

## Ovule ontogenesis and megagametophyte development in *Onobrychis schahuensis* Bornm. (Fabaceae)

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**Abstract:** The ovule ontogenesis and the megasporogenesis events in *Onobrychis schahuensis* Bornm. were studied with light microscopy. The primordium is tetra-zonate and gives rise to an anatropous ovule. The archesporium may consist of one or more archesporial cells, but only one of them undergoes meiosis, forming a linear shaped tetrad. Normally, only a single megaspore is functional, which is located in the chalazal position, while the others degenerate very soon. The young ovule is hemianatropous but the mature one is anatropous, crassinucellar, and bitegmic; integuments form a zig-zag micropyle. A 7-celled embryo-sac is formed corresponding to the *Polygonum* type. The present study provides the first report on embryological characteristics in *O. schahuensis*. Based on our results and in comparison with prior studies, several characters may have potential taxonomic significance for the *Hymenobrychis* section, such as the tetra-zonate ovule primordium, anatropous ovule type, dermal origin of the integuments, asymmetrical initiation of the outer integument, linear shaped tetrad with the presence of one functional megaspore, and having 2 young ovules but degeneration of one of them.

**Key words:** Embryology, Fabaceae, megaspore, *Onobrychis*, *Hymenobrychis*, ovule

### Introduction

The genus *Onobrychis* belongs to the tribe *Hedysareae*, with nearly 170 species mainly distributed in the northern temperate regions but centres of its diversity are in the eastern Mediterranean area and West Asia; a few taxa are cultivated as fodder or ornamentals (Lock & Simpson, 1991; Yakovlev et al., 1996; Mabberley, 1997; Aktoklu, 2001). In Flora Iranica, Rechinger (1984a, 1984b) treated 77 species under 9 sections, viz. *Dendrobrychis* (7 species), *Lophobrychis* (5), *Onobrychis* (14) *Laxiflorae* (3), *Anthyllium* (7), *Afghanicae* (3), *Insignes*

(3), *Heliobrychis* (21), and *Hymenobrychis* (12), with 2 species remaining unassigned. Morphological characters of the ovules and details of megasporogenesis can be used in systematic studies for defining the circumscription of the genus. Although pollen morphology was used as the most important microscopic character in the taxonomy of *Onobrychoidei* (Pinar et al., 2009), there are also some reports about taxonomically important characters in the Fabaceae family, such as variability in the larger megaspore of tetrads, ovule type, number of archesporial cells, number of parietal layers, and the

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alignment pattern of the integuments (Rembert, 1966, 1969a, 1969b, 1971; Davis, 1966; Prakash, 1987; Yeung & Cavey, 1990; Dute & Peterson, 1992; Johri et al., 1992; Johansson & Walles, 1993; Chamberlin et al., 1994). Based on our bibliographical studies *Onobrychis* is not studied regarding embryological characteristics. The purpose of this work was to investigate in detail the ovule ontogeny, the megasporogenesis, and the female gametophyte development in *Onobrychis schahuensis*.

## Materials and methods

The voucher specimen is deposited at the Bu-Ali Sina University Herbarium (BASUH 1098) and labelled as follows: Iran, Kermanshah province, 15 km from Javanroud to Taze-abad, alt. 1250 m. The flowers and buds were removed in different stages of development, fixed in FAA<sub>70</sub> (formalin, glacial acetic acid, and 70% ethanol, 5:5:90 v/v), stored in 70% ethanol, embedded in paraffin, and sectioned at 7-10 µm with a Micro DC 4055 microtome. Staining was carried out with PAS (Periodic Acid Schiff) according to the protocol suggested by Yeung (1984) and contrasted with Meyer's Hematoxylin (Chehregani et al., 2009). For each ovule developmental stages, several sections were studied under a Zeiss Axiostar Plus light microscope. For each stage, at least 20 flowers were studied and photomicrographs were made from the best ones.

