Cyto-embryological studies on *Astragalus glycyphyllos* L. from the areas of northern and central Poland

K. BIJOK, E. ADAMKIEWICZ, Z. GÓRAL

Institute of Biology, University of Gdańsk, and Dept. of Botany, High School of Agriculture, Olsztyn, Poland

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Abstract

The present paper reports karyological investigations on *Astragalus glycyphyllos* L. from sites in Poland, including development of pollen, embryo sac, process of fecundation, development of embryo and endosperm.

These plants have been examined by various authors, from the karyological aspect, but not from the area of Poland.

As regards embryology, this species has so far not been studied.

INTRODUCTION

Karyological and embryological studies on *Astragalus glycyphyllos* were undertaken since this species from the territory of Poland has not been elaborated, and as regards embryology it has not been studied at all.

Karyological investigations of this species were performed by Tschechow (1935), Larsen (1955b), Lipaev (1958) and Ledingham (1960).

NUMBER OF CHROMOSOMES AND THEIR MORPHOLOGY

The chromosome number in *A. glycyphyllos* n=8, 2n=16 was established in the somatic metaphases of the meristems of root tips as well as in diakinesis and the metaphase of the first and second meiotic PMC's division (Figs 1—3, 6, 8, 11).

The particular chromosomes could be arranged in eight pairs according to size and morphology (Figs. 1—2).

Two groups of chromosomes were distinguished in *A. glycyphyllos*: isobrachial ones (5 pairs) and heterobrachial ones (3 pairs) (Fig. 2).

MATERIAL AND METHODS

Seeds of *A. glycyphyllos* were collected from natural sites in northern and central Poland (Table 1), and sown on experimental plots once belonging to the Department of Genetics of the High School of Agriculture in Olsztyn.
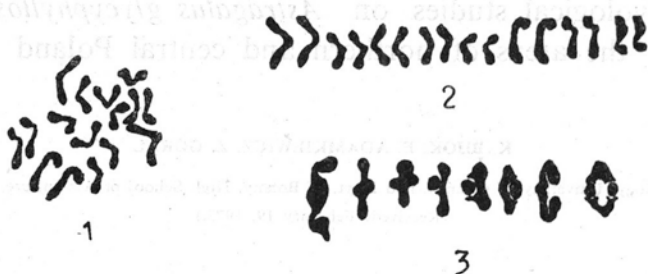

Fig. 1—3. *Astragalus glycyphallos* L. (2n = 16)

1. Somatic metaphase in root-tip (× 2000). 2. Chromosomes from metaphase plate (× 2000). 3. 8 bivalets from first metaphase in P.M.C's. (× 2000).

The material for cyto-embryological investigations, that is the root tips of flowering plants, or seeds germinated in laboratory on filter paper as well as buds and pods at variaus stages of development were collected in 1967.

The preparations were stained with 1 per cent gentian violet and differentiated with phenol solution in xylene (1:3). Before staining the material was fixed in 50 per cent Nawashin fixative, embedded in paraffin, and 15 μm sections were cut on a microtome.

Table 1

List of sites where *Astragalus glycyphyllos* L. was collected

Locality	County
Wiśniowa	Staszów
Kowale Oleckie	Olecko
Kaliszki	Pisz
Dorotowo	Olsztyn
Stare Jabłonki	Ostróda
Rogale	Gołdap
Gierłoż	Kętrzyn
Napiwoda	Nidzica
Ruszkowo	Działdowo
Szyplinki	Suwałki
Augustów	Suwałki
Słupsk	Kartuzy

RESULTS OF OBSERVATIONS

Development of anther

Topography of anther

On the cross section of an *A. glycyphyllos* young anther four loculi can be seen in which PMC's in the number of 10—14 are grouped in the centre (Fig. 4b). At this stage the anther walls had four layers: epidermis, fibrous layer, disappearing layer and tapetum.

Tapetum

In premeiotic stages of PMC's the tapetum layer consisted of mono-nuclear cells almost square in shape filled with granular cytoplasm (Fig. 4a).

