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PHENOLIC COMPOUNDS CONTENT AND MORPHOGENESIS IN THE LILAC CALLUS CULTURE (SYRINGA VULGARIS)

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Introduction. Processes of callus cultivation and morphogenesis are of special interest for the investigators in the sphere of physiology, biochemistry, genetics and plant selection because experimentally created model of callus cultivation with the further induction of morphogenesis in vitro is a suitable object for investigating the lawfulnesses of dedifferentiation and second differentiation, genetic variability of cultivated cells, mechanisms of realization of genetic information at histo – and morphogenesis [1–3]. Morphogenesis of the high metasperm plants is a combination of cell differentiation processes with the formation of specialized tissues and organs [4]. At present investigations in the sphere of plant morphogenesis have gained special importance: 1 – plant morphogenesis is a plastic and inversive process which gives an opportunity to study separate stages of development independently from each other; 2 – the usage of achievements in gene engineering for creating new plant forms requires fundamental knowledge and correspondingly investigations in the sphere of studying the mechanisms of development [4]. Intensive investigations of plant morphogenesis are perplexed by the integral character of morphogenetic processes, their dependence on many exterior and interior factors and their interactions [5, 6]. Factors, regulating the processes of morphogenesis in cell cultures of high plants, are an object of intensive investigations. Hormone and genetic regulation of morphogenetic processes for herbaceous plants has been sufficiently studied. Nevertheless the methods of cells culture for the majority of wood plants are quite difficult which is explained by the long-lasting ontogenesis process of these plants and, consequently, by the long-lasting cultural period leading to explants death [8]. As cells differentiation includes changes in genes expression on the level of transcription, consequently, there must be specific substances, determining cells competency to morphogenesis [7]. Characteristic peculiarity of higher plants is the synthesis of second metabolites (SM), determining their pharmacologic and other properties. But the role of SM in morphologic cells differentiation in culture in vitro is unclear. The most abundant group of second metabolites in higher plants is phenolic compounds (PC), which may act as regulators of plants development, growth and differentiation [9, 10]. High PC concentration is found in actively growing cells [11]. There are different points of view in the literature about the interconnection of PC presence with the processes of growth and differentiation. From the one point there is negative PC influence on the processes of cells proliferation though there is opposite point of view [12]. Characteristic peculiarity of the culture in vitro is low level of synthesis of second metabolites, that is why investigation the factors, affecting it, and the role of a number of substances, in particular PC, on the processes of morphogenesis is an actual problem.

Aim of work – determination of content total phenolics in the callus of leaf origin at cultivating in the media containing exogenic PC and their precursors, and establishment of connection between the total phenolics in the callus culture and processes of morphogeses in culture in vitro.

Materials and methods of investigation. The object of investigation has been a stable callus culture of leaf origin of the lilac (*Syringa vulgaris* L.) «M. Sholokhov» cultivar grown in the culture in the State Scientific Institution «Central Botanical Gardens of NAS of Belarus».

Callus of leaf origin has been grown on Murashige and Skoog modified medium [13] containing exogenic PC and their precursor substances: medium 1 – control medium not containing exogenic PC and their precursor substances; medium 2 – containing 6,1 mg / l rutin; medium 3 – 6,1 mg/l of quercetin; medium 4 – 1,65 mg / l of phenylalanine; medium 5 – 6,1 mg / l of rutin + 1,65 mg / l of phenylalanine; medium 6 – 6,1 mg / l of quercetin + 1,65 mg / l of phenylalanine.The period of cultivation is 60 days. 20^{th}, 40^{th} and 60^{th} days corresponding to lag-, exponential and stationary phases of the lilac callus culture growth have been taken as analyzed intervals. 10 probes have been taken at each stage of investigation.

