Embryo sac development in *Pinguicula vulgaris* L.

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INTRODUCTION

The family *Lentibulariaceae* comprising insectivorous plants has long been somewhat of a curiosity anatomically and physiologically and in relation to the variability of the environments in which the members occur.

It has also been found to possess some unique embryological characteristic such as the presence of nutritive tissue in the ovule and in the placenta, the growth of the embryo sac out of the ovule into the ovary chamber and finally into the nutritive tissue of the placenta, presence of endosperm haustoria, the elongation of the zygote to form a tube-like cell which pushes its way inbetween the endosperm cells, the lack of differentiation in the embryo (Khan 1954).

The family has been little described embryologically and most of the data available refers to the genus *Utricularia*.

The present investigation concerns *Pinguicula vulgaris* L. Members of this genus which occur in Poland have been little studied. There are three species, two of which *Pinguicula vulgaris* L. and *Pinguicula alpina* L. are considered well defined species while the third *P. bicolor* Woł. is systematically debatable. Szafer, Kulczyński and Pawłowski (1924) consider *P. bicolor* to be a variety of *P. vulgaris* while Zurzycki (1953) considers it a separate species on the basis of morphological and anatomical criteria. The chromosome number has been found to be the same for both *Pinguicula vulgaris* L. and *P. bicolor*. In *P. vulgaris* L. it is $2n=64$ (A. Löve and D. Löve 1948, D. C. Darlington 1955). In *P. bicolor* Woł. $2n=64$ (Zurzycki — 1953).

When the distinction as to the reality of species boundaries is as difficult as this an embryological investigation may well provide useful criteria. A close scrutiny of stages in embryo sac development and embryo development have in the past enabled boundaries to be drawn between orders and families as well as between smaller taxonomic units such as genera and species (S. Krupko 1960).

CHARACTERISTICS OF THE LENTIBULARIACEAE

The insectivorous family *Lentibulariaceae* includes some 300 species. Its members show a greater differentiation in the ontogeny of their vegetative organs than members of other plant families. Only the genus

Utricularia has been more extensively described (Kamieński 1877). According to Wettstein (1935) and Hegi (1954) the *Lentibulariaceae* include five genera: *Utricularia*, *Genlisea*, *Polypompholyx*, *Biovularia* and *Pinguicula*. The genus *Byblis gigantea* is also included by K. Schnarf (1929) and P. N. Schürhoff (1926).

One fossil member of this family *Utricularia berentii* has been found in old diluvial strata in Oberohr in northern Germany. It is described by Keilbah (Engler, Prantl 1895).

There is considerable differentiation in size, appearance and occurrence among these plants. They may be found growing in water as well as on land, among mosses, on rocks above water (epiphytes) and on tufts of marshland vegetation.

Anatomical and morphological characters of the members of the genus *Pinguicula* L. in Poland have been described by Zurzycki (1953).

MATERIAL AND METHODS

Young buds and isolated pistils were twice collected in the period June 4th. to 7th. 1960 and at the end of May 1961. Material was collected at two sites in the mountains Gorce both on wet peat. The younger buds were collected on the Turbacz peak while larger buds and pistils of open flowers and flowers which had already dropped their perianth parts were collected in the Łopuszna valley.

Material on which the transparency of the integuments was studied was collected at Czerwonak near Poznań.

The material was subdivided and segregated before fixation. Four different fixatives were used: Navashin (Stockholm modification), FAA, AA and the sodium uranate (2%) + chromic acid (1%). The thickness to which the sections were cut varied from 8 to 12 mikr. according to the stage of development. Most of the material was stained by the Heidenhein hematoxylin method. Ammonium ferric alum solution (3%) was used as mordant and for differentiation after staining. Fast green in clove oil was used as a second stain when it was required to see cell walls. Some preparations were stained with crystal violet and by the Feulgen method. The squash method was also used and the stain at such times was 1% or 0,5% aceto — carmine or 1% aceto — orceine.

