ACTA SACIETATIS
BOTANICORUM POLONIAE
vol. XXXIX — nr 1
1970

Cytological studies on the regenerating mature female gametophyte of *Taxus baccata* L. and mature endosperm of *Tilia platyphyllos* Scop. in *in vitro* culture

M. A. ZENKTELER and I. GUZOWSKA

INTRODUCTION

The female gametophyte in Gymnosperms and the endosperm in Angiosperms serve as the main source of nutrition for the young developing embryo. In mature seeds the cells of a female gametophyte, of the endosperm as well as of the perisperm are packed full with reserve food materials and do not show mitotic activity. The method of tissue culture *in vitro* enables us to investigate the requirements of storage tissues and to find whether they can regenerate in artificial conditions.

In recent years attempts have been made to grow immature and mature endosperm in *in vitro* culture. Successful attempts to culture immature endosperm were carried out on the following species: *Zea mays* (LaRue 1947, 1949; Tamaoki and Ulstrup 1958; Straus 1954, 1960), *Asimina triloba* (Lampton 1952), *Lolium perenne* (Norstog 1956), *Cucumis sativus* (Nakajima 1962). It is more difficult to induce regeneration of endosperm from mature seeds, however, several reports concerning the following species have also been published in the last few years: *Santalum album* (Rangaswamy and Rao 1963), *Ricinus communis* (Asha Satsagi and Mohan Ram 1965), *Exocarpus cupressiformis, Osyris wightiana, Putranjiva roxburghii, Jatropa panduraefolis* (Johri and Bhojwani 1965), *Croton bonplandianum* (Bhojwani 1966), *Oxalis dispar* (Sunderland and Wells 1968). It results from the above mentioned papers that while immature endosperm can be grown in the absence of the embryo, mature endosperm needs the association of the embryo for its proliferation. The culture of endosperm of Exocarpus cupressiformis deserves particular attention. A modified White's medium containing kinetin (1 p.p.m.), indolylacetic acid (1 p.p.m.), casein hydrolysate (400 p.p.m.) and 2% sucrose constituted the basal medium. Entire endosperm with intact embryos was inoculated and after 5 weeks of culture shoot buds developed superficially from the endosperm tissue. The shoot buds had well differentiated vascular bundles and several foliar primordia which contained chlorophyll. It was ascertained that the shoots were triploid.

There are but few papers dealing with *in vitro* culture of the female gametophyte of Gymnosperms. Callus formation was induced in explaned megagametophytes of *Zamia integrifolia* (LaRue 1948; Norstog 1965) and *Pinus lambertiana* (Borchert 1968). Investigations on *Zamia* were carried out in order to obtain haploid sporophytes. Norstog made a detailed study of the initiation of callus, embryoids, roots and leaves from the gametophyte tissue grown *in vitro*. All nuclei of the callus tissue obtained from the female gametophyte of *Pinus lambertiana* underwent diploidization during continued culture.

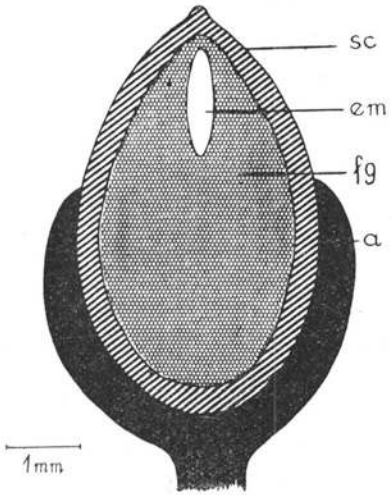
As it results from a review of the literature there are only few reports on the morphogenetic response of endosperm *in vitro*. From among Angiosperms only the endosperm of *Exocarpus cupressiformis* was induced to produce triploid shoots. There are no data as to whether these shoots were able to grow into normal triploid plants. The finding of a method for obtaining a triploid plant from the endosperm culture would be a great success not only from the experimental point of view but, above all, for practical use. The purpose of the present investigation was to culture the endosperm of several species of the Polish flora in order to study their morphogenetic potentials.

MATERIAL AND METHODS

For the preliminary experiments the following species which produce seeds with massive endosperm were used: *Taxus baccata, Larix europaea, Polygonatum multiflorum, Corydalis cava, Asarum europeum, Daphne mezereum, Helleborus niger, Tilia platyphyllos, Evonymus verrucosa.* Endosperms with embryos were inoculated on various media and cultured for several months. In these experiments it was ascertained that only the endosperm of *Tilia, Evonymus* and the female gametophyte of *Taxus* show a tendency to regeneration. The endosperm of the remaining species aborted after several weeks of culture. Since callusing of the endosperm of *Evonymus* occurred only in about 1% of the explanted cultures, this material was not included in our paper.

