ACTA BIOLOGICA CRACOVIENSIA Series Botanica 51/1: 111–117, 2009

SEED DEVELOPMENT IN *ASTRAGALUS CEMERINUS* AND *A. RUSCIFOLIUS* (FABACEAE), AND ITS SYSTEMATIC IMPLICATIONS

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Received February 17, 2009; revision accepted May 15, 2009

This study focuses on seed development in *Astragalus cemerinus* and *A. ruscifolius*, two endemic species of *Astragalus* in Iran. In both species the ovules are campylotropous, bitegmic and crassinucellate. Two polar nuclei fuse before fertilization, forming the diploid secondary nucleus. Division of the primary endosperm nucleus gives rise to coenocytic endosperm; however, part of it becomes cellular at the late globular stage. The first division of the zygote is transverse and the embryo proper forms after several divisions of the terminal cell. The mature suspensor consists of a mass of cells equal in size to the globular embryo proper, with several inflated cells towards its base. This massive suspensor seems to be plesiomorphic, as compared with the biseriate suspensor known only in section *Incani*. Abnormalities in the embryo proper as well as in the suspensor are observed at the globular stage. In both *A. cemerinus* and *A. ruscifolius*, fusion of the polar nuclei occurs in the median regions of the central cell and before fertilization occurs, as is the rule in most of the Papilionoideae, but in species of section *Incani* as in a few other species of the family, the polar nuclei approach the egg apparatus before fertilization and do not fuse until fertilization. The embryological characters of *A. cemerinus* and *A. ruscifolius* are compared with those of other species of *Astragalus*, and the taxonomic application of these characters as well as their phylogenetic significance are discussed.

Key words: Abnormal embryology, *Astragalus cemerinus*, *Astragalus ruscifolius*, megagametophyte, embryo proper, suspensor, phylogeny.

INTRODUCTION

Astragalus L. (Fabaceae: tribe Galegeae), with about 3000 species worldwide, is the largest genus of flowering plants. The high variation of morphological characters has made the taxonomic division of the genus uncertain and problematic (Liston and Wheeler, 1994; Sanderson and Liston, 1995; Sanderson and Wojciechowski, 1996; Wojciechowski et al., 1999; Zarre, 2000). Several previous systematic studies attempted to classify this huge genus into formal taxonomic groups, that is, subgenera and sections (Bunge, 1868; Podlech, 1982), but only a few groups found support from molecular systematics (Wojciechowski et al., 1999; Kazempour Osaloo et al., 2003, 2005). Detailed cladistic investigations indicated the homoplastic nature of most gross morphological characters in delimitation of subgenera and sections in *Astragalus*.

Embryological studies have provided useful characters in assessing subgeneric classifications in Papilionoideae (Rembert, 1969; Palser, 1975; Lersten, 1983; Prakash, 1987; Cameron and Prakash, 1994; Soverna et al., 2003) as well as in the genus *Astragalus* (Akhalkatsi et al., 1988; Gvaladze and Akhalkatsi, 1996; Riahi et al., 2003). However, the methods of such investigations in *Astragalus* are time-consuming and difficult to carry out due to problems in cultivating the plants under greenhouse conditions or in gardens (Riahi et al., 2003).

In this work we made a detailed embryological study of *A. cemerinus* G. Beck (sect. *Microphysa*) and *A. ruscifolius* Boiss. (sect. *Dissitiflori*), two endemic species of *Astragalus* in Iran, with the aim of providing valuable characters useful in assessing relationships within this genus.

MATERIALS AND METHODS

Flowers and young pods of *A. cemerinus* and *A. ruscifolius* were collected in May 2004 from mountains (~1580 m a.s.l.) above Mashhad Ardahal in Esfahan Province, Iran. At least 60 developing seeds and

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PL ISSN 0001-5296

ovules from *A. cemerinus* and 30 from *A. ruscifolius* were examined.

The general protocol for tissue processing was essentially the same as previously reported by Riahi et al. (2003). Ovules from flowers and young pods were dissected and fixed in FAA_{70} (formalin, glacial acetic acid and 70% ethanol, 5:5:90), stored in 70% ethanol, embedded in paraffin, and sectioned at 5–8 μm with a Leitz 1512 microtome. Staining was carried out using the Periodic Acid Schiff (PAS) techniques and counterstained with Meyer's hematoxylin. Sections were viewed with an Olympus BX-50 light microscope.

