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**REGULAR ARTICLE**

# MORPHOGENETIC MODULES FORMATION IN SUGAR BEET CALLUS TISSUES *IN VITRO*

*Oksana Leonidivna Klyachenko, Albert Likhanov, Svitlana Antoliyivna Krylovskaya**

***Adress:*** National University of Life and Environmental Sciences of Ukraine, Department of Ecobiotechnology and Biodiversity, Heroiv Oboronu st., 15, 03041, Kiev, Ukraine.

*Corresponding author: krylovskaya.sv@mail.ru

## ABSTRACT

After stocking of a sugar beet plant explants on the modificated agarized medium according to the Murashige & Skoog protocol laced with 1000 $mg.ml^{-4}$ mesoinositol, 5 $mg.ml^{-4}$ of vitamin B1, 5 $mg.ml^{-4}$ casein hydrolyzate, 10 $mg.ml^{-4}$ 6-BAP, 5 $mg.ml^{-4}$ NAA, 1 $mg.ml^{-4}$ IAA callus formation was observed on the 20-23 day. Development of callus culture accompanied by formation of two histochemicaly different zones - peripherical and inner. In the process of further differentiation in the certain sections of inner callus zones small group of cells with high proliferative potential was formed. They initiated formation of meristemoids. Their development synchronized with lignification and deposition of β (1→3)-glucan (callose) on external periclinal and anticlinal cell walls of parenchyma peripherical zone. Synthesis and deposition of callose on cell walls regulated the intensity of the intercellular ion transport and products of metabolism, creating conditions for the formation of biochemical grade in callus tissues. Morphogenic structures with developed conductive system were formed both on the callus surface and middle layers. They had spatial-structural organization in which were meristematic cones, provascular zones with hydrocites system, surrounded by several layers of small parenchymal cells. Each module was surrounded by a large sized parenchymal cells, cell walls of which had significant depositions of callose, lignin and suberins.

**Keywords:** sugar beet, callus, parenchyma, meristemoids, cell wall, lignin, callose

## INTRODUCTION

In a sugar beet (*Beta vulgaris* L.) selection methods of plant culture in vitro is widely used, which allows to grow and to investigate plant organisms and tissues in relatively controlled conditions **(Круглова *и др.,* 2005).** One of promising direction in selection of this culture is a plant regenerants through the method of indirect morphogenesis **(Mishutkina *et al.,* 2006).** It is known that the procedure of sugar beets' callus obtaining, capable to embryogenesis, is complex and has specific features **(Murasige *et al.,* 1962).** Culture requires careful selection of growth medium components, including hormones, vitamins and amino acids **(Банникова *и др.,* 1995).** There are reports about development of modified growth media **(Gürel *et al.,* 2001; Mishutkina *et al.,* 2006)**, optimal conditions for sugar beets' embryogenesis induction and viable plant regenerants obtaining **(Банникова *и др.,* 1995).** However, the less studied issue is regulation of morphogenic processes in callus, interaction between undifferentiated and highly specialized cells, spatial organization and development of meristemoids. There is conflicting data about initiation of the morphogenic zones formation in a sugar beets callus - from cells of the explants' upper epidermal layers or from deeper tissues. Morphogenesis inductors of adventive organs (buds, roots) and embryos, which have polar structure and independent from maternal tissues closed leading system, are defined. Also, the role of biopolymers, comprising cell walls, in transport of hormones and nutrient elements is studied not enough, as well as their role in processes of meristemoids formation and initiation of primary tissues differentiation. In this context the aim of our research was to explore peculiarities of meristemoids formation in a sugar beet callus tissue and to identify specific cell transformation in morphogenic zones.

The aim of present study was to determinate dose-dependent changes in activity of SOD, TAS and expression of Hsp70 in porcine ovarian granulosa cells *in vitro*.

## MATERIAL AND METHODS

Initial callus was obtained from leaf segments of a sterile sugar beet plants. In studies were used next varieties: sorts Oleksandriya and hybrids Yaltushkivskiy MS 72, Bilotserkivskiy MS 57, Ukrainskiy MS 70, Uladovo-Verhniatskiy MS 37, Ivanivskiy 33, Atamansha, Vorskla and Katyusha. Morphogenic plant callus was obtained on modified

