

## Research Article

### Floral Biology of Chinese Jujube (*Ziziphus jujube* Mill.) II: The Formation of Megasporeogenesis and Development of Female Gametes

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**Abstract:** *Ziziphus jujube* Mill is an economically important fruit crop species in China, well-known for its fruits with high dietary value and medical value. However, it is particularly prone to erratic fruit set showing very low and have little work on the reproduction biology. In order to clarify the megasporeogenesis and female gametogenesis formation in Chinese jujube, a cultivar named 'Lizao' was employed for light microscopy analysis. The results are showed that the development of embryo sac conformed to Polygonum type. Archespore under the nucellar epidermis directly developed a megaspore mother cell. The megaspore tetrad were arranged into a linear shape, the megaspore of chalazal end was functional megaspore. The functional megaspore formed 7-celled or 8-nucleate embryo sac following its three times division successively. The ovules were anatropous, bitegminous and tenuinucellate. The abnormalities that occurred during female gametophyte development resulted in ovules abortion. According to our study, a large number of abortive ovules were likely the cause of the low seed set in *Z. jujube* 'Lizao'. 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:** Female gametogenesis, floral, megasporeogenesis, *Ziziphus jujube* Mill.

## INTRODUCTION

Chinese jujube (*Ziziphus jujube* Mill.), a member of the Rhamnaceae family, has been known as a native fruit in China (Wu *et al.*, 2013). The fruit has been used in Chinese medicine for at least 4,000 years (Duan *et al.*, 2012). Jujube contains potassium, phosphorus, manganese and calcium as the major minerals. There are also high amounts of sodium, zinc, iron and copper. Jujube also contains vitamin C, riboflavin and thiamine. The vitamin and mineral content of the fruit helps to support cardiovascular health and enhance metabolism (Pareek, 2013). In 2012, the total production of Chinese jujube reached 5.4 million tons with a growing area estimated 2 million ha in China. It is also one of the most popularly used herbal medicines in India, Russia southern Europe and the Middle East (Zhao *et al.*, 2014). In addition, *Z. jujube* is tolerant to extreme environmental conditions, including drought, high temperatures and saline water (Tel-Zur and Schneider, 2009; Wu *et al.*, 2013).

As we know, the female gametophyte is critical for plant reproduction (Yadegar and Drews, 2004). The flower and fruit fallen of jujube is caused by abnormal

development of sporogenesis and gametophyte, pollination and fertilization (Yuan *et al.*, 2009). Therefore, it is significant to study the reproductive biology of jujube. However, only a few published accounts of the sexual reproduction in *Ziziphus* (Srinivasachar, 1940; Kajale, 1944; Wang, 1983; Tian and Ma, 1987; Zhang *et al.*, 2004; Yuan *et al.*, 2009; Niu *et al.*, 2011; Xue *et al.*, 2011; Chen *et al.*, 2014). 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 (Zou *et al.*, 2013a). 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. Megasporeogenesis and female gametogenesis in *Z. jujube* 'Lizao' has not

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previously been investigated. The aim of this study was to investigate megasporogenesis and female gametogenesis of *Z. jujube* 'Lizao' and provide basic information for our research projects.

## MATERIALS AND METHODS

**Plant material:** The experimental site is the non-wood 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. The experiment was conducted during the year 2010 and 2012 on 8 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' (Yuan *et al.*, 2009).

**Bud fixation and sample preparation:** The complete development of the female gametophyte was studied in *Z. jujube* 'Lizao'. 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) and were stored at 4°C prior to sectioning (Zou *et al.*, 2013c).

**Light microscopy:** The materials were dehydrated in a graded ethanol series from water through 10-20-30-50-70-85-95-100% ethanol, embedded in paraffin with a 58-60°C melting point and sectioned at a thickness of 10 µm by the microtome (Zou *et al.*, 2013b). 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). We also investigated the aborted ovules during the flowering season by microscopy. The abortive ovules was judged by the presence of darkly stain with shrunken embryo sac cells or empty embryo sac (Zou *et al.*, 2013c).

