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THE STRUCTURE OF THE OVULE OF *Sida hermaphrodita* (L.) RUSBY AFTER POLLINATION

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Abstract

The anatomical structure and the distribution of arabinogalactan proteins were investigated in the ovule of an energetic plant *Sida hermaphrodita* (L.) Rusby after pollination. Material was collected from an experimental field of the University of Life Sciences in Lublin (Felin). After embedding for immunoflurescence, semi-thin sections were stained with toluidine blue or incubated with JIM 13 or MAC 207 monoclonal antibodies for immunolabeling of arabinogalactan proteins. The structure of the ovule showed some differences in comparison to other representatives of the Malvaceae family. Conversely, the distribution of arabinogalactan proteins did not differ significantly from their distribution in the ovules of other angiosperms at the investigated stage of development.

Key words: *Sida hermaphrodita*, flower, ovule, pollination, arabinogalactan proteins, energetic plant

INTRODUCTION

Sida hermaphrodita (L.) Rusby (Virginia mallow, River mallow or Virginia fanpetals) is one of about 1000 species which belong to the cosmopolitan family of herbs, shrubs and trees widely spread from frigid to tropical zones referred to by the common name Malvaceae. The main genera among about 100 of them are *Malva, Lavatera, Hibiscus* (with 300 species in tropical and subtropical regions), Althaea, Abutilon, and Sida. Among them, there is also the most economically important genus of *Gossypium* with over 20 species (found in tropical and subtropical regions). Cultivated forms of *Gossypium* are grown in the Old and New World to provide cotton, which consists of long hairs covering the seeds. The seeds can also be crushed to obtain cottonseed oil and the oil cake is used to feed cattle. Generally, about 15 species in the family Malvaceae are of important economic value.

S. hermaphrodita is one of almost 200 species belonging to the genus *Sida* (according to some other sources the genus Sida includes more than 100 species) which are found mainly in the warmer regions of the Earth. *S. hermaphrodita*, a perennial plant, originates from the southern part of North America where it grows naturally in most riverine habitats. Because of the conspicuous destruction of its natural habitats, the species is rare in most parts of its total range, but currently is not endangered or threatened. The plant has adapted well to different types of soils and habitats (Spooner et al. 1985). For the first time, Virginia mallow was brought to Europe in 1930 and introduced in the Ukraine. Here, it was studied as a plant with a high economic potential. Since the 1950s, when it was introduced in Poland, the plant has become of interest to Polish scientists, especially at the former Agricultural Academy (currently the University of Life Sciences) in Lublin. The species is considered to be a multipurpose plant with potential application as biomass for energy generation, fodder crop, honey plant, fiber plant for the paper industry, a soil-stabilizing plant for recultivation of contaminated and devastated soils, and even a source of medication (for a smart and comprehensive review see Borkowska and Styk, 2006).

The European literature on the subject provides a range of data that can be considered evidence for the low germination potential of *S. hermaphrodita* seeds (Dmitrashko, 1972, 1973; Borkowska and Styk, 2006). Wróblewska and Kolasa (1991) as well as Wróblewska (2009) studied the biology of flowering of *S. hermaphrodita* and some other species of the Malvaceae family growing in Poland. According to our knowledge, the embryological data are limited only to a few genera of exceptionally great economic significance. For example, very

detailed embyological investigations have been carried out on cotton (*Gossypium* spp.) (Malvaceae) and cocoa (*Teobroma cacao*) representing the tropical family Stercullariaceae (order Columniferae, to which the genera Gossypium and Sida also belong) (for a review see R a o, 1954, 1955). *S. hermaphrodita* (L.) Rusby is the only member of Pseudonapaea A. Gray, a section which differs considerably from other sections in the genus (F r y x e l l, 1985). The latest molecular studies among Malvales taxa caused rearrangement of the traditionally circumscribed families in the newly proposed system of Malvaceae (B a y e r and K u b i t z k i, 2003). The authors suggest that our understanding of evolutionary relationships within many groups of Malvales is still inadequate and that further extended embryological investigations may have systematic implications (T a n g et al. 2009).

Taking into consideration mainly the exceptional economic significance of Virginia mallow and this last suggestion (T a n g et al. 2009), we decided to undertake detailed investigations of the reproduction biology of *S. hermaphrodita.* In the presented paper, we concentrate on the anatomical structure of S. hermaphrodita ovule and the distribution of arabinogalactan proteins in the ovule after pollination.

