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THE RESPONSE OF in vitro CULTURED CELLS OF *Linum grandiflorum* Desf. ON THE ACTION OF POLLUTANT AND HERBICIDE

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Abstract

Scholarly papers on in vitro culture of large-flowered flax (*Linum grandiflorum* Desf.), an ornamental crop for urban gardening, are very few and mainly elaborate morphogenesis, embryo culture, and cell line resistance issues. Heavy metals and herbicides are typical urban pollutants which cause oxidative stress. Our study for the first time reveals differences in morphophysiological characteristics and biosynthesis of phenolic compounds between in vitro cultured flaxseed cells influenced by Cd and herbicide glyphosate. With the aim to specify the mechanisms involved in stress tolerance of *L. grandiflorum* we have estimated the accumulation of antioxidants of phenolic nature, including phenylpropanoids and flavonoid in the callus culture. When studying the effect of stress factors, cadmium (60 μM), glyphosate (10 μM) or cadmium and glyphosate simultaneously (in the same concentrations) were added to the nutrient medium. Control was the standard nutrient medium. Callus cultures were analyzed at the end of the passage (day 28 of culture). Phenolic compounds extracted with 96 % ethanol from a plant material frozen with liquid nitrogen at –196 °C. The content of the sum of soluble phenolic compounds was determined with a Folin-Denis reagent, flavonoids with a 1 % solution of $AlCl_3$ at 725 and 415 nm. The concentration of phenylpropanoids was determined by direct spectrophotometric analysis of the extracts at 330 nm. To study the composition of the callus flax cultures phenolic complex compounds, the method of a thin layer chromatography on cellulose plates, solvent BUW (n-butanol + acetic acid + water in the ratio 40:12:28) were used. Callus of *L. grandiflorum* had a loose consistency, yellowish-green color, low growth index (by the end of the passage — 150 %) and high water content (95-97 %). As it grows, the content of soluble phenolic compounds and phenylpropanoids (biogenetically early polyphenols) increased 2.7 times by the end of the passage. The content of flavonoids, one of the most common representatives of phenolic compounds, in the first half of the cultivation cycle increased 2.25 times, and by the end of the passage slightly decreased. The application of cadmium caused the formation of dark colored necrotic cells on the surface of callus. In contrast, the application of glyphosate did not give such reaction. The application of cadmium did not affect the biosynthetic capacity of callus cultures in terms of the total accumulation of phenolic compounds, phenylpropanoids and flavonoids, the quantity of which was almost equal to that of the control. Under the application of glyphosate, however, the level of these secondary metabolites in cultures decreased, especially flavonoids. Under the simultaneous use of cadmium and glyphosate the total content of phenolic compounds in calluses increased, but we observed the decrease in the amount of phenylpropanoids and especially flavonoids. The data obtained show the differences in the response of large-flowered flax cells to the effect of stress factors on their morphology and the level of biosynthesis of phenolic compounds, the substances with high biological and antioxidant activity.

Keywords: large-flowered flax, *Linum grandiflorum*, callus cultures, tolerance, cadmium, glyphosate, phenolic compounds, phenylpropanoids, flavonoids

One of the problems of recent times is a change in the environmental situation due to active industrial and agricultural human activities. Technogenic pollution of the environment occurs with various pollutants, including heavy metals [1]. The most common is cadmium, which is 2-20 times more toxic to plants, animals, and humans than other metals [2]. Cd has a negative effect on the processes of photosynthesis and respiration, water regime, mineral nutrition, the functioning of the antioxidant system. Cadmium damages the light-harvesting antenna complexes of photosystem I and photosystem II [3, 4], competes with ammonium ion, thereby affecting nitrogen metabolism [5], promotes the activation of free-radical processes [6], changes the accumulation of various antioxidants [7, 8]. All of this leads to a disruption of natural plant communities, and to significant crop losses in cultivated species due to changes in growth and development [1].

In modern agroecological conditions, there is a problem of weed grass infestation of field and decorative crops, for which herbicides that can suppress plant growth are widely used [9]. These include glyphosate N-(phosphonomethyl) glycine, a post-emergent non-selective herbicide of systemic action, which ranks first in the world in production among preparations of similar action [10]. Glyphosate is a unique inhibitor of the shikimate biosynthetic pathway key enzyme for aromatic compounds 5-enolpyruvilshikimate-3-phosphate synthase, as a result of which the synthesis of proteins and secondary metabolites of a phenolic nature is suppressed in plants and deregulation of energy metabolism is also observed [11].

