

Research Article

Floral Biology of Chinese Jujube (*Ziziphus jujube* Mill.) I: The Formation of Microspores and Development of Male Gametes

¹Feng Zou, ²Jing-Hua Duan, ¹De-Yi Yuan, ¹Lin Zhang, ¹Xiao-Feng Tan and ¹Huan Xiong

¹The Key Laboratory of Cultivation and Protection for Non-wood Forest Trees, Ministry of Education, Central South University of Forestry and Technology, Changsha 410004, P.R. China

²Chinese Academy of Forestry, Beijing 100091, P.R. China

Abstract: *Ziziphus jujube* Mill is an economically important fruit tree, cultivated on marginal lands on a commercial scale, especially in China. In order to elucidate the microsporogenesis and male gametogenesis formation in Chinese jujube, a cultivar named 'Lizao' was employed for microscopy analysis. The results are showed that 'Lizao' was four ventricles in each anther and the anther wall was 4 to 5 layers. Its primary anther wall consisted of the epidermis, 1-2 layers endothecium, middle and glandular tapetum layers. The development of the anther wall conformed to the basic type. The meiosis in the microspore mother cells were belonged to a simultaneous type and most of the microspores were arranged in a tetrahedron shape in the tetrads. In the later May, the shape of pollen was a triangular germination vale with three germinal furrow and the mature pollen was of 2-cell type. Floral emergence and development lasted for 1 months-from later April to later May. Based our results, we did not find the abnormal male flower in the *Z. jujube* 'Lizao', suggesting that microsporogenesis development may not be factors in the low seed production in the *Z. jujube*. This study provides the basis for understanding the biological mechanism regulating sexual reproduction, thus expanding the prospects for *Z. jujube* breeding programs and for further molecular and genetic studies of this species.

Keywords: Floral, male gametogenesis, microsporogenesis, *Ziziphus jujube* Mill.

INTRODUCTION

Chinese jujube (*Ziziphus jujube* Mill.), a member of the Rhamnaceae family, originated from China and has cultivated for thousands of years. It is an economically important fruit crop species in China, well-know for its fruits with high dietary value and medical value (Jinghua *et al.*, 2012). Jujube fruit contains flavonoids, vitamins, amino acids, organic acids, polysaccharides and microelements and found useful in spleen diseases and nourishment of blood in Chinese system of medicine (Sunil, 2013). *Z. jujube* is also tolerant to extreme environmental conditions, including drought, high temperatures and saline water (Tel-Zur and Schneider, 2009).

As our knowledge, environment, sexual reproduction and nutrition influence the flower and fruit fallen. Sexual reproduction includes sporogenesis and gametophyte development, pollination and fertilization, embryo and endosperm development and each part will affect the fruit set percentage (Lillecrapp *et al.*, 1999). The flower and fruit fallen of jujube is caused by low temperature or cloudy and drizzly days during blossom period which affect the development of sporogenesis

and gametophyte, pollination and fertilization. The absence of nutrition also maybe the reason analyzed by cultivation theory (Hu *et al.*, 1999; Guangfang, 2014). Therefore, it is significant to study the reproductive biology of jujube. However, only a few investigation of the sexual reproduction of this species have been reported (Srinivasachar, 1940; Wang, 1983; Tian and Ma, 1987; Zhenhuai, 2004; Yuan *et al.*, 2009; Yufei *et al.*, 2011; Zhihui *et al.*, 2011). The lack of basic knowledge in sexual reproduction becomes even more evident in Chinese jujube trees. They are difficult, when not impossible, to grow in controlled conditions and seasonal flowering is not easy to overcome. This creates a strong experimental constraint and the necessity to develop most of the experimental work in just a few months. It's generally accepted that a better knowledge of the mechanisms of pollination and fertilization may result in better productivity (Feng *et al.*, 2013). Thus, knowledge of embryology on the development of reproductive organs in *Z. jujube* is essential for solving these problems.

Embryological studies are often useful in encompassing investigation of virtually all the events relevant to sexual reproduction. Microsporogenesis and

Corresponding Author: De-Yi Yuan, The Key Laboratory of Cultivation and Protection for Non-wood Forest Trees, Ministry of Education, Central South University of Forestry and Technology, Changsha 410004, P.R. China

This work is licensed under a Creative Commons Attribution 4.0 International License (URL: <http://creativecommons.org/licenses/by/4.0/>).

male gametogenesis in *Z. jujube* 'Lizao' has not previously been investigated. This present study aims to investigate microsporogenesis and male gametogenesis of *Z. jujube* 'Lizao' and provide basic information for our research projects.