## RESULTS

**Ovule development:** Floral emergence and development lasted for 1 month-from later April to later May (Table 1). In later April, a parenchyma cell beneath the epidermis of the ovule in the micropyle end developed into an archesporial cell. The cells of the nucellar epidermal layer and the subdermal layer divided anticlinally (Fig. 1A). The volume of the nucellar cells beneath the top of the subdermal layers enlarged and differentiated into a primary archesporial cell (Fig. 1B). Rapid mitosis in some nucellar epidermal cells around the base of the nucellus gave rise to the primordium of the inner integument (Fig. 1B). The outer integument primordium was initiated slightly later (Fig. 1B). Both the inner and outer integuments were composed of 2~3 layers of cells in thickness (Fig. 1B).

Table 1: Phenology of megasporogenesis development of *Z. jujube* 'Lizao' in Hunan, China in 2010-2012

Date	Developmental event in pistil
April 28 <sup>th</sup> -30 <sup>th</sup>	Ovule primordial arise
May 1 <sup>st</sup> -3 <sup>th</sup>	The nucellus started protrusions
May 4 <sup>th</sup> -7 <sup>th</sup>	Nucellur continue projection
May 8 <sup>th</sup> -10 <sup>th</sup>	Inner and outer integument initiation to elongation
May 11 <sup>th</sup> -13 <sup>th</sup>	The megaspore mother cell formation
May 14 <sup>th</sup> -16 <sup>th</sup>	The megaspore mother cell meiosis to form tetrad
May 17 <sup>th</sup> -20 <sup>th</sup>	The uninucleate embryo sac
May 21 <sup>th</sup> -24 <sup>th</sup>	Binuclear or four nuclear embryo sac
May 25 <sup>th</sup> -28 <sup>th</sup>	Eight nuclear embryo sac
May 29 <sup>th</sup> -30 <sup>th</sup>	Mature embryo sac

Both the inner and outer integuments enlongated quickly, nearly enclosed the nucellus (Fig. 1B). In the nucellus, the archesporial cell underwent periclinal division, formed a primary parietal cell (Fig. 1B). And then, the volume of both integuments increased rapidly, completely enclosed the nucellus to form the micropyle. At this time, the ovules occupied a part of the ovarian cavity (Fig. 1C).

The ovary were superior, bicarpellate and unilocular or multilocular with parietal placentae. The integuments were initiated by periclinal and oblique divisions at the base of the nucellus. The integument reached the top of the nucellus and formed a micropyle by continuous division. Ovules in *Z. Jujube* 'Lizao' were anatropous (Fig. 1C). The structure of the ovule resembled that described for other *Ziziphus* species (Srinivasachar, 1940; Kajale, 1944; Tian and Ma, 1987; Yuan *et al.*, 2009).

**Megasporogenesis and megagametogenesis:** A single archesporial cell differentiated under 1-layered epidermal cells in the young nucellus (Fig. 1B). This archesporial cells functioned directly as a megasporocyte, which was easily distinguished from other cells by its large nucellus and dense cytoplasm (Fig. 1C). The archesporial cell did not form a parietal layer, but the nucellar epidermis divided periclinally, giving rise to a subdermal layer between ovule epidermis and megaspore mother cell (Fig. 1C). Thus, the ovule was crassinucellate as named by Davis (1966).

The megasporocyte underwent meiosis to form the dyad of megaspore. A megaspore tetrad was formed after meiosis, such as metaphase (Fig. 1D), anaphase (Fig. 1E) and telophase (Fig. 1F). Finally, these processes gave rise to a liner terad of megaspores (Fig. 1G). While three megaspores of the tetrad eventually degenerated, the chalazal one became functional (Fig. 1H). The functional megaspore developed successively into a 2-nucleate embryo sac (Fig. 2A and B) after first meiotic division. Then to continue the second mitosis was formed a 4-nucleate embryo sac (Fig. 2C). Finally 8-nucleate embryo sac was formed after third meiotic division (Fig. 2E and G). When the flowers were blooming on the later May (Fig. 2D), the embryo sac began to mature. Three cells (or nuclei) were grouped together at the micropylar end and constituted the egg apparatus (Fig. 2F). The cells were initially quite similar, then one of them became an