RESULTS

Megagametogenesis and embryo development in *A. cemerinus* and *A. ruscifolius* follow similar patterns.

The ovules are campylotropous (Figs. 1, 2), crassinucellate (Figs. 1, 2), and bitegmic (Figs. 3, 4). Both integuments form the micropylar channel (Fig. 1). The outer integument is 2 or 3 cell layers thick and the inner one is 1 or 2 layers thick (Figs. 3, 4). The endothelium is adjacent to the megagametophyte (Fig. 3). In mature ovules these layers convert to Malpighian cells, hypodermal layers or mesotesta, and endotesta (Fig. 5).

The antipodal cells are arranged side by side in an inverted pyramid (Fig. 6). They are ephemeral and degenerate before fertilization (Figs. 2, 6). The two pear-shaped synergids are disposed side by side, each with a nucleus at the micropylar pole (Figs. 2, 6). The polar nuclei fuse in the median region of the central cell before fertilization, and form the endosperm mother cell (Figs. 7, 8). The mature gametophyte is composed of only five cells due to the ephemeral nature of the antipodal cells (Figs. 2, 6). After fertilization, the zygote possesses several starch grains surrounding its nucleus. The nucleus itself has its position close to the micropylar end of the cell (Figs. 7, 8). The adfunicular synergid degenerates while the abfunicular synergid persists (Figs. 7, 8). The megagametophyte is crescent-shaped (Fig. 1), soon becoming open U-shaped just after fertilization (Fig. 4).

EMBRYO PROPER DEVELOPMENT

In the two-celled embryo, the apical cell divides vertically and the basal one transversely to form a T-shaped proembryo. In the four-celled proembryo, transverse divisions of the cells result in eight cells arranged in two tiers, each tier with four cells (Fig. 9). Some starch grains occur in the proembryo. A globular embryo develops after several divisions of the proembryo (Fig. 10). A few morphological defects appear in the embryo proper of *A. cemerinus* (29 of the 60) and *A. ruscifolius* (5 of the 30) at this stage (Fig. 11).

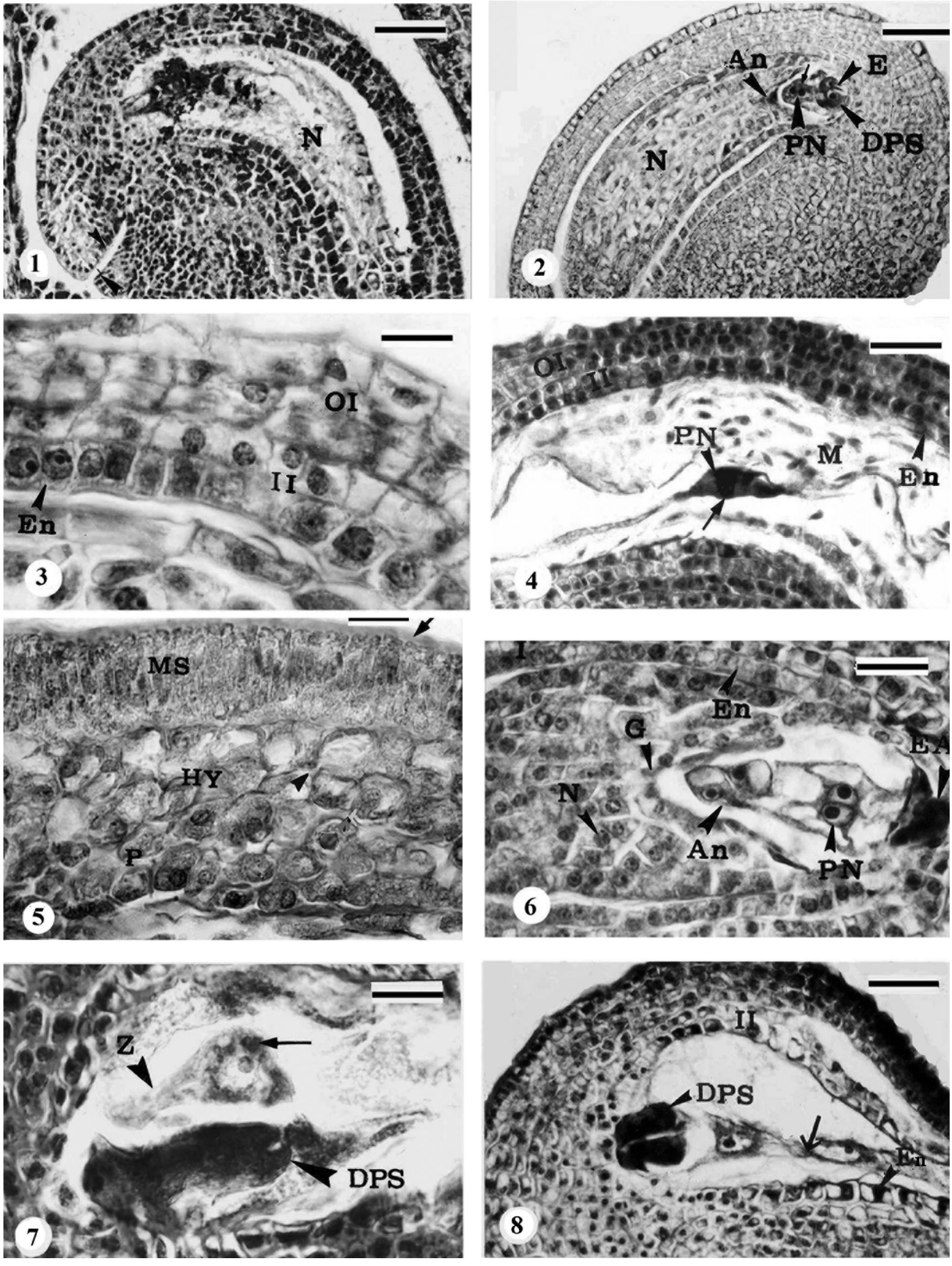
Continued cell divisions increase the size of the embryo. The heart stage embryo soon acquires distinct bilateral symmetry through the formation of the cotyledon primordia (Figs. 12, 13). No abnormal embryo was observed at this stage. The torpedo-shaped embryo develops after elongation of the embryonic axis. The cotyledons become distinguishable at this stage (Fig. 14). The mature embryo has a curved embryonic axis (Fig. 15).