MATERIAL AND METHODS

Material

The *Sida hermaphrodita* (L.) Rusby plants, investigated during this study, were grown in an experimental field of the University of Life Sciences in Lublin, located in Felin district in eastern Lublin (Fig. 1). The material for the investigations was collected during favorable weather conditions in the summer of 2009 (from June to October). First, buds and flowers at different stages of development were isolated from plants of different height (from 1 to 2.5 m). For a detailed study, presented in this paper, developing ovules at the stage after pollination were chosen.

Methods

Observation of pollination and fertilization

For observations of the pollination and fertilization processes, flowers, left for open pollination, were collected at the stages of staminate, pistillate and withering sepals and fixed in a 70% ethanol and acetic acid (3:1) mixture for 24 hours. The fixed material was stored in 70% ethanol and rinsed twice in distilled water for 1 hour before preparing slides. Next, the samples were macerated in 1 N NaOH in 60˚C for 30 minutes and washed in water. Softened pistils were placed on a microscopic slide glass in a drop of 0.01% aniline blue in phosphate buffer, pH 9.5, covered with a covering glass and gently squashed. Freshly prepared slides were examined under the NIKON fluorescence microscope. For further investigation, similar flowers as those in which the stages after pollination occurred were taken.

Flowers at the stage of withering sepals were fixed immediately after collection in a mixture of 2.5% paraformaldehyde (paraformaldehyde prepared just before use) and 0.25% glutaraldehyde in 0.1 M PBS, pH 7.2 for 24 hours, at 4˚C. Subsequently, the material was rinsed twice in 0.1 M phosphate buffer, pH 7.2, postfixed in 1% osmium tetroxide for 1 hour and washed twice in phosphate buffer. Dehydratation was performed in a series of increasing ethanol concentrations (10 – 70% ethanol) and in an acetone series (70 – 100% acetone) for 10 minutes at each concentration. The dehydrated samples were immersed for 24 hours in turn in acetone and LR white acrylic resin mixtures in the following proportions: 3:1, 1:1, 1:3, and then twice in pure LR white acrylic resin. The material embedded in the resin was placed in gelatin capsules filled with resin, closed and left for 24 hours at 55˚C for polymerization.

For anatomical examinations of the ovules, semi-thin longitudinal sections were cut, stained with 1% toluidine blue and observed under a NIKON optical microscope. Microphotographs were taken using a CANON digital camera.

Immunolabeling

Freshly prepared semi-thin sections of ovules, fixed and embedded according to the method described above, were placed on microscopic slide glasses covered with polylysine (to prevent section loss) and dried at room temperature. Subsequently, the sections were hydrated with distilled water twice for 15 minutes and preincubated with 1% bovine serum albumin (BSA) in 0.2 M PBS, pH 7.4, for 30 minutes. After washing the PBS, the sections were incubated with two different primary rat monoclonal antibodies diluted 1: 50 in 0.1% BSA in PBS, pH 7.4, overnight in a humid chamber at 4˚C. The monoclonal antibodies used (Complex Carbohydrate Research Center, University of Georgia, USA) were JIM 13, which recognizes the sugar epitope on arabinogalactan proteins containing the trisacharide β-D-glucose A-β (1→3)-d-galactose A-α (1→2)-l-rhamnose, and MAC 207, which recognizes the sugar epitope on arabinogalactan proteins α-glucose A-β (1→3)-α-galactose A-α (1→2)- rhamnose). After washing the sections in PBS (4 x 20 min.), the sections were incubated with a secondary rabbit anti-rat FITC (fluorescein isothiocyanate) conjugated antibody (whole molecule) diluted 1:200 with 0.1% BSA in 0.2 M PBS, pH 7.4, overnight in a humid, dark chamber at 4˚C (the secondary antibody was bought from Sigma Immuno Chemicals, Saint Louis, USA).

Subsequently, the slides were washed in PBS 2 x 15 min and then in distilled water 5 x 10 min. All manipulations were carried out in darkness. Finally, the sections were closed in a drop of Fluorescent Mounting Medium (The Binding Site) and immediately examined under the NIKON fluorescence microscope.

RESULTS

As mentioned earlier (chapter Materials and Methods), the buds and flowers of Sida hermaphrodita at different stages of development were isolated from plants of different height (from 1 to 2.5 m). There were no significant differences in the stages of bud and flower development among the specimens collected from plants of different height (Fig. 1). All plant shoots formed buds, flowers, and fruits simultaneously (Figs 2 – 4). Therefore, even material collected at the same time contained ovules at different stages of development.