Total phenolics content have been determined using Folin-Ciocalteu reagent [15, 16]. PC extraction has been made with 96 % ethanol in correlation callus: extragent – 1:10 (g / ml), during 18 hours, at 60 °C, in the thermostat TS – 80M. The extract has been filtered. 0,4 ml of filtrate has been mixed with 9,6 ml of water cleansed, 0,2 ml of reagent Folin-Ciocalteu and 2,0 ml of 10% Na_2CO_3. The mixture has been incubated at room temperature for 30 min. Optic density has been measured on the spectrophotometer SF – 46 in the ditch with the layer thickness 10 mm and the wave length 720 nm. In the comparing solution, containing all the components (as in the experiment), cleansed water has been added instead of the extract. Gallic acid has been used for building a standard curve. Total phenolics have been counted in mg / g of the raw callus weight [14].

Callus histologic analysis has been carried on at analyzed time intervals. For the histologic analysis the material has been fixed in 10% formalin solution, prepared on phosphate buffer pH 7, 2. The samples have been dehydrated through a row of ethanol concentrations (60, 70, 80, 96°), xilol, ethanol: xilol (1:1) and placed into hystomix. The incisions with the thickness of 6 – 10 mkm have been prepared on the microtome Leica (Germany), colored with hematoxilin – eosin and drained, being carried also through a series of ethanol various concentrations [17, 18]. Light microscopy has been carried on the microscope Leica 2500 (Germany).

Statistic analysis of the data has been carried on with a number of applied programs Statistica 6,0 (RUS) firm STATSOFT-RUSSIA [19] using the modules: parametric (t-criterion for independent selectings with different estimations of dispersions) statistics, analysis of the variants on the basis of the same factor dispersion analysis. The level of authentic probability has been determined taking into account Bonferoni rectification [19].

Results and their discussion. The check of normal allocation has been carried on during the statistic cultivation of the data on the first stage with the help of the criteria of Kolmogorov – Smirnov, Shapiro, Lilieforce. The data have been stated to have normal or close to normal allocation and this fact gave us an opportunity to use parametric methods of analysis further.

Comparing total phenolics in the callus of leaf origin, grown on the media, containing exogenic PC or their precursors, with the control group, it has been stated that indicators of total phenolics undoubtedly differ on the 20^{th} and 40^{th} day on all the media in comparison with the control (table). On the 60^{th} day authentically important are only the meanings of total phenolics in comparison with the medium 6. Media 2 and 5 are analogous in the change of the PC presence as on the 20^{th} day the PC contents increased on 28 and 29 %, and on the 40^{th} day decreased on 34 and 14 % (media 2,5, correspondingly). Analogous in accumulating PC were media 3 and 6, on which the decrease of the PC presence has been observed on the 20^{th} and 40^{th} day on 20 and 19 % (medium 3) and 112 and 33% (medium 6). On the medium 4 the PC presence increased on 125 and 30% on the 20^{th} and 40^{th} day of cultivation, correspondingly. Thus we can conclude that media 2, 4, 5 containing rutin, phenylalanine and their joining are more effective in accumulating in calli the total phenolics in comparison with the control and media 3 and 6.

In pairs comparisons matrix of total phenolics in the lilac callus on different media

Medium of cultivating	Presence of total phenolics mg/g of the raw callus weight, $X \pm s$	Medium of cultivating				
20^{th} day						
		1	2	3	4	5
1	1,491 ± 0,191					
2	1,915 ± 0,233	4,44/0,000*				
3	1,192 ± 0,382	2,21/0,010*	5,10/0,000*			
4	3,351 ± 0,285	17,12/0,000*	12,33/0,000*	14,31/0,000*		
5	1,930 ± 0,556	2,37/0,019*	0,9/0,932	3,46/0,003*	7,19/0,000*	