In no case were divisions of cell nuclei observed in the tapetum of the plants studied, nor their coalescence or an increase in the chromosome number by way of endomitosis. Thus, the nuclei of the tapetal
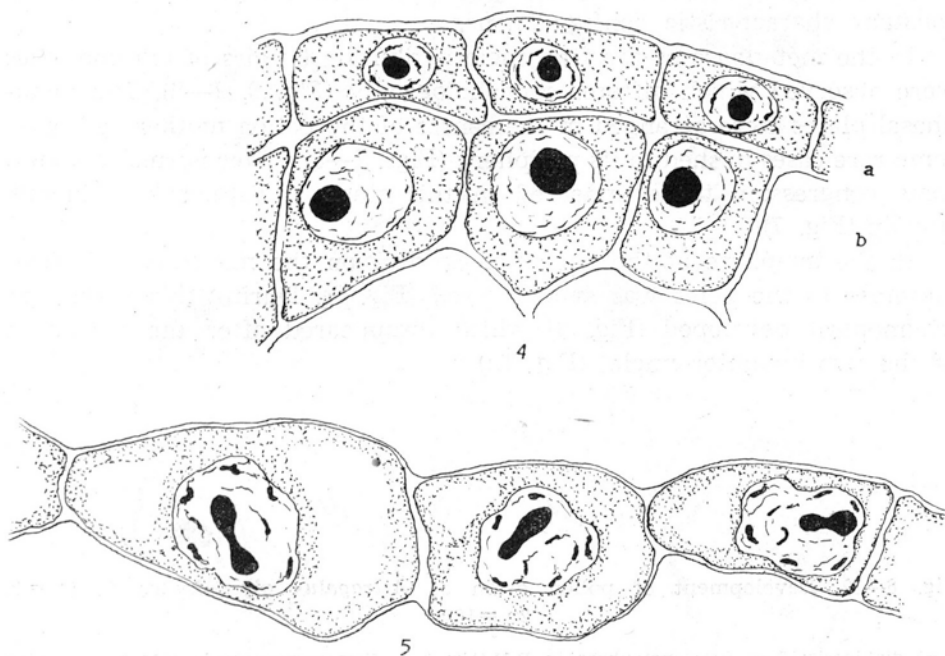

Fig. 4—5. *Astragalus glycyphyllos* L. (2n = 16). Cells of tapetal layer: 4a — tapetal cells at premeiotic stage of P.M.C.'s; 4b — P.M.C.'s; 5 — degenerated tapetal cells (× 1600).

cells were diploid. Owing to elongation growth of the anther, the tapetum cells elongated and assumed a spindle shape. In their cytoplasm at first minute vacuoles appeared which in older tapetal cells merged into one large central vacuole.

In the period of pollen grains maturation, an advancing process of their degeneration was observed in the tapetal cells (Fig. 5). This was manifested by a poor stainability of their nuclei, disappearance of cells walls, and in the end stage, degeneration of the entire cells.

Microsporogenesis

Within one anther sac PMC's occurred always at the same stage of division.

The PMC's differed distinctly from the adjacent cells of the tapetal layer (Fig. 4b). At first they adhered to one another, however, in the stage of early prophase, owing to elongation growth of the anther they lost their cohesion, became rounded and intercellular spaces appeared between them. At the stage of diakinesis they were visible as single rather loosely arranged cells in the anther sacs.

Meiosis was studied in detail from the stage of diakinesis. At this stage 8 bivalents were distinguished (Fig. 6) in *A. glycyphyllos* in certain constant characteristic configurations.

In the metaphase of the first meiotic division 8 pairs of chromosomes were observed which differed morphologically (Fig. 3, 7—8). The metaphasal plates were arranged in the centre of the pollen mother cell or in some rare cases pushed to its periphery (Figs. 7—8). After normal chromosome congression there appeared in this stage a distinct karyokinetic spindle (Fig. 7).

In the anaphase of the first meiotic division the movement of chromosomes to the poles was synchronised (Fig. 9). During this separation fragmoplast developed (Fig. 9) which disappeared after the formation of the two daughter nuclei (Fig. 10).

Fig. 6—19. Development of pollen grain of *Astragalus glycyphyllos* L. ($n = 8$, $2n = 16$):