SUSPENSOR DEVELOPMENT

In the two-celled proembryo, the basal cell divides vertically and forms a two-celled suspensor (Fig. 9). At globular stage the fully differentiated suspensor consists of a mass of cells equal in size to the globular embryo proper, with several inflated basal cells embedded in maternal tissues (Figs. 10, 11, 16).

Abnormalities in the suspensor of abnormal embryos are detected at the globular stage. These abnormal suspensors have a curved axis. They are longer and wider than the normal ones and contain more cells (Fig. 11). Both normal and abnormal suspensors possess starch grains during development. The suspensor begins to degenerate at the

Figs. 1–8. Development of megagametophyte in *Astragalus cemerinus* (Figs. 1, 4–6) and *Astragalus ruscifolius* (Figs. 2, 3, 7, 8). **Fig. 1**. Longitudinal section of campylotropus ovule with micropylar channel (arrowheads). Bar = 30 μm. **Fig. 2.** Mature megagametophyte with degenerated antipodals in chalazal region. Starch grains (arrow) close to polar nuclei. Bar = 30 μm. **Fig. 3.** Integuments of developing ovule with outer integument 2 or 3 layers thick and inner integument including endothelium. Bar = 20 μm. **Fig. 4.** Young ovule just at fertilization: fusion of one sperm (arrow) with fused polar nuclei at middle of U-shaped megagametophyte. Bar = 30 μm. **Fig. 5.** Mature testa with 3 distinct layers, i.e., Malpighian cells, two hypodermal rows or mesotesta, and 3 or 4 parenchymatous cell rows or endotesta. Arrowhead shows intercellular space. Arrow shows cuticle layer. Bar = 30 μm. **Fig. 6.** Longitudinal section of organized megagametophyte. Antipodal cells are arranged side by side in an inverted pyramid. Bar = 30 μm. **Fig. 7.** Megagametophyte at zygote stage: zygote with several starch grains (arrow). Bar = 20 μm. **Fig. 8.** First division of primary endosperm nucleus with starch grains (arrow). Bar = 20 μm. Abbreviations: An – antipodal cells; DPS – degenerative persistent synergid; E – egg cell; EA – egg apparatus; En – endothelium; G – megagametophyte wall; HY – hypodermis or mesotesta; II – inner integument; M – megagametophyte; MS – Malpighian cells; N – nucellus; OI – outer integument; P – parenchymatous cell rows or endotesta; PN – polar nuclei; Z – zygote.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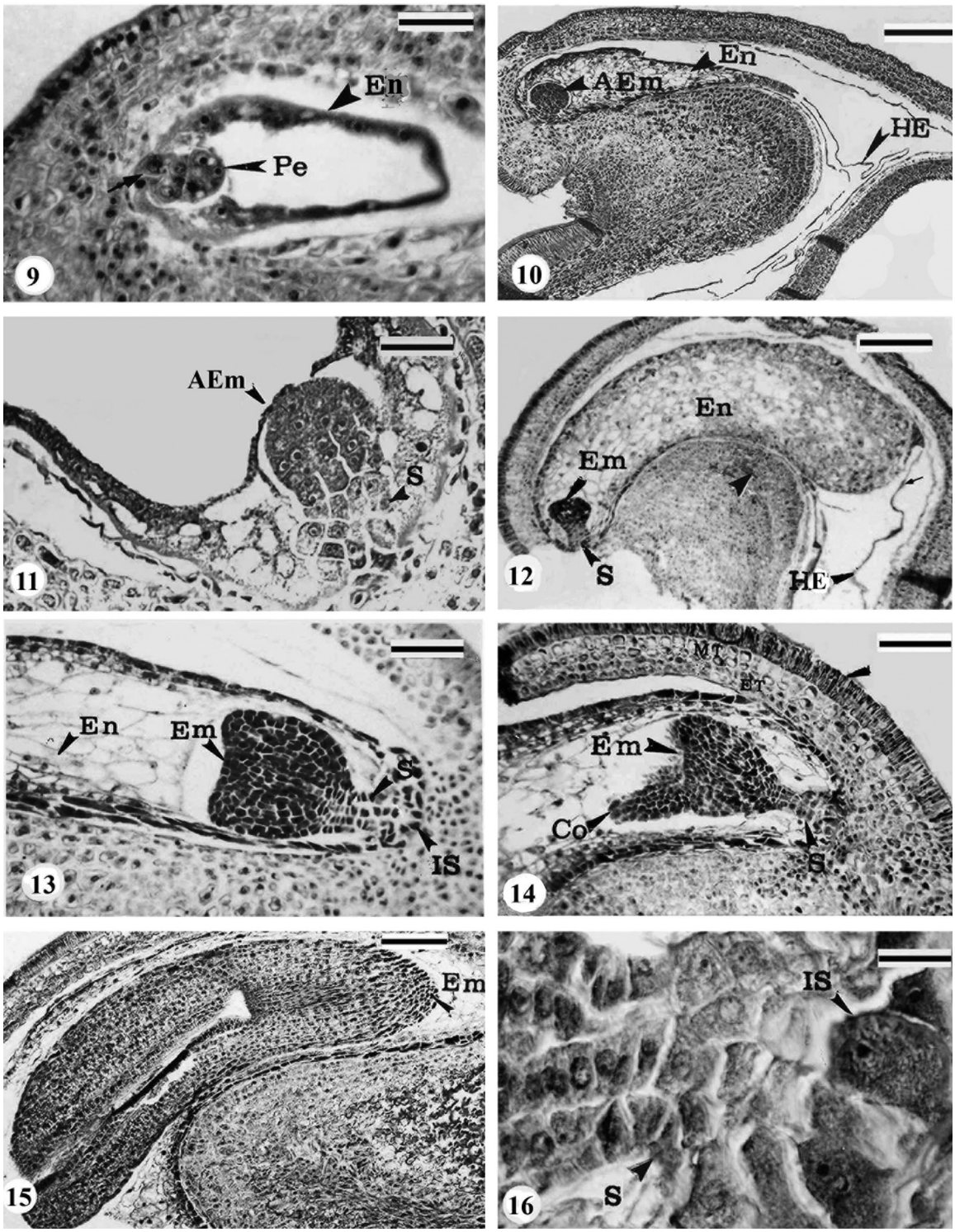
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early cotyledon stage and is absent from the mature seed (Fig. 15).

