Studies on the androgenesis in cultured anthers of *Atropa belladonna* L.

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Abstract

Embryological investigations were carried out on developing anthers of *Atropa belladonna* grown in natural conditions and on anthers which produced androgenic embryos in the *in vitro* culture. The anatomy of developing anthers was analyzed in details. Meiotic abnormalities were not detected and 36 bivalents were present at metaphase of meiosis I. About 90% of pollen grains were normally developed. Anthers inoculated at the tetrad or microspore stage and cultured on Linsmaier and Skoog medium with kinetin 4 mg/l and IAA — 2 mg/l produced androgenic embryos. Differences in the development of septum, in the morphology of pollen grains, formation of tapetum, development of proembryos and the occurrence of storage materials were recorded. The origin of autopolyploid plants from haploid cells is discussed.

INTRODUCTION

It is possible to produce haploid and autopolyploid plants from the *in vitro* cultured anthers. Experiments on the culture of anthers were undertaken by a number of workers, however, the development of embryos and plants from the male gametophyte was successful only in few genera: *Datura* (Guha and Maheshwari, 1964, 1966; Narayanaswamy and Chandy, 1971), *Nicotiana* (Nakata and Tanaka, 1968; Nitsch and Nitsch, 1969), *Oryza* (Niizeki and Oono, 1968), *Brassica* (Harn, 1971), *Asparagus* (Raquin and Pelletier, 1972), *Hordeum* (Clapham, 1971), *Solanum* (Kameya and Hinata, 1970; Zenkteler in press), *Atropa* (Zenkteler, 1971), *Lycium* (Zenkteler, 1972), *Lycopersicon* (Gresshoff and Doy, 1972).

The most suitable time for the induction of embryos from the male gametophyte is to inoculate anthers at the microspore stage. The deve-

lopment of embryos from tetrads and 2-nucleate pollen grains occurs rarely (Sunderland 1971). At present, there are no informations about the induction of embryos from the pollen mother cells at the stage of meiosis.

As it was shown in the previous report (Zenkteler 1971) anthers of *Atropa belladonna* when inoculated on a suitable medium produced embryos in a large number. Moreover, seedlings obtained from these embryos were haploids, diploids, tetraploids, triploids and pentaploids. The following work was initiated to investigate the process of androgenesis in *Atropa* by means of the anther culture. The development of anthers in natural conditions was studied also for comparative purposes.

MATERIAL AND METHODS

Flower buds at different stages of development from plants growing in the Botanical Garden of the Poznań University were collected and fixed in the Navashin fixative in August 1971. Researches of the androgenic process in the in vitro culture were carried out on anthers inoculated in May 1971 on medium prescribed by Linsmaier and Skoog (1965) with kinetin — 4 mg/l and IAA — 2 mg/l. Totally, 2000 anthers were inoculated at the stage of tetrads and microspores. About 120 anthers were fixed in FAA after the following periods: a) tetrads — 5, 10, 28 and various higher number of days till the 56-th day; b) microspores — 8, 12, 28, 49 and various higher number of days till the 56-th day. Totally, 99 anthers cultured in vitro were investigated. of these 45 were inoculated at the tetrad stage and 54 at the microspore stage.

The standard paraffin method was used for the processing of the fixed material. Microtome sections 12 μ m thick were stained by Feulgen's method with fast green counterstaining; crystal violet with orange G; iron hematoxyline after Heidenhein with fast green counterstaining. Storage materials in anthers were localized by the following cytochemical methods: 1) Proteins by applying the mercuric bromophenol blue method (Mazia 1953); Lipids by staining with Sudan IV; Starch grains by staining with a solution of iodine in potassium iodide.

RESULTS

I. The development of anthers in natural conditions

An anther of *Atropa belladonna* is composed of two lobes, each one comprising two pollen sacs separated by a septum with two small protrusions. The septum is formed by cells of the connective tissue. On the cross-section of an immature anther following layers of cells can be

Fig. 1. A cross section of an anther with pollen mother cells at the meiotic stage. *ep* — epidermis; *end* — endothecium; *ml* — middle layer; *t* — tapetum; P.M.C. — pollen mother cells

Fig. 2. A cross-section of an anther with pollen mother cells at the second meiotic division. Tapetum with nuclei having several nucleoli, two layers of cells in some parts of the anther (Explanations as in Fig. 1)

distinguished: a) one layer of epidermal cells; b) three layers of cells which at the microspore stage will start to develop fibrous thickenings, thus forming the endothecium; c) one middle layer composed of very small cells; d) one innermost layer of the tapetum comprised of cells with a dense cytoplasm, small vacuoles and two or more nuclei which