## Results

### Ovule development

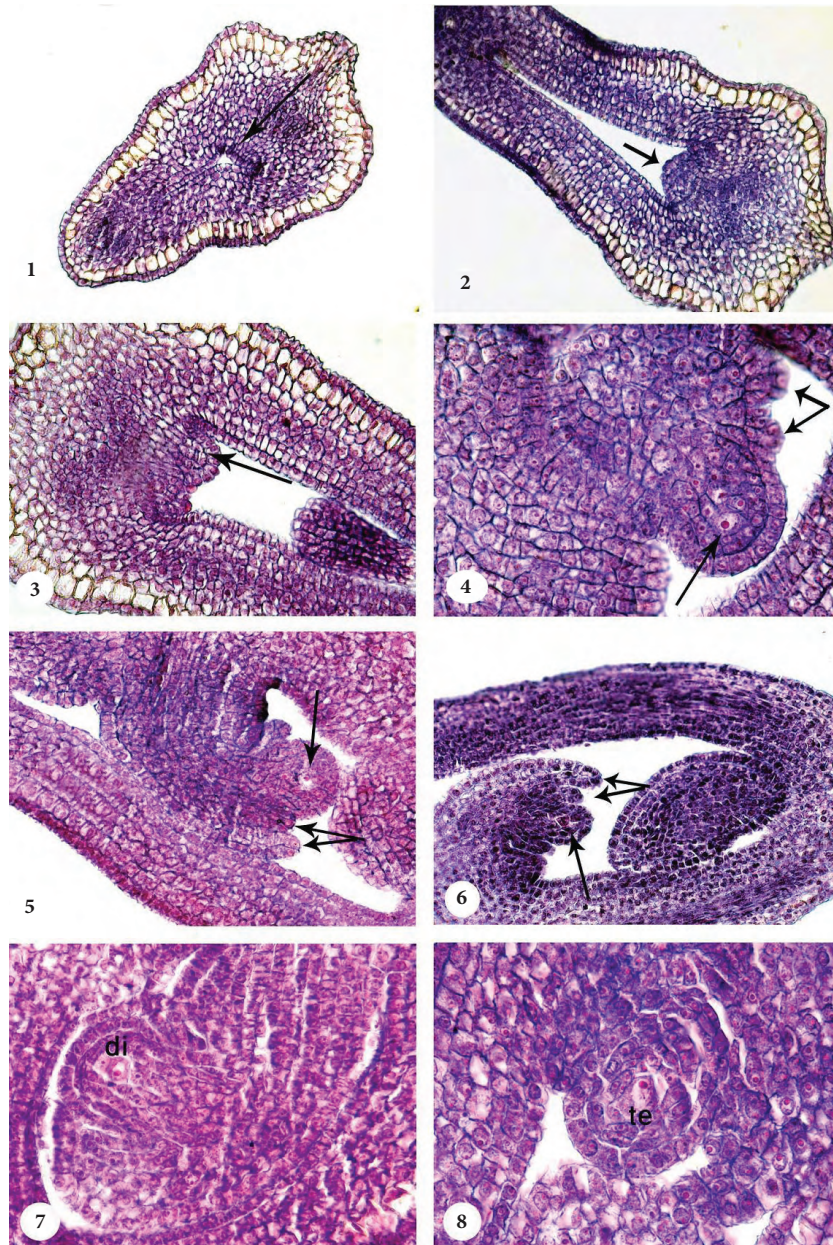
Results showed that the mature ovule is anatropous, crassinucellar, and bitegmic with a zig-zag micropyle (Figures 1-18). The carpel is already closed when the first ovular primordium appears. The ovule originates as a small protuberance (Figure 1). The ovular primordium is tetra-zonate and it is initiated by periclinal divisions in the second cell layer of the placenta (Figure 2). The initial archesporial cell is distinguished from the other nucellar cells, because it presents a larger volume, dense cytoplasm, and distinct nucleolus (Figure 3). Some of the cells of the nucellar tissue develop directly into the archesporial cells, one of which divides into a primary parietal cell and a megaspore mother cell (MMC). Then the

primary parietal cell undergoes 1 or 2 periclinal divisions (Figure 4). Simultaneously with the division of the initial archesporial cells, other sub-dermal cells also undergo periclinal divisions and increase the mass of nucellar cells. The ovule primordium starts bending at an early stage. The initiation of the integuments takes place when the ovule shows a nearly 150° curvature (Figures 4-6). The 2 integuments are initiated from periclinal and oblique divisions of dermal cells. The inner integument is 2-cell thick and differentiates simultaneously as a ring around the nucellus (Figure 4). The outer integument is 3-4-cell thick (Figure 5) and, on the opposite side of the funicular, grows asymmetrically faster (Figure 6), resulting in the exostome becoming eccentric with respect to the endostome; the 2 integuments thus constitute the zig-zag micropyle.