Mature seeds (Figs 1, 2) freshly harvested in December 1965 from *Taxus baccata* and *Tilia platyphyllos* grown in the Botanical Garden (University, Poznań) were surface-sterilized in chlorine water for 15 minutes and then washed thoroughly with sterile distilled water. The seed coats were removed aseptically and the female gametophytes as well as the endosperm containing whole embryos were planted on agar medium into 100 ml Erlenmayer flasks each containing 25 ml of medium. A modified White's medium (Rangaswamy 1961) containing 2% sucrose and 0.8% Difco bacto agar constituted the basal medium (BM). Casein hydrolysate (CH, 500 mg/l) and 2,4-dichlorophenoxyacetic

acid (2,4-D, 5 mg/l) were used as supplements. Before this medium was chosen as the most suitable for endosperm regeneration, the following supplements — separately and in various combinations — were added to BM: kinetin, indolyleacetic acid, yeast extract, casein hydrolysate, 2,4-D, and corn milk. It was found that only BM with CH and 2,4-D used in the above mentioned proportions induced callus formation in the endosperm. Before autoclaving the medium, pH was adjusted to 5.8. All cultures were kept under continuous diffused light at +24°C. In 10—15% of the cultures of *Tilia* and about 2—5% of those of *Taxus*



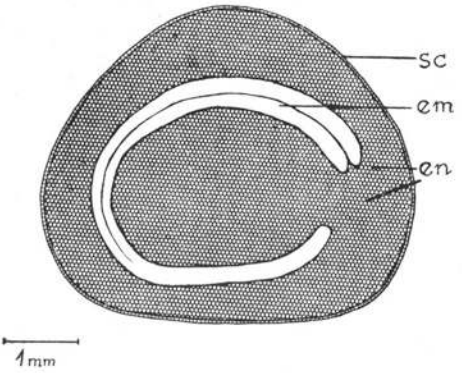


Fig. 1. Schematic drawing of mature seed of *Taxus baccata*
sc — seed coat; *fg* — female gametophyte; *em* — embryo; *a* — aril

Fig. 2. Schematic drawing of mature seed of *Tilia platyphollos*
sc — seed coat; *en* — endosperm; *em* — embryo

the endosperm proliferated and produced callus. The remaining cultures showed no signs of regeneration even after 7 months of culture. The first callus was transplanted after 18 weeks of growht, the next ones at 8—10-week intervals. Callus tissues were cultured for 3 years and during this period they were transferred 15 times (15 passages). The callus obtained from the endosperm of *Tilia* grew faster and more vigorously than that from the female gametophyte of *Taxus*.

Explants and fragments of callus from every passage were fixed in AA and FAA. The standard paraffin method was used for processing the FAA-fixed material. Microtome sections 5—18 μ thick were stained by: Feulgen's method with light green counterstaining, iron hematoxylin after Heidenhain with fast green counterstaining, Azure B, and crystal violet with orange G. Proteins were localized by applying the mercuric

bromophenol blue method (M a z i a 1953) and by staining with a solution of iodine in potassium iodide. Lipids were localized with Sudan III and Sudan IV by staining the free-hand sections. Polysacharides were demonstrated by the periodic-acid—Shiff (PAS) reaction of Hotchkins (J e n s e n 1962) and of iodine in potassium iodide. Starch grains were also observed under the polarizing microscope. In order to identify the chemical nature of the substances which filled some of the callus cells of *Taxus* the following staining reactions were applied: 1. for tannis — 5% solution of calcium hypochlorite, 1% solution of ferric sulphate in 0.1 N HCl, 10% formalin containing 2% ferric sulphate; 2. for pectic substances — an aqueous solution of ruthenium red (1:500); 3. for callose — 0.005% solution of resorcin blue (J e n s e n 1962). The material for smears was fixed in AA and stained in alcoholic hydrochloric acid carmine (S n o w 1963).

OBSERVATIONS

1. *Taxus baccata*

The mature seed contains a small embryo (1.5 mm) surrounded by a massive female gametophyte. The basal part of the thick and hard seed coat sticks in the fleshy aril (Fig. 1). Female gametophyte cells are multinucleate. The number of nuclei per one cell ranges from 2 to 10 (Fig. 8). The entire chromatine content of nuclei was condensed and formed a compact ball, therefore, after staining with crystal violet (Fig 10) nuclei can be seen as homogeneous, dark-stained bodies. By applying basic fuchsine it was much easier to distinguish the chromonema reticulum (Fig. 9). The cells are rich in reserve materials. Lipids dominate throughout the tissue, protein bodies of varying shape and volume are also present. Starch grains are not so abundant as lipids and proteins. The gametophyte forms on the periphery a distinct unicellular layer composed of mostly binucleate cells (Fig. 8).