Fig. 3. A schematic cross-section of an anther at the microspore stage. Endothecium with developing fibrous thickenings and disappearing tapetum;
m — microspores; *st* — intact stomium; *ps* — two protrusions of the septum; *vb* — vascular bundle

Fig. 4. A cross-section of an anther at the 2-celled pollen grains stage; endothecium with well developed fibrous thickenings and residues of the tapetum layer;
pg — pollen grain.

by the end of the second meiotic division may contain a dozen or more nucleoli (Figs. 1, 2). Some cells of the tapetum divide and form a second layer which is distinctly visible at the second meiotic division and at the tetrad stage (Fig. 2).

Division stages in pollen mother cells showed 36 bivalents at metaphase of meiosis I. Meiosis proceeded normally and no cytological irregularities in pollen mother cells were observed. Microspores are of a spherical or an elliptical shape, only a few of them display signs of degeneration.

During the microspore stage the endothecium develops, the middle layer is already absent, while the secretory tapetum begins to degenerate. The connective tissue, previously separating the pollen sacs, is starting to disappear and consequently the anther's lobe comprise only one big cavity with an intact stomium. In the meantime, two protrusions of the septum are enlarging (Fig. 3). At the stage of 2-celled pollen grains the endothecium cells contain well developed fibrous thickenings, four to five layers of cells are present in the region of the connective tissue, and only one layer can be distinguished in the region of the stomium. At the time of dehiscence the anther's wall is composed only of the epidermal tissue and the endothecium. Tapetum has already vanished. The 2-celled pollen grains are of a similar shape and size, about 10% of pollen grains with strongly contracted protoplasts were classified as degenerated (Fig. 4).

II. The structure and anther development in the in vitro culture

Anthers cultured on the same medium developed in two different ways:

1. The septum became contracted and in some anthers the following types of pollen grains were distinguished: a) developing into embryos, b) fully packed with starch, c) strongly vacuolized, d) forming pollen tubes of different width and length.

2. Septum enlarged strongly and pollen grains underwent a degeneration.

In natural conditions, when pollen grains reached the 2-celled stage, anther dehiscence occurred in the region of the stomium. In the culture as the process of androgenesis took place some anthers dehisced after about 6 weeks since the inoculation. In natural conditions anthers have an almost completely degenerated tapetum by the time of reaching the 2-celled pollen grain stage (Fig. 4). In culture, tapetum in the form of a narrow strand of cells rich in cytoplasm was still present after 49 days. A remnant layer of this tapetum was also observed in those anthers inside which androgenic embryos have been formed (Fig. 5). In some anthers inoculated at the tetrad stage and fixed after four weeks certain

Fig. 5. A schematic cross-section of an anther with a proembryo (p).

Explanations as in Figs. 1—3.

tapetum cells were excessively enlarged showing signs of degeneration. Those lobes comprised of normally developed pollen and groups of compact pollen with degenerated protoplasts (Fig. 6). It is not clear in which direction of development these anthers would proceed.

Anthers with a complete atrophy of tapetum but with normally developed microspores or 2-celled pollen grains also were found, which was similar to the control material. Pollen grains spherical and elliptical in shape were found in the cultured anthers (Fig. 5). Worth of stressing is the fact that pollen grains were of a different size and their nuclei, even when stained by the same method, absorbed the staining with a various intensity. In certain anthers inoculated at the microspore stage and fixed after 28 days pollen grains were empty and the tapetum was represented only by a narrow strip of degenerated cells.

As the result of the mitotic division of the generative cell 3-celled pollen grains, comprised of a vegetative cell and two male gametes were developed. Some pollen grains were strongly vacuolized and enlarged, while others were abundantly packed with starch. A number of pollen grains among those which were free of starch started to form pollen tubes. All types of pollen mentioned above differed one from another by the size and the intensity of staining of their nuclei (Figs. 7 a—n).

Fig. 6. A cross-section of an anther with an enlarged tapetum (t).

Explanations as in Figs. 1-3.

In some cases the septum enlarged excessively and pressed the normally developed pollen grains to the anther's wall giving in consequence completely degenerated pollen. In those pollen which divided mitotically and formed embryos, starch was not observed. Usually, the process of androgenesis occurred only in spherical pollen while the elliptical ones degenerated.