Phenolic compounds are secondary metabolites synthesized in all cells and tissues of plants [12]. They vary widely in structure, chemical properties and biological activity. Their functional role is diverse and associated with the processes of photosynthesis, respiration, regulation of enzymatic activity, protection of cells from stress effects [13]. The antioxidant properties of these plant metabolites are due to the presence of hydroxyl groups in their structure, which easily interact with free radicals, thereby contributing to the inhibition of radical-chain oxidation under stressful conditions [14].

Flax (class *Dicotyledoneae*, fam. *Linaceae*) is one of the most important crops of complex use [15]. It is characterized by a diversity of species groups and varieties, which opens up wide possibilities for its use in the agricultural, textile, and pharmaceutical industries, as well as in decorative and landscape agriculture [16, 17]. In the latter case, the annual decorative look of flax *Linum grandiflorum* Desf. (large-flowered flax) is used successfully as it has a well-developed vegetative part and flowering continues until autumn [18]. Since this crop is planted in urban areas where the soil and the environment are contaminated with pollutants, and various herbicides are used to control weeds, it is advisable to study its resistance to their effects.

The method of culturing cells and plant tissues in vitro makes it possible to investigate metabolic processes, as well as the response of cells to the action of stress factors, at a simpler level of organization compared to an intact plant [19, 20]. In vitro culture of flax has a long history. At the same time, the preservation of biodiversity, the study of the structure of plant cells and their resistance to stress factors [21-23] were the main areas of research. But in general, there are very few works on *L. grandiflorum*, and they mainly deal with issues of morphogenesis, embryo culture production, and study of resistance of cell lines [24, 25].

The present study is the first to identify differences in the in vitro response of cultured flaxseed flax cells to stress factors such as a pollutant (cadmium) and

a herbicide (glyphosate). This is manifested both in morphophysiological characteristics and in the biosynthesis of phenolic compounds, the substances with high biological and antioxidant activity.

The aim of this work was to evaluate the growth activity of the large-flowered callus culture of flax and the accumulation of phenolic compounds in it, including phenylpropanoids and flavonoids, as well as the culture response to the action of pollutant (cadmium) and herbicide (glyphosate).

Techniques. Callus cultures of large-flowered flax were grown on a Murashige-Skoog medium with added 2% sucrose and 2 mg/l of 2,4-dichlorophenoxyacetic acid in a factor-static chamber (Timiryazev Institute of Plant Physiology RAS) at 25 °C, relative humidity 70% and a 16 h photoperiod (illumination intensity 5000 lx).

When studying the effect of stressors, cadmium $Cd(NO_3)_2$ (60 μM), glyphosate (Monsanto, Belgium) (10 μM) or cadmium and glyphosate were added to the main nutrient medium (at the same concentrations). The control was the usual nutrient medium. Callus cultures were analyzed at the end of the passage (day 28), fixing them with liquid nitrogen for subsequent biochemical studies. Growth rate of calluses and their morphophysiological characterization were accounted. The water content in callus tissues was determined by the standard method after drying to the constant weight in a thermostat at 70 °C [26].

Phenolic compounds were recovered with 96% ethanol from plant material frozen with liquid nitrogen at –196 °C. After 45 min, the homogenate was centrifuged (16000 rpm, 15 min) and the supernatant was used for spectrophotometric measurements. The content of the sum of soluble phenolic compounds was determined with Folin-Denis reagent, flavonoids with 1% $AlCl_3$ solution at $\lambda = 725$ nm and $\lambda = 415$ nm [27]. Rutin calibration curves were built. The amount of phenylpropanoids was estimated by direct spectrophotometry of extracts at $\lambda = 330$ nm, using caffeic acid to construct a calibration curve [28].

To study the composition of the phenol complex of callus flax cultures, thin-layer chromatography was used on plates with cellulose (Merck KGaA, Germany), solvent BV (n-butanol + acetic acid + water in the ratio 40:12:28). Preliminary identification of phenolic compounds was carried out on an ultrachemiscope DESAGA UVIS (DESAGA, Holland) using specific bright blue or blue fluorescence in UV light at $\lambda = 254$ nm and $\lambda = 366$ nm. To detect phenolic compounds, chromatograms were treated with a mixture of 1% solutions of $FeCl_3$ and $K_3Fe(CN)_6$ (1:1), and phenolcarboxylic acid reagent (diazotized p-nitroaniline) was used, followed by treatment with 20% Na_2CO_3 [27].