RESULTS

Pinguicula L. flowers are bisexual and zygomorphic. They are carried on long flower stalks which emerge from a dense rosette of leaves. There are two anteriorly placed stamens. They may be seen below the lower lip of the stigma. The lower lip of the stigma curls over them

Fig. 1. *Pinguicula vulgaris* L. Sections of the flower. Fig. 1A. Longitudinal section of a flower; Fig. 1B. Transverse section of the top of the flower. $\times 50$.

per — perianth; sta — stamen; st — stigma;
ov — ovule; pl — placenta; ccn — central canal;
stl — style

forming a bowshaped covering (Fig. 1A). Each anther has four pollen grain sacs. The pistil is flask-shaped and the style has a fairly wide central canal (Fig. 1B). The stigma consists of two lip-like structures. The upper one is very small while the lower one has a number of outgrowths and is well developed. The lower lip bends backwards in a bowshaped structure, covering both stamens so protecting them from

Fig. 2. Megaspore mother cell. $\times 1120$

Fig. 3. The ovule begins turning around megaspore mother cell stage. $\times 1120$

Fig. 2

Fig. 3

self pollination. Both the outer and the inner surface of the ovary wall is covered with glandular, secretory hairs which are similar to the hairs covering the calyx. These hairs are either sessile or stalked.

The ovary is one chambered. It is formed of two carpels. The placenta is supported on a stalk growing out of the base of the ovary (Fig. 1A). The ovules are very numerous and grow over the whole surface of the spherical placenta. The placenta is spherical with the exception of the invagination where the stalk supporting it is attached. The arrangement of the ovules on the placenta is such that the oldest ones are at the apex and the youngest ones towards its base. The ovules are lined up one behind the other. Those at the top of the placenta are back to back. The young ovule primordia are no more than small protuberances on the surface of the placenta, distinguishable from the rest of the surrounding tissue by their denser cytoplasm. The ovule bends over forward as it grows and an integument develops at the front (Fig. 3). The integument grows quickly, soon passing the nucellus so that by the time the ovule is fully anatropous the micropyle faces the placenta.

Fig. 4. Diad formation. Spindle fibres visible. $\times 1250$

Fig. 5. Tetrad formation. $\times 1250$

Fig. 6. Tetrad — two top cells have begun degenerating. $\times 850$

Fig. 7. Embryo sac mother cell showing remains of degenerating tetrads. $\times 850$

Fig. 8. Two-nucleated embryo sac showing remains of degenerated nucellus cells (*m* — micropyle). $\times 850$

Fig. 9. Four-nucleated, naked embryo sac (*m* — micropyle). $\times 850$

Fig. 10. Double fertilization in the 8-nucleated embryo sac. The lower antipodals are degenerating. $\times 850$

Fig. 11. Egg cell at syngamy. $\times 1250$

The innermost layer of integumentary cells differentiates into an endothelium (integumentary tapetum) soon after the formation of the embryo sac. The endothelium lies directly in contact with the naked embryo sac. Its cells are perpendicular to the embryo sac wall. They look elongated in longitudinal section and polygonal in transverse section. They have larger nuclei and denser cytoplasm than the surrounding cells. The placental conducting tissue forms the pattern of a sparsely branched tree. The ends of the branches do not reach the ovules. They terminate in the middle of the placenta. The megaspore mother cell begins to differentiate in the hypodermis at the same time as the ovule begins to bend forward (Fig. 2). It differs from the surrounding cells in the size of its nucleus and in the density of its cytoplasm. Instances of the occurrence of two megaspore mother cells have been found. The two cells lie next to one another. At first the megaspore mother cell nuclei are larger and lie at the centre of the cell (Fig. 2). While the ovule is in the process of turning this cell elongates

Fig. 12. The antipodal chamber in the mature embryo sac. The polar nuclei have not yet fused. $\times 850$

Fig. 13. Antipodal chamber divided into two cells. $\times 850$

Abbreviations: *pt* — pollen tube; *e* — egg cell, *en* — egg cell nucleus; *pn* — polar nuclei; *sp* — sperm nucleus; *sn* — synergid nucleus; *nn* — nucellus nucleus; *ant* — antipodals

and its nucleus moves from the centre to the top (Fig. 3). It is at the megaspore mother cell stage that the ovule becomes completely anatropous. The megaspore mother cell gives rise to two diads. The metaphase and telophase stages of division leading to diad formation are shown in Fig. 4. At this stage the nucellus consists of a single layer of cells which look elongated in longitudinal section (Fig. 4). The second reduction division takes place at this time (Fig. 5) and gives rise to a tetrad of megaspores (Fig. 6). All the tetrads observed were linearly arranged. In a few cases more than one non-degenerating tetrad cells