6 — diakinesis; 7 — first metaphase in P.M.C.'s; 8 — first metaphase in P.M.C.'s — polar view; 9, 10 — first anaphase and telophase in P.M.C.'s; 11 — second metaphase in P.M.C.'s; 12, 13 — second anaphase and telophase in P.M.C.'s; 14 — microspore tetrads; 15, 16 — young pollen grain; 17 — pollen grain with vegetative and generative cell; 18 — pollen grain; disapperance of membranes between vegetative and generative nucleus; 19 — pollen grain with vegetative and generative nucleus ($\times$ 1400).
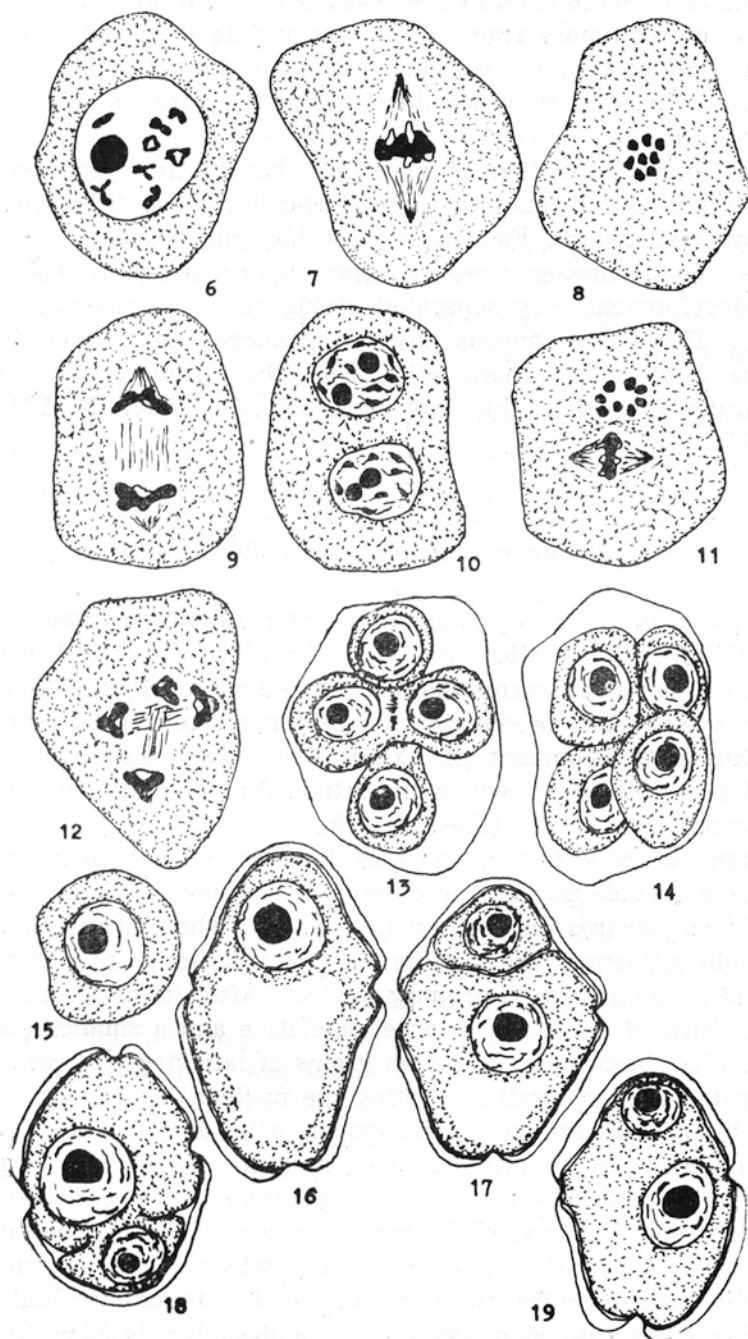
The metaphase stage of the second meiotic division occurred similarly as the stage of anaphase of the first meiotic division; here also the movement of chromosomes to the poles was synchronised. Between the separating chromosomes fragmoplast was visible (Figs. 12, 13).

In the telophase of the second meiotic division the PMC's contained four nuclei (Fig. 13). Between them six distinct fragmoplasts could be seen (Fig. 13).

It was found in further observations that in the cytoplasm of the PMC's six furrows form, and these deepening cause the formation of four protoplast masses. Each protoplast has one nucleus with a loose structure. These masses were at first connected by bridges, but in further development they separated giving rise to a microspore tetrad (Fig. 14). Thus simultaneous division occurred here, considered by numerous authors as characteristic of *Dicotyledones*. The primary pollen grains formed in this way were released when the PMC's wall dissolved.

Development of primary pollen grains

Primary pollen grains immediately after liberation from the walls of the PMC's had a single membrane — intine (Fig. 15). During their development a second membrane — exine formed (Fig. 16—19) which probably arose with the contribution of tapetal cells (M a h e s h w a r i 1950). During development of exine, three depressions (pores) were observed in it reaching down to the intine. As the exine thickened they became more pronounced (Figs. 16—19).

A large centrally located vacuole developed in the primary pollen grains. This vacuole pushes the cytoplasm and large nucleus containing one nucleous towards the cell wall (Fig. 16). The central situation of the vacuole influences the position of the spindle in the first mitotic division of the pollen grain primary nucleus. After its division the pollen grain consisted of two cells: a large vegetative and a smaller generative one (Fig. 17). Observations of pollen grains at late stages of development demonstrated that the wall separating the nucleus of the vegetative cell from that of the generative one disappears after a certain time (Fig. 18), and both the nuclei were found to lie next to one another (Fig. 19) in the vegetative cell cytoplasm. The generative nucleus, however, was surrounded by a thin layer of its own cytoplasm (Fig. 19). The vegetative nucleus was large with a loose structure, whereas the generative one was smaller, its structure was compact and its stainability higher.