Experiments were performed in 5-fold biological and 3-fold analytical replicates. Correlation and factor analysis (ANOVA) was carried out in the SigmaPlot 12.3 software (https://systatsoftware.com). The tables show the arithmetic mean values of the obtained values (*M*) and their standard errors (±SEM). Superscripts denote the statistical significance of differences in average values for the Tukey test at $p < 0.05$.

Results. The callus culture of large-flowered flax grown on the main nutrient medium was mostly yellow, although some areas were light green (Fig. 1, A). This may indicate the initial stages of the formation of chloroplasts in it, since growth occurred under the influence of light. The fact that these organelles are formed in in vitro cultures has been reported in the literature [19]. It should also be noted that the flax calluses had a loose structure, low growth during the entire passage, and high water content (Table 1). Similar characteristics of callus culture of large-flowered flax were cited by other authors [24].

In vitro plant cells retain many properties of intact tissues, including the ability to synthesize phenolic compounds [19, 20]. As the large-flowered callus

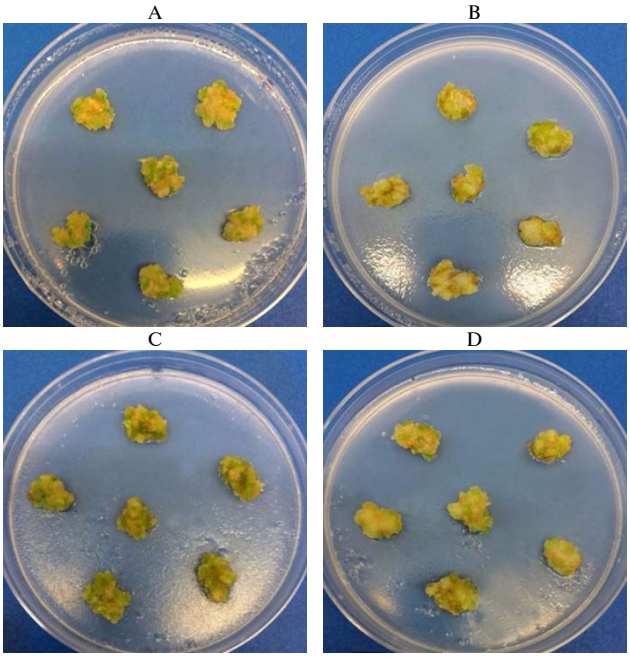

Fig. 1. The appearance of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) grown on the main nutrient medium (A)**, as well as on a nutrient medium with cadmium** (60 μM) (B)**, glyphosate** (10 μM) (C)**, cadmium and glyphosate** (D) (day 28).

flax culture grew, the total content of phenolic compounds increased (see Table 1). The most significant changes occurred from day 6 to the day 14 of culture, when the number of phenolic compounds increased 2.4 times. The maximum total content was noted at the end of the passage, being 2.7 times more compared to the initial stages of growth (day 6). Consequently, the greatest intensity of biosynthesis of phenolic compounds was confined to the first half of the culture growth cycle, which once again confirms the high ability of young plant cells to form these metabolites [12].

Phenylpropanoids are biogenetically early representatives of phenolic compounds synthesized in plant tissues [12]. Their accumulation has been reported in various members of the *Linum* genus [29]. The tendency of accumulation of phenylpropanoids in callus of large-flowered flax was similar to that for the number of phenolic compounds (see Table 1). Based on this, it can be assumed that they are the main components of the phenolic complex of cultures and determine the nature of the accumulation of phenolic compounds.

1. Morphophysiological and biochemical characteristics of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) on the main nutrient medium during passage (*M*±SEM)

Indicator	Culture age, days		
	6	14	28
Increase in the callus weight, %	115 ± 5^{A}	125 ± 6^{A}	150 ± 5^{B}
Water content, %	94.42 ± 0.48^{A}	97.27 ± 1.24^{A}	97.254 ± 1.01^{A}
Total content of phenolic compounds, mg eq. rutin/g dry weight	7.43 ± 0.31^{A}	18.07 ± 0.92^{B}	20.23 ± 0.74^{B}
The content of phenylpropanoids, mg eq. caffeic acid/g dry weight	10.58 ± 0.63^{A}	24.60 ± 0.65^{B}	28.97 ± 0.54^{B}
The content of flavonoids, mg eq. rutin/g dry weight	2.92 ± 0.74^{A}	6.56 ± 0.86^{B}	6.03 ± 0.71^{B}

N o t e. Superscripts (A, B) denote the reliability of differences in average values for the Tukey test at $p < 0.05$.

