Modeling of asymmetric division of somatic cell in protoplasts culture of higher plants

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The key result of the work is the selection of factors for the cultivation of protoplasts of higher plants *in vitro*, which allowed induction of asymmetrical cell division during the first cell cycle phase. Gibberellin has been proved to be one of the main cofactors of asymmetric division of plant cells. The objects of research were plants of the following cultivars aseptically grown in hormone-free MS medium: tobacco (*Nicotiana tabacum* L.), SR-1 line; *Arabidopsis thaliana* var. *columbia* (L.) Heynh; potato (*Solanum tuberosum* L.), Zarevo cultivar; cultivated white head cabbage (*Brassica oleraceae* var. *capitata* L.) of the following varieties: Kharkivska zymnia, Ukrainska osin, Yaroslavna, Lika, Lesya, Bilosnizhka, Dithmarscher Früher, Iyunskarannya; rape (*Brassica napus* L.) of Shpat cultivar; winter radish (*Raphanus sativus* L.) of Odessa-5 cultivar. In experiments with mesophilic and hypocotyl protoplasts of the above-mentioned plant species it has been proved that short-term osmotic stress within 16–18 hours being combined with subsequent introduction of high doses of gibberellin GK3 (1 mg/L) into the modified liquid nutrient media TM and SW led to the occurrence of pronounced morphological traits of cytodifferentiation already at the initial stages of the development of mitotically active cells in a number of higher plants. Meanwhile, in all analyzed species, there was observed the division of the initial genetically homogeneous population of mitotically active cells into two types of asymmetric division: by the type of division of the mother cell into smaller daughter cells and by the type of the first asymmetric division of the zygotic embryo *in planta*. In this case, the first type of asymmetric division occurred during unusual cytomorphism of the mother cells: a pronounced heart-shaped form even before the first division, which is inherent in the morphology of somatic plant embryo *in vitro* at the heart-shaped stage. A particular study of the effect of osmotic stress influencing protoplasts of various cultivars of white cabbage, isolated from hypocotyls of 7–9 day etiolated seedlings, revealed quite a typical consistent pattern: the acquisition and maintenance of the axis of symmetry in growing microcolonies occurred without extra exogenous gibberellin (GK3), which was added to the nutrient medium earlier. While analyzing the effect of growth regulators on the formation of microcolonies with traits of structural organization, the conclusion was made regarding the commonality of the revealed morphogenetic reactions of cells within the culture of protoplasts of higher plants *in vitro* with similar reactions studied earlier on other plants, both *in vitro* and *in planta*. Modeling of asymmetric cell division in protoplast culture *in vitro* has become possible by carrying out a balanced selection of growth regulators as well as their coordinated application through time along with changes in osmotic pressure of a nutrient medium.

Keywords: plant cell; osmotic stress; first asymmetric division; gibberellic acid (GA3); cell microcolony.

Introduction

One of the main problems in the framework of plant morphogenesis is the elucidation of the nature of the processes causing polarization and further differentiation of cells (Medvedev, 1996). Such processes include non-equivalent or asymmetric division of the mother cell, which leads to the occurrence of daughter cells of different functions, thereby the primary formation and further maintenance of the axial symmetry of multicellular structures of the plant body.

Bipolar growth is an essential trait of life activity of most plants. Essentially, the plant stem increases upwards, while the root downwards, deepening into the soil. Bipolar growth is primarily due to an increase in a number of cells through reproduction (proliferation) in special cellular structures: apical meristem located at the very top of the stem and at the root tip. In nature, plants are represented by a great variety of life forms, which are widely different in their growth features (Flindt, 1992): the number of cells in the cell chains from the tip of the shoot to the root tip, the ratio of shoot-to-root size, growth rate as well. It is determined that two

hormones – auxin and cytokinin – are necessary for the separation of plant cells (Dante et al., 2014). It is essential that these phytohormones are synthesized mainly at the plant poles: auxin is being synthesized at the crown of the stem and cytokinin – at the root apex. Through transport channels, the auxin in plants moves downward, while the cytokinins move towards them (Polevoj, 1982; Polevoj & Salamatova, 1991). The role of hormonal counterflow within the organization of growth processes is still not fully clarified. So far, it has not been possible to experimentally identify the auxin-cytokinin counterflow circuit or study its functions as a plant growth inductor. To solve this problem, it is considered quite effective to apply different kinds of simulation. In particular, the authors of one of the published works proposed a mathematical model in which the bihormonal contour controls the proliferative growth of a plant-type cell structure (Sukhoverov & Romanov, 2009).

In plants, the patterns of cell division in combination with growth and differentiation determine the hierarchical organization of plant tissues and organs. Cells can reproduce using symmetrical divisions, while asymmetrical ones are mainly related to the initiation of new cell types, neoplasms

and development patterns (Rasmussen et al., 2011; Smolarkiewicz & Dhonukshehe, 2013). In asymmetric division, the formation of the cleavage plane is often associated with cell polarity. The common paradigm is that the position of the cleavage plane will be determined by such an asymmetrically distributed determinant that will lead daughter cells to different directions of development. An alternative view is that daughter cells will initially have the same determinant, however, they will be exposed to different positional signals that will cause them to develop in various manners (Horvitz & Herskowitz, 1992). Therefore, elucidating the principles that govern symmetric and asymmetric divisions provides for better understanding the cellular bases of plant development as well as plant morphogenesis in general. The early embryogenesis of *Arabidopsis thaliana* has been deemed to be an attractive model for studying how the positions and orientation of cleavage planes are being selected. During the first generations of cells, exclusive embryo geometry is indeed organized from a single initial cell through a stereotype sequence of invariant oriented cell divisions (Mansfield & Briarty, 1991; Capron et al., 2009). Thus, the boundaries of the future direction of cell division have been established and mapped using numerous genetic and cytological reverse analyses, and these properties have been successfully used to identify the origin of developmental defects in mutant patterns (Laux et al., 2004; Palovaara et al., 2016). The influence of cell shape on the orientation and choice of cleavage planes in animal and plant cells has attracted much attention (Minc & Piel, 2012) with special emphasis on the common rules of cleavage defined as early as the late XIX century (Errera, 1888). According to the *Errera rule* (Errera, 1888), plant cells will behave as soap bubbles, so that symmetrical separations will adhere to the principle of minimum surface area. Besson & Dumais (2011) recently rearranged the rule in a stochastic version, according to which the choice of the cleavage plane between the various alternatives depending on the distribution of probabilities is associated with the plane area. It is generally accepted that the principle of minimizing surface area, according to the *Errera rule*, will a priori be a mechanism for separating plant cells in the absence of internal or external traits (Roeder, 2012). However, the vast majority of studies supporting this view have focused on symmetrical divisions in tissues that have been assimilated in 2D systems (e.g., tissues with a constant cell layer and absolutely anticlinical divisions). The question of whether geometric rules are acceptable of describing a 3D division of plant cells deserves to be asked. Recently, Yoshida et al. (2014) reported, however, that they were unable to identify the geometric rule, which underlies the sequence of 3D cell division models in the *Arabidopsis thaliana* zygotic embryos.

Other authors (Moukhtar et al., 2019) questioned the existence and nature of the rules governing cell division during early zygote embryos' stage. Using automatic image analysis of 3D embryos, they quantitatively determined cell shapes and models of cell division. It was found out that the distance between the equator of a cell and the mother cell's centroid was an invariant trait of the generations' course and spatial boundaries. Being based on the developed computer cell division model, the authors (Moukhtar et al., 2019) investigated the space of possible division planes under geometric constraints in real three-dimensional cell forms. On the basis of the proposed model, a new rule for predicting the position and orientation of division planes by the geometry of the mother cell was established, which was true for both symmetric and asymmetric cell division. The results supported the key role of the geometric feedback loop between the cell shape and the position within the cleavage plane in the self-organization of the early embryo.

The above-mentioned instances of asymmetric cell division, as well as variants of mathematical modeling of this type of division were considered on plant objects with pre-genetically determined development program at the *in planta* level, i.e. the modeling concerned those cases when the course of cytodifferential cell division were under genetic and regulatory control of a plant body.

In this regard, the culture of isolated protoplasts of higher plants *in vitro* can serve as an alternative model system for identifying and studying the cofactors of cytodifferential division and growth of plant cells as well. The feature of this model system is that it allows one to investigate the cofactor role of growth regulators as well as elements of osmotic and trophic regulation of cell division of isolated somatic plant cells *in vitro* (Butenko, 1981; Kuchuk, 2017).