At this stage the pollen sacs burst and pollen is liberated. Mean 100-pollen-grain length was 17.30 μm, and 100-pollen-grain width 13.12 μm.

Development of ovule

Topography of ovule

The ovule of *A. glycyphyllos* was amphitropous, surrounded with thin nucellus and a double integument.

The nucellus consists of one cell layer (Fig. 20). Nucellus cells in early stages of development showed on the longitudinal section of the ovule a granular cytoplasm and a large distinctly staining nucleus.

The integument cells at this stage were rectangular. Their epidermal layer did not at that time differ from the remaining integument cells. At the stage of fournucleate embryo sac the cells of this layer elongated greatly in direction perpendicular to the longitudinal axis of the ovule and formed the integumentary tapetum.

Beginning with the fournucleate stage of the embryo sac, the nucellus underwent degeneration. The cell nuclei were then hardly noticeable, and the cells were elongated and flattened (Figs. 24, 25). At the eight-nucleate stage of the embryo sac the nucellus cells were completely degenerated (Fig. 26).

The archaespore

In *A. glycyphyllos* the archaespore is multicellular (Fig. 20). Its cells develop parallelly to the longitudinal axis of the ovule, and as meiotic divisions occur they partly penetrate between the chalazal cells (Fig. 21).

Megasporogenesis

The archaespore cells were at first many-walled with large nuclei of loose structure, and they adhered to each other (Fig. 20). The primary parietal cells did not separate, and the archaespore cells transformed directly to megaspore mother cells. As meiotic division occurred in the mother cells, the archaespore cells elongated and lost their compact arrangement (Fig. 21). It was found that in this period the megaspore mother cells were in various phases of meiotic division. After the first and second meiotic divisions which occurred in all the megaspore mother cells, megaspore tetrads linearly arranged were observed (Figs 21, 22).

Development of embryo sac

Further studies revealed that in *A. glycyphyllos* the embryo sac derives from the chalazal megaspore of the tetrad nearest to the micropyle. Three megaspores of this tetrad degenerate on the micropyle side.
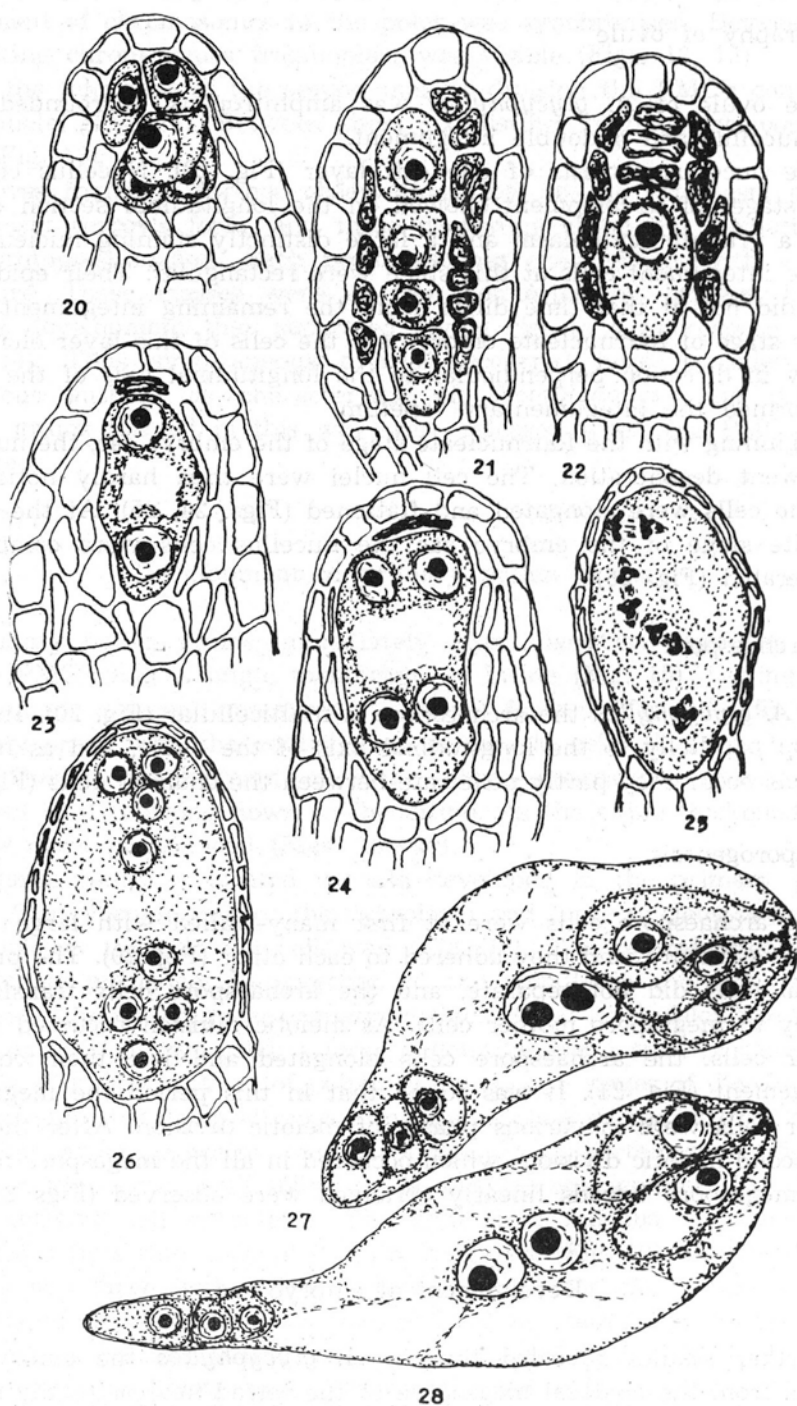
At the stage of fournucleate embryo sac they were still visible as a darkly staining mass (Fig. 22). The remaining megaspore tetrads were shifted to the sides of the ovule, exceptionally towards the chalaza where they also degenerated (Fig. 22).