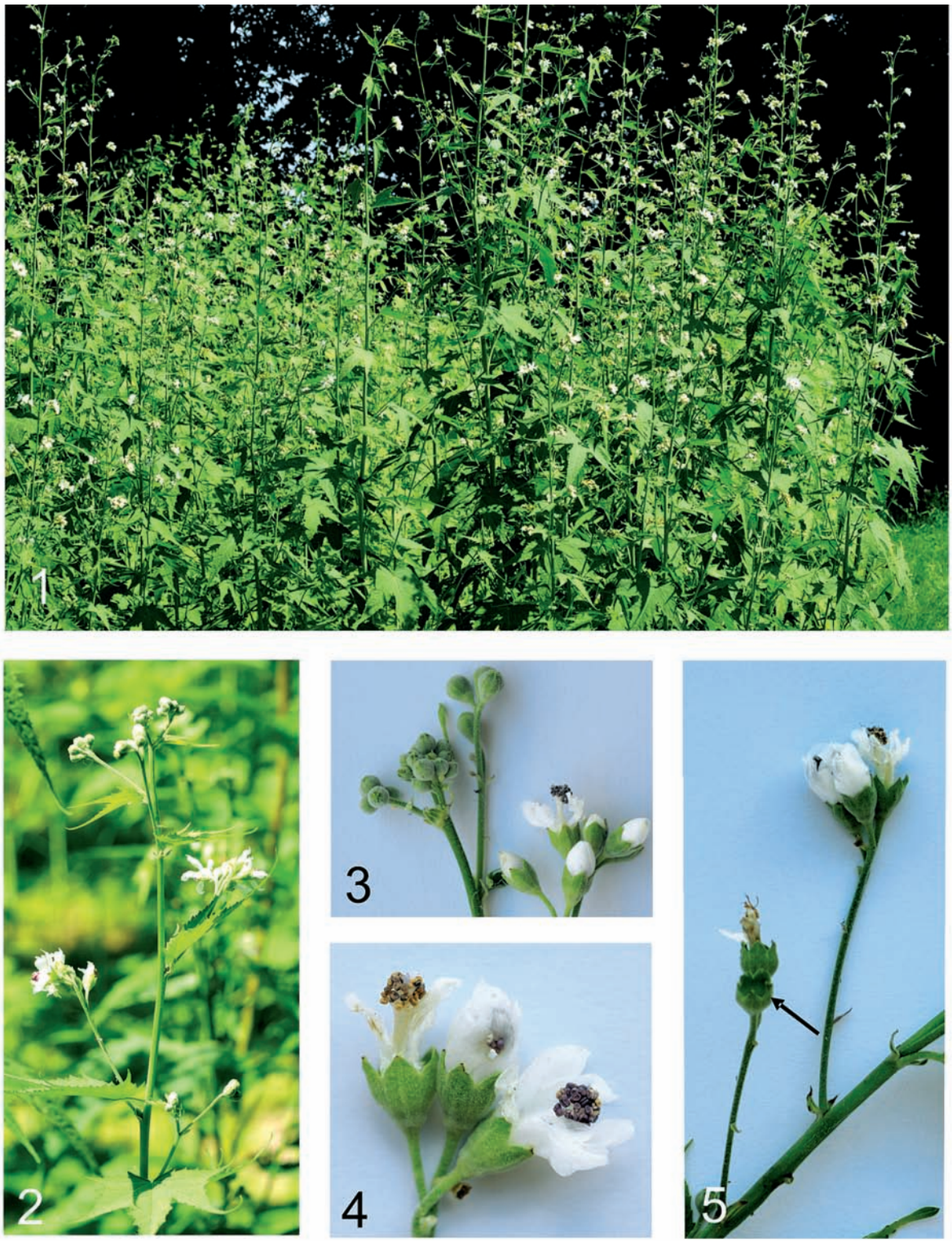
The anatomical structure of the ovule

The ovules of *S. hermaphrodita* investigated in this paper were isolated from the ovaries of overblown flowers (Fig. 5). The anatomical structure of such ovules is clearly visible in longitudinal sections (Figs 6 – 9). The ovules of S. hermaphrodita are ana-amphitropous with the micropyle directed towards the top of the loculus and the funiculus located near the ovary wall (Fig. 6). The outer integument and the funiculus are coalescent and form a dorsal raphe. The micropyle is very close to the funiculus, and the nucellus and the embryo sac are marked as curved.