ENDOSPERM DEVELOPMENT

Division of the endosperm mother cell precedes that of the zygote (Fig. 7). Endosperm formation is free-nuclear, that is, not followed by cytokinesis. In this manner the endosperm forms first at the micropylar end of the megagametophyte, then spreads towards the chalazal end and remains free-nuclear for a while (Fig. 9). In seeds with normal as well as abnormal embryos, development of cellular endosperm begins during the late globular stage. This process commences at the micropylar end of the megagametophyte and progresses toward the chalazal end (Fig. 10). During the globular stage, the endosperm haustorium (for terminology see Johri and Garg, 1959; Rembert 1969) appears at the chalazal end of the megagametophyte. It is narrow and shows a tubular structure. The endosperm haustorium contains about seven nuclei at this stage (Figs. 10, 12). At the late globular stage the wall of endosperm cells become PAS-positive in normal cases as well as in seeds with abnormal embryos. Starch grains were observed at the same stage in the endosperm. More than half of the endosperm mass becomes cellularized at the early heart stage (Figs. 12, 13), and almost completely cellularized by mid cotyledon stage (Fig. 14).

SEED COAT DEVELOPMENT

The endothelium and the inner integument begin to degenerate at the early stages of embryogenesis (Figs. 7–10). This process continues until heart stage. The testa attains its maturity at heart embryo stage.

Testa development proceeds in the manner previously described for *A. demavendicus* Bunge and *A. latifolius* Lam. (Riahi et al., 2003) and other legumes (Gunn, 1981). The mature testa is composed of a thin layer of cuticle, the outer Malpighian cell layer (epidermis), two hypodermal cell rows (sclereid layer) and 3 or 4 parenchymatous cell rows beneath them.

DISCUSSION

The major *Astragalus* classification systems (e.g., Bunge, 1868; Boissier, 1872; Podlech, 1982) variously divide the genus into 2–9 subgenera and more than 90 sections based on a limited number of morphological characters, and do not recognize convergence as a factor in the family's evolution. However, recent studies (e.g., Wojciechowski et al., 1999; Zarre, 2000; Kazempour Osaloo et al., 2003, 2006) have demonstrated the polyphyly and artificiality of almost all subgenera and some sections recognized in those earlier systems. For several reasons, including low resolution of the cladograms obtained (Wojciechowski et al., 1999; Kazempour Osaloo et al., 2003, 2006) and the homoplastic nature of morphological characters (Zarre, 2000), the application of characters from other taxonomic sources should be a goal of future studies in *Astragalus*.

The present work represents a detailed embryological study of two species of *Astragalus* belonging to two different subgenera in the classical systematics of the genus (Bunge, 1868; Podlech, 1982). The overall morphology and development of the ovule in *A. cemerinus* and *A. ruscifolius* reaffirm earlier observations on the subfamily Papilionoideae (Prakash, 1987) and on other species of *Astragalus* (Lersten, 1983; Riahi et al., 2003). Among the few *Astragalus* species studied so far (Lersten, 1983; Akhalkatsi et al., 1988; Gvaladze and Akhalkatsi, 1996; Riahi et al., 2003), differences in suspensor size and shape as well as in the timing of polar nuclei fusion have been observed.

A mass of cells with several (7 or 8) inflated cells embedded in maternal tissue (Fig. 16) form the suspensor of *A. cemerinus* (section *Microphysa* of basifixed hairy *Astragalus*; for terminology see Zarre, 2003) and *A. ruscifolius* (section *Dissitiflori* of medifixed hairy *Astragalus*). This type of suspensor is as large as or larger than the embryo proper at the globular stage. Since these two unrelated species belonging to different clades of *Astragalus* (Kazempour Osaloo et al., 2003, 2005) show the same suspensor structure at globular stage, this type of suspensor obviously represents the most

Figs. 9–16. Development of embryo in *Astragalus cemerinus* (Figs. 11, 13) and *Astragalus ruscifolius* (Figs. 9, 10, 12, 14–16). **Fig. 9.** Eight-celled proembryo, immigration of nuclear endosperm formed around embryo towards periphery of megagametophyte; suspensor marked by arrow. Bar = 30 μm. **Fig. 10.** Abnormal globular embryo, cellularization of endosperm at mid globular stage with haustorial endosperm. Bar = 140 μm. **Fig. 11.** Abnormal embryo with abnormal suspensor at late globular stage. Bar = 50 μm. **Fig. 12.** Early heart stage embryo: more than half the mass of the endosperm is cellularized; nuclei (arrow) are obvious in haustorial endosperm. Starch grains are observed in testa (arrowhead). Bar = 140 μm. **Fig. 13.** Early heart embryo stage; suspensor with its inflated basal cells. Bar = 20 μm. **Fig. 14.** Torpedo stage embryo: suspensor begins to degenerate. Bar = 100 μm. **Fig. 15.** Mature embryo with curved embryonic axis. Bar = 70 μm. **Fig. 16.** Suspensor with its inflated basal cells. Bar = 20 μm. Abbreviations: AEm – abnormal embryo; Co – cotyledon; Em – embryo; En – endosperm; HE – haustorial endosperm; IS – inflated cells; Pe – proembryo; S – suspensor.