The female gametophyte of *Taxus* was explanted alongside with the embryo. During the whole time of culture the embryo did not show any sings of germination. Regeneration of the gametophyte proceeded very slowly. After about 10 weeks, very delicate, loosely arranged friable callus cells started to appear on its surface. After 18 weeks of growth the callus tissue was transfered on a fresh medium. In cells of the first passage starch grains and few protein bodies were found. After 27 weeks of growth, only starch grains occurred in the callus cells. They were present in the tissues of all the further passages up to the last 15-th one.

The growth of very delicate, friable callus cells which observed macroscopically after 14 weeks of culture was also ascertained on histological preparations. Large loosely arranged cells of parenchymatic

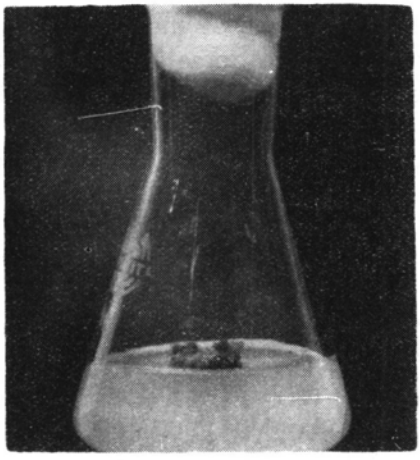


Fig. 3

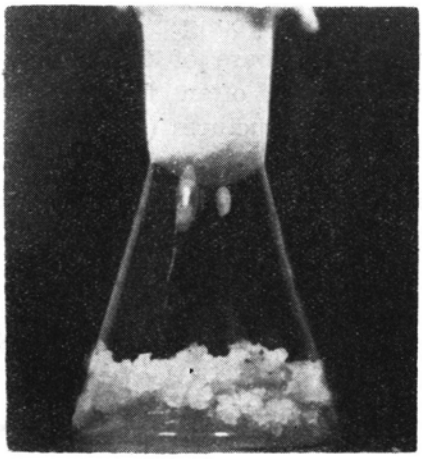


Fig. 4

Fig. 3. Fifteenth passage (3 weeks after transplantation) of the female gametophyte callus tissue of *Taxus* after 160 weeks of culture on BM + 2.4-D (5 mg/l) + CH (500 mg/l)

Fig. 4. Fourteenth passage (8 weeks after transplantation, of callus tissue of *Tilia* after 150 weeks of culture on BM + 2.4-D (5 mg/l) + CH (500 mg/l)

character were often separated from each other. They contained one nucleus and a small amount of cytoplasm disposed along the cell walls. The structure of nuclei is worth special attention. They were much bigger than those present in the explanted material, however, they stained very weakly in basic fuchsine and Azure B. The nuclei exhibited some variations in shape at all stages of the female gametophyte culture. Some of the observed shapes were spherical, elliptical, branched and even whip-like. The nucleoli also exhibited some variation in shape and size. The number of nucleoli per nucleus ranged from one to several. The presence of a big nucleus with more than one nucleolus suggests that a fusion of several uninucleolar nuclei occurred in the cells.

Among the loosely arranged cells irregularly distributed foci of small cells were present. They were rich in cytoplasm and possessed spherical nuclei which stained intensively by Feulgen method. Fig. 11 shows cell foci in the callus of the 6-th passage after 55 weeks of culture. Some cells of the callus were completely filled with homogeneous material (Fig. 11) which was also present in free spaces between cells. A test with ruthenium red reagent indicated the presence of mucilage substances of a pectic character. The number of cells with these substances was much higher in later passages. It was also found that in later passages there were more cells with thickenings than in the callus tissue of the first three passages. The single cells of the 4-th passage callus possessed spiral and reticulate thickenings. In the following passages whole groups of vascular bundles occurred (Fig. 12). Chloroplasts were

conspicuous in younger cells at the periphery zone of the callus tissue, however, the base of the callus turned brown. Some of the loosely arranged cells often formed cell-aggregates simulating embryo-like (embryoids) structures (Fig. 13).

