

A Morphological and Histological Characterization of Male Flower in Chestnut (*Castanea*) Cultivar ‘Yanshanzaofeng’

Feng Zou, Su-Juan Guo, Huan Xiong, Peng Xie, Wen-Jun Lv and Guang-Hui Li

Key Laboratory for Silviculture and Conservation, Ministry of Education, Beijing Forestry University, 100083, Beijing, P.R. China

Abstract: Chinese chestnut (*Castanea mollissima* Blume.) is a widely distributed fruit tree and well known for its ecological and economic value. In order to evaluate obstacles to male reproductive in the *C. mollissima*, a morphological and histological characterization of male flower of chestnut cultivar ‘Yanshanzaofeng’ were examined by paraffin section technique and scanning electron microscopy. The results showed that male catkins with floral primordia were formed in the buds of one-year olds shoots in later April. Later, a protoderm, ground meristem and a procambium had differentiated in young anthers. Each young anther soon developed to four microsporangia. The anther wall layers developed completely by mid-May and consisted of one-cell-layered epidermis, one-cell-layered endothecium, two or three middle layers and one-cell-layered tapetum. The tapetum was of glandular type. Microspore mother cells underwent meiosis through simultaneous cytokinesis in later May and gave rise to tetrads of microspores, which were tetrahedrally arranged. Mature pollens contained two cells with three germ pores. Anthers were dehiscent and pollen grains shed by early June. Based our results, we did not find the abnormal male flower in the *C. mollissima* cv ‘yanshanzaofeng’, indicating that male gametes were fertile and thus was considered as pollenizers.

Keywords: *Castanea mollissima* Blume, male inflorescences, microgametogenesis, microsporogenesis, ‘Yanshanzaofeng’

INTRODUCTION

Chestnut (*Castanea*) belongs to the *Fagaceae* family which includes *Quercus*, *Cyclobalanopsis*, *Castanopsis*, *Fagus*, *Lithocarpus* and *Trigonobalanus*. Chestnut has been cultivated for food and timber since ancient times (Payne *et al.*, 1983). In China, chestnut is one of the popular nuts because of positive health effects. It is a gluten-free flour source and contains essential dietary nutrients and minerals (Semih and Ilknur, 2012). Chestnut production is particularly important source of income in rural regions, especially in Yan Mountain Region of Northern China (Qian *et al.*, 2012).

Chestnut selection studies have been performed to determine superior genotypes in terms of yield and quality in Yan Mountain Region. *Castanea mollissima* cv ‘yanshanzaofeng’ was firstly selected from Yan Mountain Region and became an improved varieties in 1989 (Baotang, 2003). It was famous for ripening in the early September and developed quickly in Qianxi county of Yan Mountain Region. It cultivated area up to 42,000 ha (Yongmin, 2010). As we know, chestnuts are monoecious, bearing both staminate (male) and pistillate (female) flowers on the same tree (Botta *et al.*, 1995). Male reproductive success was considered as the key factors responsible for fruit (Beverly and

Mary, 1998; García-Mozo *et al.*, 2001). Thus, an understanding of the male flower development is of basic importance to achieve high quality and consistently high production in chestnut orchards. In recently years, there have been some reports on the characteristics of the male flower development in *Castanea* (Botta *et al.*, 1995; Ceriye and Arif, 2006; Yong-Qing *et al.*, 2011). However, there was no concomitant description on morphological characterization of this cultivar ‘Yanshanzaofeng’. Moreover, we have no information about the distribution of polysaccharides during the male gametophyte development in *Castanea*. In this study, we tried to verify the morphological and histological characterization of male flower in this cultivar, which may obtain basis information for appropriate management during flowering period and have the potential to improve nut yield and quality in the Yan Mountain Region.

MATERIALS AND METHODS

Floral buds of the chestnut cultivar ‘yanshanzaofeng’ at successive developmental stages were collected from a 12-year-old tree in Qianxi county, Hebei Province, China (40°21’57”N, 118°12’17”E), at approximately 163 m above sea level.

This site is located in the warm temperate zone and semi-humid region, with a mean annual precipitation of 744.7 mm and a mean annual temperature of 10.9°C. The samples were collected about every week from April 2011 to June 2012.