common one, and probably a plesiomorphic character for the genus. The same suspensor structure has been observed also in two outgroups of *Astragalus: Biserula pelecinus* and *Colutea persica* (Lersten, 1983). Consequently, another type of suspensor reported earlier in section *Incani* (Riahi et al., 2003), small in size and including only 2 or 3 inflated basal cells, should be a synapomorphic feature exhibited only by the species of this section. This provides additional support for the monophyly of this large section (about 110 species) of *Astragalus*, which has been suggested by molecular systematic studies based on nuclear ribosomal DNA ITS sequences (Kazempour Osaloo et al., 2005).

Another difference in the development of seeds between *Astragalus* species is the position and timing of fusion of polar nuclei. In both *A. cemerinus* and *A. ruscifolius*, fusion of the polar nuclei occurs in the median regions of the central cell and before fertilization, as has been found in most of the Papilionoideae (Prakash, 1987). However, in the species of sect. *Incani* as well as in *Trifolium alexanderinum* L., *Medicago sativa* L., *Pisum sativum* L. and *Lathyrus sativus* L. (Mocco and Mariath, 2004), the polar nuclei approach the egg apparatus before fertilization, and do not fuse until fertilization. Since there are only a few studies reporting this character, the possible significance of the timing of fusion of polar nuclei for the systematics of *Astragalus* should be assessed in future investigations of other species in the genus.

Another observation from the present study is the frequent occurrence of seed abortion and abnormalities in both species. The number of ovules is about 10 in a young pod of *A. cemerinus* and 30 in *A. ruscifolius*; mature pods of these species possess 1 or 2 and 15–20 seeds, respectively. During embryogenesis, morphological defects in both the suspensor and embryo proper are detected at the globular stage of embryo development in both species. Through this abnormal development the globular embryo attains radial symmetry and therefore cannot advance to the heart shape as it enlarges. This abnormality is accompanied by development of an abnormally curved or obliquely positioned suspensor (Fig. 11). In the species studied, morphological defects in the suspensor were not detected in the absence of defects in the embryo proper. These observations support the earlier suggestion that the primary defect in the abnormal suspensor causes the defect in the embryo proper (Schwartz et al., 1994). A similar kind of abnormality was reported in *Phaseolus* (Yeung and Meinke, 1993), mutants of *Arabidopsis thaliana*, maize (Schwartz et al., 1994), and *Epilobium obcordatum* (Seavey and Carter, 1996).

The mean percentage of seeds with abnormal embryos is about 50% and 12% in *A. cemerinus* and *A. ruscifolius*, respectively, which accounts for seed set being lower in *A. cemerinus* than in *A. ruscifolius*.

ACKNOWLEDGEMENTS

We are grateful to Dr. Abdalkarim Chehregani (Hammadan, Iran) and Dr. Behrooz Shahsavan-Behboudi for their technical assistance and useful suggestions. Prof. Dr. Dietrech Podlech (München, Germany) helped us with suggestions regarding the taxonomy of the genus and determination of specimens. We are also indebted to Prof. Dr. Hans-Juergen Tillich (München, Germany) for editing and improving the English text. This research was supported by a grant from the Research Council of the University of Tehran, and by a fellowship from Alexander von Humboldt Stiftung to Shahin Zarre.

REFERENCES

Akhalkatsi M, Beridze M, and Gvaladze G. 1988. Embryo and endosperm development in *Astragalus caucasicus* Pall. *Bulletin of the Georgian Academy of Science* 132: 601–604. (In Georgian).

Boissier E. 1872. *Flora Orientalis*, vol. 2. H. Georg, Basil and Geneva.

Bunge A. 1868. Generis *Astragali* Species Gerontageae. *Mémoires de l'Académie Impériale des Sciences de Saint Pétersbourg*, ser. 7, 11(16): 1–140.

