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THE CONTENT OF PHENOLIC COMPOUNDS AND FLAVONOIDS IN *Deschampsia antarctica* TISSUE CULTURE

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Aim. The aim of the study was to determine the quantitative and qualitative content of phenolic compounds and flavonoids in *Deschampsia antarctica* E. Desv. tissue cultures obtained from plants originating from different islands of the maritime Antarctic.

Methods. *In vitro* tissue culture, Folin-Ciocalteu method, spectrophotometry, HPLC analysis.

Results. The quantitative content of phenolic compounds and flavonoids in *D. antarctica* tissue cultures obtained from plants of six genotypes (DAR12, DAR13, G/D12-2a, Y66, R30 and L57) was determined. The highest content of phenolic compounds (4.46 and 3.75 mg/g) was found in tissue cultures obtained from root and leaf explants of plant of genotype L57. The highest amount of flavonoids (7.17 mg/g) was accumulated in G/D12-2a tissue culture of root origin. The content of the studied biologically active compounds (BACs) did not change with increasing number of subculture generations (from passage 10 to 19). HPLC analysis showed that in *D. antarctica* tissue cultures, a shift in the biosynthesis of BACs occurred towards the synthesis of more polar metabolites compared to explant donor plants.

Conclusions. It was found that the transition of cells to undifferentiated growth affected the content of BACs, the amount of which decreased 2–5 times simultaneously with a significant changes in their profile. This provided a basis for further biochemical studies, as well as "indicate the necessity" for careful selection of tissue culture of *D. antarctica* to use it as a potential source of BACs.

Key words: plant tissue culture, *Deschampsia antarctica* E. Desv., phenolic compounds, flavonoids, HPLC analysis.

Nowadays plant tissue culture is actively used as a source of environmentally friendly raw materials for the production of herbal medicines, as well as a source of high-quality food raw materials with a defined content of trace minerals, vitamins, and other biologically active compounds (BACs) [1]. The use of *in vitro* culture allows, on the one hand, to produce plant biomass in unlimited quantities in controlled conditions on artificial nutrient media regardless of geo-climatic conditions and season [2], and, on the other hand, to multiply the required amount of plant material using micropropagation technique of valuable species of plants, in particular rare or endangered plants, as well as those inhabiting hardly accessed areas [3–6].

Plant tissue culture is an effective approach for obtaining biomass for isolation of a number of valuable natural organic compounds, including pigments (anthocyanins, betacyanins), anti-inflammatory substances (berberine, rosemary acid), and antitumor agents (paclitaxel and podophyllotoxin) [2, 7].

The ability of plants to accumulate BACs is used in biotechnology and pharmacology to obtain phenolic compounds, flavonoids, antioxidants, alkaloids, saponins, and other substances [8]. Most of these metabolites are usually synthesized in response to abiotic stress, which can be caused by low or high temperatures, limited access to water and

nutrients, long periods of darkness in winter and high-level UV radiation in summer, the presence of heavy metals in soils, etc. [9, 10]. Accordingly, in plants growing under suboptimal or extreme conditions, the content of secondary metabolites may be increased due to permanent stress. One such species that has adapted to the harsh environment conditions of Antarctica is an extremophile plant *Deschampsia antarctica* E. Desv., which may be promising in terms of studying the biological activity of its secondary metabolites. Unfortunately, the inaccessibility of natural habitats and the restrictions imposed by International environmental agreements on the Antarctic territories do not allow collecting this species for large scale research. Establishment of *in vitro* culture of *D. antarctica* would allow to produce the required amount of plant material throughout the year, as well as to experiment with it under controlled laboratory conditions.

It is known that *D. antarctica* accumulates a number of BACs, in particular, flavonoids (orientin, luteolin and isoswertiajaponin (7-*O*-methylorientin) 2″-*O*-beta-arabino-pyranoside) and phenolic compounds that show antitumor activity [11], inhibit melanoma cell proliferation [12], as well as inhibit the growth of colorectal carcinoma and its metastasis to the liver [13]. Therefore, this plant is a very attractive natural source of these substances, because flavonoids also exhibit antioxidant properties. It is believed that antioxidant compounds from *D. antarctica* can be used in the pharmaceutical and food production, as well as in cosmetology [12].

Our previous studies have determined the quantitative and qualitative content of phenolic compounds and flavonoids in *D. antarctica* plants growing in nature and cultured *in vitro*, regenerated plants, and plants grown in a growth chamber [14]. The aim of this work was to determine the content of these compounds in the established tissue culture of *D. antarctica* as a potential source of these BACs.

Material and Methods

Establishment of D. antarctica tissue culture. Tissue cultures of *D. antarctica* investigated in this study were obtained from plants of six genotypes grown *in vitro* from seeds collected from five locations nearby the Ukrainian Antarctic Station Academician Vernadsky (Table).