The somatic number of chromosomes is 24 in *Taxus baccata*. Polyploidy develops in the callus cells. Variation in chromosome number from n to 8n was found in calluses of the 14-th and 15-th passages

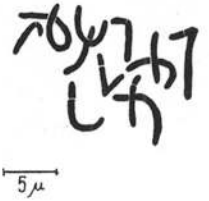


Fig. 5. Haploid metaphase plate (n = 12) in callus tissue of *Taxus baccata* (14-th passage)

(Figs 5, 14). The frequency of n and 2n was relatively low. Fig. 15 shows a diploid metaphase plate (2n = 24) in a cell filled with starch grains. The mitotic figures were common in the young peripheral cells of callus, while in the deeper regions they were much less frequent. The callus tissue has so far been maintained in an active state of growth for 160 weeks, through 15 passages (Fig. 3).

2. *Tilia platyphyllos*

The mature seed contains massive cellular endosperm and a long (0.8—1.0 cm) curved embryo (Figs 2, 16). Uninucleate endosperm cells are rich in food reserves such as proteins, oil globules and starch grains (Fig. 17). Oil globules are uniformly distributed in the cells of the whole endosperm. Proteins and starch grains are distributed more specifically. In the periphery layers of the endosperm there are many small starch grains. As regards the distribution of starch grains and protein bodies the endosperm can be divided into 3 layers: the periphery one with starch only, the transitory one with starch and proteins and the inner one with proteins only.

The embryo after one week of culture swelled and enlarged, the hypocotyl elongated, the cotyledons emerged from the endosperm and they turned green. However, the embryos never developed into seedlings. During the first 4 weeks of culture no visible changes were revealed in the structure of the endosperm cells. After 5—6 weeks the endosperm started to produce a white fragile callus. Histological preparations revealed on the endosperm surface a delicate, fluffy white tissue composed of single loosely arranged cells (Fig. 19). They originated from

cells of the peripheral layer of endosperm. Moreover, in the same layer there developed meristematic nodules composed of intensively dividing cells from which callus tissue was formed (Fig. 18). Callusing took place only from the peripheral layer of endosperm. Owing to this, in the first callussing cells and in the meristematic nodules there were only starch grains. The callus cells contained many starch grains in the first and second passage. Beginning with the third passage, there was much less starch in the cells and after 8 months of culture no storage materials were present in the callus.

In the explanted endosperm and in the callus tissue of the first passage all the cells were uninucleate. After 18 weeks of culture in many cells two nuclei were observed (Fig. 20), and they were present in all the next passages up to the 14-th which is still in culture. Endosperm

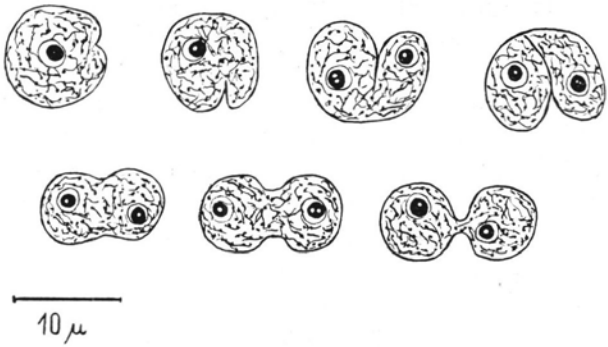


Fig. 6. Nuclei in various stages of amitosis in callus tissue of *Tilia platyphyllos* (13-th passage)

tissue has so far been maintained in culture for the last 150 weeks (forteen passages, Fig. 4). The size of the callus cells and their nuclei vary in shape and volume. Sometimes large cells with more than two nuclei were found. In Figure 22 of the four nuclei only three are visible.

After 18 weeks of culture some of the nuclei were found to undergo amitotic division or fragmentation. Usually there appeared a narrowing or constricted region in the middle of the nucleus (Fig. 21). Gradually this constriction became more marked, it grew deeper and finally the nucleus broke into two parts (upper row in Fig. 6). When a nucleolus was present in the constricted region it also underwent changes similar to those of the nucleus. In some cells nuclei became constricted in such a way that they settled into an ameboidal shape and finally they broke into separate fragments (lower row in Fig. 6).

The somatic number of chromosomes is 82 in *Tilia platyphyllos*. Alcohol-carmine smears and the paraffine preparations were examined for mitotic figures in the 1-st and 14-th passage. In the 1-st passage of

callus the analysis of metaphase plates revealed the presence of 82 (2n) and 123 (3n) chromosomes. The chromosome counts of nuclei in the 14-th passage revealed the presence of tetraploid metaphase plates, though triploid plates were also found. Sometimes the aneuploid metaphase

Fig. 7. Aneuploid metaphase plate (160 chromosomes) in callus tissue of *Tilia platyhpyllos* (14-th passage)

plates were counted and in these cases the number of chromosomes ranged from 150 to 170 (Fig. 7). Abnormalities in mitosis like the occurrence of bridges, lagging chromosomes and multipolar spindles were not observed. The chromosomes of the callus cells were quite small, nearly ovoid or only little elongated.