### Megasporogenesis and female gametophyte

The archesporial cell proper grows and differentiates directly to a megaspore mother cell, which compresses the lateral cells and becomes elongated on the nucellar major axis (Figure 6). The megaspore mother cell (MMC) divides meiotically and undergoes 2 successive divisions resulting in a linear shaped tetrad. Meiosis I forms unequally-sized dyad cells (Figure 7). Meiosis II is asynchronous, since the chalazal dyad cell divides before the micropylar one (Figure 8). The 3 micropylar megaspores degenerate, and the chalazal one develops into the megagametophyte (Figures 9-11). Three successive mitotic karyokineses give rise to an 8-nucleate embryo-sac. One central vacuole is formed and 4 nuclei are positioned in the micropylar end of the cytoplasm, and the other 4 nuclei in the chalazal end.

After the 8-nucleate stage, the coenocytic megagametophyte becomes partly cellular (Figure 12). This process is simultaneous at the micropylar and chalazal ends. The embryo-sac consists of 7 cells: the egg cell, 2 synergids, the central cell, and 3 antipodal cells (Figure 13). Simultaneously with the development of the embryo-sac, a structure consisting of a nucellar tissue resistant to the absorbing activity of the embryo-sac is observed. An endothelium is originated from the inner layer of the inner integument. The cells of this layer become radial stretched and they contain prominent nuclei and dense cytoplasm (Figure 13).