The ovules are bitegmic and crassinucellate. Without a doubt, the outer integument grows faster than the inner one and covers the whole ovule forming together with the inner integument a zigzag-shaped micropylar canal (image not shown in any figure in this paper). The outer integument is composed of 2 to 3 layers of cells and the inner integument is 4 to 5 cells thick, but in the micropylar part, forming the micropylar canal, both integuments are thicker and are composed of 7 to 8 layers of cells. The cells of the inner integument adjacent to the micropylar canal are comparatively small, filled with a dense and organelle-rich cytoplasm. The described cells are thin walled and devoid of big vacuoles, while other cells of the integument are bigger and contain big vacuoles. The cells of the inner epidermis of the inner integument are adjacent to the nucellus. The cells of the micropylar region lose their cytoplasm and are filled with tannin, which stains deeply blue after toluidine blue treatment. Some cells in this inner layer of the inner integument and in the funiculus are also filled with tannin. Between the micropylar region of the nucellus and the embryo sac, there are several layers of nucellar cells (parietal tissue). The cells along the path of the nucellus leading from the micropylar canal to the embryo sac have distinct features. They are slightly elongated, loaded with dense and organelle-rich protoplasts, and are devoid of big vacuoles. They have narrow intercellular spaces filled with a substance whose stains are deeply blue after toluidine blue treatment. Similarly stained material can be observed in the intercellular substance as well as in the space between the micropylar canal and the top part of the nucellus. The rest of nucellar cells are remarkably bigger with numerous starch grains, big vacuoles, and thin cell walls.

The mature embryo sac is 8-nucleate and is composed of the following 7 cells: the egg apparatus, at the micropylar pole, consisting of two synergids and the egg cell, two polar nuclei of the central cell in proximity of the egg apparatus (Figs 7 – 9), and three small antipodal cells localized on the chalazal pole (image not shown in any figure in this paper). The synergids are comparatively large and elongated, with a prominent filliform apparatus on the micropylar pole. They are not typically pear-shaped, but both are hooked to the micropylar pole. The cytoplasm of the two cells is dense, the nucleus in each of them is localized in the proximity of the micropylar pole, and a small vacuole is placed on the chalazal pole. On the micropylar pole, the synergids are partially surrounded with the cell wall which gradually disappears towards the chalazal part. The egg cell contains a big vacuole on their micropylar pole and a narrow strand of cytoplasm in the chalazal part, in which the nucleus is located. The cytoplasm of the egg cell contains some starch grains. Two polar nuclei that are found in the proximity of the egg cell do not fuse before fertilization (Fig. 7). They fuse at the time of fertilization. The three antipodal cells are small, ephemeral and degenerate in the mature embryo sac. The embryo sac is much wider in its micropylar part than in the chalazal part and is markedly curved. Its curvature increases during development. Around the embryo sac, there are 2 to 3 layers of lyzed and degenerated cells of the nucellus. The nucellus cells on its micropylar pole contain tannin and starch grains.

The immunofluorescence method employing the JIM 13 monoclonal antibody which recognizes epitopes of arabinogalactan proteins (AGPs) showed a strong fluorescence in the wall of the embryo sac (Fig. 10). A higher magnification showed the presence of the fluorescence not only in the area of the cell walls of the embryo sac but also in the space surrounding the embryo sac wall (Fig. 11). The fluorescence was especially strong in the chalazal part of the embryo sac, where it was widely spread, indicating or revealing the massive structure of this wall part. Markedly weaker, but still considerable, fluorescence could be observed in the egg cell and the filliform apparatus. The somatic cells of the ovule were deprived of fluorescence.



