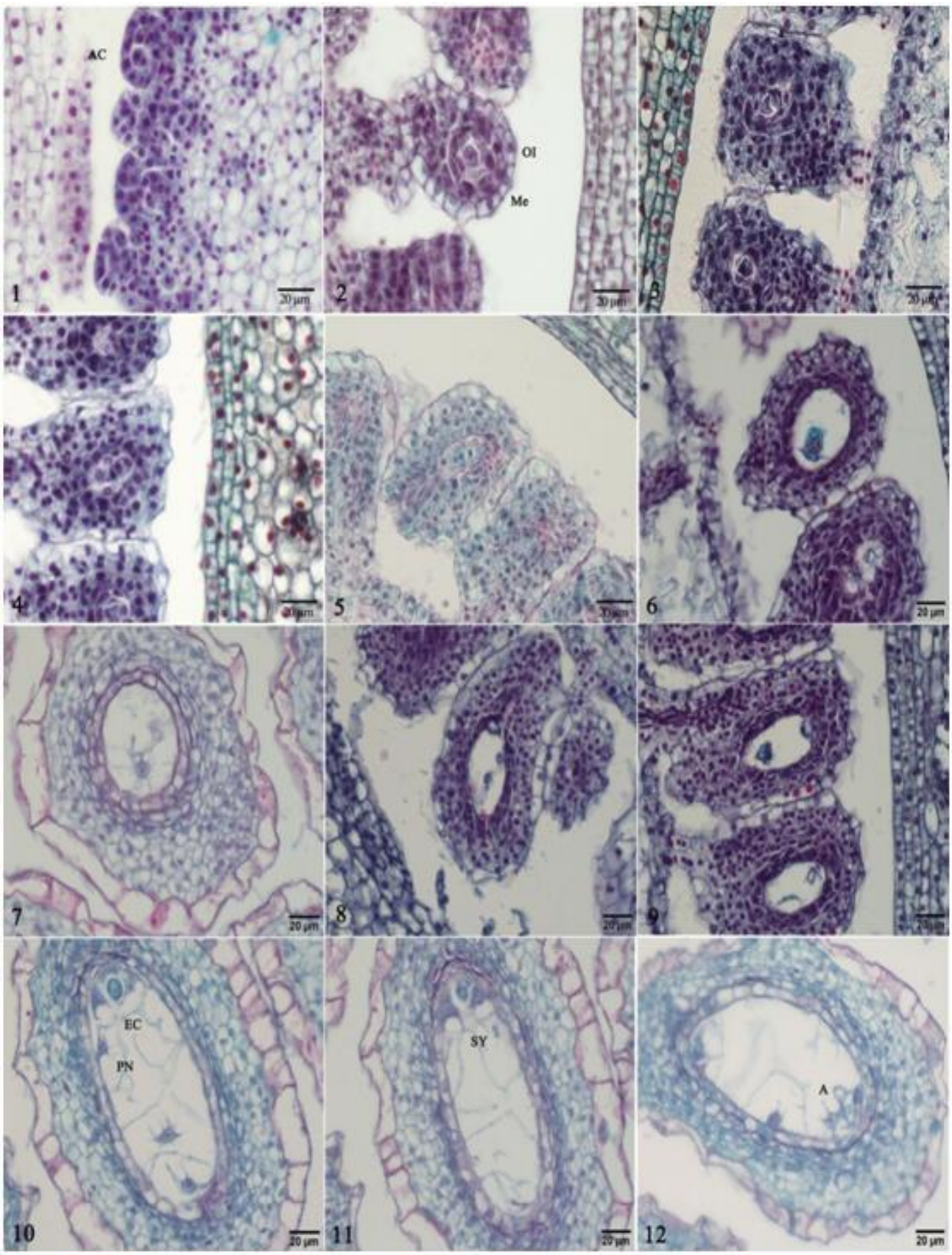

Fig. II. Megasporogenesis and development of male gametophyte of flue-cured tobacco Nanjiang 3.
1. The archesportial cell; 2. The megaspore mother cell; 3. The megaspore mother cell forms dyad after first mitosis stage; 4. The early linear tetrad; 5. The late tetrad; 6. The functional megaspore; 7. The mononucleate embryo sac; 8. The binucleate embryo sac; 9. The four- nucleate embryo sac; 10. Mature embryo sac such as egg cell, synergid cell and polar nuclei and antipodal cell; 11. Two synergid cells; 12. Three antipodal cells
AC. Archesporial cell; Me. Megaspore mother cell; OI. Outer integument; EC. Egg cell; PN. Polar nuclei; SY. Synergid; A. Antipodal cells

The division processes of the microspore mother cell of angiosperms were defined into two basic types: successive type and simultaneous type. In successive type division processes, the two cell plates are laid down in a centrifugal manner immediately after the first and the second meiotic division. And in simultaneous type, the tetrad is formed in one step after the completion of the second meiotic division (Johri, 1984; Teng *et al*., 2005).In Nanjiang 3, microspore mother cell undergoes simultaneous division with two kinds of tetrad arrangements: tetrahedral tetrad and decussate type.

Although the pollen is simple in structure, the formation of pollen grain is a complex and developmentally regulated process (Manoharan & Rudramuniyappa, 1998; Connolly & Anderson, 2003). Mature pollen grains are divided into two kinds in angiosperms: binucleate type and trinucleate type, the mature pollen grains of most species have two nuclei just like Nanjiang 3 (Brewbaker, 1967). The mature pollen belongs to 2-cell type and the pollen has 3 or 4 apertures.

Four kinds ovules have been reported for Solanaceae: anatropous, hemianatropous, amphitropous and campilotropous (Ghimire & Heo, 2012).The growth rate of funicle and other part is inconformity in the development process of ovule, thus formed the anatropous ovule in Nanjiang 3. The formation patten of embryo sac is the conventional Polygonum type and finally forms 7-celled and 8- nucleate embryo sac, which consist by one egg cell, one polar nucleus, two synergid cells and three antipodal cells. The condition is similar to that described by Cooper (1931) for *Lycopersicon esculentum* which formed a typical 7-celled embryo sac.

All performance characteristics of the sexual reproduction development process in Nanjiang 3 is consistent with the past description on the embryology of Solanaceae（Hu, 2005）, only different with chili and *Withania somnifera*, which has 7-cell and 7-cell nucleate embryo sac and dicotyledonous-type in anther wall development separately(Cooper, 1931; Ghimire & Heo, 2012). Meanwhile, it shows that the formation of macrospore, microspore and male, female gametophytes of tobacco are very conservative.

The development period of gynoecium is later than that of stamen of Nanjiang 3 and gynoecium start to development when stamen becomes tetrad. The phenomenon we found that the development of stamen is earlier than that of gynoecium, which is also consistent with the most Solanaceae species（Kaul *et al*., 2005）. The size of buds is intuitive character related to the growth of pistil and stamen, and form theoretical and practical basis for cross breeding tobacco or improving genetic trait. Embryo sac becomes mature when buds grow to 5.0-6.8 cm, which is different with the description of *Nicotiana Debneyi* varieties (Liao *et al*., 2013). In the experiment, pollen abortion is occasionally found, that is to say pollen tube is sprout, but anther not cracked. These sterility pollen can become normal pollen, but pollen can't spread normally because of non-cracked anther and pollen died due to overripe, which may be related to continuous rainy climate environment season in tobacco planting season in Guizhou.

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