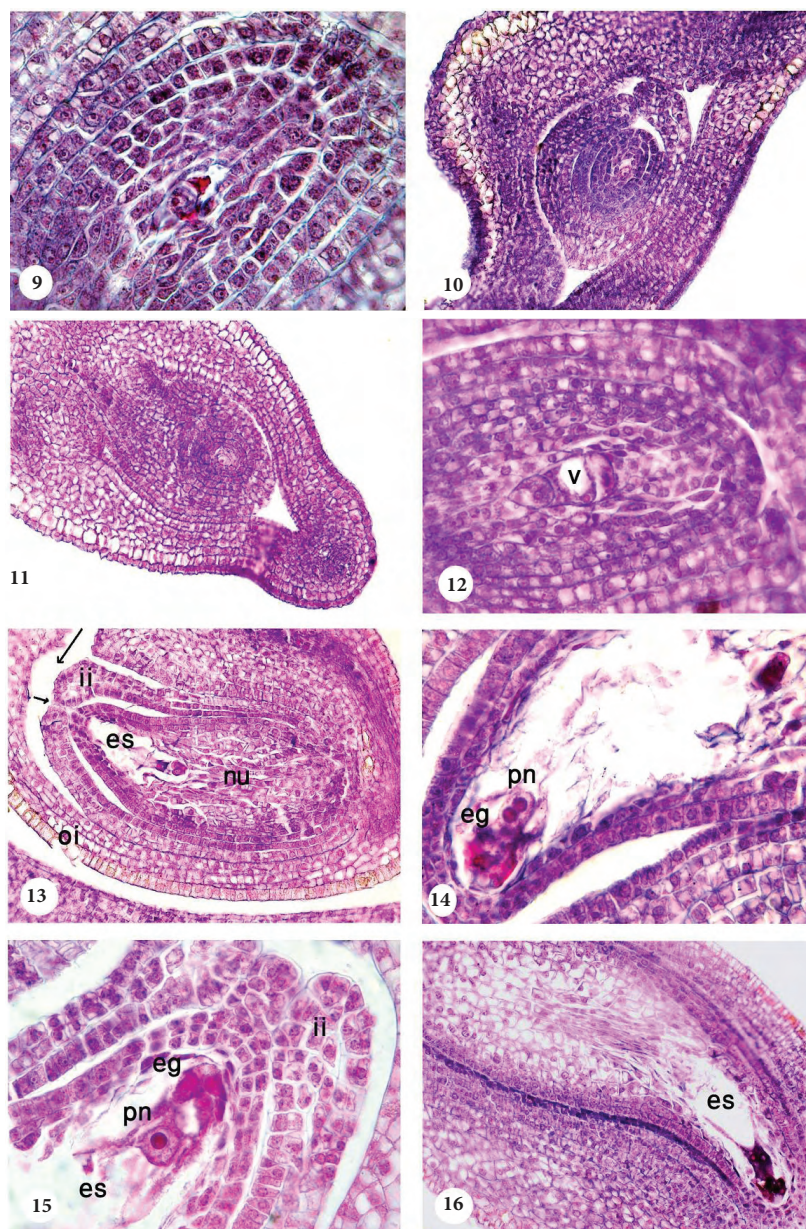


Figures 1-8. (1), Longitudinal section of a young carpel with the first (upper) ovule primordium; (2), Ovule initiation is basipetal ( $\uparrow$ ) and starts with mitotic activity in meristematic regions organized in 4 layers; (3), The ovule originates as a small protuberance ( $\uparrow$ ); (4), The initial archesporial cell ( $\uparrow$ ) is distinguished from the other sub-dermal cells; (5), The 2 integuments ( $\uparrow\uparrow$ ) are initiated from periclinal and oblique divisions of dermal cells; (6), Megaspores mother cell ( $\uparrow$ ), the outer integument (oi) grows faster than inner one (ii); (7), Unequally-sized dyad cells (di), (8), Linear tetrad of megaspores (te), Chalazal megaspore ( $\uparrow$ ).

Such an embryo-sac represents the Polygonum type. The antipodals are the smallest cells of the embryo-sac and are localized in a caecum (Figure 14). The micropylar part of the egg cell is filled by a large vacuole and the chalazal end is filled with cytoplasm

containing the egg nucleus (Figure 15). The chalazal part of the synergids is occupied by one large vacuole and the nuclei are in the micropylar region; they are hooked. The central cell is the largest cell of the embryo-sac (Figures 15-16).

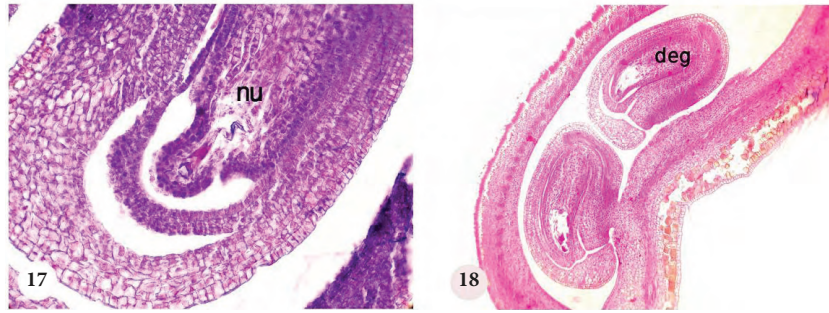




Figures 9-16. (9) Tetrads with degenerating micropylar megaspores and functional chalazal megaspore; (10, 11), One-nucleate embryo sac. The inner integument differentiates simultaneously as a ring around the nucellus; (12), Embryo sac during cellularisation with a big vacuole (v); (13). Ovule with a zig-zag micropylar channel (arrow). Antipods are distinguished in the caecum; (14), Egg apparatus (eg) and 2 polar nuclei (pn) (15), Egg apparatus (eg) and secondary nucleus resulted by the fusion of polar nuclei (pn) in the micropylar end; (16). The central cell formed the largest cell of the embryo-sac (es); (17), Degeneration of upper ovule is a characteristics of this species. Degenerative upper ovule with shrunken nucellus (nu) and embryo sac. It seems that the degeneration occurred before fertilization; (18), Two anatropous, crassinucellar, and bitegmic ovules with a zig-zag micropyle were seen in the young ovary but the upper ovule is degenerative and mature legume contains only one seed.