In experiments with tobacco culture it was shown (Nawaga et al., 1987) that the identification of endogenous gibberellin is closely correlated with the onset of morphogenesis. The results of embryological studies on all plant species concerned also demonstrate the direct involvement of endogenous gibberellins in the formation of zygotic embryos *in planta*, beginning with the heart-shaped development stage (Trigiano et al., 1987). However, in a number of already prepared studies, the physiological effect of the phytohormone was evaluated on multicellular structures, confirming a failure to fully manifest its functions. A rather convenient object for study is the culture of isolated protoplasts, where a mitotically active somatic cell may be a potential morphogenetic unit. Therefore, this cell type is an ideal experimental object for simulating different types of morphogenesis in culture *in vitro*. The purpose of our research was to determine the informative possibilities of the culture of protoplasts of higher plants *in vitro* as a model system capable of reproducing asymmetric division of somatic plant cells only due to the action of exogenous factors of growth and development controlled by a researcher.

Materials and methods

Six plant species from the Brassicaceae and Solanaceae families were chosen as research objects, which in the culture of protoplasts during gradual application of nutrient media TMmod1 (k), TMmod2 (k) and TMmod3 (k) and SWmod1 (k), SWmod2 (k) and SWmod3 (k) (Table 1) initiated the first symmetrical divisions of the cells, from which the unorganized growing colonies were then formed. The following tube stock plants were aseptically grown *in vitro* on hormone-free agarized MS medium (Murashige & Skoog, 1962): SR-1 line of tobacco (*Nicotiana tabacum* L.) (Maliga et al., 1973); wild species of *Arabidopsis* (*A. thaliana* var. *colymbia* (L.) Heynh.); Zarevo variety of potato (*Solanum tuberosum* L.); whitehead cabbage (*Brassica oleraceae* var. *capitata* L.) varieties: Kharkivska zymova, Ukrainska osin, Yaroslavna, Lika, Lesia, Bilosnizhka, Dithmarscher Früher and Iuinskarannia; Shpat variety of rape (*Brassica napus* L.); Odeska-5 variety of winter radish (*Raphanus sativus* L.).

Protoplasts of leaf mesophyll were extracted from microclonal propagated juvenile plants, with well-developed leaf area. Hypocotyl protoplasts were excreted from ethylated seedlings for 7–9 days of cultivation. Leaf or hypocotyl tissues were cut into strips 1 mm wide and incubated under darkroom conditions at 28 ℃ for 16–18 hours in an enzymatic solution containing 0.5% Macerozyme ("Calbiochem", USA), 0.5% Onozuka R-10 ("Jacult Biochemicals", Japan), 0.5 M sucrose and 5 mM $CaCl_2$ (pH 5.6). The isolated protoplasts were separated from residual tissue through metal filters with a pore diameter of 64 μm. The filtrate was transferred to centrifuge tubes (10 mL) and then centrifuged at 700 rpm for 7 min. The ring of floated protoplasts was selected by a Pasteur pipette and washed at least twice in a W-5 medium (Medgyesy et al., 1980), precipitating them each time by centrifugation for 5 minutes at 700 rpm. The number of protoplasts in the medium was calculated using the Goriaiev chamber.

In our studies, the addition of exogenous gibberellin (GK3) to the composition of nutrient medium growth regulators proved to be effective, starting from the first mitotic cell division in protoplast culture and at growth of microcolonies formed after the 5th and 6th mitosis. After tissue fermentation and isolation procedure (Sidorov et al., 1985), protoplasts of various plant varieties within the experimental variant of cultivation were transferred to the appropriate nutrient media SWmod1 (d) and Tmod1 (d) (Table 1) and cultivated on diffuse light at 22–24 ℃ for 16–18 hours under stress (hypotonic) conditions (0.36 M glucose) with a population of 10^4–10^5 cells/mL. Then the level of osmotic agents was brought to 0.5 M (modifications of SWmod2 (d) and Tmod2 (d), respectively). The increase in osmotic concentration was followed by simultaneous injection of exogenous gibberellin (1 mg/L GK3) into nutrient medium. Further, the level of osmotic agents was brought up to 0.5 M (modifications) during the first 48 hours of cultivation, more than 80% of both mesophilic and hypocotyl protoplasts of the studied plant cultivars were fully restored to the cellular membrane. The morphological criterion for such a condition in cultured protoplasts was a change in their spherical shape to elongated or oval, which is typical for suspension-cultured cells (Fig. 1). The identification of regenerating cell membranes in protoplasts was carried out by colouring them with 0.01% calcofluor (Calcofluor white, MR-2, "Serva")

on 0.1 M phosphate buffer, pH 7.2 during 5–10 minutes following the Herth method (Herth & Schnepf, 1980). For this purpose, microdrops of cell suspensions of 10–20 µL were used, which were selected from the nutrient media. Having being washed with a buffer, protoplasts were exa-

mined with the help of a fluorescent microscope ML-2, the luminescence was caused by the blue light of a mercury lamp and a light blue-green glass filter. Calculation of the percentage of protoplasts from regenerated membranes was carried out for 24 hours and 48 hours of cultivation.

Table 1

The composition of liquid nutrient media (mg/L) for cultivation of protoplasts of higher plants in the experimental (e) and control (c) variants

| Components * | The content in nutrient medium, mg/L | | | | | | | | | | | |
	TMmod1 (e)	TMmod1 (c)	TMmod2 (e)	TMmod2 (c)	TMmod3 (e)	TMmod3 (c)	SWmod1 (e)	SWmod1 (c)	SWmod2 (e)	SWmod2 (c)	SWmod3 (e)	SWmod3 (c)
Macronutrients:												
NH_4Cl	0.0	0.0	0.0	0.0	0.0	0,0	133.0	133.0	133.0	133.0	133.0	133.0
NH_4NO_3	200.0	200.0	200.0	200.0	200.0	200.0	0.0	0.0	0.0	0.0	0.0	0.0
KNO_3	1500.0	1500.0	1500.0	1500.0	1500.0	1500.0	1900.0	1900.0	1900.0	1900.0	1900.0	1900.0
$CaCl_2 \times 2H_2O$	440.0	440.0	440.0	440.0	440.0	440.0	440.0	440.0	440.0	440.0	440.0	440.0
$MgSO_4 \times 7H_2O$	370.0	370.0	370.0	370.0	370.0	370.0	370.0	370.0	370.0	370.0	370.0	370.0
KH_2PO_4	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0	170.0
Fe-helate:												
Na_2EDTA	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3
$FeSO_4 \times 7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8
Micronutrients:												
H_3BO_3	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
$MnSO_4 \times 5H_2O$	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2
$ZnSO_4 \times 7H_2O$	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
$NaMoO_4 \times 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
KJ	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
$CoSO_4 \times 6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4 \times 5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Vitamins and other biologically active components:												
Mesoinositol	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
B_1	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
B_6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PP	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Casein hydrolyzate	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Growth regulators:												
BAP (6-benzylaminopurine)	0.5	0.5	0.5	0.5	1.0	1.0	0.5	0.5	0.5	0.5	1.0	1.0
NAA (α-naphthylacetic acid)	1.0	1.0	0.1	0.1	0.1	0.1	2.0	2.0	0.1	0.1	0.1	0.1
2,4-D (2,4-dichlorophenoxyacetic acid)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
GA_3 (gibberellic acid)	0.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0	1.0	0.0
Carbohydrate sources (osmotic agents):												
Glucose	64870.0	90100.0	90100.0	90100.0	72000.0	72000.0	64870.0	90100.0	90100.0	90100.0	72000.0	72000.0
(molar concentration)	(0.36M)	(0.5M)	(0.5M)	(0.5M)	(0.4M)	(0.4M)	(0.36M	(0.5M)	(0.5M)	(0.5M)	(0.4M)	(0.4M)
Xylose	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0
The level of acidity of nutrient media: pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

Note: * – experimental nutrient media for white head cabbage, rapeseed and radish protoplasts – TMmod1(e) for cultivation of protoplasts under conditions of hypotonic stress (0.36 M glucose), TMmod2(e) for the cultivation of protoplasts after the first division (at the optimum for the initiation of mitotic activity of the osmotic content 0.5 M glucose) and simultaneous removal of osmotic stress, TMmod3(e) –for culturing cells at a later stage in the formation of micro colonies; similarly intended nutrient media for protoplasts of tobacco, potatoes and Arabidopsis – SWmod1(e), SWmod2(e), SWmod3(e).