DISCUSSION

The female gametophyte of *Taxus* and the endosperm of *Tilia* are relatively undifferentiated tissues and their role is mainly nutritive. Abundant food materials for the development of the embryo are present, therefore, at the time of inoculation these storage tissues represented a mass of cells packed with starch grains, oil globules and protein bodies. During normal germination the embryo grows at the expense of the endosperm, but in the *in vitro* culture, in the presence of a nutritive medium, the growth of the embryo was inhibited and that of the endosperm stimulated. The seed storage tissues of both species need an addition of 2,4-D and casein hydrolysate for continuous growth. Our investigations have shown that the activation of the mature female gametophyte and endosperm depends not only on the substances added to the medium but, above all, on the presence of the embryo which was inoculated together with the tissues. According to Bhojwani (1965) the activation of mature endosperm depends on some metabolites produced by the embryo. The calluses of *Taxus* and *Tillia* are capable of continuous growth, however, organ formation, has not been obtained. The differentiation of thick-walled cells and formation of embryoids

which have been observed in all passages of *Taxus* were also noticed in some other investigations concerning endosperm culture (Asha Satsangi and Mohan Ram 1965; Johri and Bhojwani 1965; Bhojwani 1966).

The calluses obtained from the female gametophyte of *Taxus* and from the endosperm of *Tilia* have been found to be suitable materials of studying the various levels of ploidy in individual passages. Owing to the shortage of material it was not ascertained which part of the female gametophyte was induced to produce the first callus tissue. The diversity of the number of nuclei in the female gametophyte explants and in the callus cells needs a special explanation. It is suggested that the uninuclear cells of the callus tissue may be derived from fusion of several nuclei which were present in the cells of primary explants. The size of the nuclei in the callus cells and their high ploidy support this presumption.

To the successful investigations on *in vitro* culture of endosperm which included cytological observations, belong those of Straus (1954) and Norstog (1956). Straus cultured the endosperm of maize and obtained chromosome numbers which were up to 7-ploid with respect to the tissue of origin. He also described the occurrence of aneuploidy, bridges, fragments, lagging chromosomes and haploid nuclei. Norstog cultured the endosperm of rye grass, and also found polyploid nuclei. The cells of callus obtained from explanted megagametophyte of *Pinus lambertiana* (Borchert 1968) showed different polyploid nuclei which ranged from C level up to 8 C. Our material also showed a high variation in ploidy, however, no chromosomal aberrations were observed. Callus cells derived from female gametophyte of *Taxus* possessed nuclei the polyploidy of which ranged from n to 8n. The variation of polyploidy in the cells of callus obtained from *Tilia* endosperm was much smaller and usually the number of chromosomes in the metaphase plates was equal to n, 2n, 3n, and 4n.

The successful results of Norstog (1965) in obtaining haploid organs when culturing the female gametophyte of *Zamia integrifolia* encourage to make further studies on other species of Gymnosperms. The megagametophyte of Gymnosperms may prove quite suitable for *in vitro* studies and as a haploid tissue may be used to produce haploid plants. In our experiments we were not able to induce the mature female gametophyte and the mature endosperm to produce organs. It is suggested that these tissues should be excised before the reserve food materials become fully deposited, that is at a stage when the cells are in the meristematic phase. The potential ability of storage tissues cells to produce organs and to reconstruct the life cycle of the plant when given the necessary nutritional and enviromental conditions should be studied further in different plants.

Plate I

Figs. 8—15. *Taxus baccata*

Fig. 8. Fragment of the female gametophyte tissue.
pl — peripheral layer composed of 2-nucleate cells; *mc* — multinuclear cells.

Fig. 9. Cell of female gametophyte.
n — nuclei stained by the Feulgen reaction (phase contrast).

Fig. 10. Cell of female gametophyte.
n — nuclei stained with crystal violet.

Fig. 11. Fragment of callus tissue (3-rd passage).
n — foci of small cells with dense cytoplasm. *m* — cells filled with mucilage substances.

Fig. 12. Vascular bundle in callus tissue (15-th passage).

Fig. 13. Cell configuration simulating embryoid in callus tissue (3-th passage).

Fig. 14. Octoploid metaphase plate (8n = 96) in the callus cell (15-th passage).

Fig. 15. Diploid metaphase plate (2n=24) in the cell filled with starch grains.