was seen. The two cells shown in Fig. 6 at the micropylar end have begun degenerating while the two remaining ones are healthy. Sometimes every alternate tetrad cell degenerates or only one tetrad cell may degenerate. The chalazal megaspore cell gives rise to the one nucleate embryo sac (Fig. 7). The one nucleate embryo sac is distinguished by its fast growth rate, a large nucleus at the centre and numerous cytoplasmic vacuoles. The nucellus cells surrounding the embryo sac begin degenerating at the first mitotic division in the embryo sac. The two nuclei resulting from the first mitotic division in the embryo sac move to the opposite poles. A large vacuole separates them (Fig. 8). Remains of degenerating nucellus cells are still visible. The four-nucleate embryo sac is much larger and its micropylar end reaches right under the micropyle. The micropylar end of the embryo sac is naked by this time since the surrounding nucellus cells have degenerated (Fig. 9). The nuclei at the micropylar end of the embryo sac lie horizontally in relation to its long axis while the nuclei at chalazal end lie parallel to it. One unusually shaped four-nucleated embryo sac was encountered. The micropylar end was wider and naked on the side facing the micropyle while the lower chalazal end was narrower and pushed right down inbetween the chalazal cells. The third mitotic division gives rise to abnormal eight-nucleate embryo sac (Fig. 10). Egg — apparatus is always centrally placed. The synergids usually lie symmetrically one on either side of the egg-cell. Their nuclei are fairly large. The polar nuclei and the nuclei of the antipodal cells are approximately equal in size (Fig. 10). The antipodal cells are large.

Two of them lie next to each other, pushed deep down into the chalazal end of the embryo sac while the third one lies above them. The two lower antipodal cell are shaped like rods widened at either end. They degenerate early. The third antipodal cell enlarges and finally fills nearly half of the embryo sac cavity (Fig. 12).

The embryo sac at this stage of development has two separate regions. The upper region contains the egg apparatus and the polar nuclei which have not yet fused. The lower region may because of its origin be re-

Fig. 14. Degenerating cells in the antipodal chamber. Egg cell at syngamy. $\times 622$

Fig. 15. Mature embryo sac. All the antipodals have degenerated. $\times 840$

Fig. 16. Five-nucleated embryo sac. Polar nuclei during fusion. $\times 840$

Fig. 17. Primary endosperm nucleus dividing. $\times 840$

Abbreviations: *e* — egg cell; *en* — egg cell nucleus; *pn* — polar nuclei; *s* — synergid; *sn* — synergid nucleus; *sp* — sperm nucleus; *d ant* — degenerated antipodal; *vn* — vegetative nucleus; *z* — zygote

Fig. 18. Two-nucleated endosperm. $\times 850$

Fig. 19. Endosperm with eight cells. $\times 560$

garded as the antipodal chamber. The nucleus in this lower region is centrally situated and larger than the nuclei of surrounding cells. It soon divides mitotically giving two equal one nucleated cells (Fig. 13). These cells push further down into the chalazal end of the embryo sac and begin to degenerate (Fig. 14). After the endosperm has formed the cells of the antipodal chamber are pushed right down into the chalazal end and degenerate (Fig. 21).

Some exceptions to the above description of the mature embryo sac have been found. In many cases all the antipodal cells of the embryo sac were found to have degenerated (Fig. 15), leaving no traces behind (Fig. 16). The polar nuclei in these embryo sacs moved lower down into a central position. They remained unfused for a long time. The synergids had large vacuoles and much smaller nuclei than those in other parts of the embryo sac (Fig. 16). One embryo sac was found in which no anti-

Fig. 20. Tube-shaped zygote pushing through the endosperm cells. $\times 570$

Fig. 21. Two celled pro-embryo. $\times 570$

Fig. 22. Twin embryo-sacs. $\times 840$

Abbreviations: *pt* — pollen tube; *sn* — synergid nucleus; *end c* — endosperm cell; *end n* — endosperm nuclei; *en* — egg cell nucleus; *z* — zygote; *embr* — embryo

podals had formed. The synergids, the egg apparatus and polar nuclei were gathered in a cluster at the micropylar end. This abnormality may have been caused by the lack of one of the normal mitotic divisions.

An 8-nucleate embryo sac just before the time of the entry of the pollen grain tube usually contains two synergids and two pollen nuclei lying next to each other and not yet fused.

The process of the penetration of the pollen grain tubes into the ovary is of special interest. The pollen tubes do not enter the wide central canal of the style immediately. They pass through the style and penetrate into the central canal at the entrance to the ovary. The pollen

tubes then grow towards the placenta and weaving in and out among the ovules finally reach the micropyle. The pollen tube may either pass next to the synergids when it enters the embryo sac through the micropyle (Fig. 10) or it may pour its contents into one of the synergids destroying the nucleus (Fig. 12). In such cases the other synergid remains intact for a long time. In one case such a synergid could still be observed at the time when the cellular endosperm was formed and the embryo had two cells (Fig. 21).