Cameron BG, and Prakash N. 1994. Variation of the megagametophyte in the Papilionoideae. In: Ferguson IK, and Tucker S [eds.], *Advances in Legume Systematics*, 97–115, part 6. Royal Botanical Gardens, Kew, England.

Gunn CR. 1981. Seeds of Leguminoseae. In: Polhill RM, and Raven PH [eds.], *Advances in Legume Systematics*, 913–925, part 2. Royal Botanic Garden, Kew, England

Gvaladze G, and Akhalkatsi M.1996. Comparative embryology of some *Astragalus* species. *Bulletin of the Georgian Academy of Science* 153: 432–434.

Johri BM, and Garg S, 1959. Development of endosperm haustoria in some Leguminosae. *Phytomorphology* 9: 34–46.

Kazempour Osaloo S, Massoumi AA, and Murakami N. 2003. Molecular systematics of the genus *Astragalus* L. (Fabaceae): Phylogenetic analyses of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene *ndh*F sequences. *Plant Systematics and Evolution* 242: 1–32.

Kazempour Osaloo S, Massoumi AA, and Murakami N. 2005. Molecular systematics of the old world *Astragalus* (Fabaceae) as inferred from nr DNA ITS sequence data. *Brittonia* 57(4): 367–381.

Lersten NR. 1983. Suspensors in Leguminosae. *Botanical Review* 49: 233–257.

Liston A, and Wheeler JA. 1994. The phylogenetic position of the genus *Astragalus* (Fabaceae): evidence from the chloroplast genes *rpo*C1 and *rpo*C2. *Biochemical Systematics and Ecology* 22: 377–388.

Mocco MCC, and Mariath JEA. 2004. Female gametophyte development in *Adesmia latifolia* (Spreng.) Vog. (Leguminosae-Papilionoideae). *Revista Brasiliera de Botánica* 2: 241–248.

Palser BF. 1975. The bases of angiosperm phylogeny: Embryology. *Annals of the Missouri Botanical Garden* 62: 621–646.

Podlech D. 1982. Neue Aspekte zur Evolution und Gliederung der Gattung *Astragalus* L. (Leguminosae). *Mitteilungen der Botanischen Staatssammlung* München 18: 359–378.

Prakash N. 1987. Embryology of the Leguminosae. In: Stirton CH [ed.], *Advances in Legume Systematics*, 241–278, Part 3. Royal Botanical Gardens, Kew, England.

Rembert DH. 1969. Comparative megasporogenesis in Papilionaceae. *American Journal of Botany* 56 (6): 584–591.

Riahi M, Zarre S, Chehregani A, and Behboudi B. 2003. Seed development in two species of medifixed hairy *Astragalus* (Fabaceae). *Flora* 198: 211–219.

Sanderson MJ, and Liston A. 1995. Molecular phylogenetic systematics of Galegeae, with special reference to *Astragalus*. In: Crisp M, and Doyle JJ [ed.], *Advances in Legume Systematics,* part 7, 331–350. Royal Botanic Garden, Kew, England.

Sanderson MJ, and Wojciechowski MF. 1996. Diversification rates in temperate legume clade: Are there so many species in *Astragalus* (Fabaceae)? *American Journal of Botany* 83: 1488–1502.

Schwartz BW, Yeung EC, and Meinke DW. 1994. Distribution of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of *Arabidopsis. Development* 120: 3235–3245.

Seavey SR, and Carter SK. 1996. Ovule fates in *Epilobium obcordatum* (Onagraceae). *American Journal of Botany* 83 (3): 316–326.

Soverna AF, Galati B, and Hoc P. 2003. Study of ovule and megagametophyte development in four species of subtribe Phaseolinae (Leguminosae). *Acta Biologica Cracoviensia Series Botanica* 45 (2): 63–73.

Yeung EC, and Meinke DW. 1993. Embryogenesis in angiosperms: development of the suspensor. *Plant Cell* 5: 1371–1381.

Wojciechowski MF, Sanderson MJ, and Hu JM. 1999. Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA *trn*L intron data. *Systematic Botany* 24: 409–437.

Zarre S. 2000. Systematic revision of *Astragalus* L. sect. *Adiaspastus*, sect. *Macrophyllium* and sect. *Pterophorus* (Fabaceae). *Englera* 18: 1–21.

Zarre S. 2003. Hair micromorphology and its phylogenetic application in thorny species of *Astragalus* (Fabaceae). *Botanical Journal of Linnean Society* 143: 323–330.