MATERIALS AND METHODS

The experiment was conducted during the year 2010 and 2012 on eight years old *Z. jujube* 'Lizao' which was planted at 2×3 m distance. The mixed planting species are *Z. jujube*. 'Guangyangchangzao', 'Jidanzao' and 'Jinsi NO.4'. The experimental site was the non timber forest garden in Zhuzhou campus of Central South University of Forestry and Technology, which longitude is eastern 113°09'50" and latitude is northern 27°55'30" and altitude is 70.00 m and the site is gentle slope with re soil (Yuan *et al.*, 2009).

We photographed the *Z. jujube* 'Lizao' from bud to bloom by the Nikon P600 camera (Japan) (Fig. 1A to H). The samples were collected about every 7 days from March to June and being representative of the jujube population. The materials were fixed in formalin-acetic acid-alcohol (FAA; 5:5:90, v/v), dehydrated through an ethyl-alcohol series, embedded in paraffin with a 58-60°C melting point and sectioned at a thickness of 10 µm by the microtome (Zou *et al.*, 2013). The sections were stained with Heidenhain's iron aium haematoxylin (Zou *et al.*, 2014). Observation and photograph of sections were carried out using an BX-51 microscope (Olympus, Japan). Some sections were stained with 0.5% decolorized aniline blue in 0.1 M K3po4 for 3~12 h and observed under a fluorescence Bx-51 microscope to investigate callose deposition during microsporogenesis (Feng *et al.*, 2013).

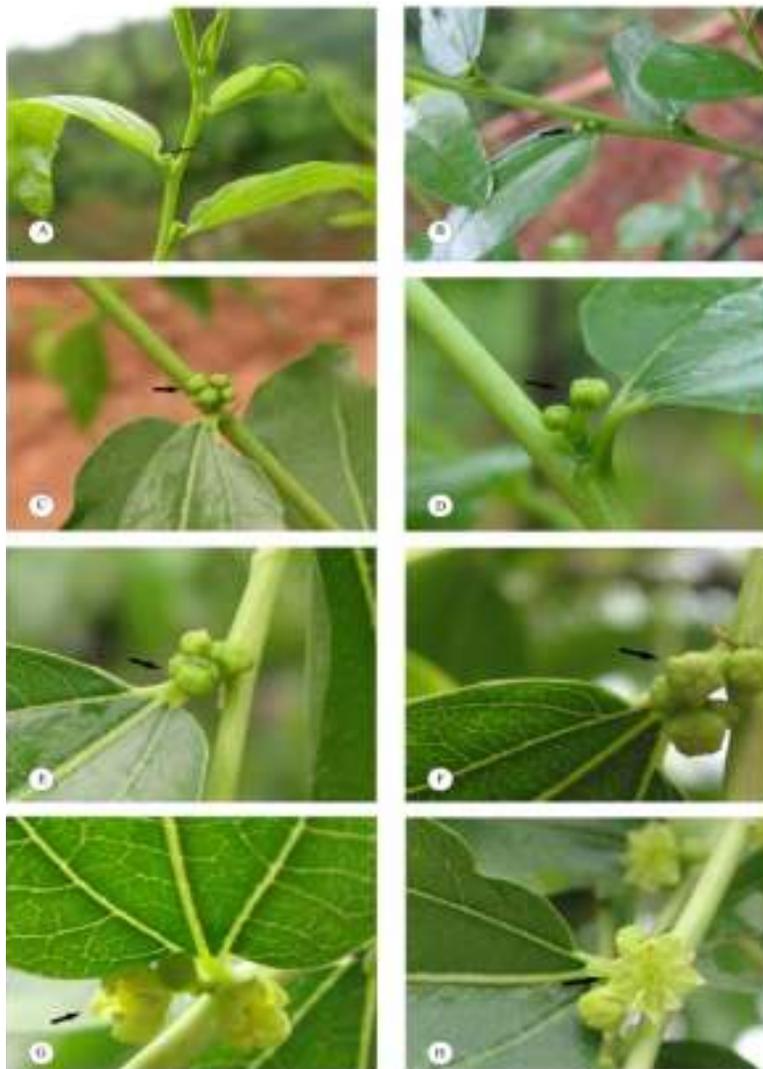


Fig. 1: The floral development of *Z. jujube* 'Lizao'; (A): The buds emerge from axillary; (B-E): The bud at alabastrum intumescence process; (F): The alabastrums are getting ready to burst; (G): The flower appearing only partially opened; (H): The flower is blooming

