HISTOCHEMICAL RESEARCHES REGARDING ABNORMALIES WITHIN THE DEVELOPMENT OF WALNUT (*JUGLANS REGIA* L.) MALE REPRODUCTIVE SYSTEM

CERCETĂRI HISTOCHIMICE PRIVIND LA ANOMALIILE ÎNTÂLNITE ÎN PROCESUL DEZVOLTĂRII SISTEMULUI REPRODUCTIV MASCULIN AL NUCULUI (*JUGLANS REGIA* L.)

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Abstract. *During the development of walnut (Juglans regia L.) male sexual system the most frequently noticed anomalies there are conditioned by low temperatures (-10 - -15^{0}C) in the meiotic period (the end of March - first half of April months). As a rule in the exterior part of inflorescences of protandrous as well as of some protoginous genotypes microsporogenesis is lead rapidly. In the microspores mother cells (MMC) appear big vacuoles, pushing slowly hypertrophied nucleus to tapetal tissue. There are observed typical phenomenon of cytomixis and picnosis with passage of fragments of nucleus and nucleols from one cell to another. Tapetal tissue is affected too, but there could be founded some sectors with normal development. Vacuolization of tapetal cells is very expressed adjacency MMC , citoplasm and nucleus are moved to parietal layer. Intensity of NA, total proteins and polysaccharides test reactions there are low. Reaction of starch test is null, and for lipids –remarkably low. Anomalies there are observed more frequently for protandrous as well as protoginous genotypes with accelerated rhythm of male flower development.*
Key words: *walnut, male flowers, microsporogenesis, abnormalies.*

Rezumat. *Pe parcursul dezvoltării sistemului reproductiv masculin la nuc (Juglans regia L.) mai frecvent sunt depistate anomalii, provocate de temperaturile joase (-10 - -15^{0}C) în perioada meiotică (sfârşitul lunii martie-prima jumătate a lunii aprilie). Din partea exterioară a amenţilor genotipurilor protandre, precum şi ale unora protogine se derulează cu un ritm rapid microsporogeneza. În citoplasma celulelor mamă microsporale (CMM) apar vacuole mari care împing nucleul puţin hipertrofiat spre ţesutul tapetal. Este foarte frecvent fenomenul tipic al citomixiei şi picnozei, cu trecerea fragmentelor de diferite mărimi de nuclei şi nucleoli, a cromozomilor incomplet formaţi dintr-o celulă în alta. Ţesutul tapetal de asemenea este afectat, dar pot fi întâlnite şi porţiuni ce păstrează o dezvoltare normală. Vacuolizarea celulelor tapetale în vecinătatea CMM ia proporţii mari, iar citoplasma şi nucleul sunt deplasate spre stratul parietal. Intensitatea reacţiilor AN, a proteinelor sumare şi a polizaharidelor insolubile este relativ joasă. Reacţia de depistare a amidonului este nulă, iar a lipidelor deosebit de slabă. Mai frecvente sunt anomaliile observate la genotipurile protande şi cele protogine cu ritm accelerat de dezvoltare a florilor masculine.*
Cuvinte cheie: *nuc, flori masculine, microsporogeneza, anomalii.*

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INTRODUCTION

The problems of knowledge and leading of optimal development of walnut male and female flowers, pollen, pollination and fruit set represent an actual goal within the formation of new direction of fruit growing in Republic of Moldova - industrial nut production. Generally it should be noticed that for a lot of crops research of male sexual structures there are much more deeply approached, as well as much more frequent utilized in the investigations of genetic engineering and for molecular biology. Walnut (*Juglans regia* L.) is much less approached comparatively with other valuable agricultural crops. Referred data could be founded only in the process of embryological approaches, base on cyto-morphological and histochemical methodologies (Pîntea et al., 2004). For walnut histochemical researches there are indispensable especially for progressively transition to the genetic engineering investigations with utilization of morphophysiological potential for diversification, enrichment and programmed changeability of genetic fond within the family *Juglandaceae* (Pîntea, 2004). The foregoing investigations there are indissolubly linked with the practical problems, considering the establishment of the most efficient pollinators for walnut industrial orchards.

MATERIAL AND METHOD

Experiments where effectuated in the frame of Experimental Station of Research and Practical Institute for Horticulture and Alimentary Technologies. Biologic material (male inflorescence –aments and separately flowers) where collected from 27 walnut varieties and elites, including protandrous, protoginous and homogamous ones. In the quality of fixative solutions where utilized fixators Carnoy, Navashin, Modilevskii, Newcomer. The blocks of paraffin coating material where sectioned at thickness 8 mk depending of developmental faze. Preparates with slides, effectuated to morphological investigations where colored with hematoxlin according Heidengain method, as well as with basic fuxin according Modilevskii method. Cito- and histochimistry tests for determination the localization and intensitaty manifestation of nucleic acids reactions (total and separately deoxiribonucleic acid), total proteins and separately only basic ones, ascorbic acid, starch, insoluble polysaccharides, lipids, calloze, total heteroauxins and for enzyme series there are fulfilled according approved methods for investigation of angiosperms plants (Jensen, 1965; Cociu and Oprea, 1989, Pîntea, 2004). Microscopic studies where effectuated using microscope DN-816 (MEOPTA) conformable the methods approved for the respective domain.