medium according to Murasige & Skoog's protocol **(Murasige *et al.*, 1962)** laced with 1000 $mg.ml^{-4}$ mesoinositol, 5 $mg.ml^{-4}$ of vitamin B1, 5 $mg.ml^{-4}$ casein hydrolyzate, 10 $mg.ml^{-4}$ 6-BAP, 5 $mg.ml^{-4}$ NAA, 1 $mg.ml^{-4}$ IAA **(Gürel *et al.*, 2001).** Callus was cultured in the darkness at 25°C **(Mishutkina *et al.*, 2006).** For cytological and histochemical studies morphogenic sugar beet callus, cultured during three passages (9 weeks), was selected. Plant material was fixed for 24 h according to Chamberlain's protocol **(Паушева, 1988).** Tissue sections were fixed with iron hematoxylin according to Heidenhain's protocol **(Паушева, 1988).** Callose depositions was found through fluorescence microscopy by using fluorochrome aniline blue (dilution - 1:10000) with microscope Axioscope A-1 Carl Zeiss. Callus tissue was fixed with fluorochrome during 30 min in phosphate buffer (pH - 12.0), then washed in buffer twice for 5 min. Photographic materials and digital experimental data processing were made in AxioVision 40V Carl Zeiss.

**Statistical analysis**

Statistical analysis and regression analysis of the obtained data was made in Microsoft Office Excel. Descriptive statistical characteristics were evaluated. The level of significance was set at ($P < 0,05$).

**RESULTS AND DISCUSSION**

In natural surroundings, the main function of plant callus is to restore the integrity of plant tissues of the body after traumatic injury, which appeared as a result of physical (physical stress, gravitational forces, soil shears, thermal damages, sunburns, lightning strikes etc.) or biotic (direct impact of herbivores or pathogens) factors. On synthetic media callusogenesis has different dynamics and vector of development. As a result of active cell division preconditions for microclonal reproduction of new plants can be spontaneously created. In our experiments, the first signs of callusogenesis on the surface of injured sugar beet leaves appeared at 20-23 day. Active inducer of the callus cell proliferation was selected components of nutrient medium in every manner, which included 1000 $mg.ml^{-4}$ mesoinositol, 5 $mg.ml^{-4}$ of vitamin B1, 5 $mg.ml^{-4}$ casein hydrolyzate, 10 $mg.ml^{-4}$ 6-BAP, 5 $mg.ml^{-4}$ NAA, 1 $mg.ml^{-4}$ IAA.

On initial stages of cultivation, mesophile and parenchyma cells of covers in leaves' vascular bundles took part in callusogenesis initiation. Cells actively divided, but cytokinesis

directions hadn't distinct spatial orientation and synchronization in time. Due to this friable diffuse interstitial tissue with weak bonds was formed. Non-morphogenic callus cells were parenchymal type, with thin cell walls, strongly vacuolated, spherical or ellipsoidal shape with length - 50-150 microns. In non-morphogenic callus tissues, as a result of significant, spatially non-synchronized stretching of cell walls, intercellular transport of substances is made mainly through the apoplast. Simplast connection in initial callus tissues of a sugar beet complicated by the lack of the close link between cells, although, it plays a key role in highly specialized plant tissues in selective transport of ions and the formation of substances concentration gradients, including hormones.