RESULTS

Formation of anther wall: Floral emergence and development lasted for 1 month—from later April to later May (Fig. 1 and Table 1). The archesporial stage of microsporogenesis was first observed in buds collected in later April (Fig. 1A). The archesporial cells appeared at the corners of the young anther (Fig. 2A). The anthers were tetrasporangiate in the early May (Fig. 2B). At an early stage of development, archesporial cells (which were recognizable by their large volume with conspicuous nuclei) differentiated below the epidermis of anthers. These cells divided periclinally to

Table 1: Phenology of microsporogenesis development of *Z. jujube* ‘Lizao’ in Hunan, China in 2010-2012

Date	Developmental event in stamens
April 28 th ~30 th	Young anther
May 1 th ~3 th	Microspore mother cell stage
May 4 th ~7 th	Meiosis of microspore mother cell stage
May 8 th ~10 th	Microspore tetrad stage
May 11 th ~13 th	Central nucleus microspore stage
May 14 th ~16 th	The late uninucleate stage
May 17 th ~20 th	Binucleate stage
May 21 th ~24 th	Two-celled pollen
May 25 th ~28 th	Mature pollens
May 29 th ~30 th	Pollen grains released

form outer primary parietal cells and inner primary sporogenous cells (Fig. 2B). The epidermal layer

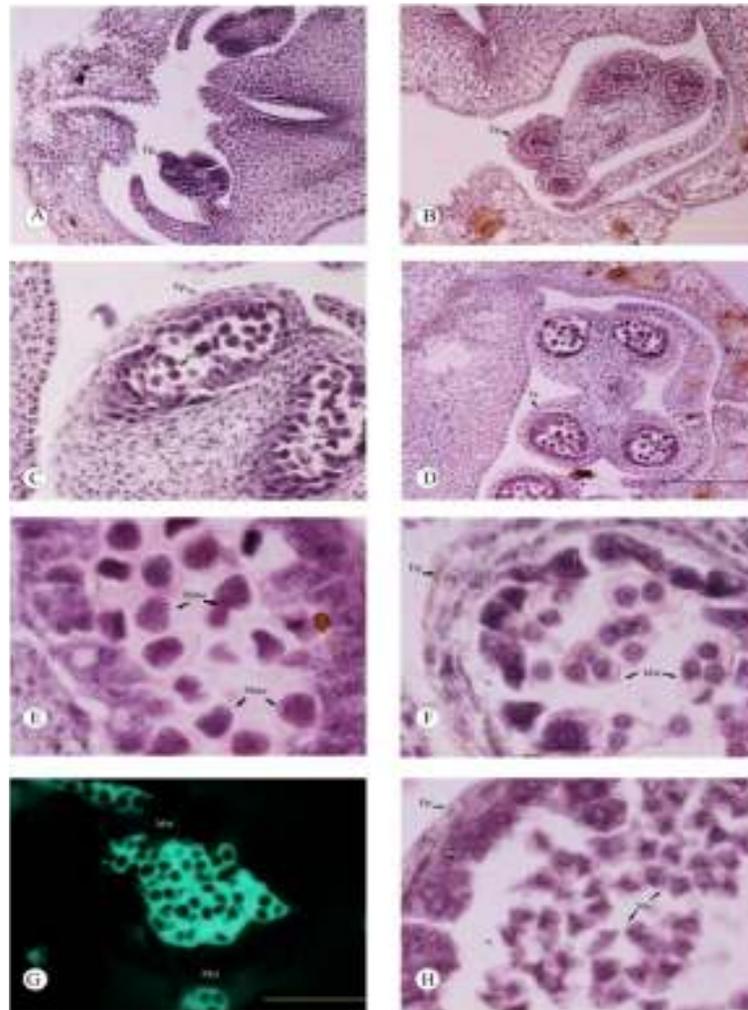


Fig. 2: Formation of microspores and development of male gametophyte in *Z. jujube* ‘Lizao’; (A): The archesporial cells appeared at the corners of the young anther (filament, petal); (B): Detail of an microspore mother cell wall showing the microspore mother cell coat and thin layer of callose (epidermis layer, endothecium, two middle layers and inner tapetum); (C): Detail of a late microspore mother cell wall at meiosis I telophase; (D): Detail of a late microspore mother cell wall at meiosis II metaphase; (E): High magnification of D showing microspore mother cell w+ all at meiosis II telophase; (F): Details of microspores showing microspore tetrads were tetrahedral and enclosed in callose; (G): Details of anther showing microspore tetrads were enclosed in the callose layer under the fluorescence microscope; (H): Details of anther showing at free microspores cell stage