Young ovaries have 2 ovules in early developmental stages that seem to be synchronous, but it seems that upper ovule degenerates before the fertilization (Figures 17-18). Thus, mature pods

contain only one seed. When the embryo-sac starts its development, the ovule is completely inverted, so the nucellus and integuments lie alongside the funiculus (Figure 18).



Figures 17-18. (17), Degeneration of upper ovule is a characteristics of this species. Degenerative upper ovule with shrunken nucellus (nu) and embryo sac. It seems that the degeneration occurred before fertilization; (18), Two anatropous, crassinucellar, and bitegmic ovules with a zig-zag micropyle were seen in the young ovary but the upper ovule is degenerative and mature legume contains only one seed.

## Discussion

Bouman (1974, 1984) compared cellular organization in the ovule primordium with the shoot apical meristem. The ovular primordium in *O. schahuensis* Bornm. is tetra-zonate. The ovule is anatropous according to the ontogenetic classification of Bocquet and Bersier (1960). This anatropous curvature, as in other legumes, is usually related to unequal growth at the funicular region (Bocquet, 1959; Bocquet & Bersier, 1960; Bor, 1978; Bouman & Boesewinkel, 1991). Some authors classified this type of ovule as campylotropous (Reeves, 1930; Pal, 1960; Rembert, 1967; Ojeaga & Sanyaolu, 1970; Oomman, 1971; Deshpande & Bhasin, 1976) or anacampylotropous (Prakash & Chan, 1976). Such controversy is certainly due to the outer integument growth, displacing the exostome to near the hilum and forming a zig-zag micropylar channel (Figures 13 and 18). Other aspects are also considered important for the typology of the ovules in angiosperms, such as the origin and sequence of integument formation (Bouman, 1971, 1974, 1984).

Bouman (1974) cited the *Papilionoideae* as characterized by an outer integument with sub-dermal derivation, except for *Arachis* L. (cf. the drawings of Smith 1956), *Melilotus* Mill. (Cooper, 1933), and *Adesmia latifolia* (Spreng.) Vogel (Moco & Mariath, 2003). Based on our results in *O. schahuensis*, the both integuments are from dermal origin, but the inner one develops first. The sequence of the inner vs. outer integument initiation in the Fabaceae is extremely variable. In some taxa the inner integument develops first (Cooper, 1933; Roy, 1933; Pantulu,

1945; Dnyansagar, 1954; Smith, 1956; Dnyansagar, 1957; Rembert, 1967; Lim and Prakash, 1994). However, in *Medicago sativa* L., *Vicia americana* Muhl., *Trifolium pratense* L., *T. hybridum* L., *T. repens* L. (Martin, 1914), *Cajanus* DC. and *Lathyrus* L. (Roy, 1933), *Tamarindus* L. (Paul, 1937), *Glycine* Willd. (Rembert, 1977; Kennell & Horner, 1985), and *Adesmia latifolia* (Moco & Mariath, 2003) the first to develop is the outer one. Other characters of the ovules are important, such as the number of cell layers in each integument and the participation of integuments in micropyle formation. The inner integument in *O. schahuensis* as in the most Fabaceae, consists of 2 cell layers (Cooper, 1933; Roy, 1933; Samal, 1936; Paul, 1937; Smith, 1956; Dnyansagar, 1957; Hindmarsh, 1964; Deshpande & Bhasin, 1974; Rembert, 1977; Ashrafunnisa & Pullaiah, 1994, 1999). However, in *Glycine javanica* L., *Tephrosia* Pers., *Clitoria ternata* L., *Pongamia glabra* Vent. (Anantaswamy Rau, 1951), *Teramnus labialis* (L.f.) Spreng. (Anantaswamy Rau, 1953), *Psophocarpus tetragonolobus* (L.) DC. (Lim & Prakash, 1994) and also in species of *Cassia* L. (Pantulu, 1945), it is formed by more than 2 layers. An outer integument with several cell layers, as in the ovules of *O. schahuensis*, is also common in Fabaceae, except for a few *Mimosoideae* (Dnyansagar, 1954) and *Glycine max* L. (Prakash & Chan, 1976), in which the outer integument has only 2 layers. Recently, studies on the genetic and molecular control of ovule development have concluded that the 2 integuments have an independent origin from an evolutionary standpoint (Angenent & Colombo, 1996; Schneitz et al., 1998). This could explain the great variability found in the



structure of these layers. The nucellus of the Fabaceae ovules is crassinucellate (Prakash, 1987; Galati et al., 2006; Faigón Soverna et al., 2003), which was also found in *O. schahuensis*. This type has been considered to be the plesiomorphic condition in angiosperms (Sporne, 1969).