Plate II

Figs. 16—22. *Tilia platyphyllos.*

en — endosperm; *em* — embryo.

Fig. 17. Storage materials in endosperm cells
s — starch grains; *p* — protein bodies; *l* — lipid droplets.

Fig. 18. Meristematic nodules in the peripheral layer of endosperm after 6 weeks of culture.

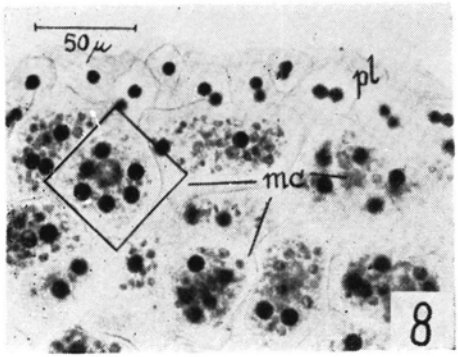
Fig. 19. Development of callus tissue from the peripheral layer of endosperm after 6 weeks of culture.
c — callus cells; *en* — endosperm.

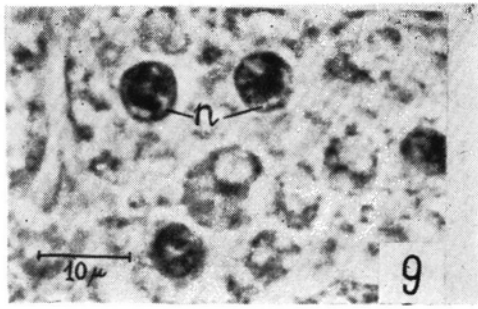
Fig. 20 Callus cell with two nuclei (13-th passage).

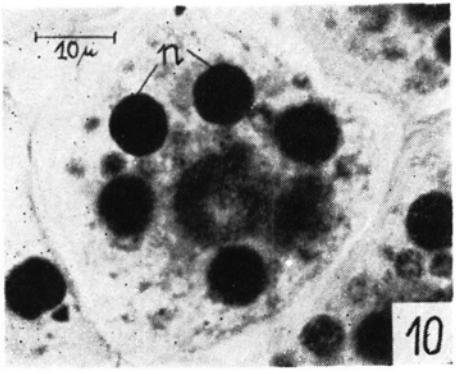
Fig. 21. Nucleus in amitotic division (14-th passage).

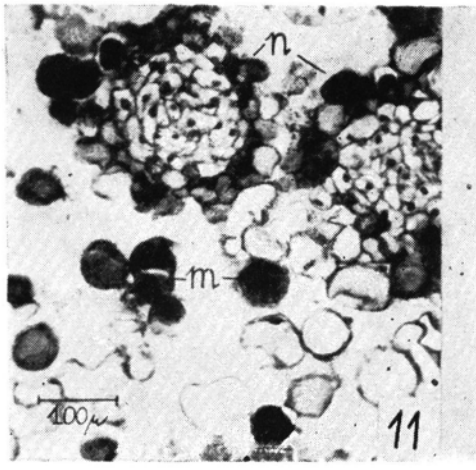
Fig. 22. Giant cell of callus tissue with 4 nuclei; 3 nuclei are visible (2-nd passage).