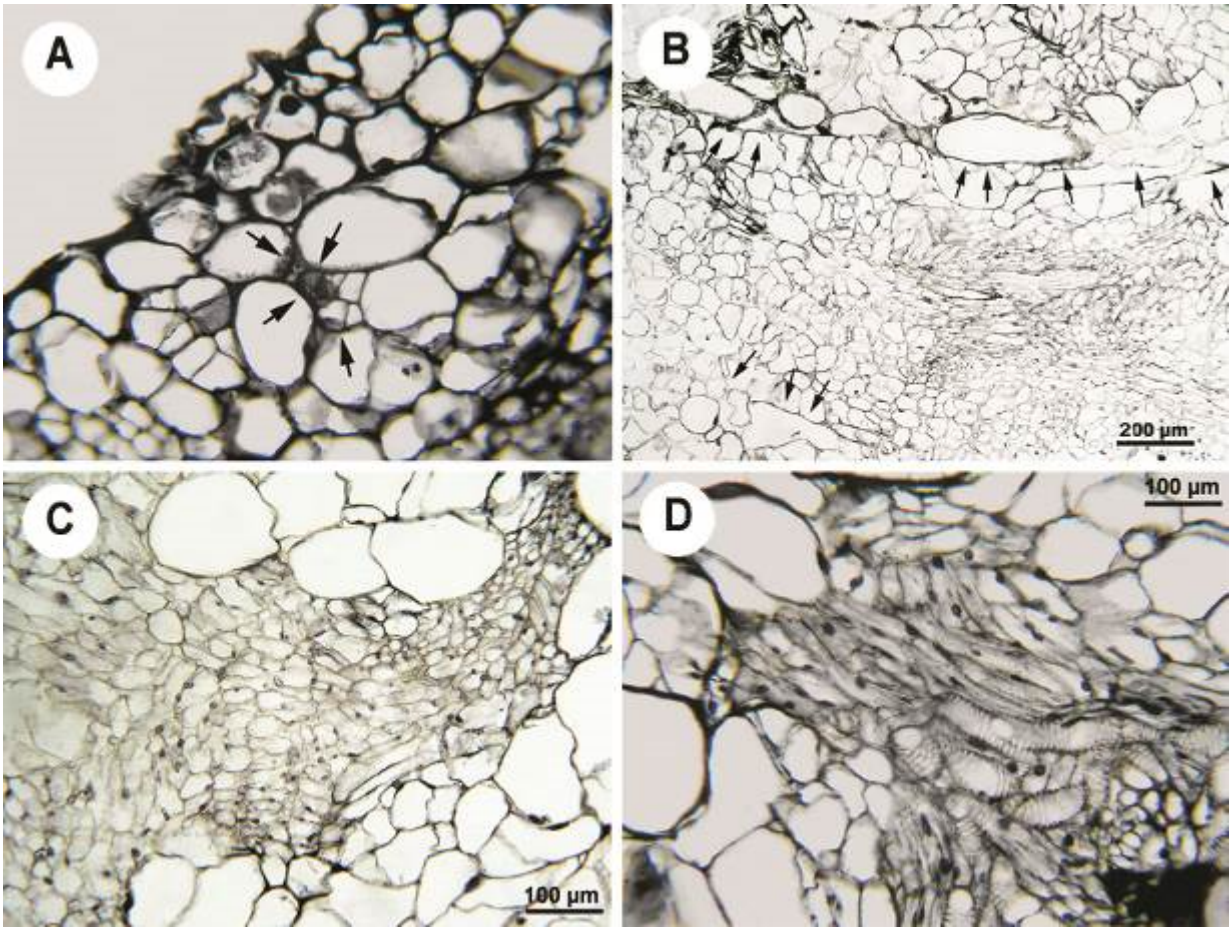


**Figure 1** Differentiation of cells in sugar beet callus tissues of Katyusha cultivar in vitro: A - formation of cell groups (indicated by arrows) with high proliferative potential, B, C - provascular zone in callus cells surrounded by lignificated parenchyma (arrows mark area of lignin components deposition), D - differentiation of callus cells.

In the second callus passage (5-6 week) parenchyma cell walls gradually transformed. In outer layers of cells intense deposition of lignin components is taken place. Thus in non-

morphogenic callus gradually formed two histochemical heterogeneous zones - peripheral and internal. Following histochemical differentiation of cells in certain areas of the callus inner zone separated small groups of cells with high proliferative activity. Proliferation of these cells is not accompanied by strong stretching and water absorption. They have a dense basophilic cytoplasm and a high nucleocytoplasmic ratio (N/C), which is distinguishing for meristem cells (Figure 1, A).

We have found out that the most compact packed and structured were callus cells, which were surrounded by the large (150 - 180 microns) non-nuclear parenchyma cells with significant deposits of callose and lignin components in secondary cell walls. In case of optimal cultivation conditions small cells with thick plasma initiate meristemoid's formation. During investigation of sugar beet morphogenic callus it was found that functionally active meristemoids can form both on the surface of callus tissue and in their middle (Figure 1, B). However, the formation of new gradated cellular structures is not always finishes with development of axial organs, hemo-or rhizogeny.

Primary meristematic zone of morphogenic callus usually consist of a small group of 4 – 8 small (12-15 microns) tightly aligned cells, which development depends on the culture medium composition, the spatial position (contact with the nutrient medium, adaxial or abaxial explants side), functional status and constitutional properties of cells that surround them. Plane and intensity of initial cell division cause the initial morphology of a newly formed structure, which eventually became an active synthesis center of hormones and bioactive substances and thereon began to function as a regulatory center, which synchronizes the division and differentiation of cells. When cells in morphogenic structures differentiate by the vascular type hydrocites - highly elongated cells are formed, in which dradually formed mesh and spiral thickenings of secondary cell walls (Figure 2, A and B). The degree of the cells stretching depended on their spatial position and decreased in the centrifugal direction. The process of the cell walls stretching is known to be regulated by hormones, mainly auxin **(Полевой, 1989)** or its synthetic analogues, that is why according to intensity and direction of cells elongation, specific symplast growth, centers of hormones synthesis and growth factors concentration gradient can be determined. In peripheral areas of sugar beet callus of Katyusha and Atamansha varieties parenchyma cells were characterized by a significant increase in size indicators, but unlike provascular chords cells this process was relatively in all directions (Fig. 2).