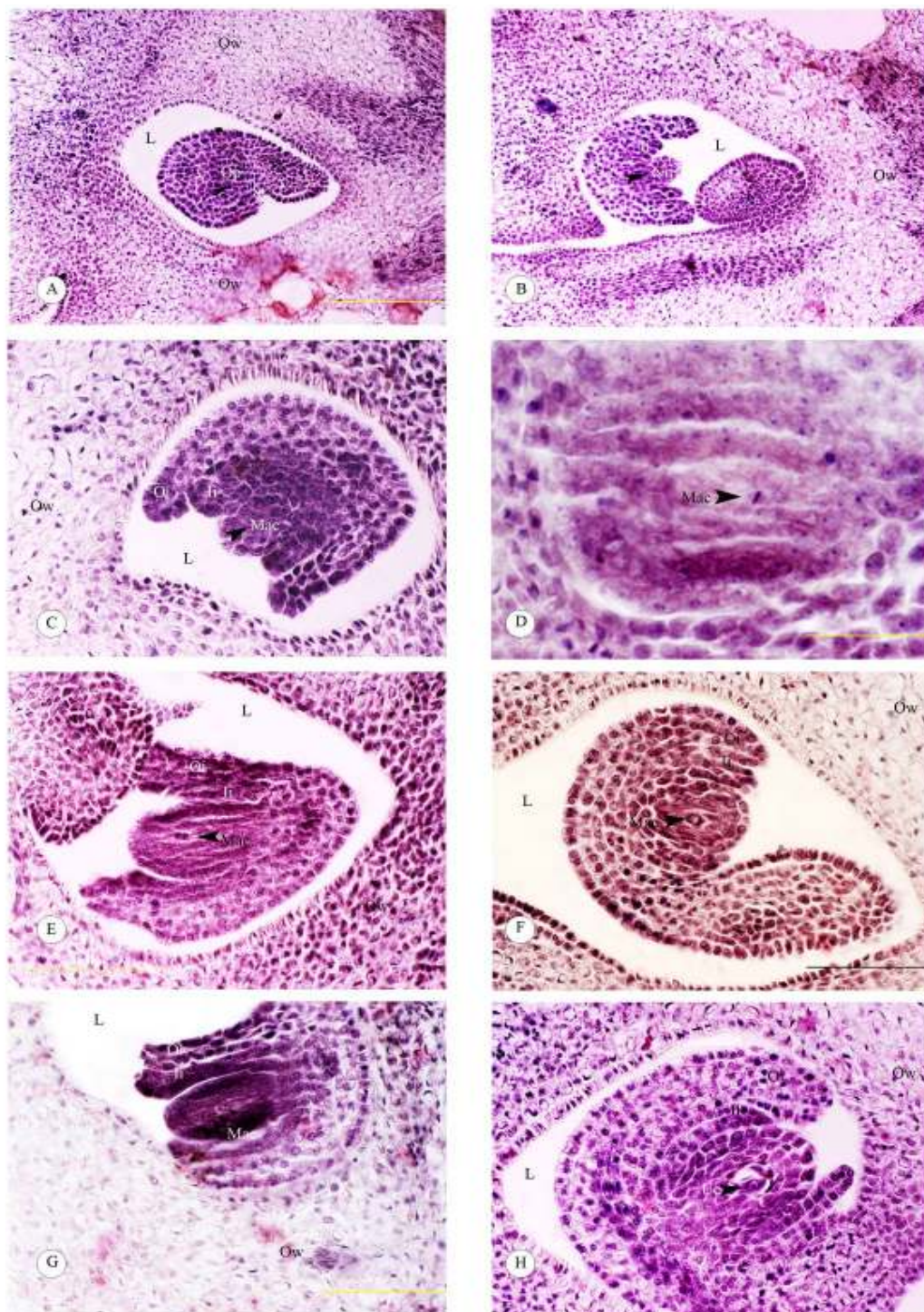


Fig. 1: Formation of megaspores and development of female gametophytes in *Z. jujube* 'Lizao'; (A): Longitudinal section showing ovule primordium, locus and ovary wall; (B): Longitudinal section showing initiation and development of the inner and outer integuments, nucellus, locus and ovary wall; (C): Longitudinal section showing a megaspore mother cell, inner and outer integuments and ovary wall; (D): Longitudinal section showing a megaspore mother cell at meiotic metaphase stage; (E): Longitudinal section showing a megaspore mother cell at meiosis anaphase stage; (F): Longitudinal section showing a megaspore mother cell at meiosis telophase stage; (G): Longitudinal section showing a megaspore mother cell at tetrad stage; (H): Longitudinal section showing a uninuclear embryo sac, inner and outer integuments and ovary wall

II: Inner integument; L: Locus; Mac: Megaspore mother cell; Nu: Nucellus; Oi: Outer integument; Op: Ovule primordia; Ow: Ovary wall; Ue: Uninuclear embryo sac