Mitotic division of the nuclei in the embryo sac occurred synchronously (Fig. 25). The megaspore giving rise to the embryo sac elongated considerably. At various stages of embryo sac development two-, four- and eightnucleate embryo sacs were noted in *A. glycyphyllos* (Figs. 22—26). In the two-, four- and eight-nucleate embryo sac a vacuole is seen between the nuclei on the micropyle side, and those on the chalazal side (Figs 23, 24, 26). The embryo sac in *A glycyphyllos* was eightnucleate of monospore type (Figs 27, 28).

Structure of mature embryo sac

The structure of the egg apparatus of *A. glycyphyllos* was typical, it consisted of one egg cell and two synergids (Figs 27, 28). The egg cell lies immediately below the micropyle. Its cytoplasm is coarse-grained and accumulated mainly in the lower, widened part of the cell surrounding the loose-structure nucleus. The vacuole in the egg cell lies usually in the constricted and elongated part on the micropyle side (Fig. 28).

The synergids have a greatly elongated upper part on the micropyle side. The nuclei of the synergids are surrounded by cytoplasm and located in the upper part of the cell. In the lower one, unlike the egg cell, there is a vacuole (Figs 27, 29).

The secondary nucleus of the embryo sac with a very loose structure has two strongly staining nucleoli (Fig. 28). It arises by the fusion of two polar nuclei which lie more or less at one third of the embryo sac length on the micropyle side (Fig. 27).

The clavate antipodials in the number of three cells contained vacuolised cytoplasm and one nucleus each (Figs 27, 28).

It should be stressed that *A. glycyphyllos* always developed only one embryo sac. Typical embryo sacs developed in all ovules. No ovules were observed with incompletely developed embryo sacs.

Fig. 20—28. Development of embryo sac of *Astragalus glycyphyllos* L. (n = 8, 2n = 16):

20 — multicellular archespore; 21 — tetrads of megaspores; 22 — mononucleate embryo sac; 23 — binucleate embryo sac; 24 — fournuncleate embryo sac; 25 — mitoses in four nuclei; 26 — eightnuncleate embryo sac; 27 — embryo sac with synergid cells, polar nuclei and antipodal cells; 28 — embryo sac with egg apparatus secondary embryo sac nucleus and antipodal cells that degenerate ($\times$ 370).

Fertilization

Numerous pollen tubes penetrated into the style. Of these 9—10, exceptionally 12 reached the ovary. The growth of the other pollen tubes ended at various levels of the style, mostly at one fourth of its length.

In the end part of the pollen tubes reaching the ovules, a distinctly visible pollen tube nucleus was found, and over it, linearly arranged two sperm nuclei surrounded by their own cytoplasm, distinctly demarcated from the pollen tube content.