Within 60–72 hours after about 50% of cells started the first cell division, the suspensions were diluted with liquid media TMmod3 (d) and SWmod3 (d) with a low auxin content (0.1 mg/L NAA) and a high one of gibberellin (1 mg/L GK3) in order to reach the final density of 10^2–10^3 cells/mL. Phenological observations of the state of culture were carried out on a daily basis, while the number of formed microcolonies from one mitotic active cell and their morphological analysis for 15 days of cultivation were counted. The registration of the state of cell as well as microcolony development *in vitro* cultures of mesophilic and hypocotyl protoplasts of various plant varieties was carried out within two months – a sufficient period at which in the applied experimental option of cell cultivation the traits of structural organization of microcolonies and their complete transition to unorganized growth were completely lost. During the first 20 days, cell culture was grown in the darkroom conditions, then transferred to diffuse light to gradually grow the existing callus clones to an optimum size of 3–4 mm in diameter, which is necessary for their migration to agarized nutrient media. Photo fixing of various stages of growth and division of floating cells in nutrient media of protoplasts and plant cells after the cellular membrane and microcolonies repair process was carried out by photographing them on black and white film with the use of a Zenith camera from the photonhead to an inverted microscope of the Olympus CKX53. The mitotic activity of cells in protoplast culture was assessed by fixation of callus and cell aggregates in a mixture of

ethanol – acetic acid – chloroform (6 : 1 : 3). The material was dyed in 1% aceto-orecin and cells at different stages of mitosis were counted under a microscope on squash material. The mitotic index was determined in 5-fold repetition when analyzing 5–7 thousand cells for each fixation point. The above cytological parameter was determined using the following formula:

$$Mitotic\ index = \frac{100\% * \sum of\ cells\ at\ mitosis\ stage}{\sum of\ cells\ in\ total} \quad (1)$$

The analysis of the experimental data was carried out using the ANOVA method. The differences between the values in the different variants of experience in the cultivation of somatic cells of plants were determined using the Tukey criterion, where the differences were considered reliable at P < 0.05 (with consideration of the Bonferroni correction). The numerical data in the tables are presented as x ± SD (n = 5).

Results

When using the control variant of cultivation of mesophilic protoplasts – a combination of initial isosmotic conditions (0.5 M glucose) and high doses of auxin (1 mg/L NAA for Brassicaceae plants and 2 mg/L for Solanaceae), we observed, primarily, the initiation of symmetrical division of daughter cells and further formation of mini-clones in the majority of cases (Fig. 1). Application of low osmotic pressure of the nutrient medium

during the first 16–18 hours of cultivation led to changes in the morphology of the protoplasts even before full reparation of the cell wall. Namely, under such conditions of cultivation, physiological reaction to osmotic stress (OS) represented excessive vacuolization of protoplasts with formation of large vacuole, which polarized cell cytoplasm (Fig. 2a). Subsequently, a similar cytomorphosis persisted until the beginning of the mitotic activity of the cells and led to the initiation of the first asymmetric divisions with the appearance of daughter cells with clearly visible cytoplasmic optical density – transparent with excessive vacuolization and dense, saturated with cytoplasmic elements (Fig. 2b). Previously, when conducting methodological studies in order to select optimal concentrations of the auxin regulator NAA, we observed the phenomenon of vacuolization in the culture of higher plant protoplasts at the regulator's doses exceeding 2 mg/L and the initial isosmotic conditions. However, the prolonged exposure to the mentioned dose of auxin only increased the growth of cells by

stretching, neutralizing their mitotic activity for too long. Thus, the stimulation of asymmetrical first cell division can be explained by the synergistic effect of high doses of auxin and the initial low, compared to the control variant, osmotic pressure of the nutrient medium. In the application of OS on the protoplasts of potatoes, tobacco and *Aarabidopsis*, the first divisions with unequal distribution of cytoplasmic elements in formed daughter cells were observed. A characteristic feature of the protoplasts of rapeseed, cabbage and radish after the OS stage was the stimulation of mitotic active cell division by the type of cleavage into smaller daughter cells without determination of cytoplasmic elements. After removal of the initial OS protoplasts of all plant species formed disorganized growing cell clones morphologically identical to the minicalli of the control variant of the experiment without any traits of cytodifferentiation (Fig. 3). A prolonged use of hypotonic influence of the nutrient medium (for a day or more) led to the final loss of mitotic activity and death of cell populations.
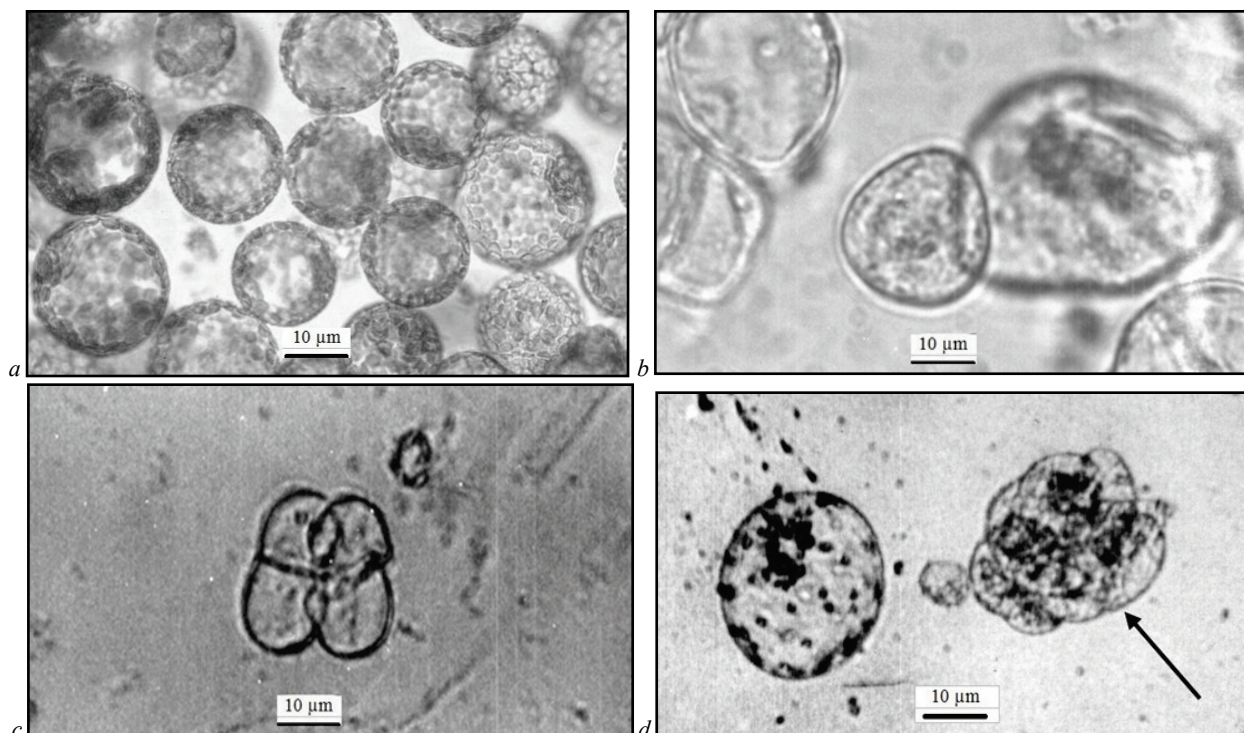

