ON THE REPRODUCTIVE BIOLOGY OF *TRIBULUS TERRESTRIS* L. (*ZYGOPHYLLACEAE*): EMBRYOLOGICAL FEATURES; POLLEN AND SEED VIABILITY

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ABSTRACT

An embryological study of *Tribulus terrestris* from the Bulgarian flora was carried out. The anther is tetrasporangiate and its wall, the development of which follows the Dicotyledonous type, consists of epidermis, fibrous endothecium, two middle layers and glandular tapetum. Different types of microspore tetrads are formed after simultaneous microsporogenesis. The mature pollen grains are two-celled. The mature ovule is anatropous, bitegmic, crassinucellate with one-celled archesporium. The development of the embryo sac follows the Polygonum (monosporic) type. The formation of endothelium and hypostase were reported. The legitimate embryo and endosperm develop after double fertilization. Initially, the endosperm is nuclear. The embryogenesis runs according to the Solanad type. A high pollen and embryo viability was estimated.

Materials and Methods

A Bulgarian native population of *T. terrestris* from the Thracian Lowland, the valley of Karlovo, near to Vedrare Village was studied.

For the embryological study and the pollen viability test, flower buds and flowers at different developmental stages were collected and fixed in FAA mixture (formalin: glacial acetic acid: 70% alcohol, in a 5:5:90 ratio), embedded in paraffin, cut into 8-20 \( \mu m \) sections with a rotary microtome and treated according to the classical paraffin methods (22). The sections were stained with Heidenhain’s haematoxylin and embedded in Entelan.

To estimate the embryo viability, a quick 24-hour viability test was applied. The isolated seed embryos from three consecutive years were preliminary incubated in water at 30-35°C temperature and then in a diluted (1%) solution of 2,3,5-triphenyltetrazolium chloride for 24 h – Tetrazolium test, according to Peters (14). Initially, the tetrazolium solution is colorless but changes to red when it comes into contact with the hydrogen (reduction process) derived from the enzymes of the respiration process of the seed. The embryos showing active respiration turn red and are considered as viable (the darker the color, the greater the respiratory activity in the seed). Light pink indicates an embryo with lower viability than the embryo stained dark red. Depending on the intensity of the staining and the location of the colorless parts, the embryos were estimated as viable or nonviable and classified in four...
classes: Class I – embryos stained 100% (dark red colored – Fig. 1a); Class II – embryos stained 80% (light red colored – Fig. 1b); Class III – embryos stained 10% (only root or tip of the cotyledons stained in red – Fig. 1c); Class IV – colorless embryos (Fig. 1d). According to the criteria for interpretation of the tetrazolium test results given by Moore (10), viable embryos are represented by the color patterns of Classes I and II.

**Fig. 1.** Embryo viability after tetrazolium testing  
(a) 100% stained- viable embryo; (b) 80% stained- viable embryo; (c) 10% stained- nonviable embryo; (d) colorless embryo- nonviable; Magnification 40x

Observations were made and photographs were taken using LM “Olympus” CX21 and “Infinity lite” Digital Camera 1,4 Mpix, respectively.

**Results and Discussion**

**Anther and development of the male gametophyte**

The anthers of *T. terrestris* are tetrasporangiate. The anther wall develops according to the Dicotyledonous type (5) and prior to maturation usually comprises five layers: epidermis, endothecium, two middle layers and tapetum, which are clearly distinguishable from each other, as early as the time of Prophase I of the meiosis in the microspore mother cells (MMCs) (Fig. 2a).

The epidermal cells are one-nucleate, almost rectangular, tangentially lengthened during the ontogenesis and rounded up on the outside. The endothecial cells are also one-nucleate and significantly enlarge during the anther ontogenesis. Later on (after the formation of one-celled pollen in the anthers), they develop fibrous thickenings – typical for the family *Zygophyllaceae*. The middle layers are relatively ephemeral and degenerate during the meiosis in MMCs. Only a few darkly stained remnants of the middle layers are present at the one-nucleate pollen stage. The tapetum is glandular, consists of a row of one-nucleate large, rectangular or quadratic cells. During the anther ontogenesis, as result of mitotic divisions, a multiplication of the nuclei in the tapetum cells takes place and they become two-, four-nucleate. About the time of microspore tetrads formation, the tapetum begins to disintegrate and completely degenerates at the stage of one-nucleate pollen. The anther dehiscent is realized longitudinally from the lateral walls of the thecae. This type of anther dehiscence in *T. terrestris* is named “Latrorse” anther type by Saad-Ul-Islam (18).