Table continued

Medium of cultivating	Presence of total phenolics mg/g of the raw callus weight, $X \pm s$	Medium of cultivating				
6	0,167 ± 0,013	21,86/0,000*	23,68/0000*	8,48/0,000*	36,24/0,000*	10,0/0,000*
40th day						
1	1,749 ± 0,097					
2	1,159 ± 0,306	5,81/0,000*				
3	1,588 ± 0,188	2,40/0,003*	3,77/0,001*			
4	2,267 ± 0,480	3,35/0,003*	6,16/0,000*	4,17/0,000*		
5	1,511 ± 0,261	2,70/0,014*	2,77/0,010*	0,75/0,46	4,38/0,000*	
6	1,164 ± 0,120	11,96/0,000*	0.04/0,963	5,99/0,000*	7,06/0,000*	3,82/0,000*
60th day						
1	1,216 ± 0,249					
2	1,120 ± 0,292	0,79/0,44				
3	1,319 ± 0,190	1,05/0,309	1,89/0,087			
4	1,293 ± 0,489	0,45/0,658	0,96/0,348	1,154/0,879		
5	1,100 ± 0,252	1,033/0,315	0,164/0,871	2,196/0,04	1,113/0,28	
6	0,806 ± 0,115	4,74/0,000*	3,175/0,005*	7,32/0,000*	3,074/0,000*	3,365/0,003*

N o t e: In the numerator signification of t – is criterion, in the denominator, p – is signification, X – is average signification, s – is standard deviation.

*Significations are authentically important ($P < 0,01$).

The data of dispersion analysis are represented in the fig. 1. While analyzing the data, it has been stated that during callus cultivation of leaf origin on the media, containing exogenic PC, maximum accumulation of the PC sum has been observed on the 20th day on the media 2, 4, 5, in comparison with the control and media 3 and 6. Thus, the presence of the PC predecessor – phenylalanine, glycoside – rutin and of the joining rutin – phenylalanine in the medium contributed to more intensive PC accumulation in comparison with aglicone – quercetin or joining quercetin – phenylalanine.

Callus morphologic characteristics has shown that callus staining and structure was variable on different media. On the 20th day of cultivation the callus on all the media had light brown staining with sole white-yellow formations, the callus structure was loose, except the callus on the medium 5, characterized by the presence of thickenings at the callus base. On the 40th day of cultivation on the media 2, 3, 5,

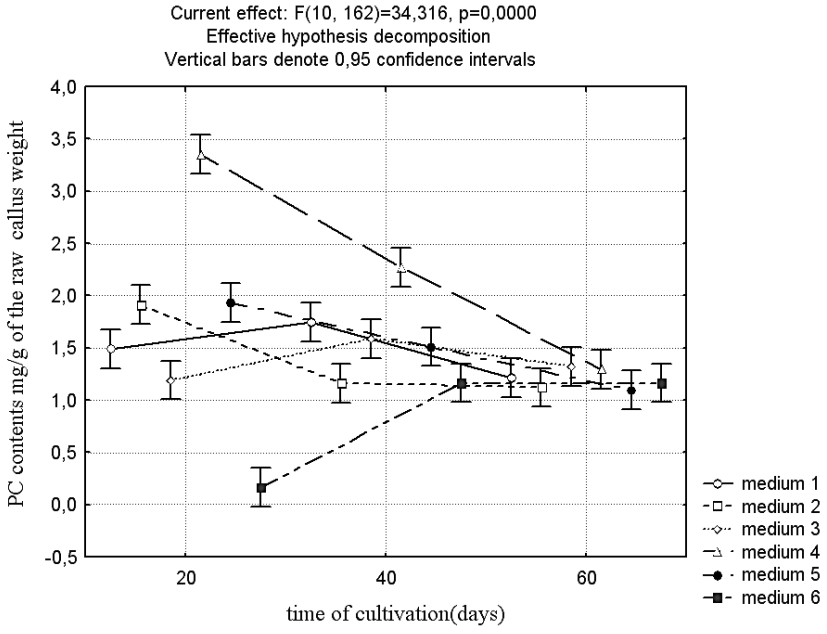

Fig. 1. The presence of total phenolics in the callus of the lilac leaf origin in dynamics of cultivation on the media of different composition

the callus was thick, full of hillocks, on the callus surface the formation of white-yellow thick structures, taking 2/3 of its surface was observed. On the rest media during this period the callus morphology was the same as on the 20th day. On the 60th day the structures, formed on the callus surface became yellow-green, and took almost all the callus surface on the media 2, 5, while on the medium 4 callus became loose, almost black with white and white-green formations on the surface. Callus darkening is probably connected with the PC oxidation and the formation of chinons, which exert toxic action on the callus, destroying it [20].