Fig. 1. Typical cell growth in the culture of protoplasts of the studied plant species *in vitro* under the control variant of cultivation: *a* – recently isolated mesophilic protoplasts of rapeseed Shpat (*Brassica napus* L.), sown in a liquid nutrient medium TMmod1 (k) (1 mg/L NAA; 0.2 mg/L 2.4-D, 0.5 mg/L BAP, 0.5 M glucose); *b* – cell wall repair process within the first 48 hours of cultivation of hypocotyl protoplasts of white cabbage Kharkivska zymnia (*Brassica oleraceae* var. *capitata* L.); *c* – the first symmetrical cell division of the mother cell (hypocotyl protoplasts of white cabbage Lika cultivar (*Brassica oleraceae* var. *capitata* L.)); *d* – unorganized microcolony growth (highlighted with an arrow) on the nutrient media SWmod3(k) in the culture of mesophilic tobacco protoplasts *in vitro* of the SR-1 line (*Nicotiana tabacum* L.)
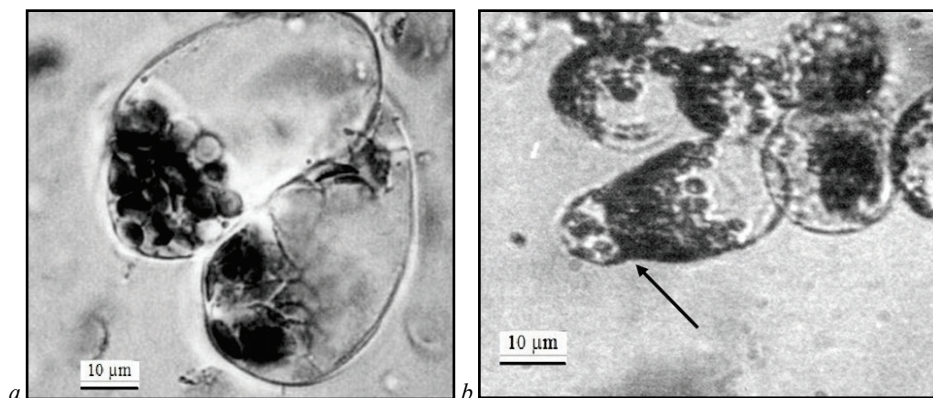

Fig. 2. Cell response to OS in the experimental variant of mesophilic protoplasts cultivation of higher plants *in vitro*: a – excessive cellular vacuolization and polarization of cytoplasmic elements of mitotic active cells after the stages of cell wall repair process (*Nicotiana tabacum* L); b – the first asymmetrical cell division (highlighted with an arrow) with unequal cytoplasmatic elements' distribution (*Arabidopsis thaliana* var. *colymbia* (L.) Heynh.)

Another phenotypic demonstration was observed if the introduction of gibberellin GK3 (1 mg/L) into liquid media was followed by simultaneous osmotic stress relief and reduction of NAA auxin concentration to 0.1 mg/L after the first cell division. In all the analyzed plant species under this variant of cultivation the division of the original population of mitotically active cells into two types of unequal division was observed (by the type of the first asymmetric division of the zygotic embryo *in planta* as well as by the type of cleavage of the mother cell into smaller daughter cells). In the experimental variant of cultivation in the selected species of higher plants the level of manifestation of the first type of unequal division was within 3.1–54.1%, while the second type was within 15.0–40.6%. The symmetrical divisions were 8.5–64.9% (data of the diagram in Figure 4).

A characteristic feature of the first type of division was an unequal distribution of cytoplasmic elements between daughter cells, among which one cell was of a large size with optically dense cytoplasm, while the second cell was of a smaller size and quite vacuolated with an optically light cytoplasm. Subsequently, there was a development of two types of microcolonies, which were formed after asymmetric cell division. One of them had a clearly expressed part consisting of compact small cells in the form of globules and long vacuolated cells, which were divided along the axis (Fig. 5). The second one represented a cone-shaped microcolony, which, as it grew, acquired forms morphologically identical to the heart-shaped zygotic embryo *in planta* (Fig. 6). Thus, in this case there was an analogy between the first asymmetric division of somatic cells in mesophilic protoplasts culture *in vitro* and the first one, determined during the germinal axis, direction of zygote separation *in planta*, after which apical and basal cells were formed (Tvorogova & Lutova, 2018). The second

feature of the development of microcolonies with primary traits of cytodifferentiation was the determination of the symmetry axis of the second order, which was formed as a result of bilateral division of cells that had optically dense cytoplasm after the first asymmetric division (Fig. 7). The above-mentioned trajectory of microcolonies development seemed to be typical for tobacco and *Arabidopsis* protoplasts.
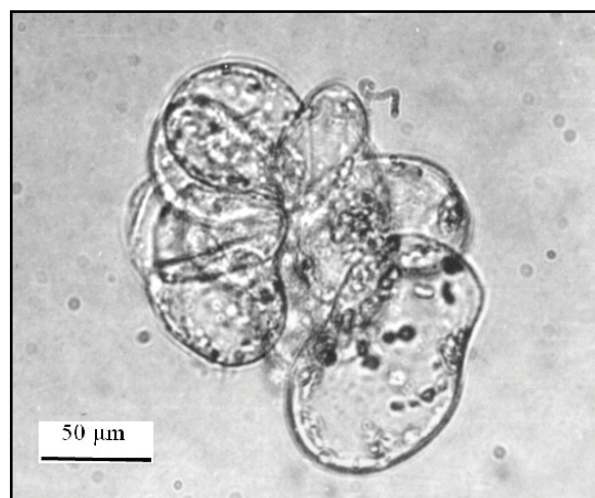

Fig. 3. Unorganized microcolony cell growth in the control variant derived from mesophilic protoplasts (*Nicotiana tabacum* L.)
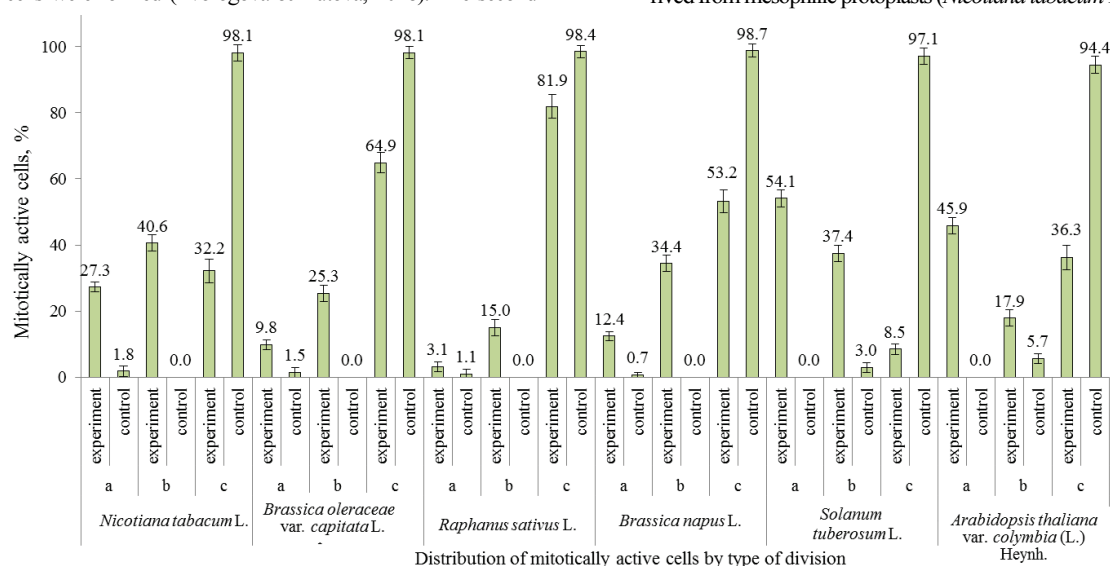

Fig. 4. Percentage ratio of different directions of division of mitotic active cells in both control and research variants in mesophilic protoplasts culture of 6 Brassicaceae and Solanaceae plant species: *a* – the first asymmetric zygotic embryo *in planta* division type; *b* – division of the mother cell cleavage type into smaller daughter cells; *c* – symmetrical divisions; the numerical data in the diagram are presented as x ± SD (n = 5)
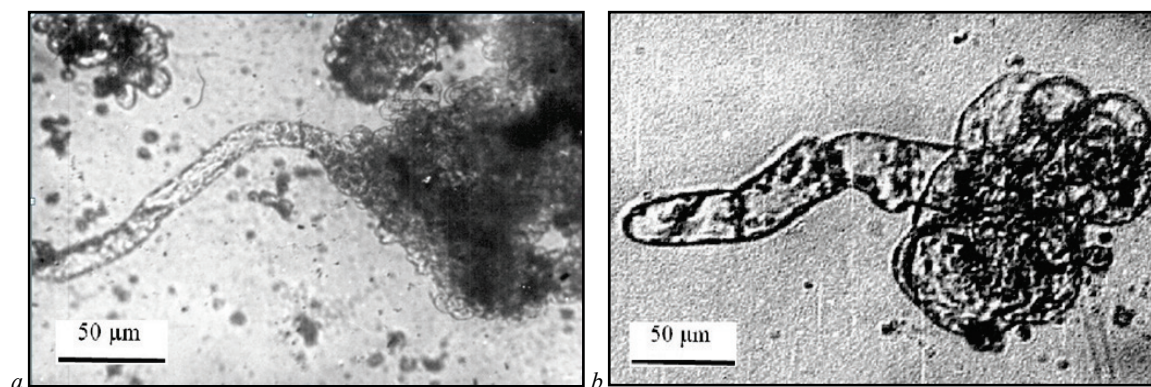

Fig. 5. Polar morphology of microcolonies in the experimental variant of the cultivated cells derived from mesophilic protoplasts: *a* – potato of Zarevo cultivar (*Solanum tuberosum* L); *b* – wild form of *Arabidopsis* (*A. thaliana* var. *columbia* (L.) Heynh.)