**Fig. 2.** Anther and development of male gametophyte  
(a) Five-layered anther wall and MMCs in prophase I of the meiosis; (b) Microspore tetrads in the anther sac; (c) Mature pollen grains; (d) One-celled pollen and anther wall with still nonfibrous endothecium; (e) Mature pollen grains with endothecium; (f) Mature pollen grains and anther wall; *aw*– anther wall; *epi*– epidermis; *end*– endothecium; *ml*– middle layer; *tap*– tapetum; *pg*– pollen grain; Magnification- 400x for all figures

The primary sporogenous cells undergo meiosis, forming a two-, three-layered sporogenous tissue consisting of small, one-nucleate polygonal cells with dense cytoplasm that were tightly packed with one another. Later on, the sporogenous cells grow, round up, separate from each other and differentiate into MMCs (Fig. 2a). The meiosis in MMCs runs almost normally with insignificant deviations: presence of chromosomes lagging behind the achromatic spindle; chromosome bridges (predominantly in heterotypic division); asymmetrical disposition of spindles in the homeotypic meiotic division. After simultaneous microsporogenesis, tetrahedral, isobilateral, decusate and sporadically linear tetrads form in the anthers (Fig. 2b). It is important to note that when one-
celled pollen grains form in the anther locules, in the ovule one-nucleate archesporium is present (proterandry).

The mature pollen is predominantly two-celled (Fig. 2c), rarely three-celled. Kamelina (8) has described the mature pollen in Zygophyllaceae as two-celled and has reported three-celled pollen only in the genus Fagonia L. and T. terrestris. Watson and Dallwitz (23) have also observed T. terrestris three-celled mature pollen. The fibrous thickenings of the endothecium cells that form after one-nucleate pollen (Fig. 2d) are clearly expressed at the stage of the mature pollen (Fig. 2e) when the epidermis and tapetum are still present in the anther wall.

The mature pollen grains are almost morphologically uniform and only a limited amount of small size pollen grains (result of the insignificant meiosis deviation) are present (Fig. 2f). In turn, this fact leads to the high percentage viable pollen grains in the anthers estimated by the pollen viability test (96.85%, Table 1 and Fig. 4). This result corresponds with the high fertility percentage of the mature pollen grains reported in T. terrestris from the Pakistan flora (1).

On the basis of regularity of the meiosis in the MMCs and the morphological uniformity of the mature pollen, Hilu (7) defined three morphological-eytological groups in T. terrestris. Our observations allow us to refer the studied population of T. terrestris to the first of Hilu’s three groups, comprising the robust individuals with normal meiosis and pollen grains.

**Ovule and development of female gametophyte**

The gynoecium is syncarpous. The ovary is superior, initially two-locular with numerous ovules, short style and papillate stigma (Fig. 3a). The ovary locules secondarily divide by “false septa”, described also for this species by Watson and Dallwitz (23). Multiloculate (up to five-loculated) ovary in T. terrestris has been observed by Belyaeva (2).

The well-developed ovule of T. terrestris is anatropous, bitegmic, crassinucellate, with the micropyle formed by the two integuments, whereas Belyaeva (2) and Kamelina (8) have reported hemitropous ovule in this species. In the still young orthotropous ovule, a unicellular archesporium forms (Fig. 3b). The archesporogenesis in the megasporocyte passes with formation of one to three parietal cells, which Poddubnaya-Arnoldi (15) and Kamelina (8) describe as a typical characteristic of the family Zygophyllaceae. After meiosis in the megasporocyte, a linear tetrad forms (Fig. 3c). Usually, its chalazal megaspore functions as a megaspore mother cell (MMC). After three consecutive mitotic divisions in it, two-, four-, and eight-nucleate embryo sac (ES) forms. Thus, the development of the female gametophyte follows the Polygonum (monosporic) type.