Penetration of the pollen tube into the embryo sac occurred through the micropyle (porogamy). When the pollen tube approaches the embryo sac, the vegetative nucleus desintegrates. The pollen tube penetrating into the embryo sac usually destroys one of the synergids (Fig. 29), although in five cases from among the 100 ovules investigated the pollen tube passed between the egg cell and one synergid. After penetration
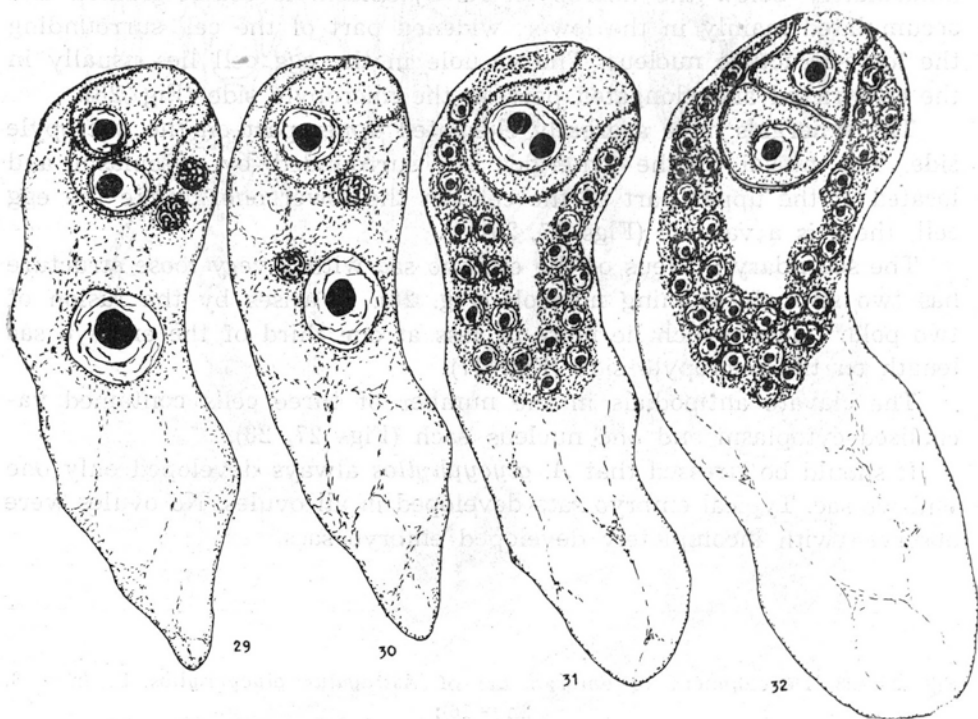
Figs. 29—32. Sperm nuclei in embryo sac (29) and sperm nuclei oriented towards egg cell and secondary nucleus of embryo sac (30). Egg zygote in nuclear endosperm (31). Bicellular embryo in nuclear endosperm (32). (Magn. × 600).

of the pollen tube into the embryo sac, the sperm nuclei were discharged into its contents. At this time the cytoplasm surrounding the sperm disappeared.

In the 100 embryo sacs examined from various plant individuals, no differences were found in the size and shape of the sperm nuclei
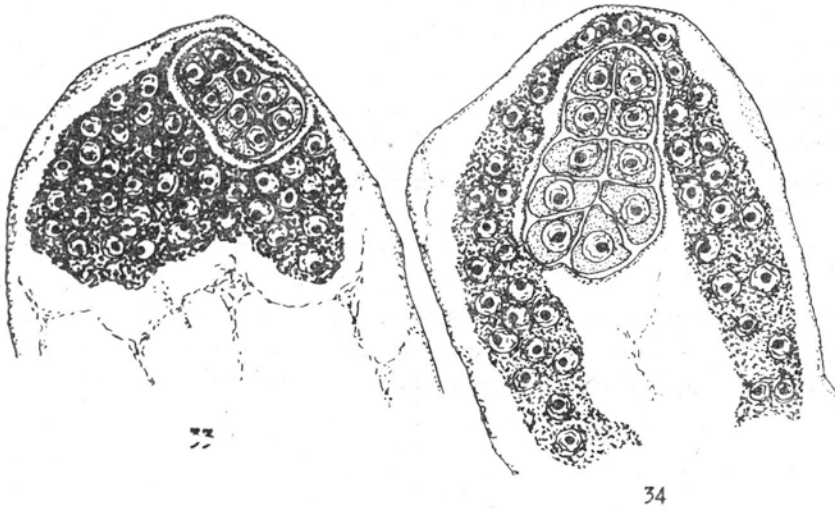
Figs. 33—34. Young embryos in nuclear endosperm ($\times$ 400).

travelling to the egg cell and of the secondary nucleus of the embryo sac (Figs 29, 30).

Fertilization processes were not observed. In several cases the sperm nuclei lay close to the egg cell and secondary nucelus of the embryo sac (Fig. 30). At this stage the remaining synergids degenerated.

In no case was degeneration of the embryo sacs observed.

Development of embryo

Embryonal development in *A. glycyphyllos* (*Papilionaceae*) was followed from the moment before the fertilised egg cell divided and when the fertilised secondary nucleus of the embryo sac had already undergone multiple divisions (Fig. 31). Some time after fertilization the egg zygote divided into two cells: a basal larger and a terminal smaller one (Fig. 32). It resulted from further observations that the basal cell does not take part in the development of the embryo, and gives rise

to a short massive suspensor (Figs 33—35). Development of the embryo occurred as the result of terminal cell divisions (Figs. 33—35).