Light Microscopy (LM). The materials were fixed in FAA (formalin: acetic acid: 70% ethyl alcohol = 5:5:90 v/v), dehydrated through an ethyl-alcohol series, embedded in paraffin and sectioned at a thickness of 10 µm by Leika RM2265. The sections were stained with Safranin-O/Fast Green (Hollender *et al.*, 2012) or PAS (Agadi, 2012). Observation and photograph of sections were carried out using a microscope (BX51, Olympus, Japan).

Scanning Electron Microscopy (SEM). The materials were fixed in 2.5% glutaraldehyde, dehydrated through an ethyl-alcohol series (Zhuogong and Li, 2010) and then by freeze-drying treatment (Hitachi ES-2030, Japan). The materials were mounted on SEM stubs, coated with gold-palladium (Hitachi E-1010, Japan) and examined with a SEM (Hitachi S-3400N, Japan).

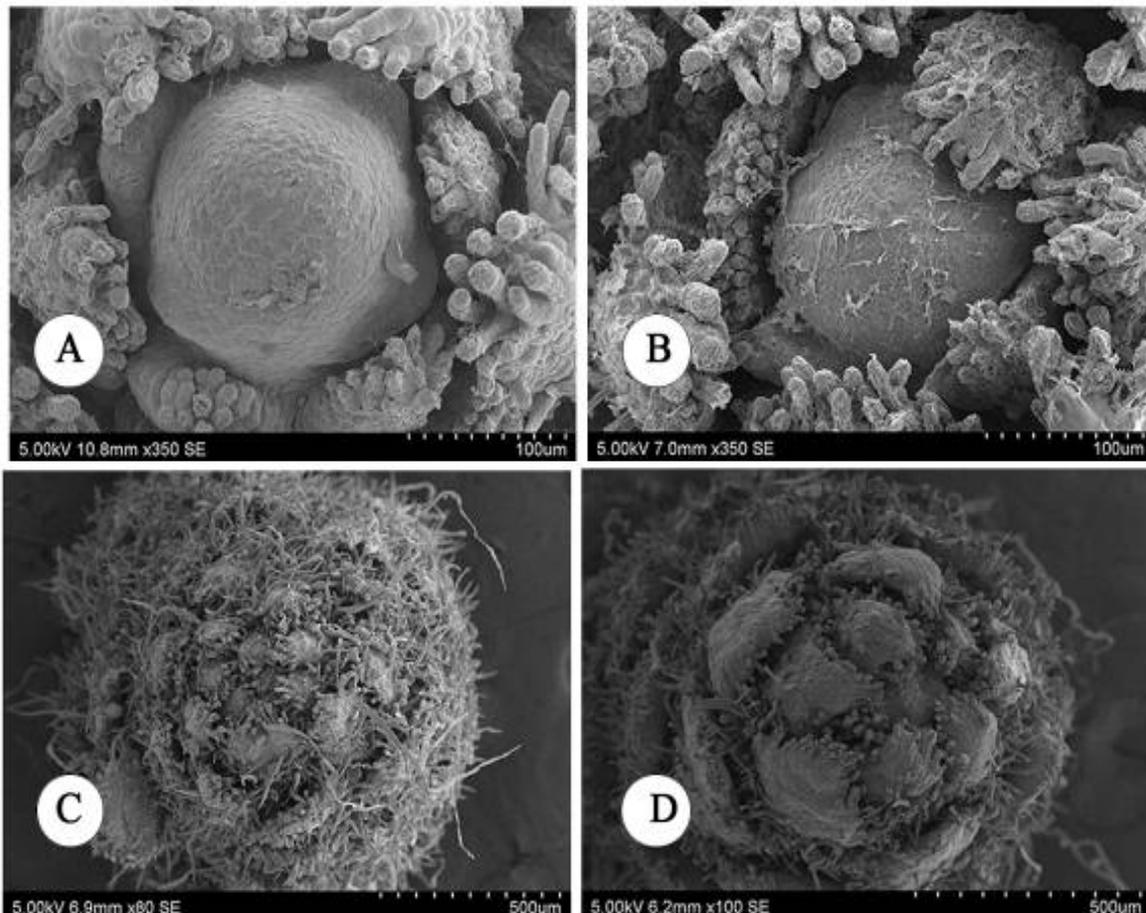
RESULTS

Morphological of male inflorescences: Initial development of staminate inflorescence primordial was