The process of androgenesis started to occur 8 to 10 days after the inoculation. The first step of this process was the mitotic division of the vegetative and generative nucleus and the formation of a 4-nucleate pollen grain. Two small compact nuclei devoided of nucleoli have been considered as the male gametes, the two remaining ones, much larger and having nucleoli, probably derived from the vegetative nucleus (Figs 7 c, d). These large nuclei underwent a mitosis division. (Fig. 7 e). A division of the male gametes was never observed and due to this fact it is assumed that in the process of androgenesis only the vegetative cell participated. In the early stages of androgenesis pollen grains were multinucleate and the cell walls appeared later. It is difficult to determine at which stage of the embryo development the cell walls were

formed, however, usually at the stage of 6-nuclei, cell walls were already present.

Multinucleate pollen grains with the cytoplasm stained almost as intensively as nuclei were found in those anthers which have been cultured longer than 49 days. In anthers inoculated at the microspore stage and fixed after 49 days several-celled proembryos were laying inside the lobe. It is worth to note that some small proembryos, composed of 22 cells, were still enclosed by the exine, whereas other very young proembryos composed of only 8 cells, were almost completely deprived of exine (Figs. 8 a, b).

Proembryos differed one from another not only by the number of cells and the presence or absence of the exine but by the size and shape of nuclei and the number of nucleoli. All proembryos can be classified into 4 groups according to the following features: a) a finely vacuolized cytoplasm, small nuclei, each one containing one nucleolus (Figs. 8 a, 9); b) a highly vacuolized cytoplasm and small ameboidal — like nuclei (Fig. 8 c); c) a finely vacuolized cytoplasm, medium-sized nuclei with one nucleolus (Fig. 8 e); d) a finely vacuolized cytoplasm, large nuclei of an irregular shape and size, each one containing several nucleoli (Fig. 8 b). Some proembryos and embryos have suspensor — like structures. In the case of the globular proembryo this structure can be still enclosed by the exine, while in the later stages of development exine was absent (Fig. 10).

Considerable variations were observed in regard to the size of the proembryos and embryos. Usually in one anther lobe several androgenic proembryos and embryos at different stages of development were present. While some pollen grains had already developed into plantlets, others were still at the proembryonic stage. During their early ontogeny they were of a spehiral or an ellipsoidal shape, later they differentiated into the heart, torpedo and cotyledonary stages. The development of embryos was not always a synchronous process in both lobes. Sometimes in one lobe pollen grains were completely degenerating, while in the other one numerous embryos were formed.

Fig. 7. Pollen grains in anthers after 8 days of culture.

a — 2-celled pollen grain with a vegetative (v) and generative (g) nucleus
b — the same as a; small vacuoles are present; c — 2-celled pollen grain with both nuclei, the vegetative and the generative at the mitotic division; d — 4-nucleate pollen grain, where the two larger nuclei originated from the vegetative cell while the two smaller ones male gametes (mg) from the generative nucleus; e — 6-nucleate pollen grain, three nuclei are at the metaphase stage, one at prophase, two smaller nuclei — male gametes; f-g — 2-celled pollen grains with one nucleus undergoing the mitotic division; h-j — three-celled pollen grains with nuclei of different sizes, two nuclei without a nucleoli are considered as male gametes; k-m — two and three-celled pollen grains with pollen tubes of different width and length, the cytoplasm variously vacuolized; n — an excessively enlarged and vacuolized pollen grain with a vegetative nucleus and two male gametes

Fig. 7

Fig. 8. Proembryos at their early stage of development.

a-e — 6 to 14-celled proembryos

Fig. 9. A 15-celled proembryo enclosed by the exine.

Fig. 10. A globular proembryo with a suspensor — like structure surrounded by the exine.

Storage materials in cultured anthers and in the control

1. Starch: Starch appears in the epidermal cells, in the endothecium (some cells were completely filled up with starch) and in the connective tissue. In the latter starch was present in the region of the vascular bundle and the septum as well. Starch was also observed in some pollen grains and in the mature androgenic embryos. There was no evidence of starch in the tapetum, the middle layer, stomium cells, proembryos and young embryos.

2. Proteins and lipids: There was no indication of the presence of storage proteins and lipids in anthers cultured *in vitro* and in the control material as well.

DISCUSSION

Thanks to the discovery that pollen grains can produce plants, we have now some knowledge about the process of androgenesis in the *in vitro* culture. It is of a great importance that during the course of androgenesis plants of different ploidy can develop. Narayanaswamy and Chandy (1971) interpret the process of forming poliploid plants from pollen in the following ways: 1) Prior to embryogenesis endoduplication may occur in the haploid pollen grain and as a consequence of this diploid plants can develop. 2) Fusion between the two male gametes with the vegetative nucleus may take place in pollen grain thus forming a triploid cell, functional in embryogenesis, thereby giving rise to a triploid plant.