The development of the *A. glycyphyllos* embryo always was sexual, in no case were embryos formed by way of apomixis or was degeneration of proembryos observed.

Endosperm development

Endosperm development in *A. glycyphyllos* began earlier than embryo development. Before the first division of the egg zygote several tens of endosperm nuclei surrounded the embryo in the form of a coat in the embryo sac cytoplasm (Figs 31—34). Endosperm nuclei divisions occurred simultaneously in the entire embryo sac. All the nuclei in stages of interkinesis or of meta- and anaphase were observed in many embryo sacs (Figs 31—34, 36—37).

At the time when the cotyledones began to develop in the embryo, the nuclear endosperm transformed to cellular endosperm. Formation of the endosperm cell walls progressed from the walls of the embryo
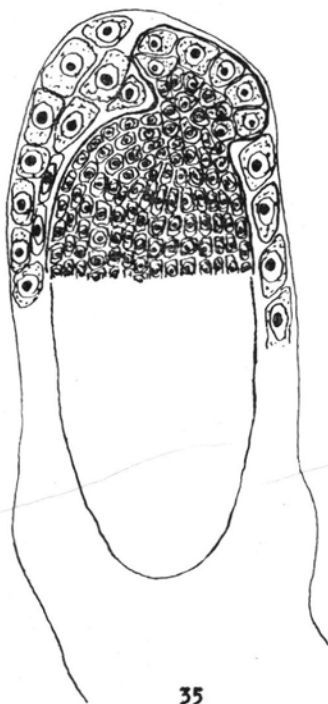
35

Fig. 35. Elder embryos in cellular endosperm (× 250).

36   37

Figs. 36—37. Mitotic divisions of endosperm nuclei ($\times$ 950).

sac towards its interior. It should be noted that the distribution of the endosperm nuclei in the embryo sac, at first in the form of a coat around the embryo, extended with its development so that at the moment of transformation of the nuclear to cellular endosperm the nuclei filled the entire embryo sac. During further growth of the embryo the endosperm was gradually consumed up to complete absorption by the embryo.

DISCUSSION

The chromosome number $2n = 16$ observed in the mitotic divisions of *A. glycyphyllos* from sites in northern and central Poland agrees with the results reported for this species by T s c h e c h o w (1935), L i p a e v (1958) and L e d i n g h a m (1957, 1960). The $n = 8$ number has also been confirmed in this species in investigations on meiosis in PMC's by L a r s e n (1955).

The tapetal cells in *A. glycyphyllos* were always uninucleate with diploid nuclei. An increase of the chromosome number by way of mitotic division with cytokinesis inhibition or endomitosis were not observed in this species. A number of authors, reported the formation of multi-nucleate cells in the tapetum. Their nuclei showed varying degrees of ploidy, particularly in plants from the family *Compositae*, less frequently in other families (M a y e r, 1935; C h i a r u g i, 1930; R a d o l i c o, 1930;

Copper, 1933; Hartwick, 1936; Avanzi, 1942; Benerji, 1942; Witkus, 1945; Carniel, 1952, 1954; Mechelke, 1952; Wcisło, 1952; Wunderlich, 1954; Skalińska, 1958; Trela, 1958; Turała, 1958; Bijok, 1960; Jaworska, 1963; Adamkiewicz, 1966; Bijok, Sulma, Tokarz, 1968; Walter, Kuta, 1971).

Observations of the course of meiotic division in the PMC's demonstrated that it is normal, therefore degenerated pollen was not found. The papers of Bergman (1935, 1942) show that pollen degeneration is closely linked with apomictic propagation.

Meiotic division in the multicellular archaespore of *A. glycyphyllos* occurred in all the megaspore mother cells of the ovule. In this way several megaspore tetrads formed, of which only one, and pratically its chalazal spore, gave rise to the embryo sac. The remaining megaspore tetrads degenerate as observed earlier by Bijok, Góral and Góral (1970a, 1970b), Bijok, Góral (1970) in *Trifolium hybridum, T. pratense* var. *spontaneum* and *T. repens*. In the material investigated development of larger numbers of embryo sacs was not noted. In *A. glycyphyllos* always one eightnucleate embryo sac of monosporus type was found. A similar picture was observed in *T. pratense, T. pratense* var. *spontaneum* and *T. repens* — plants of the same family as *A. glycyphyllos* — by Martin (1914), Poliakowa, Sołomina (1952), Poliakowa (1956), Kazubowska-Mackiewicz (1964), Bijok, Góral and Góral (1970, 1970) and Bijok, Góral (1970).