Figs 1 – 5. *Sida hermaphrodita* (L.) Rusby

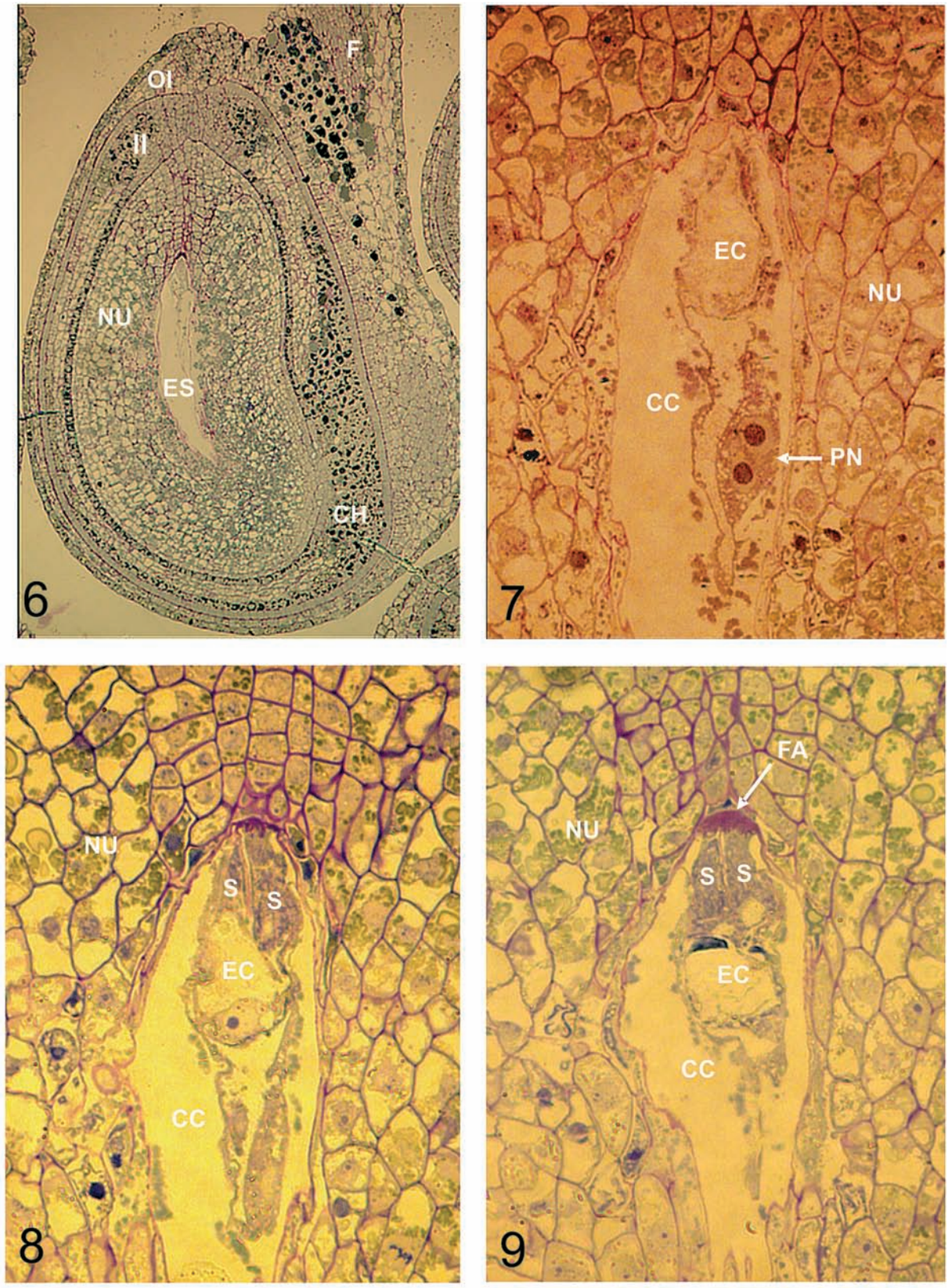
Fig. 1. Plants growing in an experimental field of the University of Life Sciences in Lublin, located in Felin district in eastern Lublin Figure shows plants up to 2.5 m high