Morphogenesis of hydrocites system in vitro has characteristics of more complex cell differentiation, which provided the formation of a system suitable to specialized functions

performance: storage and transport of substances. Hydrocites and tracheid parenchyma have a higher ratio of cell length to their diameter, which is also can be due to hormonal inductions and presence of a sufficient water amount. Spatially, hydrocites system of sugar beet callus usually ended at the basal meristematic cone that under optimal cultivation conditions transformed into apical meristem of adventive buds or roots.

Meristematic cones that function as attractants and centers of hormones synthesis, surrounded by lignificated parenchyma envelopes, create conditions for cell differentiation by vascular type. Thus, in the callus cells culture, the major structural elements of the axial organ are gradually highlight.

In the third callus passage (8-9 week) of Uladovo-Verhniatskiy MS 37 sugar beet cultivar, microsprouts with fully-formed axillary meristem typical of this type of structure are formed (Figure 3, A). According to the nucleocytoplasmic index, cytoplasm density, morphology and gradated zonal distribution of cell, apical meristem differs significantly from meristemoids. In meristem cells from tunics to column, size of cells have a distinct trend of the morphological parameters increasing. Cells of apical meristemoids are determined by the less expressed dependence of their size on the morphogenic tissues location in the structure (Fig. 4, B).

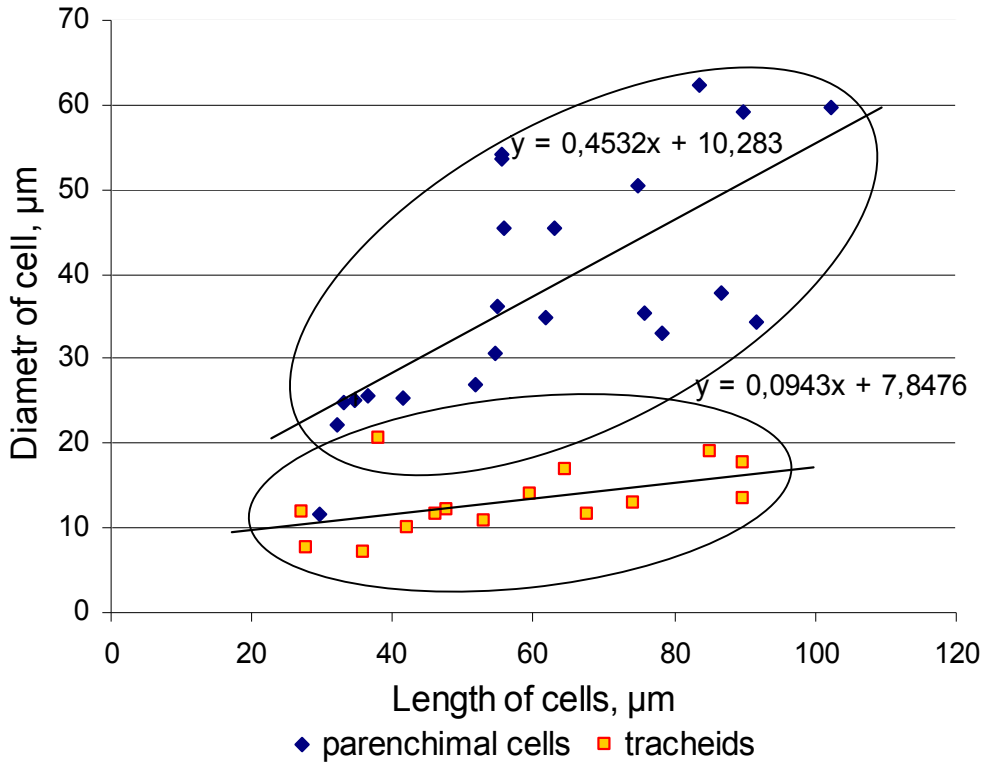


**Figure 2** Correlation of cells linear size of sugar beet callus in Ukrainskiy MS 70 cultivar

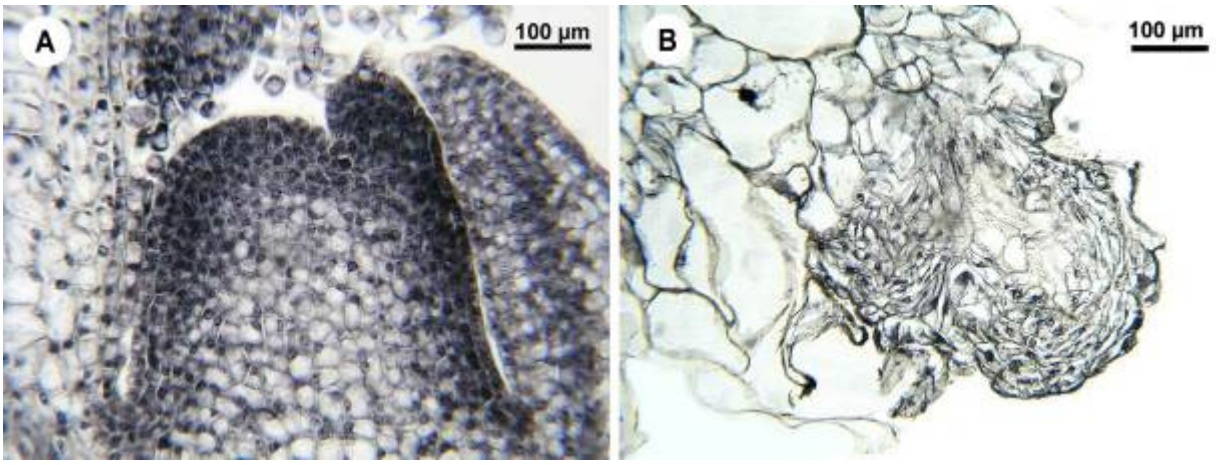


**Figure 3** Axillary apical meristem (A) and meristematic structure (B), which is formed on the surface of callus in the third passage of Uladovo-Verhniatskiy MS 37 sugar beet cultivar

Important indicators of the cells functional specialization are their morphology, linear sizes, shape and size of the nucleus. Comparative estimation of cytometric datas showed significant differences between cells of peripheral parenchyma cells and provascular chords, apical meristems and apical meristemoids (Figure 4).

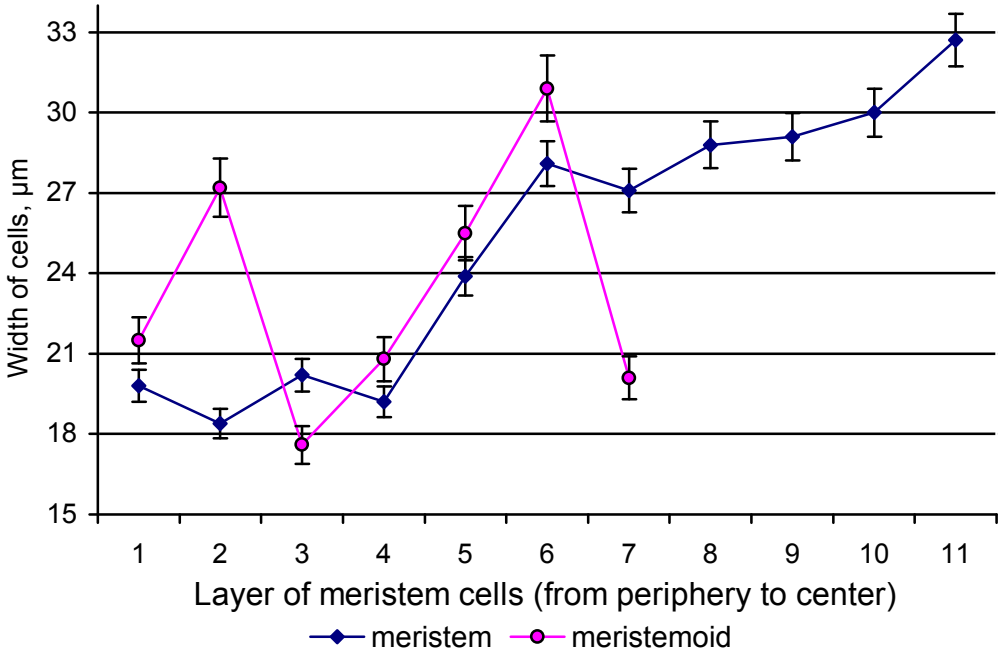


**Figure 4** Comparative characteristics of the spatial dependence of cells linear sizes in apical meristem and sugar beet meristemoids in Uladovo-Verhniatskiy MS 37 sugar beet cultivar