observed in material collected in late April (Fig. 1A and B). By early May, the apex of the structure which subsequently developed into a staminate inflorescence arose laterally on the shoot axis between the development leaf primordial and innermost bud scales. Within two weeks, primary, secondary and tertiary bracts were regularly present and six or so of the quaternary or perhaps higher order bracts, although these latter occur only in a median position on the upper and lower sides (Fig. 1C and D). By later May, then anthers were almost totally covered by tepals (Fig. 1E). In the early June, the staminate inflorescence has lengthened and appeared closer to its mature shape (Fig. 1F). At maturity, the tepals of the staminate flowers were reflexed, completely exposing the conspicuously lobed anthers (Fig. 1G). A few days prior to dehiscence, the mature anther was filled with pollen grains (Fig. 1G). The pollen grains were three germ pores (Fig. 1H).

Male flower development:

Formation of anther wall: The male flowers were tetrasporangiate anthers. At early stage of development, archesporial cells, which were recognizable by their large volume and conspicuous nuclei, differentiated below the epidermis of anthers (Fig. 2A).



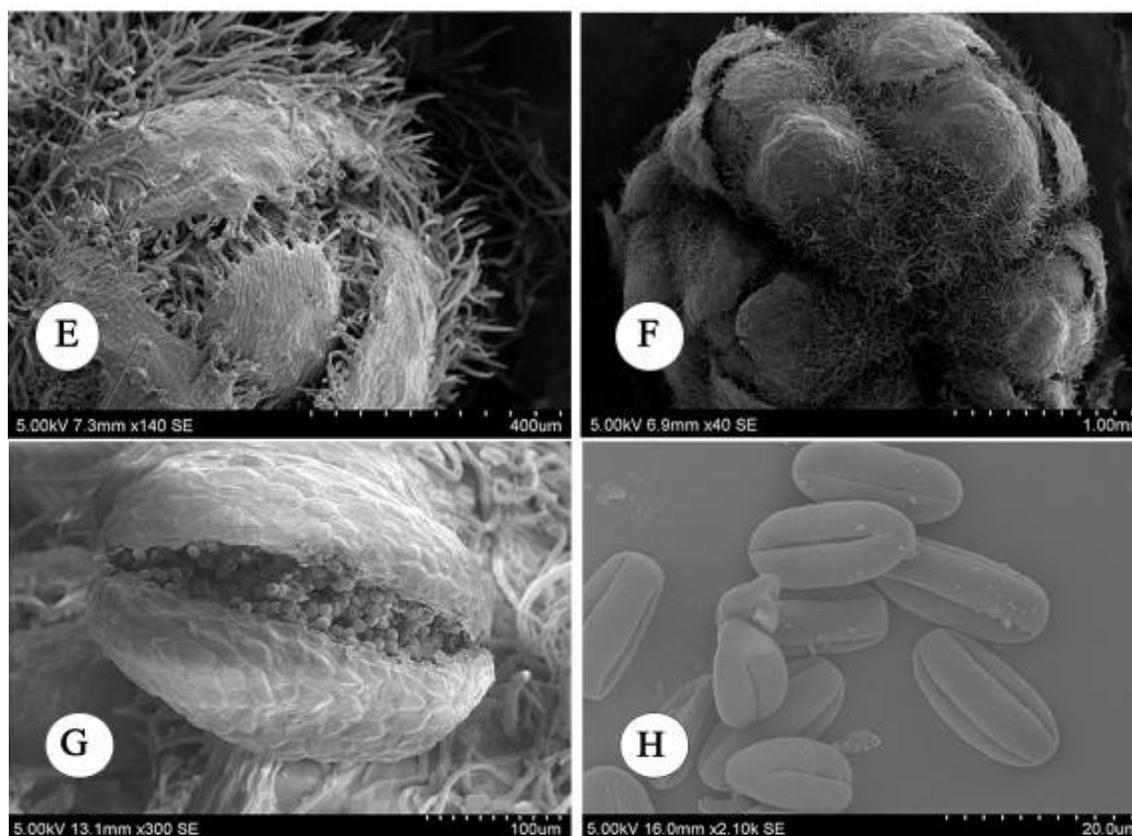


Fig. 1: The morphological of male inflorescences in *Castanea* cultivar 'Yanshanzaofeng' A-B, Top view, late April, showing the staminate flower primordium. C, Early May, showing the anthers covered by primary, secondary and tertiary bracts. D, Middle May, showing the anthers covered by primary, secondary and tertiary bracts. E, Late May, showing the anthers covered by bracts. F, Top view, late May, is showing a mature staminate flower. G, Early June, a mature anther, showing pollen grains. H, Early June, mature pollen grains