Double fertilization was seen in an 8-nucleate embryo sac. One of the sperm nuclei was visible next to the wall of the egg cell while the other sperm nucleus was in contact with one of the polar nuclei (Fig. 10). The polar nuclei lay side by side during fertilization. They did not fuse for a long time. In this instance the fertilization of the polar nuclei and of the egg cell nucleus took place at the same time. In other preparations the embryo sac could contain two endosperm cells while the egg cell was still undergoing syngamy (Fig. 11). It also happened that the polar nuclei did not fuse while the egg cell underwent syngamy and the second sperm nucleus stayed in the pollen tube (Fig. 14). Fertilization is probably post — mitotic in this case (Gierassimowa-Navashin). The sperm nucleus lies next to the egg cell nucleus after it enters the egg cell and does not fuse until its size approaches the size of the egg cell nucleus (Fig. 11, 14).

The primary endosperm nucleus divides before the fertilized egg cell nucleus. The first division in the endosperm nucleus can be either perpendicular or parallel to its long axis. The first division in the endosperm nucleus is always longitudinal in embryo sacs which have an antipodal chamber. In embryo sacs in which the antipodals have degenerated the first division is in a transverse plane (Fig. 17, 18). Following this the plane of division changes with each successive division, the next division being longitudinal while the following one is transverse. Two rows of cells result (Fig. 19). The process of endosperm formation is not always as regular as this. Endosperm cells are not always arranged in a given order. The size of their nuclei may differ. When division is regular two endosperm cells travel very far into the micropylar end and replace the synergids on either side of the zygote (Fig. 19).

The zygote divides a long time after the primary endosperm nucleus. For a long time it occupies the position of the egg cell. At a stage when there are eight endosperm nuclei in the embryo sac the zygote has not begun dividing (Fig. 19). The zygote elongates in the course of its development pushing through the endosperm cells and reaching the middle of the embryo sac (Fig. 20). It forms a tube. The zygote nucleus lies in the tip of this zygote tube at this stage. It is of the same size as the endosperm nuclei. The zygote is still undivided when the number of endosperm

nuclei reaches twelve (Fig. 20). The first division in the zygote is transverse. The apical cell is small and the basal cell is long and tube shaped (Fig. 21).

Twin embryos are of rare occurrence in *Pinguicula vulgaris* L. One such case was found. The two embryo sacs lay above one another. One was at the one-nucleate stage while the other one was at the 2-nucleate stage (Fig. 22). They probably originated from two sister megaspores of the same tetrad.

Some cases of retarded development in embryo sacs were noted. One embryo sac no different in size and appearance to the neighbouring ones, in which fertilisation already occurred in fact was at the one-nucleated stage of development i.e. in the course of its first mitotic division.

The proliferating cells found in one of the ovaries might be considered a type of abnormality. They arose from the cells of the inner layer of ovary wall due to abnormally fast growth. These cells had big dimensions but their nuclei were not very large. They grew deep down in between the ovules. These proliferating cells developed only at the entrance to the central canal.

Since the nucellus degenerates very soon in *Pinguicula* and since the embryo sac is naked i.e. surrounded by only one integument, the integuments were supposed to be transparent. A closer investigation using the smear technique and vital staining did not confirm this.

DISCUSSION

Embryo sac development in *Pinguicula vulgaris* L. is of the *Polygonum* type. It is similar to that in other members of this family. There are some generic differences in the structure of the reproductive organs. The ovules are spread over the whole surface of the spherical placenta. The ovules are anatropous, tenuinucellate and have a single integument. The nutritive tissue of the placenta which occurs in *Utricularia* and *Polypompholyx* does not occur in *Pinguicula vulgaris*. The conducting vessels which reach the nutritive tissue of the placenta in *Utricularia*, end blindly in the placental tissue in *Pinguicula*. The integument grows over the nucellus very quickly. The ovule becomes completely anatropous at the megaspore mother cell stage. Its integument grows past the nucellus forming a typical micropyle as in the genus *Genlisea* (Merl 1915). The one-layered nucellus degenerates very soon leaving a naked embryo sac at the 2-nucleated stage. The embryo sac does not extend past the integument as it does in *Utricularia* (Maheshwari 1950) and *Polypompholyx* (Schnarf 1931, Lang 1901). It remains within the ovule immediately under the micropyle.