The developmental pathway inherent mainly in rapeseed, radish, cabbage, and potato cells in the presence of gibberellin was to initiate cell division, in which the mitotically active cells were fragmented into smaller daughter cells. In this case, protoplasts, after the cell wall was repaired, were morphologically similar to somatic embryos in the heart-shaped stage of development (Fig. 8). Such cytomorphosis was observed even before the first division and only in the presence of exogenous gibberellin. During the first and subsequent mitosis, daughter cells continued to preserve the initial morphology of the mother cell, however, their cell division had no clear orientation throughout the initial axis of symmetry. Nevertheless, within the process of further development, such microcolonies held for a long time the heart-shaped form inherited from the mother cell (Fig. 8 and 9). Except for mitotic active cells, which formed microcolonies with morphological traits of structural organization in all plant species under study, there were cells that divided in an isopolar way, forming microcolonies without traits of structural organization after OS exposure and subsequent injection of exogenous gibberellin into nutrient media (Fig. 3). Consequently, in experiments with the culture of mesophilic protoplasts of higher plants *in vitro* it was shown that osmotic stress followed by the subsequent introduction of high doses of gibberellin GK3 into the nutrient medium led to the appearance of morphological traits of cytodifferentiation already at the initial stages of development of somatic cell microcolonies in a number of higher plants.
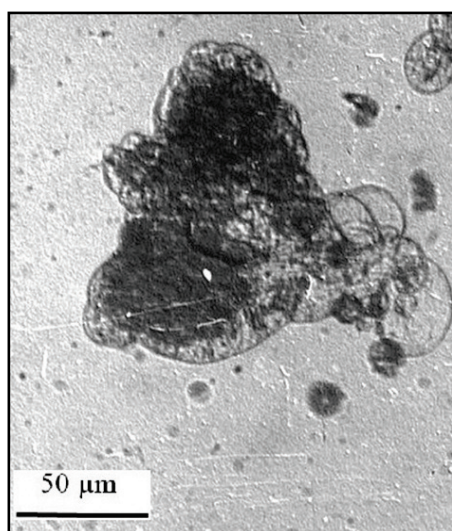

Fig. 8. Structural form of microcolonies of cells in which the mother cell has taken the morphological form of a somatic embryoid at the heart-shaped stage of development even before the first division begins (*Brassica napus* L.)

Fig. 6. Heart-shaped microcolony with morphological traits of structural organization (*Nicotiana tabacum* L.)
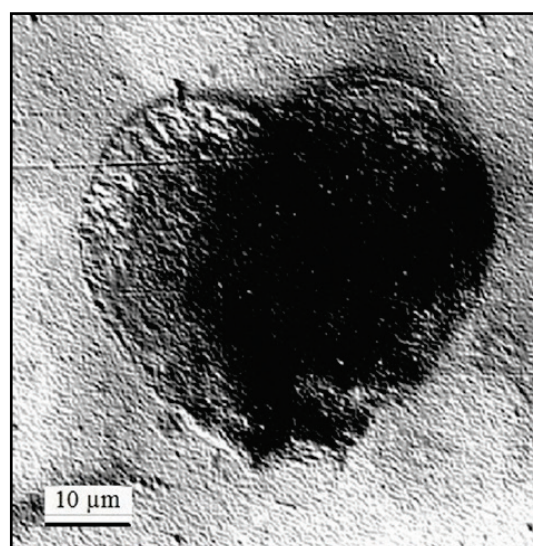
Fig. 9. Structural form of microcolonies of cells in which the mother cell has taken the morphological form of a somatic embryoid at the heart-shaped stage of development even before the first division begins (*Brassica oleracea* var. *capitata* L.)

A separate study of the effect of OS on hypocotyl protoplasts of different varieties of white cabbage isolated from tissues of 7–9 day etiolated seedlings revealed one characteristic feature: the determination of cytoplasmic elements in this type of cell and the subsequent development of differential morphology of microcolonies occurred without additional injection of exogenous gibberellin (GK3) into the nutrient medium TMmod2 (k). That is, the hypocotyl protoplasts, having undergone a cell membrane repair process, morphologically resembled somatic embryos at the heart-shaped stage of development in the absence of exogenous gibberellin (cytomorphosis similar to the one presented in Figure 9 for white cabbage cells derived from mesophilic protoplasts). This fact, as well as preliminary data obtained on mesophilic protoplast culture, gave us grounds to assume that plant gibberellin has the same regulatory effect as GK3. It has been established that during the period of germination of zygote embryo, the endogenous content of different forms of gibberellin in different plant species increases by several orders of magnitude (Blume et al., 2012), which probably significantly affected the phenotypic reactions of hypocotyl protoplasts in terms of the action of osmosis. In the next series of experiments for this type of cells, the evaluation of asymmetrical division was conducted from the standpoint of phytohormonal regulation

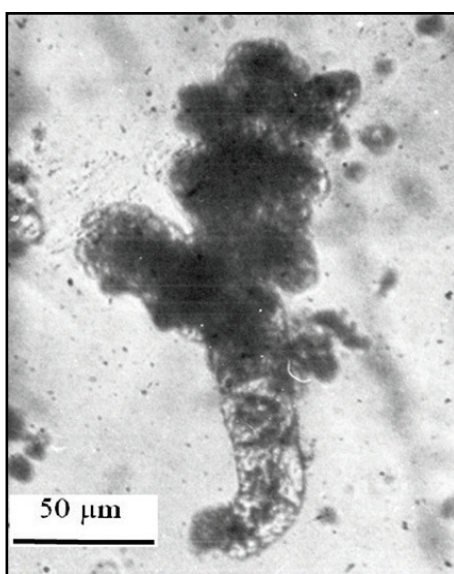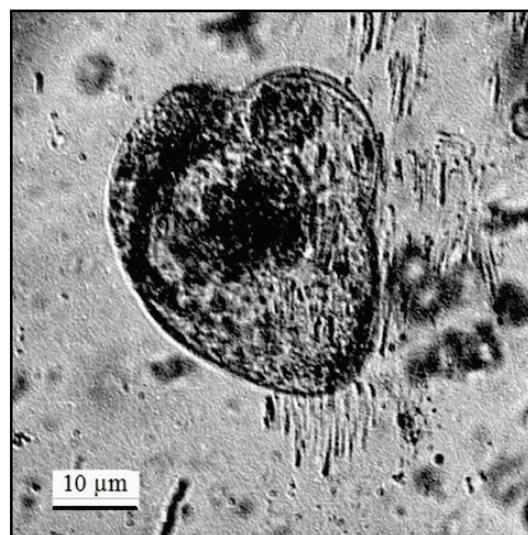**Fig. 7.** Bilateral symmetry of microcolonies with morphological traits of structural organization (*Arabidopsis thaliana* (L.) Heynh.)

of proliferation and cell differentiation. Figure 10 shows the diagram reflecting the percentage ratio of different types of division of mitotic active cells in the culture of hypocotyl protoplasts of 8 varieties of white cabbage after the application of control and trial cultivation, similar to that of mesophilic protoplasts of higher plants (Fig. 4).