Characteristic of the morphogenic callus cell polymorphism is a high coefficient of the nucleus size variability. Thus, the maximum diameter of the nucleus in parenchyma cells, which surround morphogenic zone (modules), almost three times more than in polar elongated cells of tracheal elements. At the same time, the size of nucleuses and apical meristem apical meristemoids is slightly differ. Highly informative in the functional aspect is an correlation ratio of nucleus and cytoplasm sizes (N/C) (Table 1). This index was the highest for apical meristem cells - 26.3%, the lowest for parenchyma of peripheral zone - 1.8%.

**Table 1** Cytometric indexes of morphogenic callus in apical meristems of Uladovo-Verhniatskiy MS 37 sugar beet cultivar

| Type of cells | Elements of cell | Metrical index | | M ± m |
|---|---|---|---|---|
| Cells of provascular zones (hydrocites) | Nucleus | Diameter, μm | min. | 6,3 ± 0,32 |
| | | | max. | 8,5 ± 0,46 |
| | | Perimeter, μm | | 23,9 ± 1,27 |
| | | Square, μm$^2$ | | 46,3 ± 5,27 |
| | Protoplast | Diameter, μm | min. | 17,6 ± 1,57 |
| | | | max. | 36,7 ± 2,97 |
| | | Периметр, мкм | | 93,0 ± 6,83 |
| | | Square, μm$^2$ | | 551,8 ± 83,2 |
| | N/C index*, % | | | 8,4 ± 1,31 |
| Parenchyma of peripheral zone | Nucleus | Diameter, μm | min. | 10,5 ± 1,17 |
| | | | max. | 22,7 ± 1,57 |
| | | Perimeter, μm | | 57,8 ± 2,50 |
| | | Square, μm$^2$ | | 197,4 ± 16,38 |
| | Protoplast | Diameter, μm | min. | 83,7 ± 12,5 |
| | | | max. | 159,3 ± 7,47 |
| | | Perimeter, μm | | 425,9 ± 17,2 |
| | | Square, μm$^2$ | | 10964,0 ± 438,9 |
| | N/C index, % | | | 1,8 ± 0,24 |
| Cells of apical meristem | Nucleus | Diameter, μm | min. | 9,0 ± 0,31 |
| | | | max. | 11,5 ± 0,33 |
| | | Perimeter, μm | | 33,5 ± 0,86 |

| | | | | |
|---|---|---|---|---|
| | | Square, μm$^2$ | | 86,8 ± 4,86 |
| | Protoplast | Diameter, μm | min. | 17,0 ± 0,55 |
| | | | max. | 24,0 ± 1,18 |
| | | Perimeter, μm | | 68,4 ± 2,72 |
| | | Square, μm$^2$ | | 341,2 ± 27,9 |
| | N/C index, % | | | 26,03 ± 1,09 |
| Cells of apical meristemoid | Nucleus | Diameter, μm | min. | 6,8 ± 0,47 |
| | | | max. | 9,9 ± 1,16 |
| | | Perimeter, μm | | 27,4 ± 2,52 |
| | | Square, μm$^2$ | | 60,1 ± 11,4 |
| | Protoplast | Diameter, μm | min. | 19,7 ± 1,49 |
| | | | max. | 40,0 ± 4,40 |
| | | Perimeter, μm | | 105,3 ± 8,70 |
| | | Square, μm$^2$ | | 652,0 ± 77,42 |
| | N/C index, % | | | 9,38 ± 1,21 |

Note* – nucleocytoplasmic index, (%)

In the most investigated samples of a sugar beet callus tissues small cells with dense cytoplasm were surrounded by a large cells with large deposits of β-(1 → 3)-glucan (callose) in cell walls (Figure 5, A-F).