These cells divided periclinally to form outer primary parietal cells and inner primary sporogenous cells (Fig. 2A). The epidermal layer consisted of slightly elongated cell that developed into compressed epidermis cells (Fig. 2B). The primary parietal cells formed a subepidermal endothecium and two middle layers by periclinal and anticlinal divisions (Fig. 2B). The outer secondary parietal cells by periclinal and anticlinal divisions. The endothecial cells elongated gradually, acquiring fibrous thickenings at the time of anthesis (Fig. 2K). The middle layers had a common histogenetic origin with the endothecium and it persisted until the tetrad stage and degenerated before forming two-celled pollen grains (Fig. 2J). The innermost layer gave rise to the tapetum, which partly originated from the ground tissue near the connective tissue and some of the tapetum cells had one to two prominent nuclei at the microspore mother cell phase (Fig. 2C). At the tetrad stage, tapetal cells elongated and lost close contact but still remained in their original position (Fig. 2H). They began to degenerate at uninucleate pollen stage and disintegrate at binucleate pollen stage. The endothecium comprised one layer. The middle layers

appeared to undergo further divisions to form three layers. The anther wall prior to maturation was usually comprised of (5 to 6) cell layers, i.e., an epidermis, an endothecium, two or three middle layers and a layered tapetum. Thus, the tapetum was of the glandular type and wall formation conformed to the basic type as defined by Davis (1966).

Microsporogenesis and microgametogenesis: A row of sporogenous cells, produced by archesporial cells, gave rise to a mass of microspore mother cells by several mitotic divisions (Fig. 2B). Simultaneously with changes taking place in the wall of the microsporangia, the primary sporogenous cells underwent mitosis to form secondary sporogenous cells, from which microsporocytes were derived. Microsporocytes were identifiable by their large volume, dense cytoplasm and conspicuous nuclei (Fig. 2B). Meanwhile, a few polysaccharides appeared in the sporogenous cells (Fig. 2L). The microsporocyte underwent meiosis and the process of meiosis involved two cell divisions, such as meiosis I includes the leptoneuma (Fig. 2C), the diakinesis (Fig. 2D), the prophase (Fig. 2E) and