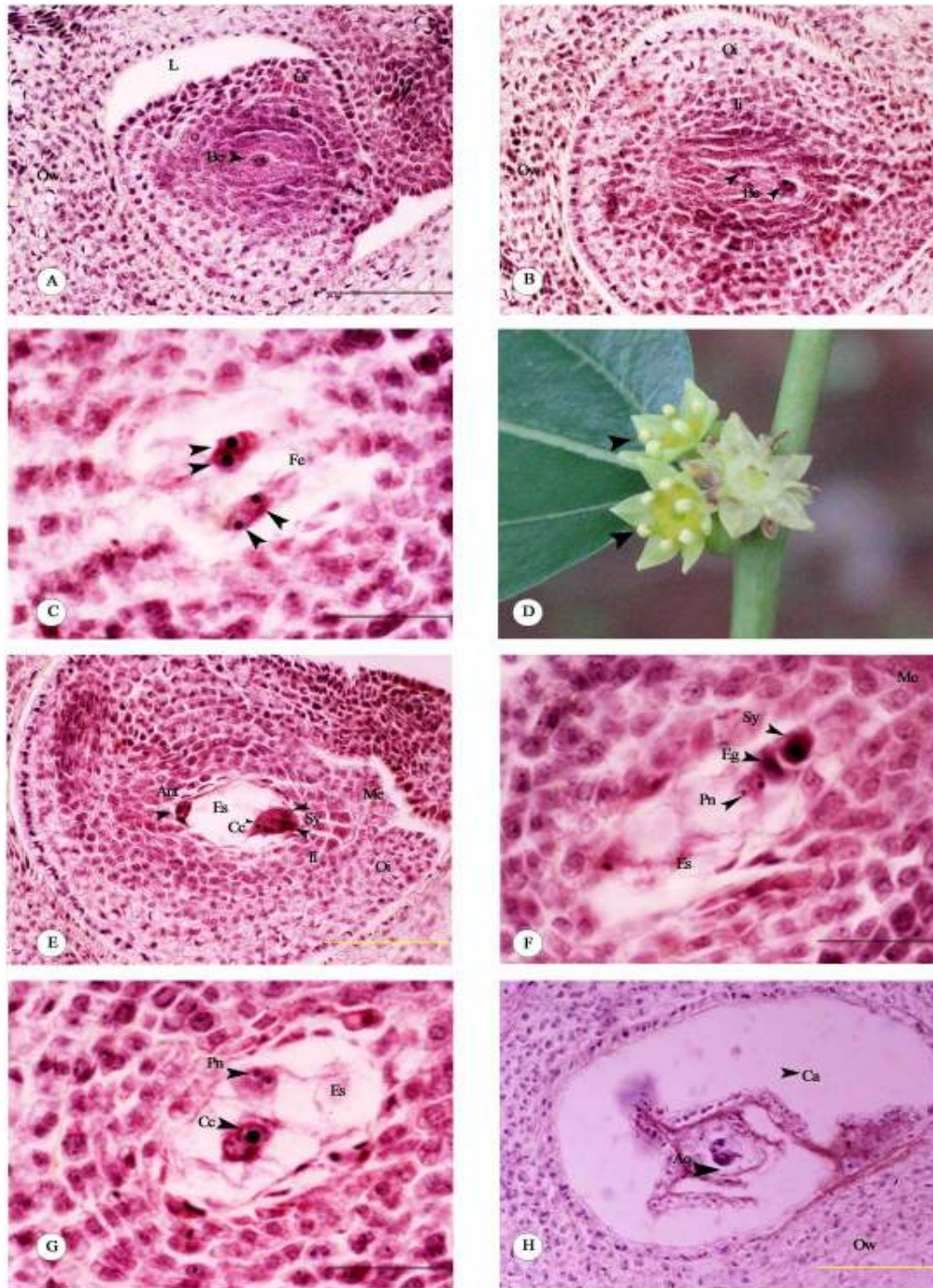


Fig. 2: Formation of megaspores and development of female gametophytes in *Z. jujube* 'Lizao'; (A): Longitudinal section showing a binuclear embryo sac, inner and outer integuments, locus and ovary wall; (B): Longitudinal section showing a binuclear embryo sac, inner and outer integuments; (C): Longitudinal section showing a four embryo sac, inner and outer integuments; (D): The flowers are blooming on the later May; (E)-(G): Developmental stages of a mature embryo sac; (E): Longitudinal section showing two synergid cells at the micropylar end, three antipodal cells at the chalazal end, a central cell and inner and outer integuments; (F): Longitudinal section showing an egg cell at the micropylar end, a synergid cell, two central polar nuclei and the inner and outer integuments; (G): Longitudinal section showing a central cell, two central polar nuclei and inner and outer integuments; (H): Longitudinal section showing an abortive ovule, cavity of the embryo sac and ovary wall;

Ant: Antipodal cells; Ao: Abortion ovule; Be: Binuclear embryo sac; Cc: Central cell; Ca: Cavity embryo sac; Eg: Egg cell; ES: Embryo sac; Fe: Four-nucleate embryo sac; II: Inner integument; L: Locus; Me: Micropylar end; Oi: Outer integument; Op: Ovule primordial; Ow: Ovary wall; Pn: Polar nuclei; Sy: Synergid

egg cell and the other 2 developed into triangular-shaped synergid cells (Fig. 2E). Two central polar nuclei (positioned close to each other) were fused into a central cell before fertilization (Fig. 2G), while the three antipodal cells were ephemeral at the chalazal end (Fig. 2E) and degenerated soon after fertilization. Thus, the mode of embryo sac formation conformed to the Polygonum type as defined by Davis (1966) (Table 1).

**Abortive ovules during development:** Abortion happened both before and after the development of the embryo sac. We examined about 25.80% abortive ovules in 1856 ovules. The aborted ovules were darkly stained with shrunken embryo sac cells or cavity embryo sacs (Fig. 2H). Thus, it was reasonably to assume that these ovules were going to abort.

## DISCUSSION

The embryological feature of *Z. jujube* 'Lizao' are summarized as follows. Archegonium under the nucellar epidermis directly develop a megaspore mother cell. The megaspore tetrad are arranged into a line shape, the megaspore of chalazal end is functional megaspore. The functional megaspore form 7-celled or 8-nucleate embryo sac following its three times division successively. The ovules are anatropous, bitegminous and tenuinucellate. The development of embryo sac conform to Polygonum type.