Cytologic investigations have shown that the lilac callus structure of leaf origin, cultivated on different media, is a heterogenic cellular population. Cells of meristematic type, cells of parenchymic type of different sizes and forms, elements of the vascular system are present in it. Analyzing callus, cultivated on the control medium, it has been stated that on the 20th and 40th day the basic tissue mass formed loosely placed cells of different form (round, oval, polyhedral) – parenchymic cells, among which there have been observed cells of small sizes, izodiametric with large nuclei placed excentrically, and also meristematic cells. On the 60th day of cultivation callus became thicker and was represented by the cells with twisted membranes, but the cellular composition at the same time didn't change (fig. 2, *A* – 1, 2).

On the medium 3 (quercetin) on the 20th day of the cultural cycle, in the callus culture among large non-nucleated cells of different size and form, there has been observed the presence of morphogenic sections in the form of meristematic aggregates forming embryogenic complexes of the cordated structure (fig. 2, *B* – 3). These complexes consisted of small nonvacuolysed, with the dense cytoplasm cells. The cells of meristemoids showed polarity and had a nucleus pressed out to the cell membrane. The cells of the inner callus part were involved into the formation of embryogenic complexes, the outer callus part was tightened, at the same time characteristic formations were nodular knots. On the 40th day on the medium 2, 5 it was seen that the outer callus surface was also involved into the process of embryogenesis forming globular form structures (fig. 2, *C* – 4). And besides, cells traces are seen, which probably represent the elements of the vascular system (fig. 2, *C* – 5). The mentioned structures are distinctly seen on both media. On the 60th day the decrease of the number of local accumulations of the cells of meristematic type on the media 2, 3 and 5 was marked, at the same time callus cultures consisted mostly of the cells of the parenchymic type of different sizes and form. Embryoid – like structures weren't practi-