Fig. 2. The habit of the top part of an *S. hermaphrodita* plant shoot. 0.4 x

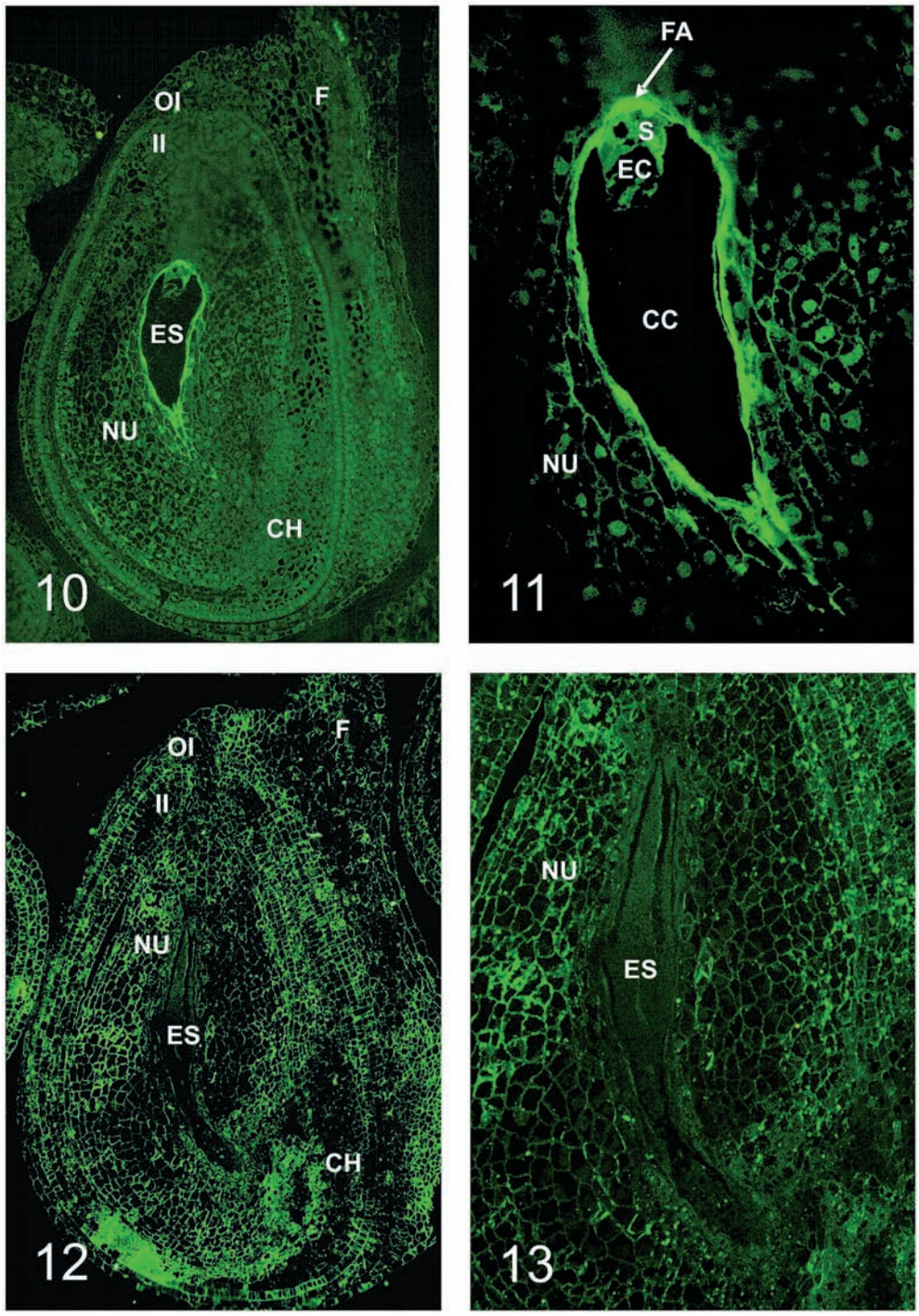
Fig. 3. Flower buds on the top of the inflorescence at different stages of development. 2 x

Fig. 4. Flowers at different stages of development. 4 x

Fig. 5. Fragment of a shoot with an overblown flower (arrow). 2 x



Figs 6 – 9. Longitudinal sections of *S. hermaphrodita* ovules isolated from the flowers after pollination, stained with toluidine blue.
Fig. 6. 120 x; Figs 7-9. 450 x
OI – outer integument, II – inner integument, NU – nucellus, F – funiculus, ES – embryo sac, CH – chalaza, EC – egg cell, CC – central cell, PN – polar nuclei, S – synergid, FA – filliform apparatus



Figs 10 and 11. Immunofluorescence of *S. hermaphrodita* ovules isolated from the flowers after pollination, labeled with the JIM 13 monoclonal antibody recognizing epitopes of arabinogalactan proteins (AGPs); positive reaction visible in the cell wall of the embryo sac, in the egg apparatus and the filliform apparatus; fluorescence is not visible in somatic cells of the ovule. Fig. 10. 120 x; Fig. 11. 450 x

Figs 12 and 13. Immunofluorescence of *S. hermaphrodita* ovules, isolated from flowers after pollination, labeled with the MAC 207 monoclonal antibody recognizing epitopes of arabinogalactan proteins (AGPs); positive reaction scattered in the cell walls of all somatic cells of the ovule; reaction absent in the cell wall of the embryo sac, in the egg apparatus and the filliform apparatus. Fig. 12. 120 x; Fig. 13. 450 x

OI – outer integument, II – inner integument, NU – nucellus, F – funiculus, ES – embryo sac, CH – chalaza, EC – egg cell, CC – central cell, S – synergid, FA – filliform apparatus

The immunoreaction made using MAC 207, which recognized a different (than JIM 13 monoclonal antibody) epitope of AGPs as the primary antibody, revealed the prescence of AGPs in the cell walls of all somatic cells of the ovule (Fig. 12). The labeling was stronger in both the integuments and the external part of the nucellus. Markedly weaker fluorescence could be observed in the epidermal cells of the outer integument, the central part of the funiculus and the inner part of the nucellus. A higher magnification of the central part of the ovule in Fig. 12 shows the absence of fluorescence in the cell walls of all the cells in the embryo sac (Fig. 13).