Some striking features are found in *Ziziphus*:

- The formation of the embryo sac of *Ziziphus* conforms to the Polygonum type. Davis (1966) classified the development of the embryo sac into eleven types: Polygonum, Oenothera, Allium, Endymion, Plumbago, Peperomia, Fritillaria, Drusa, Adoxa, Penaea and Plumbagella. The observation of the embryo sac of *Ziziphus* was the Polygonum type as Srinivasachar (1940) previously described. However, Kaiale (1944) and Tian and Ma (1987) investigated that the development of embryo sac was of Allium type. Because sufficient data on embryology characters of Rhamnaceae families are lacking, the pattern of formation megagametogenesis of other taxa in *Ziziphus* is unknown.
- In angiosperms generally, three patterns of the arrangement of megaspores in a tetrad are recognized: two column, linear and T-shaped (Davis, 1966). From the sections, we observed that the tetrads of *Ziziphus* was linear.
- Some abnormalities that occurred during megagametogenesis in *Z. jujube* 'Lizao'. Abnormalities of the reproductive structures commonly have a negative effect on reproduction. It is generally known that the normal ovule development is important for high fruit set in orchards. Zeng *et al.* (2009) observed that embryo sac abortion was one of the major reasons for sterility in indica/japonica hybrids in Rice. Zou

*et al.* (2013a) reported that the low fruit set in *Castanea mollissima* 'Yanshanzaofeng' could be partially explained by abortive ovules in the female gametophytes development. In our anatomical investigation, although most of the female gametophytes developed normal, some ovules were abortive in *Z. jujube* cultivar 'Lizao'. The absence of embryo sacs and the occurrence of empty embryo sacs accounted for abortion in other ovules. The observed anomalies in *Z. jujube* also were consistent with previous description (Yuan *et al.*, 2009). To our knowledge, the ovule is the source of the megagametophyte and the progenitor of the seed (Reiser and Fischer, 1993). Poor fruit set has been attributed to an undesirable environmental conditions or male sterility or female sterility (Julian *et al.*, 2010; Guerra *et al.*, 2011). However, in our study, we found 'Lizao' not only grew under a good hydrothermal condition in orchards, but also exhibited male fertility (preliminary results). Thus, the low fruit set in 'Lizao' could be partially explained by abortive ovules in the female gametophytes development and the high percentage of abortive ovules were identified as the major factor causing female sterility and possibly influenced the fruit production in 'Lizao' orchards. But 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.

## CONCLUSION

In the present study we clarify the stages of the reproductive cycle and consider similarities with other *Z. jujube* plants. In *Z. jujube*, the megasporogenesis results in Polygonum type as has been observed in other genera in *Ziziphus*. Cytological and embryological characteristics of *Z. jujube* 'Lizao' were studied for the first time. Abnormal embryo sacs or abortive ovules were observed in the ovary, suggesting that the same cellular mechanisms might act in somatic as well as in embryo rescue and embryogenesis induction (Liang *et al.*, 2014). A large number of abortive ovules may influence yield in *Z. jujube*. Because of a lack of sufficient data from each family of Rhamnaceae, we need to clarify embryological attributes for each family in the future. Further studies should be performed to delineate whether the abortive ovules were caused by resources limitation or programmed cell death in the future.