The mature ES comprises three-celled egg apparatus, two polar nuclei and three antipodals. The two synergids are pear-shaped with filiform apparatus (Fig. 3d), which Poddubnaya-

**TABLE 1**

<table>
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<th>Tribulus terrestris L.</th>
<th>Total pollen grains from 30 anthers</th>
<th>Viable</th>
<th>Nonviable</th>
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**Fig. 3.** Ovule, female gametophyte development, embryo- and endospermogenesis

**Fig. 4.** Estimated pollen viability
Arnoldi (15) has shown as a typical feature of the Zygophyllaceae family. The polar nuclei fuse before fertilization, forming a big central cell, situated close to the egg cell. The three antipodals are usually ephemeral and degenerate before the fertilization as in most Zygophyllaceae representatives (5, 8, 15). The integumentary tapetum (endothelium) differentiates from the innermost layer of the inner integument to the stage of two-nucleate ES and its cells are radially elongated and vacuolated. Formation of a hypostase, which is a specific ovule structure of the Angiosperms, is observed under the antipodal cells. Davis (5), Poddubnaya-Arnoldi (15) and Kamelina (8) have noted the hypostase as a typical feature in Zygophyllaceae.

The pollen tube penetrates through the micropyle, destroys one of the synergids and discharges its content (two sperm cells) into the ES cavity. The legitimate embryo and endosperm form after double porogamous fertilization. The embryogenesis follows the Solanad type. The young globular embryo is characterized with a short suspensor consisting of 3-4 one-nucleate cells that arrange in a row (Fig. 3e). The mature embryo is straight with two equal cotyledons and occupies the whole length of the seed.

The endospermogenesis begins before the first division of the zygote and undergoes a free-nuclear stage. The transformation of the nuclear endosperm into cellular one proceeds from the micropylar end of the ES to the chalaza when several hundred free nuclei have already formed (Fig. 3f). The mature seeds are non-endospermic.

Estimation of the embryo viability

The embryo viability was estimated by the tetrazolium test. According to the intensity of staining, the embryos were classified into four classes (Table 2 and Fig. 5). The majority of the embryos fell into Class I, representing the dark-red colored embryos (52.94%) estimated as viable, and the minority – into Class IV, representing the colorless nonviable embryos (11.76%). According to the used test, the embryos of Class II, representing the light-red colored embryos (17.65%), were estimated as viable too. Thus, the viable embryos in T. terrestris were 70.59% and the viability can considered as relatively high. Nikolova (12) has also established high embryo viability for this species based on tetrazolium testing of the seeds.

The results of the test showed that the viability of the embryos estimated at the moment of maturation of the seeds slightly decreased to 64.71% (Table 2 and Fig. 5) and up to 2/3 (23.53%) in the second year of the seed collection.

Conclusions

As result of the study on the reproductive biology of Tribulus terrestris carried out, in particular on its embryology, many embryological features typical for the genus Tribulus and the family Zygophyllaceae were established: tetrasporangiate anthers; Dicotyledonous-type of anther wall formation; simultaneous microsporogenesis; different types of microspore tetrads; two-celled mature pollen; anatropous, bietegmic, crassinucellate ovule with endothelium and hypostase; archesporogenesis with formation of parietal cells; Polygonum-type development of the ES; Solanad-type embryogenesis; nuclear endosperm. Some embryological features shown as specific ones for T. terrestris were confirmed: three-celled mature pollen and multiloculate ovary that form in initially two-locular one by setting of “false septa”.

The revealed embryological characteristics of the studied population of T. terrestris show that it consists of strongly proterandrous and sexually reproducing individuals. The high pollen and embryo viability established as well as the sexual reproduction are significant determinants that T. terrestris has sufficiently high reproductive capacity and its effective realization provides a stability of its populations.

Acknowledgements

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<table>
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<tr>
<th>Year</th>
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<th>Class I (%)</th>
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