The integumentary tapetum or endothelium forms a layer connecting the naked embryo sac and the integument. This is so in all the members of the *Lentibulariaceae* family. The endothelium is the only nutritive tissue in *Pinguicula vulgaris*. In the linear megaspore tetrad the chalazal megaspore cell is the one which gives rise to the embryo sac in the same way as it does in the genera *Utricularia* and *Polypompholyx*. The mature embryo sac is 8-nucleated. The position of the nuclei is normal. The antipodals are an exception. Two of the antipodals are pushed right down into the chalazal end while the third one lies above them. The antipodal cells in *Pinguicula vulgaris* do not behave uniformly as they do in *Utricularia* where there is early degeneration (Khan 1954). Two antipodals in *Pinguicula vulgaris* degenerate while the one which lies above them enlarges to fill half the embryo sac. Its nucleus also enlarges and finally divides giving two cells. This third antipodal cell has been called the antipodal chamber due to its origin. Its function is obscure. It has been suggested that it is a chalazal haustorium which originated from the antipodal instead of the division of the primary endosperm nucleus as it does in *Utricularia*, *Polypompholyx* and *Genlisea*. This may be the explanation for the report by Khan on a chalazal haustorium in *Pinguicula vulgaris*. This is not very likely since the cells of the antipodal chamber degenerate quickly. It is possible that two cells of haploid endosperm are formed after division in the antipodal chamber. These cells are destroyed by the true triploid endosperm when it develops.

The endosperm in *Pinguicula vulgaris* does not form three chambers as stated by Merz (1897). These chambers should later give rise to endosperm haustoria and the endosperm proper as happens in *Utricularia*, *Polypompholyx* and *Genlisea*. The endosperm is arranged in two rows of cells. In *Pinguicula vulgaris* there are not any haustoria nor a nutritive tissue in the ovule. In the endosperm division occurs much sooner than in the zygote. In all members of the family the zygote elongates to form a long tube which pushes between the endosperm cells. The first division in *Pinguicula* is transverse. The resulting zygote tube has small apical cell and long basal cell. Only the apical cell seems to participate in the formation of the embryo proper. This would be similar to the course of embryo development in *Utricularia*, although it is difficult to be certain of the exact course of events in either species since there is no differentiation in the mature embryo (Johansen 1950).

In some respects the course of embryo sac development is simpler in *Pinguicula vulgaris* than in other representatives of this family. It is the least specialized species in this family. The endothelium is the only nutritive tissue produced. The embryo sac remains within the ovule and the endosperm does not develop either micropylar or chalazal haustoria.

The feature distinguishing the course of embryo sac development in *Pinguicula vulgaris* from that in other representatives of the *Lentibulariaceae* is the ability to form an antipodal chamber. The possible function of the antipodal chamber is unknown.

SUMMARY

Pinguicula vulgaris ovules cover the whole surface of the spherical placenta and are anatropous. The nucellus degenerates at the 2-nucleated stage of the embryo sac. The ovule is tenuinucellate. The embryo sac is naked. It remains within the ovule. There is one integument which gives rise to the endothelium. The endothelium is the only nutritive tissue in *Pinguicula vulgaris*. The embryo sac development follows the *Polygonum* type. Twin embryos occur rarely. The antipodals either all degenerate or one develop further to form the antipodal chamber. The polar nuclei fuse after the entry of the pollen tube into the embryo sac. Fertilization is post-mitotic. The zygote develops into a long tube before dividing. This tube passes in between the endosperm cells. The endosperm is cellular.

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Rozwój woreczka zalążkowego u *Pinguicula vulgaris* L.

Streszczenie

Zalążki *Pinguicula vulgaris* umieszczone są na całej powierzchni kulistego łóżyśka; są one anatropowe. Nucellus typu cienkoosrodkowego degeneruje już w stadium dwujądrowego woreczka zalążkowego, przy czym nagły woreczek nie wychodzi poza obręb mikropyle, lecz pozostaje wewnątrz zalążka. Woreczek zalążkowy otoczony jest przez pojedynczy integument wykształcający endothelium, które jest jedyną tkanką odżywczą występującą u *Pinguicula vulgaris*. Rozwój woreczka przebiega według typu *Polygonum*. Bliźniacze woreczki występują bardzo rzadko. Antypody degenerują wszystkie, albo zachowuje się tylko jedna z nich, w postaci tzw. komory antypodalnej. Jądra polarne zlewają się po wnikięciu łagiewki do woreczka zalążkowego. Zapłodnienie jest postmitotyczne. Zygota przed podziałem rozwija się w długą rurkę wnikałą między komórki bielma, które jest typu komórkowego.