Flavonoids are among the most common representatives of phenolic metabolism in plant tissues [12]. Their accumulation in callus cultures increased in the first half of the cultivation cycle and by the 14th day was 2.25 times higher than the same indicator in a 6-day culture. By the end of the passage, the content of flavonoids was slightly reduced, but remained higher than at the beginning of the passage (almost 2 times). That is, at the final stages of culture growth, namely during the stationary phase, there was a tendency to a decrease in the content of these metabolites, which is also characteristic of plant tissues [13].

The impact of stress factors such as cadmium and glyphosate leads to an increase in the formation of reactive oxygen species in cells [2, 11]. Under these conditions, an important role belongs to phenolic compounds, the low-molecular-

weight components of the antioxidant defense system [14, 31]. They are able to "interrupt" chain oxidation reactions caused by stress factors, as well as form complexes with heavy metals, preventing their toxic effect [1]. In this regard, the next task was to study the effect of cadmium and glyphosate on the growth of large-flowered callus crops, as well as the accumulation of phenolic compounds in them, which was estimated at the end of the passage, that is, during their maximum accumulation.

2. Morphophysiological and biochemical characteristics of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) on media with cadmium (60 μM) **and glyphosate** (10 μM) (*M*±SEM, days 28)

Indicator	Stressor		
	Cd	glyphosate	Cd + glyphosate
Increase in the callus weight, %	135±7^{A}	165±8^{B}	150±6^{B}
Water content, %	97.20±1.01^{A}	95.90±1.01^{A}	97.71±1.12^{A}
Total content of phenolic compounds, mg eq. rutin/g dry weight	19.45±0.36^{A}	15.93±0.72^{A}	25.23±0.94^{B}
The content of phenylpropanoids, mg eq. caffeic acid/g dry weight	30.22±0.74^{A}	27.68±0.63^{A}	15.46±0.34^{B}
The content of flavonoids, mg eq. rutin/g dry weight	6.38±0.71^{A}	3.48±0.29^{B}	2.77±0.14^{B}

N o t e. Superscripts (A, B) denote the reliability of differences in average values for the Tukey test at $p < 0.05$.

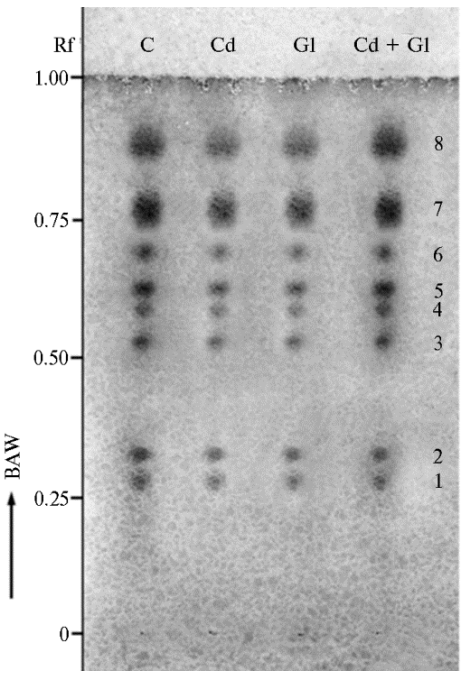

Fig. 2. Chromatographic separation of ethanol extracts from callus cultures of large-flowered flax (*Linum grandiflorum* Desf.) grown on the main nutrient medium (C) **and on media with cadmium** (Cd)**, glyphosate** (Gl) **and their combination** (Cd + Gl)**:** 1-8 – discovered substances of a phenolic nature. Thin layer chromatography on plates with cellulose, solvent composition (BAW) is n-butanol + acetic acid + water (40:12:28). The values of Rf (the ratio of the distance traveled by the substance to the distance traveled by the solvent) are indicated.