Evidence suggests that these cultures, in the framework of the experimental variant, were dominated by maternal cells' being divided into smaller daughter cells. The greatest number of them was registered in

early-ripening cultivars: Dithmarscher Früher and Iyunskayarannyaya at 71.5–79.0% (Fig. 10). In other cultivars, this figure ranged between 25.3–67.9%. Division by type of cleavage occurred in those cells, which after OS and subsequent administration of exogenous gibberellin (GK3) acquired initial morphology with traits of structural organization (heart-shaped one). In late ripening varieties of white cabbage, the highest percentage of such cells (more than 50%) was observed in Kharkivska zymnia, Lika, Lesya and Bilosnizhka cultivars (Fig. 10).
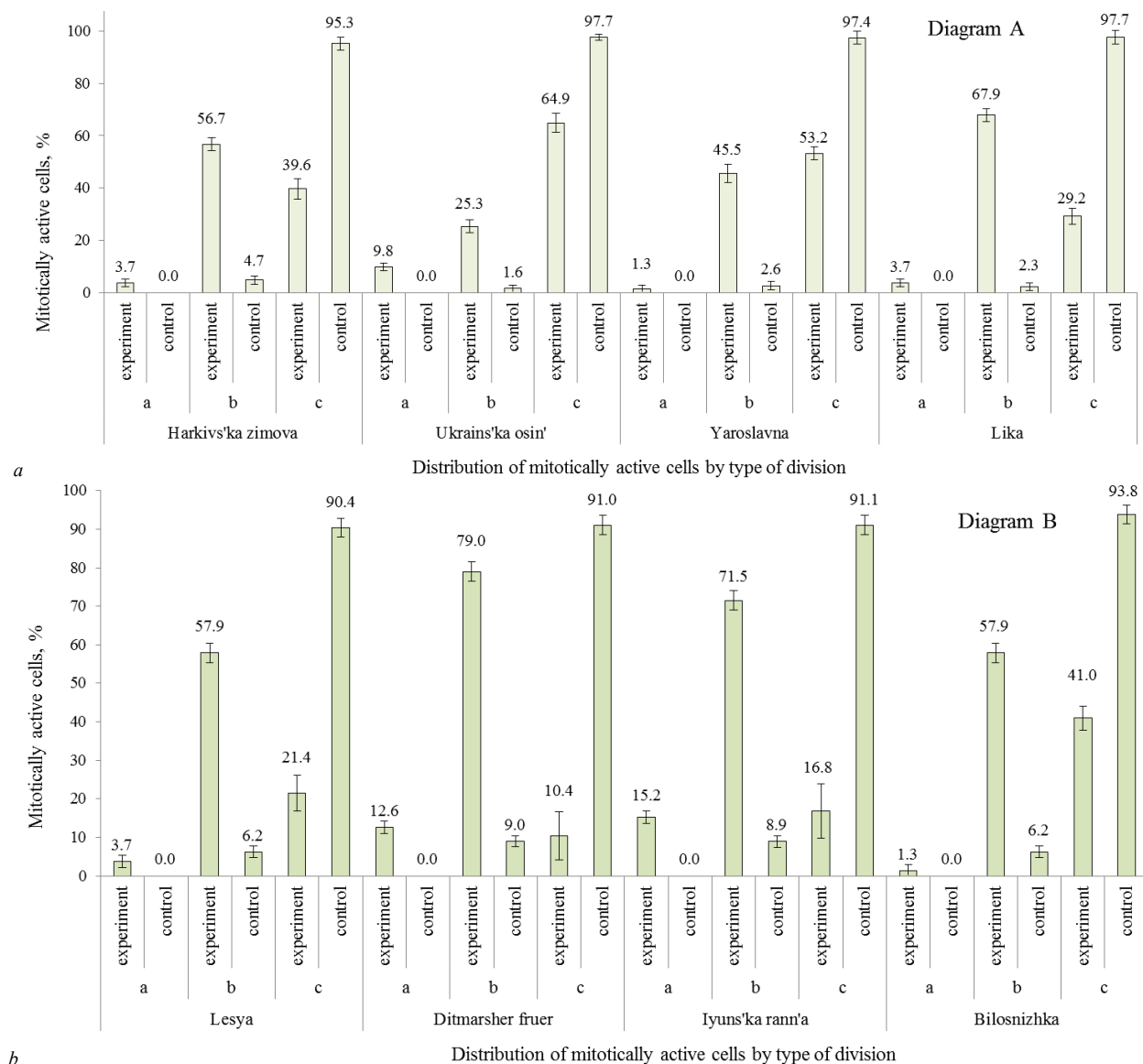

Fig. 10. The percentage ratio of different types of division of mitotic active cells in both control and research variants in culture of hypocotyl protoplasts *in vitro* of white cabbage cultivars (*Brassica oleraceae* var. *capitata* L.): *a* – the first asymmetric zygotic embryo *in planta* division type; *b* – division of the mother cell cleavage type into smaller daughter cells; *c* – symmetrical divisions; the numerical data in the diagrams are presented as x ± SD (n = 5)

It is common practice when at the stage of their cultivation up to the nutrient media TMmod3(d) is applied, they continued to preserve the differential morphology. To support mitosis, the culture was transferred to the optimal growth medium for microcolonies TMmod3 (d) with modified content of growth regulators (1 mg/L BAP, 1 mg/L GK3, 0.2 mg/L 2.4-D and 0.1 mg/L NAA), glucose (0.4 M) as well. In the process of further cultivation, microcolony growth was followed by a gradual loss of differential morphology and a transition to unorganized growth. Moreover, the further presence of gibberellin (1 mg/L GK3) in the growth regulators of TMmod3 (d) nutrient medium had a negative impact on cell development, increasing their transition to growth by extending while reducing mitotic activity. This affected the change in the qualitative composition of cell populations, in which not meristematic, but vacuolated parenchymal cells began to predominate in an amount up to 65–70% of

the total population (Fig. 12). The dynamics of mitotic activity of cells, which took place during the 2-month period of cell cultivation with the use of different options of osmotic and hormonal regulation of plant cell culture growth, derived from hypocotyl protoplasts of white cabbage, is reflected in the graph in Figure 13. The obtained results show that the value of mitotic index reliably differs in the two experimental variants of cell cultivation with the presence and absence of GK3 in the composition of growth regulators of TMmod2 (e) nutrient medium. Moreover, the value of this statistical indicator was higher in the options of cultivation, where gibberellic acid was not used. Cell proliferation in the presence of the auxin regulator 2,4-D in the control variant of cultivation affected the maximum number of peaks of mitotic activity of cells. Altogether, the obtained results indicate that changes in osmotic and hormone regulation had a peculiar effect on growth processes in cultivated plant cells.
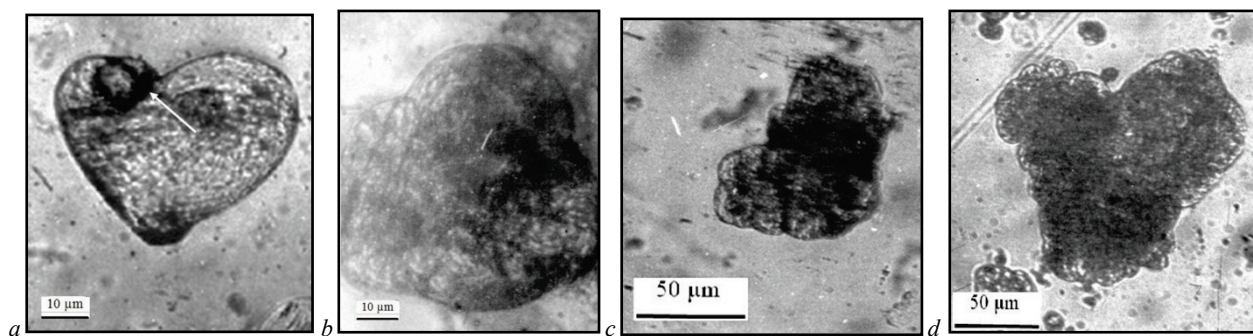
Fig. 11. Typical microcolony development with morphological traits of structural organization in hypocotyl protoplasts' *in vitro* culture of white cabbage Kharkivska zymnia, Ukrainska osin, Yaroslavna, Lika, Lesia, Bilosnizhka, Dithmarscher Früher and Iyunskarannia (*Brassica oleraceae* var. *capitata* L.): *a* – the first cell division (indicated by an arrow), which initiates the onset of division of a more vacuolated mother cell into smaller daughter cells; *b* – growth of microcolonies of cells after their transfer to the nutrient medium TMmod3(d); *c, d* – gradual transition to unorganized microcolony growth with primary traits of structural organization on the TMmod3(d) nutrient medium

Decrease in mitotic cell activity in the framework of variants with the use of gibberellic acid correlates well with the beginning of cell growth by extension and inhibition of cell division. By the number of mitotic active cells, the research variant of cultivation with application of hypotonic stress only took an intermediate place between the control and experimental variants with application of a high dose of gibberellin in the TMmod3(e) nutrient medium. This intermediate state was preserved up to about 20 days of cultivation, and the mitotic index value was almost equal to the control one (Fig. 13).