In the material examined, ovules deprived of embryo sacs or with degenerate ones were not observed.

The process of penetration of the pollen tubes through the style into the ovary and embryo sacs was observed in *Trifolium pratense, T. pratense* var. *spontaneum, T. hybridum, T. repens* and their experimentally bred polyploid forms (Martin 1913; Silov 1931; Kazubowska-Mackiewicz 1964; Bijok, Góral, Grygorczyk, in press). In all the forms investigated, as in *A. glycyphyllos*, these authors found in the style several pollen tubes. Most of them, however, did not reach the ovary. Their growth ceased at about 1/3 or 1/4 of the style length so that only three or four pollen tubes penetrated into the ovary.

Kazubowska-Mackiewicz (1964) observed in *Trifolium pratense* and its polyploid form differences in the size of the sperm nuclei when they had reached the embryo sac. This has not been noted in *A. glycyphyllos* or in other investigated species of the genus *Trifolium* (Bijok, Góral, Grygorczyk — in press).

Numerous authors studied embryo development in various species of the family *Papilionaceae* (Guignard, 1881; Martin, 1914; Cook, 1914; Soueges, 1927a, b, c, 1929, 1946a, b, c, d, e; Cooper 1933, 1935, 1938; Bijok 1962; Kazubowska-Mackiewicz 1964; Wojciechowska 1971; Bijok, Góral, Grygorczyk — in press). No

investigations on embryo development in *A. glycyphyllos* have been published to date.

Kazubowska-Mackiewicz (1964) and Bijok, Góral, Grygorczyk (in press) noted in *Trifolium pratense, T. pratense* var. *spontaneum, T. hybridum* and *T. repens* withering of the embryo sacs and proembryos with the embryo sacs, or seed formation without embryos in *T. pratense* var. *spontaneum, T. hybridum, T. repens* and their polyploid forms. Such disturbances were not found in *A. glycyphyllos*. The embryo development in the latter plant corresponded to that described by Souege (1927) in *T. minus* and occurred according to the type Onagard variety *Trifolium*.

Apomictic development of the embryo did not occur in *A. glycyphyllos*.

The endesperm developed in *A. glycyphyllos* according to the nuclear type. Division of endosperm nuclei occurred simultaneously in the entire endosperm, and could be observed at the same stage. In the period of cotyledone formation in the embryo of *A. glycyphyllos* the nuclear endosperm transformed to cell endosperm. The *A. glycyphyllos* seeds had no endosperm since it was resorbed by the developing embryo.

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Studia cyto-embriologiczne u Astragalus glycyphyllos
z terenów północnej i środkowej Polski

Streszczenie

W toku badań potwierdzono liczby n = 8, 2n = 16 u *Astragalus glycyphyllos* z terenów północnej i środkowej Polski.

W stadium diakinezy i metafazy pierwszego podziału mejotycznego występowały zawsze biwalenty.

Komórki tapetum były jednojądrowe o jądrach diploidalnych.

Podziały mejotyczne w komórkach macierzystych pyłku miały przebieg normalny i dlatego w przeprowadzonych badaniach nie obserwowano zdegenerowanego pyłku. Dojrzałe ziarna pyłku były dwujądrowe — zawierały jądro wegetatywne i jądro generatywne.

Archespor u *A. glycyphyllos* był wielokomórkowy. Komórki archesporu przekształcały się bezpośrednio w komórki macierzyste megaspor. Podziały mejotyczne zachodziły we wszystkich komórkach macierzystych megaspor. Chalazalna megaspora tetrady megaspor najbliższej okienka dawała początek woreczkowi zalążkowemu.

U *A. glycyphyllos* wykształcał się w zalążkach zawsze tylko jeden woreczek zalążkowy, ośmiojądrowy typu monosporowego. Woreczek zalążkowy posiadał budowę normalną.

W badanym materiale nie obserwowano zalążków pozbawionych, względnie o zdegenerowanych woreczkach zalążkowych.

U *A. glycyphyllos* wnikało kilkanaście łagiewek pyłkowych w szyjkę słupka. Do zalążni dochodziło jednak 9 do 10, a wyjątkowo 12 łagiewek pyłkowych. Wzrost pozostałych łagiewek kończył się na różnych wysokościach szyjki słupka, najczęściej jednak w 1/4 długości szyjki.

Zapłodnienie zachodziło we wszystkich zalążkach. Nie stwierdzono obumierania woreczków zalążkowych i zarodków, nie obserwowano także tworzenia się nasion pozbawionych zarodków.

Rozwój zarodka przebiegał normalnie i odbywał się według typu *Onagrad*. odmiany *Trifolium*.

Bielmo rozwijało się według typu jądrowego.