Ac: Archesporial cell; An: Anther; En: Endothecium; Ep: Epidermis; Fi: Filament; Mi: Middle layers; Mmc: Microspore mother cell; Ms: Microspore; Mst: Microspore tetrad; Pe: Petal; Se: Septum; T: Tapetum

consisted of slightly elongated cells that gradually developed into compressed epidermis cells (Fig. 2B). The archesporial cells divided by periclinal divisions to form an outer layer of primary parietal cells and inner primary sporogenous cells. The primary parietal cells divided repeatedly by periclinal and anticlinal divisions to form a subepidermal endothecium and two middle layers (Fig. 2B). Cells of the outer layer again divided periclinally and anticlinally to form the massive nucellus. The inner cells form the sporogenous cells (Fig. 2B). The endothecial cells elongated gradually,

acquiring fibrous thickenings at the time of anthesis (Fig. 3G). The middle layers had a common histogenetic origin with the endothecium and it persisted until the tetrad stage (Fig. 2F) and degenerated before forming two-celled pollen grains (Fig. 3F). The middle layers appeared to undergo further divisions to form three layers. The rich starch in cells of middle layers was digested and gradually it disappeared in the process, providing nutrition for the pollen development. The walls of endothecium became thickened after microspore formation (Fig. 2H) and