The definitions of nucellus types, however, do not take into account some aspects that Rutishauser (1982) considered to be important in the evolution of the archesporium, such as the origin of its cells and the moment when the vegetative potential is converted to a reproductive one, as previously emphasized by Warming (1878). The crucial point in Rutishauser's work (1982) was the demonstration that the archesporium is composed only of cells that will undergo meiosis. In many studies on megasporogenesis in Fabaceae species, erroneous interpretations were made about archesporial cells, differentiated into sub-hypodermal or variable position (Roy, 1933; Samal, 1936; Rembert, 1969a, b; Prakash, 1987). Our results showed that archesporial cells formed 2-3 layers lower in dermal cells (Figure 4). This misinterpretation of the archesporial cells position is probably due to the difficulty in distinguishing the initial archesporial cells during the early stages of development. Studies of megasporogenesis in the Fabaceae have shown great variability in tetrad. Our results indicate the linear shaped tetrad in *O. schahuensis*, but T-shaped tetrads were reported in most legumes (Chehregani & Majd, 1992; Faigón Soverna et al., 2003; Riahi et al., 2003). Variations in the position of the functional megaspore have been reported in *Trifolium repens* L. (Martin, 1914) and *Vicia faba* L. (Mitchell, 1975) with an epichalazal position, and in *Milletia ovalifolia* Kurz (Pal, 1960), *Trifolium hybridum* L. (Kazimierski & Kazimierski, 1979) and in some Australian species of the tribe Mirbelieae (Cameron & Prakash, 1994), where it may be in a chalazal, micropylar, or epichalazal position. Our results indicate that functional megaspore in *O. schahuensis* is chalazal (Figures 8-9). Hindmarsh (1964) recorded vacuolation in all 4 megaspores of the tetrad in

*Trifolium pratense*, but only the chalazal one became larger, while the others degenerated. In *Cassia abbreviata* Oliv. the chalazal megaspore is functional but the epichalazal one persists for a while and is the last one to degenerate (Rembert, 1969a, 1971).

In *O. schahuensis* the embryo-sac consists of 7 cells: the egg cell, 2 synergids, the central cell, and 3 antipodal cells (Figures 13-16). Such an embryo-sac represents the *Polygonum* type that is common in this family (Kazimierski & Kazimierski, 1979; Chehregani & Majd, 1992; Cameron & Prakash, 1994; Riahi et al., 2003; Galati et al., 2006). A new finding of Bakar Buyukkartal (2009), detailed ultra-structural changes in egg apparatus, is in accordance with our observations partly.

Results showed that young legumes have 2 ovules in early development stages that seem be synchronous, but the upper ovule degenerate before fertilization, thus mature legume contains only one seed. Seed abortion was also reported in some other members of this family that took place after fertilization (Arathi et al., 1999). Seed abortion seems therefore to be a result of competition between the 2 seeds for maternal resources. The evolutionary significance of single-seeded pods in *P. pinnata* is discussed with respect to possible dispersal advantage enjoyed by such pods (Arathi et al., 1999). Algan and Bakar (1990) reported that seed abortion in tetraploid *Trifolium pratense* might be due to several factors. There may be some problems in microspore and megaspore formation due to difficulties in male and female gametophyte or in the fertilization and post-fertilization stages.

Although many studies showed that anatomical characters have taxonomic importance (Abdel-Khalik et al., 2008 and references therein) but this is the first report of taxonomical importance of ovule and megagametophyte structure. Based on our bibliographical studies this is also the first report about the ovule ontogeny, the megasporogenesis, and the female gametophyte development in *Onobrychis schahuensis*.

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