RESULTS AND DISCUSSIONS

According the analysis of the results of our investigations of walnut male reproductive system development get through about one calendar year. Starting from this consideration there is incontestable fact that certain structures could be affected by unfavourable environmental factors. Thus, our observations and investigations, effectuated during a lot of years permit to presuppose that extremely high or low temperatures could provoked anomalies during the initiation of archesporium, corresponding in the month of August, September,

October. Results of researches shows that in the years, than during the month August - October there are high temperatures for long time (more than one weak), namely, higher than 25-28^{0}C, protandrous varieties with early flower period like Ivaşcenco 4/7, Costiujenski there are observed hipertrophied nuclei and nucleoli of 1-5 arhesporial cells of anthers of exterior part of male flowers/inflorescences. Citolplasma of those cells is very dens, intensively colored when is used as colorant hematoxilin. These cell groups there are distinguished by suddenly intensification of test-reaction for nucleic acids, total proteins and, especially, basic proteins. Phosphatase acid activity is estimate to 4 points (1-5 scale). Biological rest period of these already affected cell zones pass without any susceptible morphological and cytochemical changes. It could be presupposed that in such cases there are reparative metabolic processes at the level of integral walnut male sexual system.

In the table 1 and 2 there are presented some general metabolic differences, established by histochemical tests during normal and anomalous (tab. 2) microsporogenesis processes. Accordingly effectuated investigations it is highlighted that if in the period of January-February there are suddenly temperature decreasing in the limits of –20 .... -25^{0}C within the affected in autumn cells there is produced a total necrosis. During the meiotic divisions there are also observed some anomalies principally at the stages: metaphase telophase, anaphase even in the case of normal temperature regime. It is important to accentuate the fact that in such cases tapetal cell layer supports considerable disorders, in some places totally being absent (fig. 1 A, B).

Table 1

General walnut microsporogenesis histochemical characterization (1-5 points).

Component/ Studied structures	Nucleic acid (AN)	Total proteins	Basic proteins	Insoluble polysaccharides
Sporogen tissue in the winter	4,5	5,0	4,8	3,0
Arhesporium	4,8	4,9	4,7	4,0
Tapetum	4,9	4,9	4,8	3,0
Microspores mother cells	5.0	5,0	5,0	3,5
Tapetum	4,9	4,7	4,9	3,5
Dyads, tetrads, pentads of microspores	4,8	4,5	4,6	3,8
Tapetum	4,9	4,4	4,8	3,8
Isolated juvenile microspores	4,7	4,0	4,5	4,0
Residual tapetum	4,2	4,6	4,7	4,3
Mature pollen during dissemination	5, 0	5,0	5,0	4,5

Furthermore it should be noticed that formation of callosecapsule also is completely out of order, being represented by irregular and around the

incompletely developed layer of microspores mother cells (MMC). In the cytoplasm of microspores mother cells (MMC) appear big vacuoles, pushing slowly hypertrophied nucleus to tapetal tissue. Within the MMC population there are established an evident polarity of this phenomenon (fig. 1 A, B).

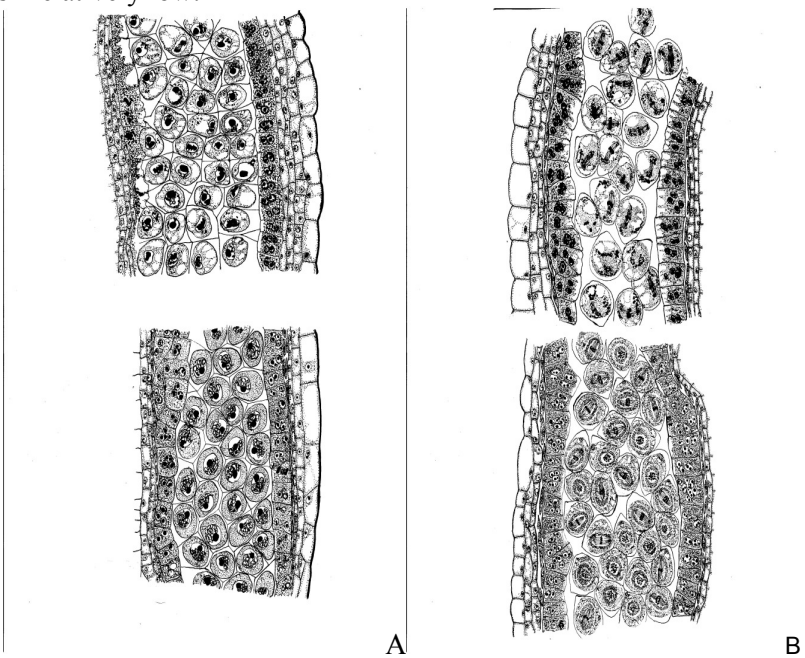
Table 2

Citochimical characterization of main structures during anomalous microsporogenesis (1-5 points)