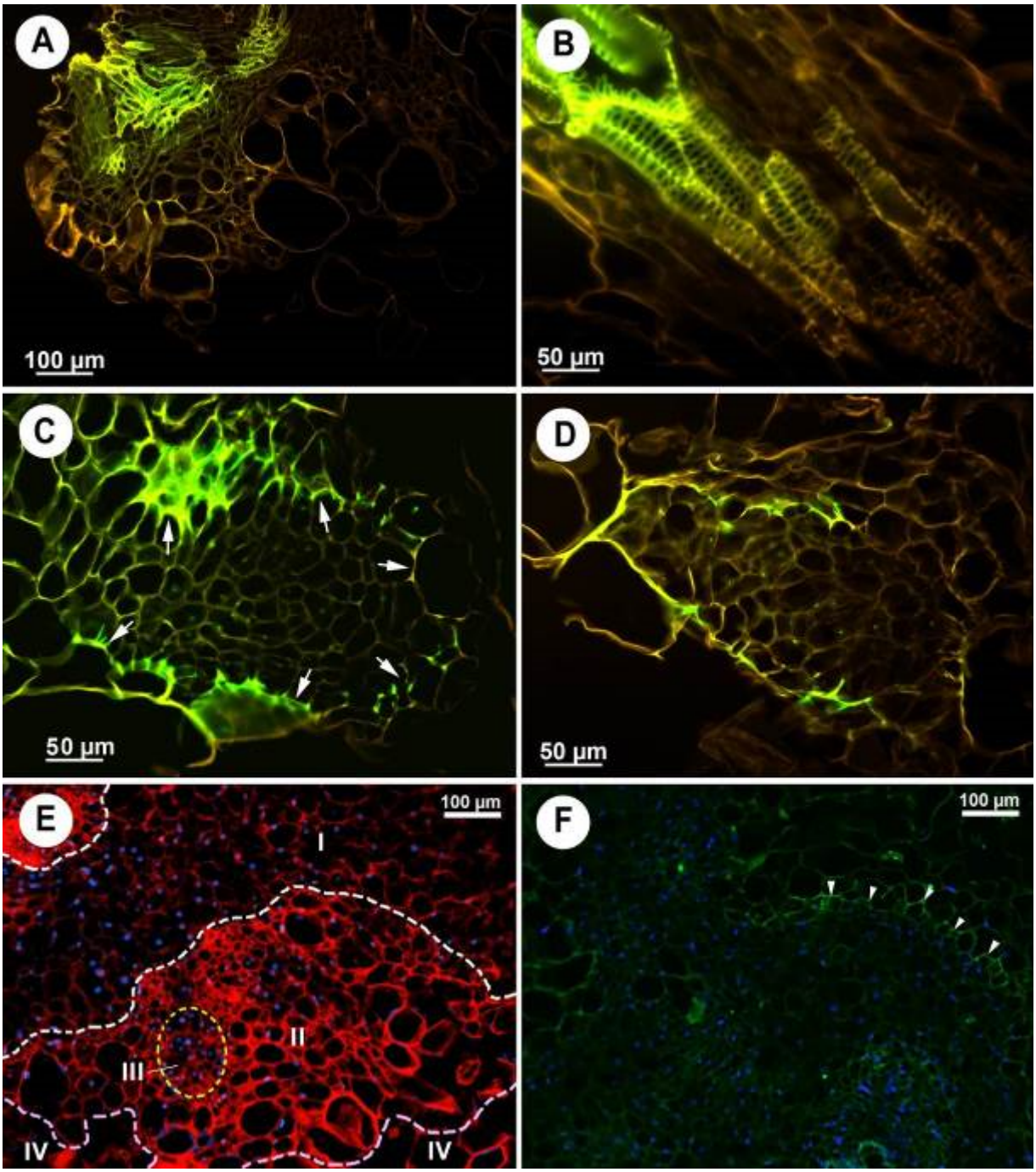


**Figure 5** Callose deposition (green fluorescence), cellular differentiation and meristemoid zones formation (red fluorescence) in callus tissues of: Katyusha sugar beet cultivar - (A, B), Uladovo-Verhniatskiy MS 37 - (C) Bilotserkivskiy MS 57 - (D). Staining with aniline blue (1: 10000); (E) – heterogenic structure of morphogenic sugar beet callus (color staining: DAPI – DNA definition in cell nucleuses; safranin for definition of cell walls lignification): I – non-morphogenic callus with a large sized nucleuses; II - peripherical zone of lignificated cells; III – meristemoid zone surrounded by lignificated cells; IV – zone of cells necrotization; (F) – formation of heterogenic zones in callus with high and low density of nuclear DNA (color staining: DAPI and aniline blue for the callose revelation; arrows mark depositions of callose)

It is known that callose in plant organism performs protective and regulatory functions **(Горшкова, 2007).** Its synthesis is induced increase in the total pool of calcium ions in the cytoplasm of plant cells **(Kauss, 1985),** action of elicitors **(Емельянов *и др.*, 2008),** mechanical effects **(Jaffe *et al.*, 1985).** Deposition of callose as a dynamic component of the plants cell wall, regulate transport of assimilates in tissues, create conditions for partial or complete isolation of protoplasts from cells from external factors **(Курсанов, 1976).** Polyfunctional polysaccharide creates preconditions in sugar beet callus for selective transport and gradient redistribution of organic substances, products of primary and secondary metabolism, and as a result causes histochemical heterogeneity of tissues.

