Research Article
Floral Biology of Chinese Jujube (*Ziziphus jujube* Mill.) II: The Formation of Megasporogenesis 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 megasporogenesis 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 chazlazal 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 megaspore of chazlazal end was functional megaspore. 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, megasporogenesis, *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. Megasporogenesis and female gametogenesis in *Z. jujube* ‘Lizao’ has not...
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’, ‘Jidanzaao’ 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 *Z. 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>
<thead>
<tr>
<th>Date</th>
<th>Development event in pistil</th>
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<tbody>
<tr>
<td>April 28-30</td>
<td>Ovule primordial arise</td>
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<tr>
<td>May 1-3</td>
<td>The nucellus started protrusions</td>
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<tr>
<td>May 4-7</td>
<td>Nucellur continue projection</td>
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<tr>
<td>May 8-10</td>
<td>Inner and outer integument initiation to elongation</td>
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<tr>
<td>May 11-13</td>
<td>The megaspore mother cell formation</td>
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<tr>
<td>May 14-16</td>
<td>The megaspore mother cell meiosis to form tetrad</td>
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<tr>
<td>May 17-20</td>
<td>The uninnucleate embryo sac</td>
</tr>
<tr>
<td>May 21-24</td>
<td>Binuclear or four nuclear embryo sac</td>
</tr>
<tr>
<td>May 25-28</td>
<td>Eight nuclear embryo sac</td>
</tr>
<tr>
<td>May 29-30</td>
<td>Mature embryo sac</td>
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Both the inner and outer integuments elongated 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 periclinaly, 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 second 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 primordial; 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
Some striking features are found in conform to Polygonum type. The development of embryo sac successively. The ovules are anatropous, bitegminous haploid megaspores occur in a tetrad and the megaspore of chalazal end is functional megaspore. The megaspore tetrad are arranged into a line shape, the megaspore mother cell. Archespore under the nucella r.

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. Archespore under the nucellar epidermis directly develop a megaspore mother cell. The megaspore tetrad are arranged into a line shape, the megaspore of the 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 defined by Davis (1966) (Table 1).

- 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 *indical japonica* hybrids in Rice. Zou *et al.* (2013a) reported that the low fruit set in *Castanea mollissima* ‘Yanshanzhoufeng’ 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 are 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