Nitsch and coworkers (1969 a) have used various cytokinins for obtaining polyploid cells from haploid callus of *Nicotiana tabacum* and *N. sylvestris*. Many fertile dihaploids were formed in both species. The callus technique has also been used in *Oryza* with a good effect. Plants including diploids, triploids, tetraploids and pentaploids have been obtained (Nishi and Mitsuoka, 1969).

The reports mentioned above give us informations about the possibility of getting plants of different ploidy from the haploid callus cells, however, they do not explain what factors induce this process. As suggested by Sunderland (1971), Narayanaswamy and Chandy (1971) and as it ensues from our observations pollen grains give rise to poliploid plants due to endomitosis. There were no informations till now at what stage of the embryo development this process can occur. As it is shown in our work, endoduplicated nuclei can be observed in a several-celled proembryo. At this stage nuclei of some proembryos became enormously enlarged which may indicate that endoduplication have already occurred. The mechanism by which endoduplication was evoked remains to be ascertained and clarified. We have still not enough data for suggesting that it happened due to the presence of kinetin.

Further work is in progress to clarify this supposition. Our observations did not reveal the fusion of male gametes with the vegetative nucleus and thus the formation of autopolyploid nuclei. On the contrary, it was clearly visible that the male nuclei degenerated early and did not participate in the formation of embryos.

According to Sunderland and Wicks (1971) pollen grains of *Nicotiana tabacum* when filled with starch never formed pollen tubes inside anthers cultured *in vitro*. The same was observed in *Atropa*. Rybchenko (1963) has stated that the presence of starch in pollen grains of species belonging to the *Solanaceae* family indicates their degeneration. The lack of starch was noticed in pollen grains, proembryos and young embryos of *Atropa*, but as for the mature embryos starch was present. When starch occurred in pollen the process of androgenesis was never noticed, similarly an excessively vacuolated and enlarged pollen did never divide and degenerated.

The development of anthers in natural conditions proceeds similarly as in other species of this family. A high percentage, about 90%, of fully developed pollen grains shows that the course of microsporogenesis was normal. Tapetum in the form of a thin strip of cells was present in anthers cultured *in vitro*, even in those anthers which produced androgenic embryos. In some cultured anthers tapetum became outgrown in the direction of the inside of the lobe and compressed completely the microspores. So far, it is dubious to understand what kind of function, if any, has the tapetum in the process of androgenesis. Tapetum at the stage of 2-celled pollen grains is almost completely consumed and therefore its function as a nourishing tissue for the developing embryos seems to be very unimportant or none at all.

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REFERENCES

- Clapham D., 1971, *In vitro* development of callus from the pollen of *Lolium* and *Hordeum*, Z. Pflanzenzücht. 65: 285—292.
- Gresshoff P. M. & Doy C. H., 1972, Development and differentiation of haploid *Lycopersicon esculentum* (tomato), Planta 107: 161—170.
- Guha S. & Maheshwari S. C., 1964, *In vitro* production of embryos from anthers of *Datura*, Nature 204: 497.
- Guha S. & Maheshwari S. C., 1966, Cell division and differentiation of embryos in the pollen grains of *Datura* *in vitro*, Nature 212: 97—98.
- Harn C., 1971, Studies on the anther culture in *Solanum nigrum*, SABRAO Newsletter 3: 39—42.