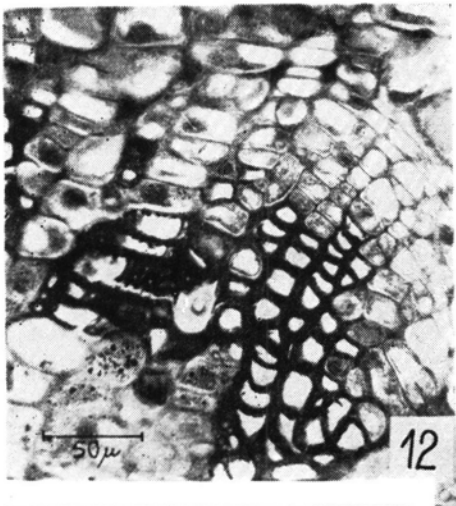
Plate I

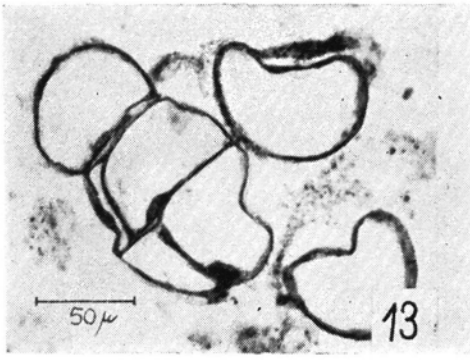


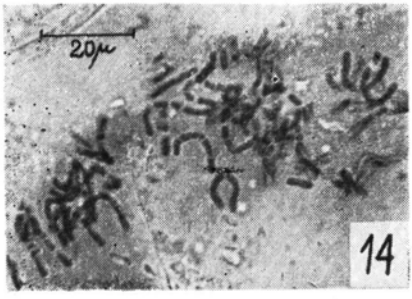


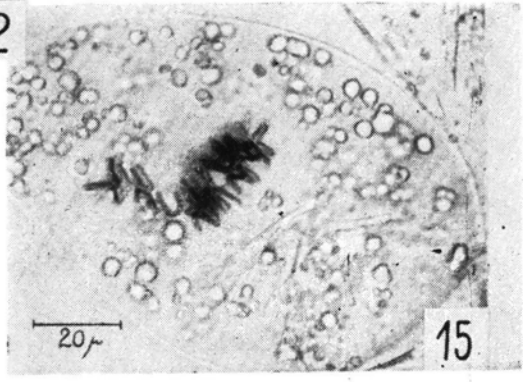












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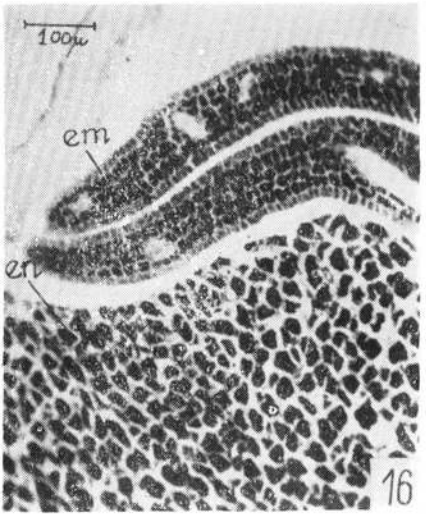

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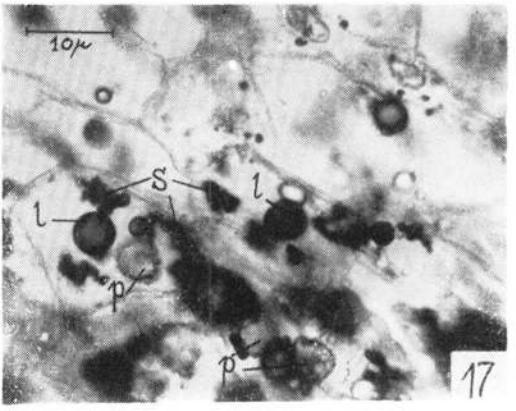

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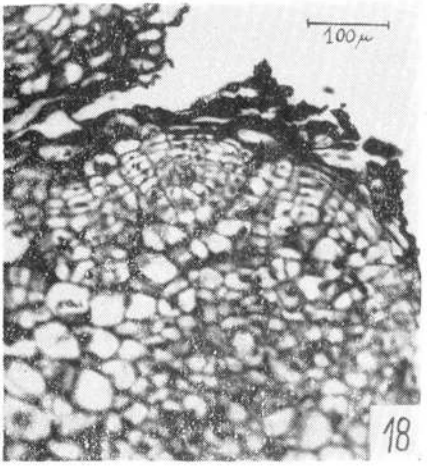

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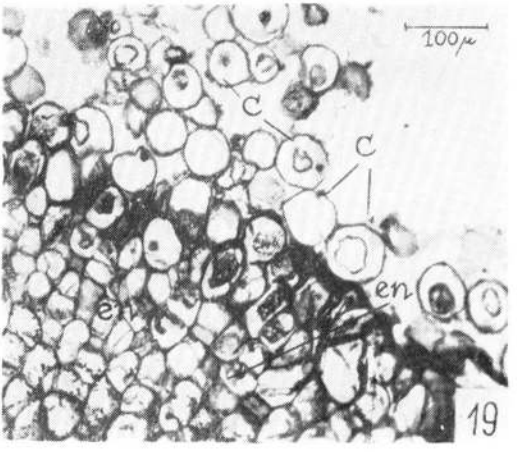

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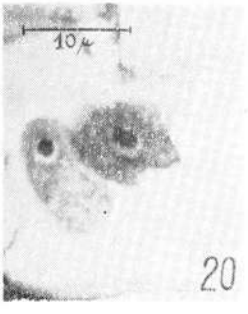