It is possible that in merystemoids cones and small groups of cells that function as attractants and synthesis centers of hormones in the parenchyma surrounded by huge deposits of callose in cell walls starts a cascade of molecular and genetic processes responsible for the regulation of vascular system and gradual differentiation of tissues, which are the main structural elements of the axial organ. For spatial structural organization morphogenic zone in the callus tissue of sugar beet can be considered as separate modules (Figure 5).

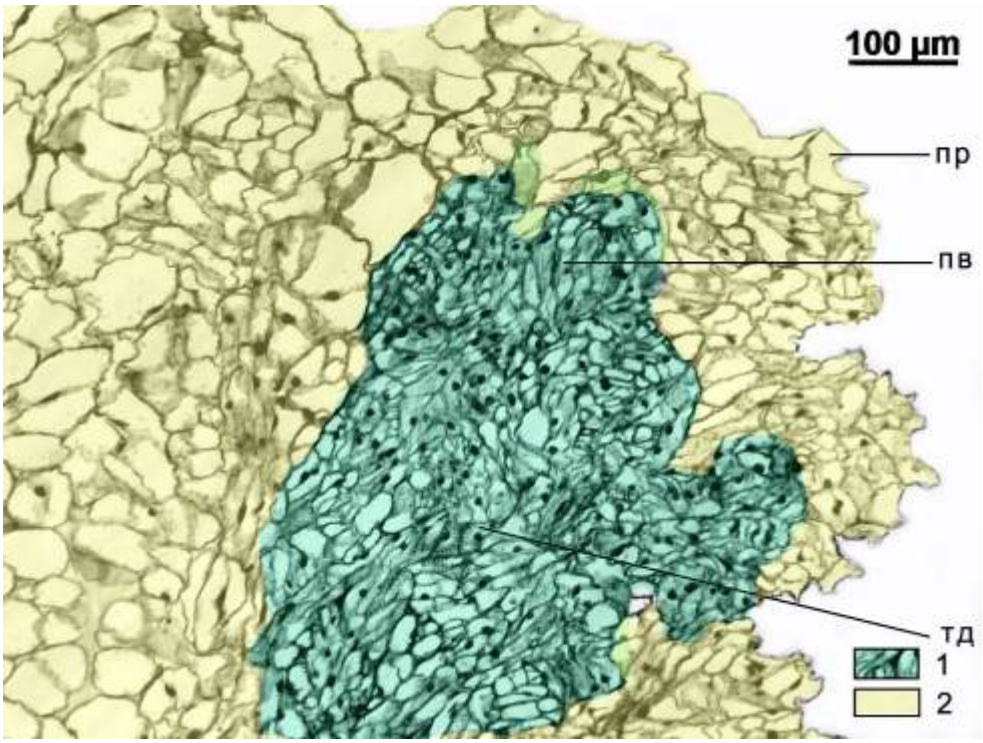


**Figure 5** Formation of modules in morphogenic callus in Ukrainskiy sugar beet varieties: 1 - morphogenic module with provascular chords; 2 - parenchymal cells of callus; pr - parenchyma, pv - provascular cells, td - tracheoid elements (hydrocits)

Each of these modules partially or completely separated from the non-morphogenic callus area with large parenchyma cells with developed secondary cell walls. Further realisation of structures' morphogenic potential, which formed de novo, depend on cultivation conditions, as well as on the functional activity of meristematic zones.

## CONCLUSION

1. It was found that formation of meristemoids in a sugar beet callus synchronized withlignification and significant callose deposition on parenchyma cell walls.
2. It was clarified that formation of highly differentiated morphogenic structures with developed hydrocites system on the surface or inside callus tissues of sugar beet begins with formation of clusters, which are formed by a group of small aligned cells with thick cytoplasm.
3. It was proposed the spatial-structural model of primary morphogenic structures initiation in sugar beet callus tissues as morphogenic modules that consist of meristemoids cone (or group of cells with high proliferative activity), provascular zone with hydrocites system and several layers of small parenchyma cells. Each morphogenic module is surrounded by large cells with large deposits of callose, lignin and suberin on cell walls.

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