Discussion

Characterizing the effect of growth regulators of nutrient media on the development of microcolonies of cells, we should note the generality of our results obtained in earlier studies of regulatory action of phytohormones at the *in planta* level. Cells in higher plants are known to lack mobility due to the presence of cell walls, so plant morphogenesis is largely dependent on regulated cell division in a strictly defined direction (Medvedev, 1996). Accurate determination of the cell division area is extremely important for many forming processes, with the structural components of cell cytoskeleton playing an important role in this process, among which microtubes (MT) perform the most important function (Medvedev, 1996). The first indication that the cell begins to divide in this direction appears immediately after interphase, as the MTs reorient in the process of preparing for mitosis. There is evidence that processing with auxin leads to transverse (relative to the long axis) orientation of MT in cells of higher plants (Blume et al., 2012). It was found out that the impact of the auxin on the orientation of MT is after the preliminary processing of the GK3 (Blume et al., 2012). Gibberellins, like auxin, induce transverse (relatively long axis) location of MT cells (Blume et al., 2012). Short-term cell extension under the influence of gibberellin is not followed by MT retargeting. GK3 pre-treatment leads to MT reorientation if the gibberellin combines with

auxin. Data from other studies show that the orientation of MT also depends on the cytokinins. It has been determined that action of kinetin on plant cells can change the location of these structural components of cytoskeleton from transverse to longitudinal (Blume et al., 2012). Thus, under the influence of phytohormones there is a dynamic modification of cortical MT system in mitotic active cells *in planta*. In the presence of all the above-mentioned phytohormone analogs in the nutrient medium for the cultivation of white cabbage protoplasts, we observed similar phenomena in the growth of cell clones. However, the induction of differential cell division required a balanced selection of growth regulators as well as their coordinated application in time together with the regulation of osmotic pressure in the nutrient medium.
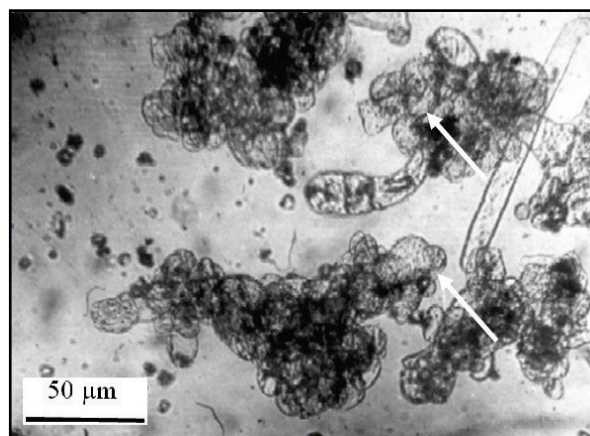

Fig. 12. Transition to cell growth by stretching (indicated by arrows) in microcolony in the TMmod3(d) nutrient medium (culture of hypocotyl protoplasts *in vitro* of white cabbage of Kharkivska zymnia cultivar)
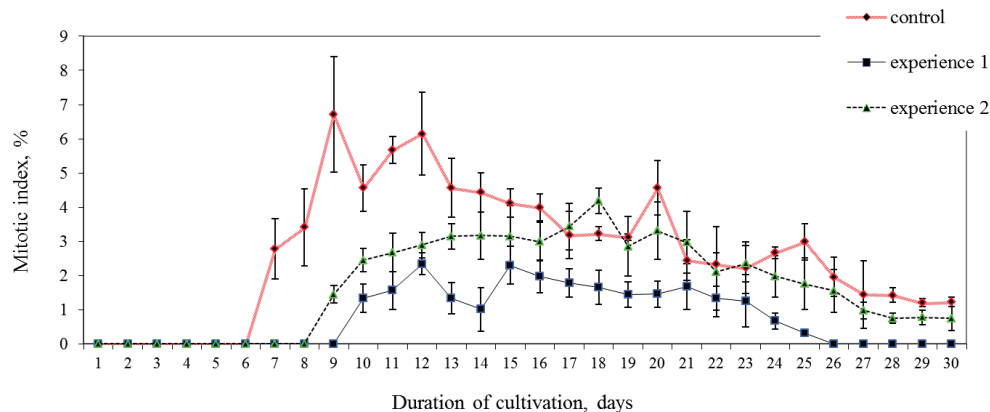

Fig. 13. Features of the mitotic cell activity identified at different variants of cultivation of hypocotyl protoplasts (averaged data for 8 varieties of white head cabbage): experiment 1 – cultivation mode with osmotic stress and subsequent introduction of gibberellin GK3 into nutrient medium; experiment 2 – osmotic stress-only cultivation mode; the numerical data in the graph are presented as x ± SD (n = 5)

Assessing the contribution of osmotic stress to cytodifferentiation processes, it should be noted that previously various types of stress agents were widely used *in vitro* culture for embryogenesis induction. For example, cold and osmotic stresses are often used for androgenesis induction in the cultivation of isolated microspores (Ochattet al., 2009; Islam & Tuteja, 2012). The addition of abscisic acid (AA) to nutrients as one of the stress agents has been applied to induce cytodifferentiation in carrot globular embryos (Jameel & Abdulaziz, 2012). In our case, one of the possible mechanisms of osmotic stress influence is the emerging of a powerful diffuse gradient, which led to the intake of excess water and growth regulators (primarily auxin) into the cells. Forceful hydration of both membrane and cytoplasmic proteins could also contribute to the "unblocking" of the functional activity of a number of enzyme systems as well as to the mobilization of the genome of cells as a whole (Komaki & Sugimoto, 2012). It is worth noting that when using a system of isolated protoplasts, the heterogeneity of the original cell population is determined by the absence of synchronization in the phases of the cell cycle. Thus, within the framework of our researches we observed a different ratio of cell population being developed by cytodifferentiation. The morphology of the microcolonies obtained in the result of our study was common with the early stages of development of protoplasts of both *Gentiana kurroo* (Niedziela & Rybczyński, 2007) and *G. straminea* (Shi et al., 2016), which developed through direct somatic embryogenesis in specially selected plant lines in the deficiency of exogenous gibberellin. Related morphology has been observed, also in the initial division of the zygote *in planta* (Zhao & Sun, 2015) as well as polarization (formation by root-like projection) in the brown algae (Bogaert et al., 2013). As already proven, in the lower algae, such polarization is due to the polar transport of auxin. In terms of our research, the morphological diversity of microcolonies according to the differential types may be explained by the accelerated intermutation of various gibberellins by cell enzyme systems (Hedden & Sponsel, 2015), while the determinacy of symmetry axes – by the effect of GK3 on the orientation of cytoplasm microtubes of mitotic active cells in a certain direction, e.g. along the axis of growing microcolonies (Wenzel et al., 2000). It is worth highlighting that gibberellin GK3 is rarely used in plant culture *in vitro*, while more often at the last stage of regeneration (Edwin et al., 2007) and is almost out of use in cultivated media at early stages of protoplast culture development. Assessing the possible role of gibberellin in cytodifferentiation processes, it should be noted that plants have predominantly the same type of auxin (IAA) and cytokinin (zeatine), but more than 130 different forms of gibberellin, most of which exhibit their activity specifically and only at specific stages of plant development (Binenbaum et al., 2018). Besides, gibberellin synthesis level *in planta* increases significantly in the process of *de novo* formation of all organs of higher plants and, especially, in the development of the zygotic embryo, which is quite convincingly demonstrated in embryological studies on rape (Hays et al., 2001). Nevertheless, studies of the phytohormone content in the unorganized growth of tobacco callus revealed a complete absence of endogenous gibberellins (Nawaga et al., 1987). Our results show that gibberellins directly participate in regulation of somatic cell differential division. Based on the above, there is every ground to assume that of all phytohormones, it is the gibberellin that is the hormone determinant of morphogenetic processes in plants. To verify the current hypothesis, we require more detailed studies and clarifications of the regulatory functions of this phytohormone in plants. Our morphological evaluation of microcolonies that underwent the OS stage provides additional evidence in favour of the hypothesis, since even the presence of only one compound from the class of gibberellins (GK3) in the hormonal composition of the nutrient medium led to the formation of microcolonies with initial morphological traits of structural organization. The generality of reaction on presence of gibberellin in mesophilic protoplasts of the species under study gives the grounds to assume that the phenomenon described by us represents the general physiological regularity inherent in higher plants as a whole. Summing up the effect of GK3 gibberellin at the early stages of development of hypocotyl protoplasts of different varieties of white cabbage genotypes, it ought to be remarked that the effect of this phytohormone is more negative than positive. According to Butenko (1981), when growing an explant plant tissue in the dark-room conditions with the use of gibberellic acid, it is possible to suppress the function of auxin oxidase, which may adversely affect the mitotic activity

of cells due to the growth of endogenous auxin. Since under conditions of optimal proliferation of cells, their cultivation during the first 20 days was carried out in the dark-room conditions, the above-mentioned negative impact of this phytohormone on the mitotic activity of cells could naturally occur.