Component/ Structures	Nucleic acid (AN)	RNA Separatly	DNA separatly	Total Proteins	Basic Proteins	Insoluble polysac-charides
Sporogen tissue in the winter	4,5	4,5	5,0	4,8	4,8	3,0
Arhesporium Tapetum	4,8 4,7	4,8 4,7	4,6 4,5	4,7 4,6	4,7 4,8	4,0 3,0
Microspores mother cells Tapetum	4,9 4,8	4,9 4,8	4,9 4,9	4,9 4,9	5,0 4,9	3,5 3,5
Dyads, tetrads, pentads of microspores Tapetum	4,3 4,7	4,3 4,7	4,5 4,8	4,6 4,8	4,6 4,8	3,8 3,8
Isolated juvenile microspores Residual tapetum	0,0-4,8 4,3	0,0-4,8 4,3	0,0-5,0 4,0	0,0-5,0 4,6	0,0-5,0 4,6	3,0-4,0 4,5
Mature pollen during dissemination	0,0-4,9	0,0-4,5	0,0-5,0	0,0-5,0	0,0-5,0	0,0-4,0

It is obviously also secretor character of those structures. Hence hereafter nucleus membrane there is shrunked. In the same time hypertrophied nucleolus there is shooting in some parts, which continue to be connected (fig.2). Nevertheless meiotic division is produced in the non transformed part of CMM. Therefore during meiosis there are observed different kind of anomalies: restitutions and accelerated movement of chromosomes, asynchronic formation of chromatides etc. Adjacent sectors of tapetal likewise suffer anomalous transformations (fig.1 A, B). It is observed, that cytoplazmic content is strongly granulated, polarisant vacuoliosation nearly MMC is produced. We suppose that those results could confirm hypothesis, proposed by Reznicova (1984). According this author activity of tapetum there are programmed for the development of different stage of male reproductive system.

Among the observed anomalous a typical phenomenon of citomixis and picnosis with passage of different fragments of nucleus and nucleolus, as well as of incomplete developed chromosomes from one cell to another there are frequently established (fig. 2). Adequate changes there are noticed for tapetum. In such cases vacuolization is strong nearly MMC, citoplasma and nucleus are moved to parietal (fig. 1 A, B). Intensity on NA reaction, total proteins and polysaccharides test reactions there are low. Reaction of starch test is null, and for lipids –relatively low.



A B

Fig.1 - A. Appearance of zones of necrotic archesporial cells: A- primary archesporium, cv.Tihomirov ; B - in MMC, at the corner of pollen bags. Ob.20^{x}. / up - anomalous development; down – normal development

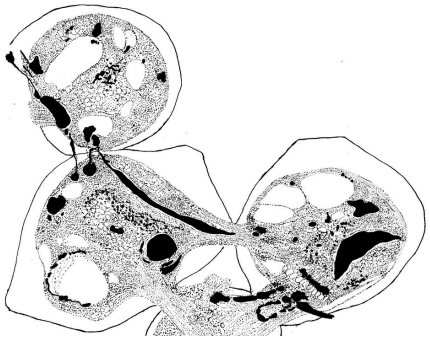


Fig. 2 - Manifestation of **c**itomixis within walnut microsporogenesis process Ob.90^{x}

Our investigations shows also that during tapetum degeneration some portions of citoplasma could be delimitated in the form of fascicules with disparity of cells membranes. In those fascicules there are found up discharge of nucleolus and nuclear cromatin. In the cases, when in small portions there took place process of meiosis, formed pollen grains are sterile or with high morphophysiological disorders. Partially aberrant microsporogenesis where noticed not only for some part of male inflorescence as well as for hermaphrodite flowers within secondary flowering (in summer period).

In spite of often manifestation of anomalous microsporogenesis within some genotypes, basically this phenomenon could not play an primordial role of walnut pollination in the frame of conditions of Republic of Moldova. This conclusion could be explain by the fact that walnut, produce an enormous pollen quantity. Thereby walnut anemophily pollination is plentiful ensured, locally being large cultivated by seedless. In the case of establishment of industrial plantations, based on concrete grafted cultivars, it is necessary to utilize in the quality of pollinator for basal commercial cultivar a variety with good resistance of male flower to unfavourable temperatures for non compromised efficient fruit setting. Generally, male sterility, especially male cytoplasmic sterility (CMS) is described for more than 140 species of angiosperm plants. Manifestation of sterility depends on species and of degree of anomalous development. In our case a significant importance is related to diminution of anther cracking power, resulting pollen degeneration at different microsporogenesis stages. In our opinion different phenomenon of male sterility manifested within introduction of some varieties from family *Juglandaceae* could play an important role for genetic transformation and general evolution of *Juglans regia L.* species.

CONCLUSIONS

1. In the frame of walnut dichogamy the most sensible male flowers to extremly lower temperatures there are observed more frequently for protandrous as well as protoginous genotypes with accelerated rhythm of male flowers development.

2. Tapetal layer of walnut anther could have an direct influence to normal or abnormal microsporogenesis advancement.

3. Citomixis there is a phenomenon which obligatorily is manifested during anomalous microspogenesis awaked by extreme temperatures within *Juglans regia L*. varieties.

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