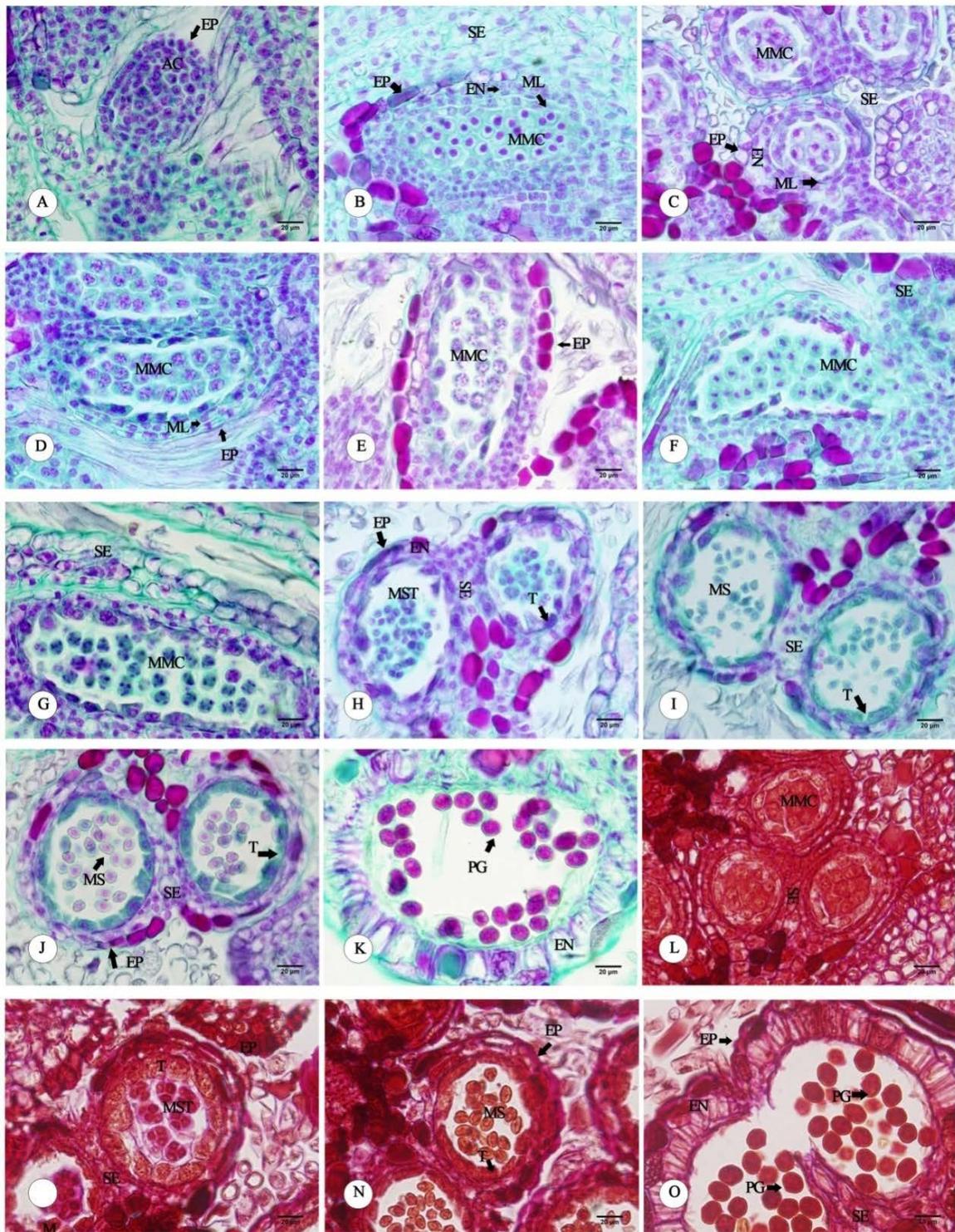


Fig. 2: Formation and development of microspore and male gametophyte in *Castanea* cultivar 'Yanshanzaofeng'. A, The corners of the young anther development aarchesporial cell. B, Anther showing microspore mother cell with developed anther wall: epidermis layer, endothecium, two or three middle layers and inner tapetum. C, Anther showing central microspore mother cell at leptonema stage. D, Anther showing central microspore mother cell at diakinesis. E, Anther showing central microspore mother cell at meiosis I prophase. F, Anther showing central microspore mother cell at meiosis I metaphase. G, Anther showing central microspore mother cell at meiosis II metaphase. H, Anther showing microspore tetrads arranged tetrahedrally and enclosed in callose. I, Anthers showing the microspores were released from tetrads (early microspore stage). J, Anthers at uninucleate microspore stage. K, Anthesis showing anther wall with stretched

epidermis, fibrous thickened endothecium, relic of middle layers, degenerated tapetum and mature pollen grains. L, Anthers showing at the sporogenous cell stage, a few polysaccharides appeared in sporogenous cells. M, At the tetrad stage many polysaccharides increased in tapetal cell. N, Anthers showing at the microspore stage, a few polysaccharides in microspore cells. O, At the mature pollen, tapetal cells degenerated and many polysaccharides were accumulated in pollen wall. AC = Archesporial Cell; CAL = Callose; EN = Endothecium; EP = Epidermis; ML = Middle Layers; MMC = Microspore Mother Cell; MS = Microspore; MST = Microspore tetrad; PG = Pollen grains; SE = Septum; T = Tapetum

metaphase (Fig. 2F). As a result, a microspore dyad was formed. A microspore tetrad was formed via the prophase II, for example metaphase II (Fig. 2G). At the tetrad stage many polysaccharides increased in the tapetal cell (Fig. 2M). Most of the microspores in the tetrads were tetrahedral (Fig. 2H). Callose deposition occurred at the onset of meiosis of pollen mother cells, reached a peak at metaphase II or anaphase II by enveloping the pollen mother cells or micro tetrads (Fig. 2H) and disappeared at end of meiosis. It was considered that the cycling of callose during microspore development played an important role in the protection of the pollen mother cells and released of microspores from the micro tetrads (Scott *et al.*, 2004). In contrary, simultaneous cytokinesis took place after pollen mother cell meiosis.