- Kameya T., Hinata K., 1970, Induction of haploid plants from pollen grains of *Brassica*, Jap. J. Breed. 20: 82—87.
- Linsmaier E. M. & Skoog F., 1965, Organic growth factor requirements of tobacco tissue culture, *Physiol. Plantarum* 18: 100—127.
- Mazia D., Brewer P. A., and Alfert M., 1953, The cytochemical staining and measurement of protein with mercuric bromophenol blue, *Biol. Bull.* 104: 57—67.
- Nakata K. & Tanaka M., 1968, Differentiation of embryoids from developing germ cells in anther culture of tobacco, Japan. J. Genet. 43: 65—71.
- Narayanaswamy S. & Chandy L. P., 1971, In vitro production of haploid, diploid, triploid androgenic embryoids and plantlets in *Datura metel* L., *Annals of Botany* 35: 535—542.
- Niizeki H. & Oono K., 1968, Induction of haploid rice plant from anther culture, *Proc. Japan. Acad.* 44: 554—557.
- Nishi T. & Mitsuoka S., 1969, Occurrence of various ploidy plans from anther and ovary culture of rice plant, Japan J. Genet. 44: 341—346.
- Nitsch J. P. & Nitsch C., 1969, Haploid plants from pollen grains, *Science* 1963: 85—87.
- Nitsch J. P., Nitsch C. and Hamon S., 1969 a, Production de *Nicotiana* diploides à partir de cals haploides cultivés in vitro, *C. R. Acad. Sci. Ser. D, Sci. Natur.* 269: 1275—1278.
- Raquin Ch., Pelletier G., and Simon G., 1972, La culture in vitro d'anthers d'Asperge (*Asparagus officinalis*), *C. R. Acad. Sci. Paris* 274: 848—851.
- Rybchenko O. I., 1963, Citoembriologichne doslidzhenia rozvitku cholovichogo gametofita w rodini paslonowikh, *Ukrain. Bot. Zhur.* 20: 3—14.
- Sunderland N., 1971, Anther culture: a progress report, *Sci. Prog. Oxford* 59: 527—549.
- Sunderland N. & Wicks F. M., 1971, Embryoid formation in pollen grains of *Nicotiana tabacum*, *Journal of Exper. Botany* 22: 213—226.
- Zenkter M., 1971, In vitro production of haploid plants from pollen grains of *Atropa belladonna*, *Experientia* 27: 1087.
- Zenkter M., 1972, Development of embryos and seedlings from pollen grains in *Lycium halimifolium* Mill. in the in vitro culture, *Biol. Plant.* 14: 420—422.
- Zenkter M., In vitro development of embryos and seedlings from pollen grains of *Solanum dulcamara*, *Zeitschrift für Pflanzenphysiol.* (in press).

Badanie androgenezy w pylnikach Atropa belladonna hodowanych in vitro

Streszczenie

W pracy zbadano rozwój pylnika *Atropa belladonna* w warunkach naturalnych oraz wczesne stadia androgenezy w pylnikach hodowanych in vitro na pożywce Linsmaiera i Skooga z dodatkiem kinetyny 4 mg/l i IAA — 2 mg/l.

Mikrosporoogeneza przebiegała bez zakłóceń, liczba biwalentów wynosiła 36, w dojrzałych pylnikach obserwowano ok. 90% prawidłowo wykształconych ziaren pyłkowych. Szczegółowo prześledzono rozwój ściany pylnika, specjalnie zwrócono uwagę na tapetum, endothecjum i łącznik.

Celem uzyskania androgenicznych zarodków wyszczepiono pylniki w stadium tetrad i mikrospor. Rozwój pylnika na tej samej pożywce przebiegał w dwóch kierunkach: a) w kierunku powiększania się przegrody przy jednoczesnej degeneracji ziaren pyłkowych; b) w kierunku zmniejszania się przegrody i równoczesnym

wykształcaniu się zarodków z ziaren pyłkowych. Tapetum występowało w postaci wąskiego pasemka komórek; niekiedy komórki tapetum nadmiernie się powiększały. Jądro komórki wegetatywnej pyłku odgrywa decydującą rolę w procesie powstawania zarodków. W wyniku podziału jądra wegetatywnego powstają kilkujądrowe ziarna pyłkowe, następnie dopiero pomiędzy jądrami zakładają się ściany komórkowe i pyłek przekształca się w prazarodek. Kilkunastokomórkowy prazarodek jest z początku otoczony egzyną, w miarę dalszego powiększania się zarodka egzyna zanika. W wyniku podziału jądra komórki generatywnej powstają dwie gamety. Na podstawie dotychczasowych obserwacji przypuszcza się, że gamety już się zwykle dalej nie dzielą i nie współuczestniczą w powstawaniu androgenicznego zarodka. Androgeniczne zarodki różnią się w obrębie jednego pylnika pod względem wielkości, kształtu, stopnia wakuolizacji, wielkości jąder i liczby jąderek. Stwierdzono znaczne powiększanie się jąder u niektórych kilkukomórkowych prazarodków, co może wskazywać, że endoduplikacja zachodzi we wczesnych stadiach androgenезы, tym zjawiskiem należałoby tłumaczyć występowanie homozygotycznych poliploidalnych roślin. Prześledzono zakładanie się skrobii w dojrzałych androgenicznych zarodkach oraz w niektórych tylko ziarnach pyłkowych. Skrobii nie stwierdzono w tych ziarnach pyłkowych, które przekształcały się w zarodki.