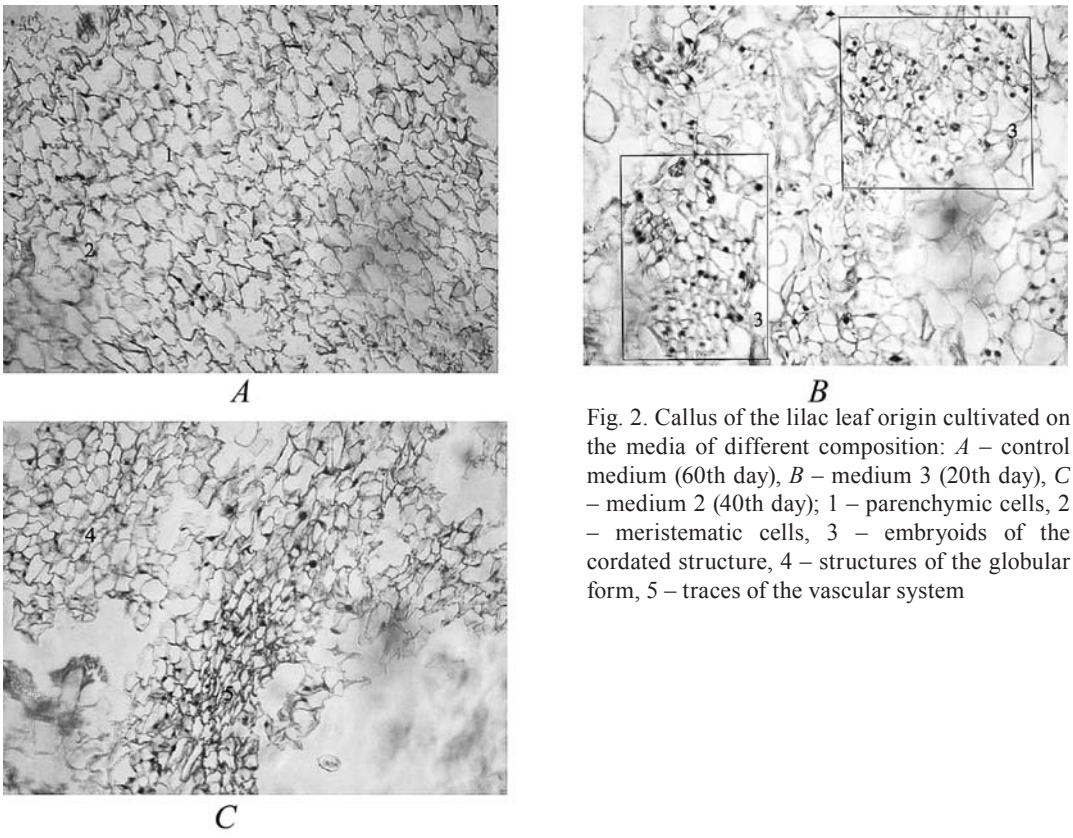

Fig. 2. Callus of the lilac leaf origin cultivated on the media of different composition: *A* – control medium (60th day), *B* – medium 3 (20th day), *C* – medium 2 (40th day); 1 – parenchymic cells, 2 – meristematic cells, 3 – embryoids of the cordated structure, 4 – structures of the globular form, 5 – traces of the vascular system

cally revealed. On the media 4 and 6 the callus structure during the cultural cycle differed from the callus, grown on the control medium, only by the increase in the number of meristematic cells which were placed not only in the centre of the callus but also occupied its periblestic layers. The cells of the meristematic type insignificantly varied in sizes, their diameter was 15–30 mkm.

Analyzing the processes of morphogenesis, it has been stated, that the formation of the embryogenic structures in the callus of the lilac leaf origin «M. Sholokhov» cultivar is observed at low contents of the PC sum. According to the data of histologic analysis dark calli, with soft structure, were related to non-embryogenic. Light, dense calli – on the media 2, 3, 5 – are related by us to embryogenic.

The results of investigations have shown that morphogenetic changes depend on the presence of the PC sum in the callus culture, cultivation medium and time.

Conclusion. Including in the medium of cultivation exogenic PC and their precursor substances allows to increase total phenolics in the callus culture of leaf origin and abridge cultivation time. Maximum total phenolics have been observed on the medium, containing 1,65 mg / l of phenylalanine on the 20th day of cultivation.

Formation of the embryogenic complexes takes place at low concentration of total phenolics in callus culture. Callus cultivation with the presence of exogenic PC and their precursor substances allows to reduce the time of cultivation up to three weeks to form embryogenic structures.

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PHENOLIC COMPOUNDS CONTENT AND MORPHOGENESIS IN THE LILAC CALLUS CULTURE (SYRINGA VULGARIS)

Summary

The purpose of the given investigation was establishing of interrelation between the content of total phenols and ability for morphogenesis of the callus of leaf origin of the lilac «M. Sholokhov» cultivar at cultivating the media containing exogenous PC and their precursor substances. In the process of the investigation it has been established, that the greatest total phenolics were seen on the 20-th day of cultivation on the media containing of rutin (medium 2), phenylalanine (medium 4), rutin + phenylalanine (medium 5). The results of this investigation show, that the period of cultivating callus, containing exogenous PC for obtaining culture with the high contents of PC, can be reduced up to three weeks (20 days). Morphogenetic changes were seen in callus cultivated on the media having low total phenols on the 20-th and 40-th days of cultivation, such media were medium 3 containing quercetin (20th day), medium 2 – containing rutin, medium 5 – containing rutin + phenylalanine (40th day).