Callus culture grown on nutrient media with the addition of cadmium and glyphosate had a light-yellow color (see Fig. 1, B-D). There was a slight greening, but to a lesser extent than in the control version. In addition, small dark brown areas were formed on the surface, which could indicate cell necrosis, characteristic of other in vitro cultures in the presence of cadmium [30]. At the same time, the morphophysiological characteristics of calluses grown on the medium with glyphosate were better than in the control variant. They were denser and more compact, yellowish-green in color, with good growth (Table 2). The water content of the cultures in all the experimental variants had close values, approximately equal to those in the control.

The presence of cadmium in the medium had practically no effect on the biosynthetic ability of callus cultures (see Table 2). The total accumulation of phenolic compounds, the amount of phenylpropanoids and flavonoids were similar to those in the control (see Table 1). Most likely, *L. grandiflorum cells* are resistant to the studied metal concentration. This may be due to the fact that flax belongs to the group of accumulator plants, in which the toxic effect of the metal is expressed at higher concentrations of pollutant compared to other cultures, which are mainly exclusives that accumulate heavy metals in

Glyphosate serves as an inhibitor of one of the enzymes of phenolic metabolism responsible for the initial stages of the biosynthesis of these secondary metabolites [10]. When it entered the plants, in some cases a decrease in their accumulation was noted [31]. In the current experiment, a similar trend was observed, especially with respect to flavonoids (see Table 2). It can be assumed that glyphosate inhibited predominantly the flavonoid biosynthesis pathway for phenolic compounds and, to a much lesser extent, phenylpropanoid.

Accumulation of phenolic compounds in callus cultures was the most pronounced under the influence of combined cadmium and glyphosate. In this case, the total content of phenolic compounds significantly increased while reducing the amount of phenylpropanoids and flavonoids, which suggests the activation of the formation of other classes of phenolic compounds, in particular lignans, the compounds of a phenolic nature characteristic of flax plants [29].

To understand the peculiarities of the formation of phenolic compounds in plant cells, it is important to study not only their content, but also their composition [12].

3. Composition of the phenolic complex of ethanol extracts of callus cultures of large-flowered flax (*Linum grandiflorum* Desf.) in the control and all test variants (Murashige-Skoog medium)

Fraction No.	Rf	I	II
1	0.28	+	+
2	0.35	+	–
3	0.53	+	+
4	0.59	+	+
5	0.62	+	+
6	0.70	+	+
7	0.75	+	+
8	0.89	+	+

Note. Thin-layer chromatography on plates with cellulose was used for the separation, the solvent is n-butanol + acetic acid + water (40:12:28). Rf is the ratio of the distance traveled by the substance to the distance traveled by the solvent, I and II are the manifestation of a reagent for phenolic compounds and phenylpropanoids, respectively.

A thin-layer chromatography method revealed no differences in the composition of the complex of ethanol extracted phenolic compounds in the control and test variants (Fig. 2, Table 3). In all extracts, eight compounds of a phenolic nature were present, of which three dominated. Most phenolic compounds, as per their mobility, were conjugates of phenol carboxylic acids, i.e. p-hydroxybenzoic (compounds 1, 6, 8), p-coumaric (compounds 3, 7), ferulic (compound 4) and caffeic (compound 5) [27]. The presence of these substances in the phenol complex of flax plants was also reported by other authors [29, 30]. Since thin-layer chromatography allows only a preliminary estimate of the composition of the phenolic complex of plant cells and tissues and does not provide a complete picture, these studies will be further continued.

Thus, cultured in vitro cells of the large-flowered flax have the ability to form phenolic compounds, the biologically active substances with antioxidant activity, the greatest accumulation of which occurs at the end of the culture cycle. Their content increased rapidly in the first half of the cycle, reaching the highest values by the end of the passage, which points to the importance of the formation of phenolic compounds, including phenylpropanoids and flavonoids, in plant cells not only in vivo, but also in vitro. The heavy metal cadmium, when added to the culture medium, affected the morphophysiological characteristics of the callus culture; however, no changes in the accumulation of phenolic compounds, including phenylpropanoids and flavonoids, were observed. This once again confirms the significant resistance of flax cells to the action of heavy metals. The presence of glyphosate herbicide reduces the ability of the large-flowered flax culture to accumulate phenolic compounds, which is more pronounced for flavonoids. With the combined action of these two factors, the total content of phenolic compounds in cultures increases, and the amount of phenylpropanoids and especially flavonoids decreases, which may be due to the

formation of other representatives of phenolic compounds. Our findings confirm once again the species-specific response of plant cells to stress factors.

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