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## REFERENCES

- Chen, X.Y., J.X. Wang, Y.M. Pei, X.H. Kang and J.Y. Peng, 2014. Observation of pollination and fertilization of *Ziziphus jujuba* Mill. 'Jinsixiaozao' and *Z. jujuba* Mill. 'Wuhexiaozao' [J]. J. Agric. Univ., Hebei, 37(6): 28-32 (In Chinese with English Abstract).
- Davis, G.L., 1966. Systematic Embryology of the Angiosperms [M]. John Wiley and Sons, Inc., New York, pp: 283-505.
- Duan, J.H., F.D. Li, H.Y. Du, L. Zhang, D.Y. Yuan, Y.Z. Feng, H.X. Long and L.Y. Ma, 2012. Cloning and expression analysis of a LFY homologous gene in Chinese jujube (*Ziziphus jujube* Mill.) [J]. Afr. J. Biotechnol., 11(3): 581-589.
- Guerra, M.E., A. Wünsch, M. López-Corrales and J. Rodrigo, 2011. Lack of fruit set caused by ovule degeneration in Japanese Plum [J]. J. Am. Soc. Hortic. Sci., 136(6): 375-381.
- Julian, C., M. Herrero and J. Rodrigo, 2010. Flower bud differentiation and development in fruiting and non-fruiting shoots in relation to fruit set in apricot (*Prunus armeniaca* L.). Trees (Berl.), 24: 833-841.
- Kajale, L.B., 1944. A contribution to the life-history of *Zizyphus jujuba* Lamk [J]. Proc. Nat. Inst. Sci. India, 10(4): 387-391.
- Liang, C.L., J. Zhao and M.J. Liu, 2014. A high-efficiency regeneration system for immature embryo of *Ziziphus jujuba* 'Dongzao' at very early stage [J]. Acta Hortic. Sinica, 41(10): 2115-2124 (In Chinese with English Abstract).
- Niu, Y.F., J.Y. Peng and L. Li, 2011. Study on Morphology of flower organ and anatomical characteristics of microspore different developmental period in Chinese Jujube and wild Jujube [J]. J. Plant Genetic Resour., 12(1): 158-162 (In Chinese with English Abstract).
- Pareek, S., 2013. Nutritional composition of Jujube fruit [J]. Emir. J. Food Agric., 25(6): 463-470.
- Reiser, L. and R.L. Fischer, 1993. The ovule and the embryo sac [J]. Plant Cell, 5: 1291-1301.
- Srinivasachar, D., 1940. Embryological studies of some member of Rhanaceae [J]. Proc. India Acad. B, 11: 107-116.
- 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 parthenocarp Jujube [J]. Acta Botanica Sinica, 29: 29-33 (In Chinese with English abstract).
- Wang, Q.Y., 1983. The development of embryo and endospermic of Chinese Jujube [J]. Acta Bot. Sin., 25(6): 526-531 (In Chinese with English Abstract).
- Wu, C.S., Q.H. Gao, R.K. Kjellgren, X.D. Guo and M. Wang, 2013. Yields, phenolic profiles and antioxidant activities of *Ziziphus jujube* Mill. in response to different fertilization treatments [J]. Molecules, 18: 12029-12040.
- Xue, Z., P. Liu and M. Liu, 2011. Cytological mechanism of 2n pollen formation in Chinese jujube (*Ziziphus jujuba* Mill. 'Linglingzao') [J]. Euphytica, 182: 231-238.
- Yadegar, R. and G.N. Drews, 2004. Female gametophyte development [J]. Plant Cell, 16: S133-S141.
- 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 *Zizyphus jujuba* Mill. cv. 'Hunanjidanzao' [J]. ISHS Acta Hortic., 840: 347-355.
- Zeng, Y.X., C.Y. Hu, Y.G. Lu, J.Q. Li and X.D. Liu, 2009. Abnormalities occurring during female gametophyte development result in the diversity of abnormal embryo sacs and leads to abnormal fertilization in indica/japonica hybrids in Rice [J]. J. Integr. Plant Biol., 51(1): 3-12.
- Zhang, X., S. Peng and Z. Guo, 2004. Studies on the pollination, fertilization and embryo development of Chinese jujube [J]. Sci. Silvae Sin., 40(5): 210-213 (In Chinese with English Abstract).
- Zhao, J., J. Jian, G. Liu, J. Wang, M. Lin, Y. Ming *et al.*, 2014. Rapid SNP discovery and a RAD-based high-density linkage map in jujube (*Ziziphus* Mill.) [J]. PLOS One, 9(10): e109850.
- Zou, F., S.J. Guo, P. Xie, W.J. Lv, H. Xiong and G.H. Li, 2013a. Sporogenesis and gametophyte development in the Chinese chestnut (*Castanea mollissima* Blume.) [J]. Taiwan J. For. Sci., 28(4): 171-184.
- Zou, F., D.Y. Yuan, J.H. Duan, X.F. Tan and L. Zhang, 2013b. A study of microsporogenesis and male gametogenesis in *Camellia grijsii* Hamce [J]. Adv. J. Food Sci. Technol., 5(12): 1590-1595.
- Zou, F., D.Y. Yuan, X.F. Tan, P. Xie, T. Liao, X.M. Fan and L. Zhang, 2013c. Megasporogenesis and female gametophyte development of *Camellia grijsii* Hance [J]. J. Chem. Pharmaceut. Res., 5(11): 484-488.
- 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.