The microspore tetrads soon separated from each other and released from the microspores (Fig. 2I). A few polysaccharides existed at the microspore stage (Fig. 2N). Each microspore had a dense cytoplasm, conspicuous wall and a prominent and centrally placed nucleus (Fig. 2I). As the central vacuole developed, the nucleus took a peripheral position. At this stage, the microspore was uninucleate (Fig. 2J) which underwent a mitotic division to give rise to a binucleate cell. However, at the mature pollen, tapetal cells fully degenerated and many polysaccharides were accumulated in pollen wall (Fig. 2O). Mature pollen grains contained two cells with three germ pores (Fig. 1H). Anthers were dehiscent and pollen grains shed approximately on June 10.

DISCUSSION

The male development characters of *C. mollissima* cv 'yanshanzaofeng' are similar to *Cyclobalanopsis* (Hann-Chung and Tzu-Ming, 2005), *Castanea* (Cevriye and Arif, 2006) and *Quercus* species (Deng *et al.*, 2008). The anther of this cultivar 'Yanshanzaofeng' is microdiodanges. The mature anther wall comprises an epidermis, a layer of endothecium, two middle layers and a single-layered tapetum. The tapetum during development of the anther wall is of the heteromorphic and glandular type. This type of tapetum was reported for some species in *Quercus* (Stairs, 1964; Robert, 1985; Deng *et al.*, 2008). At about the time of pollen tetrads, the walls of the tapetal cells became indistinct and the tapetal cells degenerated at their original site. The tapetal cells degenerated completely at the stage of one-nucleate pollen grains and the nucleus near the wall. The cytokinesis of microspore mother cell meiosis is modified simultaneous type and tetrads are

tetrahedral. The pollen grain is two-celled type with three germ pores.

The distribution of polysaccharides during the male gametophyte development of the *C. mollissima* cv 'yanshanzaofeng' displayed some distinct features. There was a close relationship between the change of polysaccharide with the supplement of nutrition during the development of pollen (Albertini *et al.*, 1981). At first, a few polysaccharides appeared in the sporogenous cells. At the tetrad stage many polysaccharides increased in the tapetal cell. Subsequently, the tapetum began to degenerate. A few polysaccharides existed at the microspore stage. However, at the mature pollen, tapetal cells fully degenerated and many polysaccharides were accumulated in pollen wall. Thus, tapetal cells play the important role of providing nutrition substances for the development of microsporocytes (Scott *et al.*, 2004).

Cytoplasmic male sterility should be taken into account as a factor influencing variations in chestnut production (Mckay, 1939; Jaynes, 1963; Omura and Akihama, 1980; Soyly, 1992; Sisco *et al.*, 2001; Cevriye and Arif, 2006). Cytoplasmic male sterility are usually accompanied by anomalies tapetum (Kaul, 1988). In our anatomical investigation, we did not find the anomalies tapetum during microsporogenesis in the *C. mollissima* cv 'yanshanzaofeng'. Therefore, it was indicated that male gametes were fertile and thus was considered as pollenizers.

CONCLUSION

This study provided basic information on different male reproduction aspects of *C. mollissima* and could shed light on the embryogenesis in this species. In *C. mollissima* cv 'yanshanzaofeng', the microgametogenesis results in binucleate pollen as has been observed in other genera in *Castanea*. We did not find the abnormal male flower in the *C. mollissima* cv 'yanshanzaofeng', indicating that male gametes were fertile and thus was considered as pollenizers.

ACKNOWLEDGMENT

The project was supported by the National Science and Technology Support (Grant No. 2013BAD14B0402), the Doctoral Foundation of Ministry Education (Grant No. 20120014110011) and the Public Welfare Project of Forestry Industry (Grant No. 201204401).