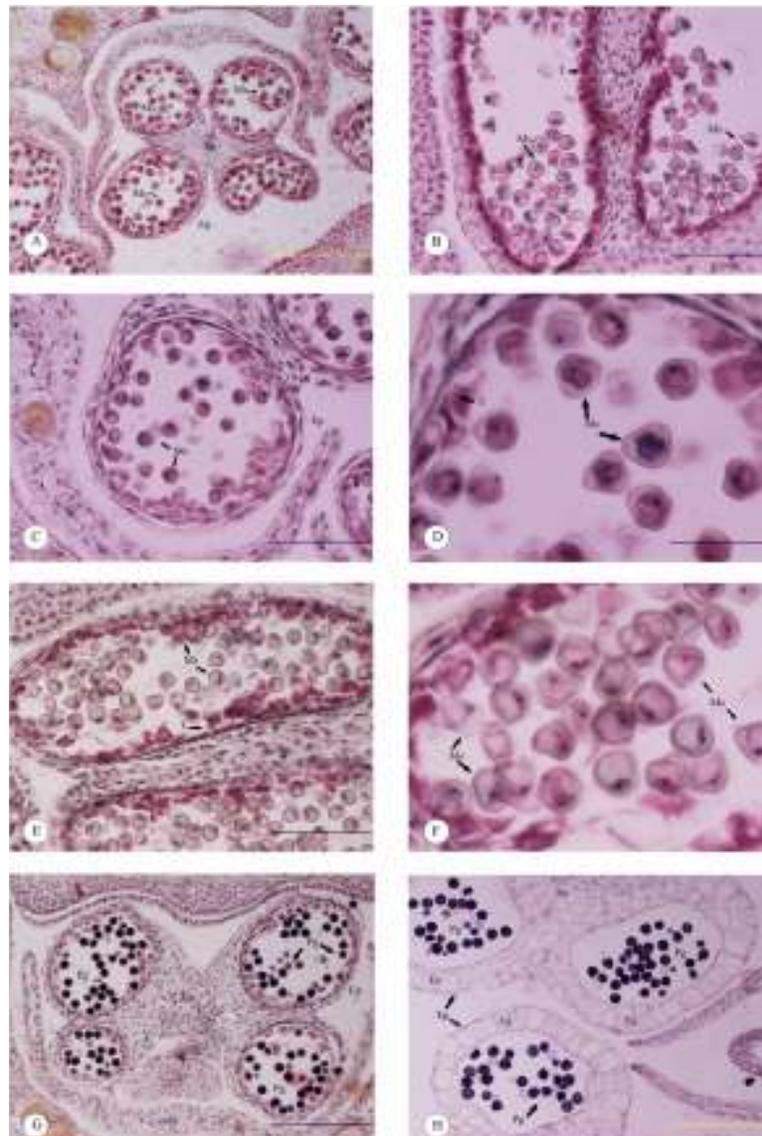


Fig. 3: Formation of microspores and development of male gametophyte in *Z. jujube* 'Lizao'; (A): Details of anther showing at microspores cell stage; (B): Anthers at uninucleate microspore stage with stretched epidermis, fibrous thickened endothecium, relic of middle layers; (C): Details of anther showing at stage of central nucleus microspore; (D): High magnification of C showing at stage of central nucleus microspore; (E): Details of anther showing at stage of late-nucleus microspore; (F): High magnification of C showing at stage of late-nucleus microspore; (G): Details of anthesis showing anther wall was composed of epidermis, endothecium and mature pollen grains; (H): High magnification of G showing epidermis, endothecium and mature pollen grains
En: Endothecium; Ep: Epidermis; Ms: Microspore; Pg: Pollen grains; Se: Septum; T: Tapetum

when pollen matured, the endothecium showed one layer (Fig. 3H). The anther wall, prior to maturity, was usually comprised of five or five cell layers, i.e., a single epidermis, an endothecium, two middle layers and the tapetum. Thus, the wall formation was of the basic type (Davis, 1966).

Tapetum characteristics: 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 or two prominent nuclei at the microspore mother cell meiosis stage (Fig. 2C to E). The tapetal cells were uninucleate or binucleate and posed abundant cytoplasm (Fig. 3B). The tapetum reached maximal size during meiosis of microspores. At the tetrad stage, tapetal cells elongated and lost close contact but still remained in their original position (Fig. 2F to G). However, they began to degenerate at the stage of uninucleate microspore (Fig. 3C) and has totally disintegrated at the mature pollen grain stage (Fig. 3H). Therefore, the tapetum conformed to the glandular type as named by Davis (1966).

Microsporogenesis and microgametogenesis: The anther primordium began to form as a teat, composing of meristematic tissue enveloped by an epidermal layer. Simultaneously with the changes taking place in the wall of the microsporangia, four groups of hypodermal cells differentiated near each of the four corners of the young anther (Fig. 2A). From the sections, a row of sporogenous cells deriving from 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, forming secondary sporogenous cells, from which microsporocytes were derived. Microsporocytes were discernable by their large volume, dense cytoplasm and conspicuous nuclei (Fig. 2B). The microsporocyte underwent meiosis which involved two cell divisions. Meiosis I includes the prophase, metaphase, anaphase and telophase (Fig. 2C). As a result, a dyad of microspores was formed. A microspore tetrad was formed during prophase II, metaphase II, anaphase II and telophase II (Fig. 2D to E). Meiosis II in each dyad resulted in a microspore tetrad. A majority of the tetrads were tetrahedral (Fig. 2F). There was a thick callose wall surrounding a microspore tetrad (Fig. 2G). We found that 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 microtetrads (Fig. 2G) and disappeared at end of of meiosis. The callose cycling (or cellulose synthesis) during microsporogenesis might play important roles in protecting the pollen mother cells from various environmental stresses and release of microspores from the microtetrads. In all sections, we observed that only

the second divisions occurred with the formation of wall between microspores, thus the microsporogenesis was simultaneous.

The microspores were soon separated from each other and released from the tetrads as uninucleate free microspores (Fig. 2H). Each microspore released from the tetrads had a dense cytoplasm, conspicuous wall, with a prominent and centrally placed nucleus (Fig. 3A). During further development, uninucleate microspores gradually increased their volumes and had more vacuoles (Fig. 3B to D). As the central vacuole developed, the nucleus took a peripheral position (Fig. 3E to F). The microspore development of could be divided into three periods: early-or mid-uninucleate (Fig. 3C to D), late-uninucleate (Fig. 3E to F) and binucleate. At this stage, the uninucleate microspore underwent an asymmetric mitotic division (the microspore mitosis) to give rise to two cells with distinct fates-the vegetative cell and the generative cell. The nucleus of the vegetative cell was round and large and generally confined at the center of the pollen grain, whereas the smaller generative cell was crescent-shaped and located close to the pollen wall. In the later May, mature pollen grains contained two cells with three germ pores (Fig. 3G to H). Anthers were dehiscent (Fig. 1G to H) and pollen grains were shed approximately on May 29th (Table 1).