DISCUSSION

In the development of the reproductive structures of flowering plants, it is possible to notice periodization. According to B a t y g i n a and V a s i l i e v a (2003), some periods seem to be critical and can be considered as the stages of ontogenesis in which the structures display the greatest sensitivity to unfavorable enviromental factors. These authors divided the postmeiotic periods in ovule development into four stages: 1) the coenocytic phase of the megagametophyte; 2) cellularization and gametogenesis; 3) the fully cellularized megagametophyte not yet ready for fertilization; and 4) the mature embryo sac with complete specialization of its elements, ready for fertilization. Our results, presented in this paper, concern the fourth, most crucial stage of development of the ovule of *Sida hermaphrodita*.

From the analysis of the anatomical structure we can conclude that the ovules of *S. hermaphrodita* are not typically anatropous or campylotropous. Anatropous ovules are described in over 200 families but probably more than 75% of angiosperms have ovules which, to a lesser or greater degree, turn back toward the placenta (L e r s t e n , 2004). *S. hermaphrodita* has a bitegmic ovule in which, as shown in this paper, the nucellus as well as the funiculus are slightly curved. Ovule curvature is quite large at ovule maturity, and the embryo sac within the nucellus is also curved, as is the mature embryo in the seed. In the literature, this variant of the anatropous ovule is termed campylotropous, which means "curved turning" (L e r s t e n , 2004). In their typical form, campylotropous ovules also occur in such families as Chenopodiaceae (e.g. in spinach) or Fabaceae.

Bitegmic and crassinucellate ovules with all three parts, the nucellus, the funiculus and integuments, in the structure are typical in angiosperms. In *S. hermaphrodita* the outer integument and the funiculus are coalescent, forming a dorsal raphe, and the micropyle is situated very close to the funiculus, like in the anatropous type of ovules, whereas the nucellus and the embryo sac are markedly curved. The funiculus of the investigated species forms an outgrowth directed towards the micropyle, but its cells are not rich in cytoplasm; conversely, they usually lose their cytoplasmic content and accumulate tannin, so this structure cannot be considered an obturator. Generally, the cells of the ovule parts (elements), such as the inner and outer integuments, the funiculus and the nucellus, differ in some features concerning their spatial arrangement, shape, size, contents of differently sized vacuoles, or presence of chemicals such as tannin and starch.

The embryo sac in *S. hermaphrodita* develops according to the *Polygonum* type, and at its mature stage consists of 8 nuclei or 7 cells. This type of embryo sac development is described in over 80% of angiosperms. The cells bulding the embryo sac seem to be typical of the majority of angiosperms, although attention should also be paid to the synergids. In the investigated species, they are comparatively large and elongated, with a prominent filliform apparatus on the micropylar pole. The synergids are not typically pear-shaped, but are hooked on the micropylar pole. As it was mentioned in the chapter Results of this paper, the three antipodal cells are small, ephemeral and degenerate in the mature embryo sac. Because they disappear before fertilization, we do not present a magnified chalazal pole of the embryo sac in this paper.

Among the Malvaceae species, and even among the *Abutilon* alliance to which *Sida* belongs, some structural features of ovules, including position of micropyle, type of ovule, shape of synergids, presence of filliform apparatus and number of antipodals, are very variable (R a o , 1954; T a n g et al. 2009). Some differences in the ovular structure can also be noted within the genus *Sida*, among species such as *S. cordifolia, S. veronicaefolia, S. carpinifolia studied* by R a o (1954) and S. hermaphrodita presented in this paper. On the similarities side, the ovules of all the abovementioned species of *Sida* are bitegmic, crassinucellate, pendulous, campylotropous (or even amphitropous) with the micropyle directed towards the top of the loculus with a dorsal raphe and a markedly curved nucellus. The embryo sac in the nucellus is also bent. Usually the micropylar canal is in a zigzag form and the polar nuclei fuse at the stage of fertilization. The differences include the shape of synergids, the presence of the filliform apparatus and obturator as well as types of antipodals. Hooked synergids with a well-formed filliform apparatus and an absence of obturator have been observed in *S. hermaphrodita*, *S. carpinifolia* and *S. cardifolia.* In contrast, in *S. veronicaefolia* the synergids are pear-shaped without a filliform apparatus, but the obturator is well-formed. Antipodals in *S. hermaphrodita* are ephemeral and not visible in the

mature embryo sac, while in the other studied *Sida* species antipodals are numerous and persist till the late embryogenesis stage.

From our additional investigations (not presented in this paper) it appears that in all the ovaries that we studied 8 ovules were formed, which, however, at the late anthesis stage, showed significant differences in size and developmental stage of the embryo sacs. Over 30% of the ovules remained in their juvenile stage at the time when pollen tubes reached the ovary. These ovules were probably unfertlizable, did not attract the pollen tubes and underwent degeneration before or during fruit development. Other studies on the biology of flowering of *S. hermaphrodita* in Polish conditions showed that as many as 95% of seeds were set (Wróblewska and Kolasa, 1991). These discrepancies can be connected with seasonal differences. Unfertilizable and degenerating ovules with normally formed embryo sacs are common among Malvaceae species, e.g. in Malvaviscus arboreus Cav., Kydia calycina, and Thespesia populnea (Rao, 1955).