REFERENCES

- Agadi, S.N., 2012. Anthers of *Chlorophytum orchidastrum* Lindl. Exhibit rare pattern of distribution of starch. *Biosci. Int.*, 1(4): 94-96.
- Albertini, L., H. Grenetauberger and A. Souvire, 1981. Polysaccharides and lipids in microsporocytes and tapetum of rhoeo-discolor hance-cytochemical study. *Acta Soc. Bot. Pol.*, 50(1-2): 21-28.
- Baotang, D., 2003. New varieties in Jingdong Chinese chestnut. *Hebei Fruits*, 6: 28-29. (In Chinese)
- Beverly, D.D. and V.A. Mary, 1998. Factors influencing male mating success in bur oak, *Quercus macrocarpa*. *New Forests*, 15: 161-180.
- Botta, R., G. Vergano, G. Me and R. Vallania, 1995. Floral biology and embryo development in chestnut (*Castanea sativa* Mill.). *Hort. Sci.*, 30(6): 1283-1286.
- Cevriye, M. and S. Arif, 2006. Flower and stamen structures of male-fertile and male-sterile chestnut (*Castanea sativa* Mill.). *J. Am. Soc. Hort. Sci.*, 131(6): 752-759.
- Davis, G.L., 1966. *Systematic Embryology of the Angiosperm*. Wiley, New York, pp: 283-505.
- Deng, M., Z.K. Zhou, Y.Q. Chen and W.B. Sun, 2008. Systematic significance of the development and anatomy of flowers and fruit of *Quercus schottkyana* (Subgenus cyclobalanopsis: Fagaceae). *Int. J. Plant Sci.*, 169(9): 1261-1277.
- García-Mozo, H., P.J. Hidalgo, C. Galán, M.T. Gómez-Casero and E. Domínguez, 2001. Catkin frost damage in mediterranean cork-oak (*Quercus suber* L.). *Israel J. Plant Sci.*, 49: 41-47.
- Hann-Chung, L. and H. Tzu-Ming, 2005. Microsporogenesis in *Cyclobalanopsis glauca*. *J. Exp. For. Nat. Taiwan Univ.*, 19(1): 55-68.
- Hollender, C.A., A.C. Geretz, J.P. Slovin and Z. Liu, 2012. Flower and early fruit development in a diploid strawberry, *Fragaria vesca*. *Planta*, 235: 1123-1139.
- Jaynes, R.A., 1963. Male sterility in *Castanea*. *Proceeding of the International Congress on Genet. The Hague, The Netherlands*, pp: 203. (Abstract).
- Kaul, M.L.H., 1988. *Male Sterility in Higher Plants*. Springer-Verlag, Berlin.
- Mckay, J.W., 1939. Male sterility in *Castanea*. *Am. Soc. Hort. Sci. Proc.*, 37: 509-510.
- Omura, M. and T. Akihama, 1980. Male sterility in chestnuts: A tentative plan for the seed propagation of fruit trees. *Gamma Field Symp.*, 19: 77-89.
- Payne, J.A., R.A. Jaynes and S.J. Kays, 1983. Chinese chestnut production in the United States: Practice, problems and possible solutions. *Econ. Botany*, 37(2): 187-200.
- Qian, W., S. Shuchai, Z. Di *et al.*, 2012. Study on varieties combination suitable for mutual pollination of Yan Mountain Chestnut. *Nat. Res.*, 3: 66-70.
- Robert, B.K., 1985. Reproductive morphology of *Quercus* (Fagaceae). *Am. J. Bot.*, 72(12): 1962-1977.
- Scott, R.J., M. Spielman and H.G. Dickson, 2004. Stamen structure and function. *Plant Cell*, 16: S46-S60.
- Semih, O. and S. Ilknur, 2012. Phenolic compounds and antioxidant activities of chestnut (*Castanea sativa* Mill.) fruits. *Qual. Assur. Safety Crop Foods*, 4: 199-205.
- Sisco, P.H., F.V. Hebard, Y. Shi *et al.*, 2001. Cytoplasmic male sterility resulting from interspecific crosses in chestnut (*Castanea* spp.). *Proceeding of the Plant and Animal Genome Conference*.
- Soylu, A., 1992. Heredity of male sterility in some chestnut cultivars (*Castanea sativa* Mill.). *Acta Hort.*, 317: 181-185.
- Stairs, G.R., 1964. Microsporogenesis and embryogenesis in *Quercus*. *Botan. Gaz.*, 125(2): 115-121.
- Yongmin, S., 2010. Study on the development of chestnut industry in Yan Mountain Region. *Minzu University of China, Beijing*. (In Chinese)
- Yong-Qing, F., S. Yuan-Yue, Q. Ling *et al.*, 2011. Short cakin1, a novel mutant of *Castanea mollissima*, is associated with programmed cell death during chestnut staminate flower differentiation. *Sci. Horticult.*, 130: 431-435.
- Zhuogong, S. and X. Li, 2010. Stigmatic morphology of Chinese chestnut (*Castanea mollissima* Blume). *Hort. Sci.*, 45(6): 981-983.