The generalization of the factors that influenced the asymmetric division of cells derived from mesophilic and hypocotyl protoplasts of higher plants is presented in Table 2. The table shows in more detail the main types of asymmetric cell division of the studied plant species, depending on the time-coordinated factors of the osmotic and hormonal regulation of the division process. At the stage of the sixth mitosis in dividing cells, microcolonies switched to unorganized growth, while losing morphological traits of structural organization. In our opinion, the reason for such a transition to the unorganized growth of microcolonies is the incorrect choice and balance of growth regulators in the composition of nutrient media TMmod3 (e) and SWmod3 (e). The possibility of implementing a program of somatic embryogenesis in protoplast culture *in vitro* for the studied plant species requires additional study of the factors of cell differentiation.
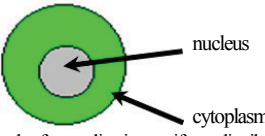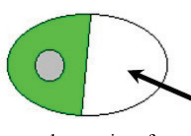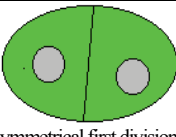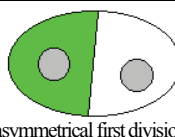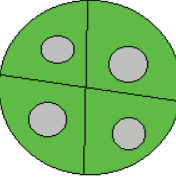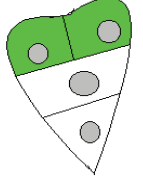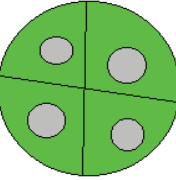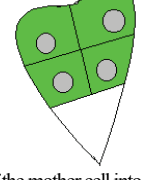
As is known, isolated protoplast is the most disintegrated structure of the plant body. Therefore, this cell type is an ideal experimental object for studying the regularities of the "cell-plant" development cycle when simulating morphogenetic processes *in vitro* culture. Currently, significant progress has been made in studying plant protoplasts, but the issue of their cultivation and regeneration of plants on their basis is still of fundamental importance. This is mainly due to the fact that the protoplast is a component of a complex system of development, and in addition, generally, it is subject to key stress factors when cultivated. It is known that different types of stress can influence the regulation of genes through their expression or derepression. According to Cocking (1972), the protoplast is a damaged, with a missing cellular shell, isolated plant cell, which is under osmotic stress. The principal physiological feature of protoplasts is the ability, under certain conditions of cultivation, to regenerate the cellular cover and begin to divide, forming cell colonies or even plants (Davey et al., 2005). Having undergone cellular repair process, plant cells are formed in the same way as in a conventional suspension culture (Sidorov et al., 1985), therefore the conditions of their cultivation are subject to change. It is required as follows: reduction of osmotically active concentration in substances, gradual change of hormonal balance being dominated by cytokinins. Existing experimental practice requires an empirical selection of all three cultivation regulatory factors (Sidorov et al., 1985), thus a certain number of protoplast nutrient media options have been developed that have been applied to a limited number of genotypes or plant cultivars. Violation of any of the above-mentioned elements of the regulation of cultural growth as well as development leads, commonly, to cell death. A special role in maintaining cell growth and differentiation in protoplast culture belongs to the regulators auxin, among which we should mention the special role of 2,4-D as an activator of reprogramming the genome of special somatic cells, after which they become totipotent and achieve their development potential, just as it occurs in egg cells after fertilization (Peterson et al., 2016).

In our study, the occurrence of the regulator 2,4-D was obligatory as in its absence cultivated cells of plants did not restore or completely lost their mitotic activity. It has been established that the regulator belongs to the class of phytohormonal herbicides, a quite toxic substance, which negatively affects the ontogenesis of plants. This has been proven in numerous studies on many plant species (Song, 2014). In particular, the toxicity of this substance is manifested in chromosomal disorders and the growth of meristematic cells in apical zones (Song, 2014). 2,4-D is poorly disposed of by plants. It provides grounds to assume that while implementing the program of plant morphogenesis *in vitro*, the regulator may disrupt regeneration processes in the cultivated objects due to the herbicidal effect, especially in the determination of stem organogenesis in dicotyledonous plants.

Thus, the question of the final determination of the principles of osmotic, trophic and hormonal regulation of morphogenesis *in vitro* of higher plant protoplasts requires further study. The identified common consistent pattern of the development of somatic cells an early stage of the growth of protoplast culture of higher plants *in vitro* served as a basis for the authors' developed method of the effective plant regeneration in protoplast *in vitro* culture of white cabbage through organogenesis. The results of these studies are planned to be presented by us in the next article.

Table 2

Exogenous factors of plant cell cultivation before and after cell wall repair that influenced the asymmetric cell division
in the culture of mesophilic and hypocotyl protoplasts of six plant species of the Brassicaceae and Solanaceae families

Stage of cell development	Cultivation variants for mesophilic and hypocotyl protoplasts			
	control variant		experimental variant	
	growth regulators and glucose osmotic content	the phenotypic response of cells	growth regulators and glucose osmotic content	the phenotypic response of cells
Cell wall repair in protoplasts, the beginning of growth processes in cells	1–2 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	lack of vacuolization, uniform distribution of cytoplasmic cells elements	1–2 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.36 M glucose (during 16–18 hours and followed 0.5 M glucose)	unequal separation of cytoplasmic elements of cells due to the growth of vacuoles
I mitosis	1–2 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	symmetrical first division	1–2 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	asymmetrical first division
II-VI mitosis	0.1 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	disorganized growth of microcolonies	1 mg/L GA₃, 0.1 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	microcolony growth with traits of structural organization: 1) formation of cell division directions by type of zygotic embryo development *in planta*;
	0.1 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	disorganized growth of microcolonies	1 mg/L GA₃, 0.1 mg/L NAA, 0.5 mg/L BAP, 0.2 mg/L 2,4-D, 0.5 M glucose	2) division of the mother cell into smaller daughter cells.

Conclusion

The key result of the research carried out is the determination of the development process for the cultivation of protoplast of higher plants that provide for an indication of the process of asymmetric cell division *in vitro*. Exogenous gibberellin has been proved to be one of the initiators of such a division type. As has been shown above, this class of phytohormones significantly influences the initiation of morphogenetic processes in higher plants, therefore, in our experiments, the action of gibberellic acid (GK3) has been studied in more detail at the initial stage of cultivation of mesophilic protoplasts of six species of plant belonging to the Brassicaceae and Solanaceae families.

In experiments with mesophilic protoplasts it was shown that short-term osmotic stress within 16–18 hours in combination with the subsequent administration of high doses of gibberellin GK3 into the nutrient medium led to the appearance of morphological traits of cytodifferentiation already at the initial stages of cell microcolonies in a number of higher plants. At the same time in all analyzed plant species there was observed the division of the original population of mitotic active cells into two types of asymmetric division: by the type of cleavage of the mother cell into smaller daughter cells and by the type of the first asymmetric division of the zygotic embryo *in planta*. A special study of the effect of osmotic stress on hypocotyl protoplasts of various cultivars of white cabbage, isolated from hypocotyl 7–9 day etiolated seedlings, revealed a rather characteristic feature: the determination of cytoplasmic elements in this type of cells and the subsequent development of differential morphology of microcolonies occurred without additional injection of exogenous gibberellin (GK3) into the nutrient medium. This fact, as well as the data obtained on mesophilic protoplasts, gave us grounds to assume that phytogenic gibberellin, localized in juvenile etiolated seedlings, which were the source of protoplasts, had a regulatory effect similar to that of GK3.

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