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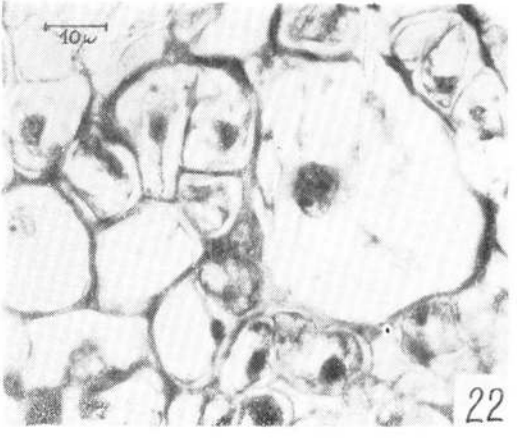

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SUMMARY

Female gametophyte from mature seeds of *Taxus baccata* and endosperm tissue from mature seeds of *Tilia platyphyllos* were planted on White's medium with 2,4-D and casein hydrolysate. Continuously growing tissues have so far been maintained for 160 weeks, through 15 passages. Callus tissue derived from female gametophyte was composed of large, loosely arranged cells of a parenchymatic nature, with irregularly distributed foci of small cells. Some cells of the callus were packed full with mucilage substances. The differentiation of thick-walled cells with spiral and reticulate thickenings, the formation of vascular bundles and embryo-like structures have also been noticed in callus tissue. Callus cells derived from the endosperm of *Tilia* were composed of parenchymatic cells only. Organ formation has not been obtained either in female gametophyte of *Taxus* or in the endosperm of *Tilia*. Cytochemical reactions have been carried out in order to study the distribution of reserve food materials in the explants and in individual passages. In callus obtained from the female gametophyte the cells were uninuclear and the chromosome number ranged from n to 8n. Callus cells derived from the endosperm of *Tilia* possessed nuclei the polyploidy of which ranged from n to 4n. Amitotic divisions were observed in some nuclei, and more than two nuclei occurred in large cells.

The authors wishes to express their gratitude to Professor A. Szweykowska, Head of the Department of General Botany, for the facilities provided during the course of this work and for reading criticaly the manuscript. Our thanks are due to Professor St. Krupko for helpful discussions.

Department of General Botany,
Adam Mickiewicz University,
Poznań, Poland

(Entered: August 25, 1969.)

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Badania cytologiczne nad regenerującym w hodowli in vitro dojrzałym gametofitem żeńskim Taxus baccata L. i dojrzałym bielmem Tilia phatyphyllos Scop.

Streszczenie

Żeński gametofit (prabielmo) *Taxus baccata* i bielmo *Tilia platyphyllos* pochodzące z dojrzałych nasion hodowano wraz zarodkiem na pożywce mineralnej White'a zawierającej witaminy wg Rangaswamy i 2% sacharozy z dodatkiem 2,4-D (5 mg/l) i hydrolizatu kazeiny (500 mg/l).

Po około 6 tygodniach u *Tilia* i po 10 tygodniach u *Taxus* rozpoczął się proces regeneracji tkanki bielmowej i prabielmowej, w wyniku czego otrzymano kalusy charakteryzujące się nieograniczonym wzrostem, które w ciągu 160 tygodni hodowli przeszczepiono 15 razy. Przeprowadzono badania cytologiczne eksplantatów i transplantatów z poszczególnych pasaży. Wyszczepione wraz z bielmem zarodki okazały się konieczne dla kalusowania bielma, ale same nie rozwijały się i nie wytwarzały tkanki kalusowej.

Zastosowane w pracy reakcje cytochemiczne wykazały obecność w eksplantatach dużych ilości materiałów zapasowych takich jak: białka, tłuszcze i skrobia.

Tkanka kalusowa otrzymana z prabielma *Taxus* zbudowana jest z luźno ułożonych 1-jądrowych komórek o charakterze miękiszowym, zawierających duże

ilości ziaren skrobi, wśród których znajdują się 1) gniazda małych ułożonych zwarcie komórek wypełnionych gęstą cytoplazmą i dużymi kulistymi jądrami, 2) pojedyncze komórki wypełnione całkowicie śluzami pektynowymi, 3) grupy silnie zdrewniałych elementów z spiralnymi lub siateczkowatymi zgrubieniami ścian, 4) utwory przypominające embrioidy.

W kalusie otrzymanym z bielma *Tilia* następował od trzeciego pasażu stopniowy zanik materiałów zapasowych.

W komórkach kalusa *Taxus* liczba chromosomów wynosi od 12 (n) do 96 (8n). Komórki kalusa *Tilia* są jedno-, dwu- lub kilkujądrowe. W niektórych jądrach obserwowano podziały amitotyczne. Liczba chromosomów wynosi od 42 (n) do 168 (4n). Występowały także płytki o aneuploidalnej liczbie chromosomów.