DISCUSSION

The embryological feature of *Z. jujube* are summarized as follows. The anther of *Z. jujube* 'Lizao' is microdiodanges. The mature anther wall layers comprises an epidermis, one layered endothecium, two middle layers and a single-layered tapetum. The cytokinesis during meiosis of its microspore mother cell is modified simultaneous type and tetrads are tetrahedral. The formation of the anther wall conforms to the basic type. The pollen grains are monocolpate and two-celled with three germ pores at shedding. Some striking features are found in *Ziziphus*:

- The anther wall was usually comprised of five or five cell layers, i.e., a single epidermis, an endothecium, two middle layers and the tapetum. Davis (1966) classified the development of the anther wall into four types: basic, dicotyledonous, monocotyledonous and reduced. The formation of the anther wall of *Ziziphus* conforms to the basic type. Because sufficient data on embryology characters of Rhamnaceae families are lacking, the pattern of formation of anther walls of other taxa in *Ziziphus* is unknown. Tel-Zur and Schneider (2009) described that the main cellular events happened synchronously in the female and the male structures in *Z. mauritiana*, but still lack of

sufficient details in the male gametophyte development process.

- In angiosperms generally, five patterns of the arrangement of microspores in a tetrad are recognized: tetrahedral, isobilateral, linear, T-shaped and decussate (Xue *et al.*, 2005). From the sections, we observed that the tetrads of *Ziziphus* is tetrahedral.
- The tapetum during the anther wall development is of the heteromorphic and glandular type. This type of tapetum was reported in some species of *Ziziphus* (Tel-Zur and Schneider, 2009). 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 when anthers dehisced. Tapetum's major functions include producing pollen wall components, nutrients for pollen development and enzymes for microspore release from tetrads. All nutrients reaching the sporogenic cells must pass through tapetal cells (Yan *et al.*, 2010). In addition, callose deposition occurred at the onset of meiosis of pollen mother cells and disappeared at end of meiosis. The callose cycling (or cellulose synthesis) during microsporogenesis might play important roles in protecting the pollen mother cells from various environmental stresses and release of microspores from the microtetrads (Li and Ma, 2006).
- Microsporogenesis of *Ziziphus* is successive. As we known, male sterility might be an important factor influencing fruit production (Feng *et al.*, 2013). Male sterility is the result of failure of formation or development of functional stamens, microspores or gametes. According to Yan *et al.* (2010), there were some instances of failure to develop normal pollen, probably caused by the abnormal development at three levels: the sporogenous tissue, tapetum layer and microspore. Male sterility are usually accompanied by abnormal callose deposition or anomalies in tapetum (Kaul, 1988). However, in our anatomical observation, we did not find the abnormal callose deposition and anomalies in tapetum during microsporogenesis in the *Z. jujube* 'Lizao', indicating that male gametes were fertile and male sterility was not the major cause of the low seed set in the *Z. jujube* cultivar 'Lizao'. Because of a lack of sufficient data from each family of Rhamnaceae, we are need to clarify embryological attributes for each family in the future.

CONCLUSION

In the present study we clarify the stages of the reproductive cycle and consider similarities with other *Z. jujube* plants. In *Z. jujube* 'Lizao', the microgametogenesis results in binucleate pollen as has

been observed in other genera in *Ziziphus*. Cytological and embryological characteristics of *Z. jujube* 'Lizao' were studied for the first time. The reproductive biology of the *Z. jujube* species is still poorly understood and various aspects remain to be studied in depth. Only when we know more about the reproductive biology of the Rhamnaceae will it also become possible to understand and control fruit yield and quality in Chinese jujube.

ACKNOWLEDGMENT

The authors received assistance from many individuals during the course of the work and express appreciation to all, especially Ting Liao, Chao Gao, Yanzhi Feng, Weihua Duan and Xiao Zhang. The research was supported by the National Science and Technology Pillar Program (2013BAD14B03) and the Special Funds for Forestry Scientific Research in the Public Interest (201204407).