S. hermaphrodita is known in the literature as a plant with low seed germination potential. In Europe many author have dealt with this problem because of possible industrial uses of this plant (for a review see Borkowska and Styk, 2006). Many methods for improving the germination potential have been proposed, including physical and chemical treatment. In Polish conditions the highest recorded germination success was 77%, using seeds stored for 12 months (Borkowska and Styk, 2006). However, the germination potential of S. hermaphrodita seeds showed variability depending on the vegetative season and in many experiments the maximum number of germinating seeds was about 30-40%. For this reason, in Europe this plant is reproduced mainly in a vegetative way.

Arabinogalactan-proteins (AGPs) are a large family of highly glycosylated hydroxyproline-rich glycoproteins occurring mainly on the cell surface (Showalter 2001). They are widely spread in the plant and animal kingdoms; classical AGPs are comonly distributed in flowering plants. These glycoproteins accompany the processes taking place during plant growth and development (Showolter, 2001).

The proteins immunolocalized in our experiments were observed earlier in different cells of leaves, roots, and seeds (Fincher et al. 1983; Nothnagel, 1997). In the different floral parts, they were observed in the transmitting tissue of the style and in the pollen tube, including the generative cell (Jauh and Lord, 1996; Coimbra and Salema, 1997; Roy et al. 1998; Coimbra and Duarte, 2003; Chudzik et al. 2005). It was documented that a specific AGP molecule was necessary for female gametophyte development in Arabidopsis (Acosta – Garcia and Vielle – Calzada, 2004; Coimbra et al. 2007). Nonetheless, for the first time in this study, the localization of AGPs was shown in *S. hermaphrodita.*

Two kinds of monoclonal antibodies were employed in the localization of AGPs in the present study: JIM 13 and MAC 207. JIM 13 recognizes sugar epitopes on arabinogalactan proteins containing the trisacharide β-D-glucose A-β (1→3)-D-galactose A-α (1→2)-L-rhamnose and MAC 207 recognizes the sugar epitope on arabinogalactan proteins: α-glucose A-α (1→3)-α-galactose A-α (1→2)-rhamnose, but the two monoclonal antibodies bind to different AGPs molecules and show variable labeling in tissues (Knox et al. 1991; Yates et al. 1996). The antibodies used during our investigations detected AGPs in the wall surrounding the embryo sac (JIM 13) and in the cell walls of all somatic cells of the ovule (MAC 207). It was documented in other plant species that AGP molecules recognized by the JIM 13 monoclonal antibody are specific for generative cells, while these labeled with the MAC 207 monoclonal antibody are scattered among somatic tissues. AGP molecules can be therefore considered as the molecular marker for somatic and generative cells (Pennell and Roberts, 1990; Coimbra et al. 2007). The results showing the presence of AGPs in the ovule of S. hermaphrodita obtained by us are not especially unusual or atypical.

From the investigations presented in this paper, it cannot be concluded clearly that the features observed during the investigated stage of Sida hermaphrodita ovule development have any influence on seed germination of the species. The ovule structure, including the distrbution of arabinogalactan proteins, seems to be proper and is characteristic of a transition stage towards the future step in development – fertilization. Nonetheless, it should be underlined that the present paper is the first in the series of studies in which we plan to undertake the problem of the embryology of this promising energetic species.

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Struktura anatomiczna zalążka *Sida hermaphrodita* (L.) Rusby po zapyleniu

Streszczenie

Badano budowę anatomiczną i rozmieszczenie białek arabinogalaktanowych w zalążku rośliny energetycznej *Sida hermaphrodita* (L.) Rusby po zapyleniu. Materiał zbierano z poletka doświadczalnego Uniwersytetu Przyrodniczego w Lublinie (Felin). Po procedurze zatapiania do metody immunofluorecencyjnej, skrawki półcienkie barwiono błękitem toluidyny lub inkubowano z monoklonalnymi przeciwciałami JIM 13 lub MAC 207 w celu immunodetekcji białek arabinogalaktanowych. Budowa zalążka wykazuje różnice w porównaniu z innymi przedstawicielami rodziny Malvaceae. Przeciwnie, rozmieszczenie białek arabinogalaktanowych nie różni się istotnie od ich rozmieszczenia w zalążkach innych roślin okrytozalążkowych w badanym stadium rozwoju.