REFERENCES

- Davis, G.L., 1966. Systematic Embryology of the Angiosperms [M]. John Wiley and Sons Inc., New York, pp: 283-505.
- Feng, Z., G. Su-Juan, X. Peng, X. Huan, L. Wen-Jun *et al.*, 2013. Sporogenesis and Gametophyte Development in the Chinese Chestnut (*Castanea mollissima* Blume.) [J]. Taiwan J. For. Sci., 28(4): 171-184.
- Guangfang, Z., 2014. The Key Technology for Green and Efficient Production of Jujube [M]. Jinan: Shangdong Technology Publicshing House, China.
- Hu, F.M., He Y.H. and Xie B.X., 1999. The law of *Ziziphus jujube* flowers drop and fruit drop and the control measures [J]. J. Cent. South Fores. Univ., 19: 19-22 (In Chinese with English abstract).
- Jinghua, D., L. Fangdong, D. Hongyan, Z. Lin, Y. Deyi *et al.*, 2012. Cloning and expression analysis of a LFY homologous gene in Chinese jujube (*Ziziphus jujube* Mill.) [J]. Afr. J. Biotechnol., 11(3): 581-589.
- Kaul, M.L.H., 1988. Male Sterility in Higher Plants [M]. Springer-Verlag, Berlin, pp: 3-96.
- Li, W. and F. Ma, 2006. Reproductive Biology of Sexual Hybridization in Woody Plants: An Atlas [M]. Science Press, Beijing, pp: 1-16.
- Lillecrapp, A.M., M.A. Wallwork and M. Sedgley, 1999. Female and male sterility cause low fruit set in a clone of the 'Trevatt' variety of apricot (*Prunus armeniaca*) [J]. Sci. Hortic., 82: 255-263.
- Srinivasachar, D., 1940. Embryological studies of some member of Rhanaceae [J]. P. Indian Acad. Sci. B, 11: 107-116.
- Sunil, P., 2013. Nutritional composition of jujube fruit [J]. Emir. J. Food Agric., 25(6): 463-470.

- Tel-Zur, N. and B. Schneider, 2009. Floral biology of *Ziziphus mauritiana* (Rhamnaceae) [J]. Sex Plant Reprod., 22: 73-85.
- Tian, H.Q. and D.Z. Ma, 1987. The embryological observation of a kind of parthenocarpy Jujube [J]. Acta Bot. Sin., 29: 29-33 (In Chinese with English abstract).
- Wang, Q.Y., 1983. The development of embryo and endosperm of Chinese Jujube [J]. Acta Bot. Sin., 25: 526-531 (In Chinese with English abstract).
- Xue, C.Y., H. Wang and D.Z. Li, 2005. Microsporangogenesis and male gametogenesis in *Musella* (Musaceae): A monotypic genus from Yunnan, China [J]. Ann. Bot. Fenn., 42: 461-467.
- Yan, Q., Y. Hong, Z. Xiang-Ling, X. Yun-Feng and C. Xin-Qi, 2010. A study of microsporangogenesis and male gametogenesis in *Psammosilene tunicoides* (Caryophyllaceae) [J]. Ann. Bot. Fenn., 47: 175-189.
- Yuan, D.Y., S. Wang, Z.Y. Gu, J.H. Duan and F.D. Li, 2009. Blossom and fruit drop and anatomic observations on embryonic development of *Ziziphus jujuba* Mill. cv. Hunanjidanzao [J]. ISHS Acta Hort., 840: 347-355.
- Yufei, N., J. Peng and L. Li, 2011. Morphology of flower organ and anatomical characteristics of microspore different development period in Chinese Jujube and Wild Jujube [J]. J. Plant Genet. Res., 12(1): 158-162 (In Chinese with English abstract).
- Zhenhuai, Z.X.P.S.G., 2004. Studies on the pollination, fertilization and embryo development of Chinese Date 'Wu_He_Zao' (*Ziziphus jujuba*) [J]. Sci. Silvae Sinicae, 40(5): 210-213 (In Chinese with English abstract).
- Zhihui, X., L. Ping and L. Mengjun, 2011. Cytological mechanism of 2n pollen formation in Chinese jujube (*Ziziphus jujuba* Mill. 'Linglingzao') [J]. Euphytica, 182: 231-238.
- Zou, F., D.Y. Yuan, J.H. Duan *et al.*, 2013. A study of microsporangogenesis and male gametogenesis in *Camellia grijsii* Hance [J]. Adv. J. Food Sci. Technol., 5(12): 1590-1595.
- Zou, F., S.J. Guo, P. Xie, H. Xiong, W.J. Lv and G.H. Li, 2014. Megasporogenesis and development of female gametophyte in Chinese chestnut (*Castanea mollissima*) cultivar 'Yanshanzaofeng' [J]. Int. J. Agric. Biol., 16(5): 1001-1005.