The plants *in vitro* were cultured at 16–18 °C with a 16 h light/8 h dark photoperiod at a light intensity of 6 500 lux and relative humidity of 55–65% on the Gamborg's B_5 medium [15], supplemented with 0.1 mg/L of 1-naphthylacetic acid.

Conditions for induction and proliferation of *D. antarctica* tissue culture are described in detail in [16]. Root, leaf, and shoot growth point segments of *in vitro* plants were used as explants. To induce callus formation explants were placed on the Murashige and Skoog (MS) [17] or B_5 media supplemented with various concentrations of phytohormones such as 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (Kin). Tissue cultures were grown in the dark at 16–20 °C and subcultured every four weeks.

Preparation of extracts for biochemical analysis. To determine the total content of phenolic compounds and flavonoids in cultured

Characteristics of the studied tissue cultures of *D. antarctica*

No	Seed collection location	Genotype	Explant	Passage
1	Darboux Island	DAR12	roots	11
2		DAR12 regenerated plant	roots	12
3		DAR13	shoot growth point	7
4	Galindez Island	G/D12-2a	shoot growth point	10
5		G/D12-2a	shoot growth point	19
6		G/D12-2a	roots	19
7	Rasmussen Oasis	R30	roots	7
8	Great Yalour Island	Y66	roots	10
9	Lahille Island	L57	roots	6
10		L57	leaves	6

tissues, 96% ethanolic extracts of lyophilized tissues were used. Ethanolic extracts were prepared as follows: a sample of 20 mg of dried tissue was ground to a powder using a ball mill and extracted with 2 mL of 96% ethanol at 27 °C for 18–20 h, then subjected to 30 min ultrasonic extraction in an ultrasonic bath. The extracts were centrifuged and the supernatant was collected for the analysis. For HPLC analysis, the ethanolic extracts were concentrated 10-fold using Savant SpeedVac vacuum concentrator. Biochemical analysis was performed in three repetitions per callus tissues of each genotype.

Determination of total phenolic content. The total phenolic content in cultured tissues extracts was determined using the Folin and Ciocalteu assay [18], which is based on colorimetric reaction between phenolic compounds and the Folin-Ciocalteu reagent (phosphomolybdic-tungstic acid) resulting in the production of molybdenum-tungsten blue, the concentration of which was measured spectrophotometrically.

The appropriate volume of extract was adjusted to 100 µL with 96% ethanol. To the resulting solution, 200 µL of 10% (v/v) aqueous solution of Folin-Ciocalteu reagent was added and stirred for 20–30 s. Then, 800 µL of 7.5% aqueous Na_2CO_3 solution was added to create an alkaline environment optimal for the reaction. The resulting solutions were left for 2 h at room temperature before the measurement of the absorbance at 765 nm using a spectrofluorometer Fluorat®-02-Panorama in the spectrophotometer mode.

The calibration curve was constructed using standard solutions of ferulic acid. Data were expressed as mg of ferulic acid per 1 g of dry weight.

Determination of the total flavonoid content. Total flavonoids expressed as rutin equivalents were quantified using spectrophotometric assay. This technique is based on the ability of flavonoids to form a coloured complex with aluminium [19].

The appropriate volume of extract was adjusted to 1 mL with 96% ethanol. The resulting solution was mixed with 360 µL of 5% $NaNO_2$ and incubated for 5 min, then 600 µL of 2% $AlCl_3$ was added, mixed thoroughly, and left for 6 min. Then 600 µL of 1 M NaOH solution was added and incubated for 10 min. The reaction mixture changed colour to pink; the absorption of the formed complex was measured at 510 nm using a spectrofluorometer Fluorat®-02-Panorama in the spectrophotometer mode.

The calibration curve was constructed using standard solutions of rutin. Data were expressed as mg of rutin per 1 g of dry weight.

Qualitative analysis of phenolic compounds and flavonoids. Profiling of phenolic and flavonoid content in *D. antarctica* tissue cultures was performed using high performance liquid chromatography (HPLC). HPLC analysis was performed on a Shimadzu HPLC10Avp system (Japan) using a Zorbax Eclipse column (XDB-C18, 6×250 mm, 5 µm, Agilent) with a Waters Symmetry C8 pre-column. Chromatographic conditions: mobile phases were acetonitrile (B) and deionized water + 1% formic acid (A); gradient: increase from 10% B to 40% B in 22 min; the total run time: 30 min. Column temperature: 40 °C, flow rate: 0.8 mL·min^{-1}, injection volume: 20 µL, UV detection: at 318 nm.

Standards (luteolin, apigenin, orientin, rutin, quercetin, and kaempferol) were dissolved in 96% ethanol.

Statistical analysis. Descriptive statistics methods were used to analyse the data. The significance of the differences in the content of phenolic compounds and flavonoids in extracts was assessed using Student's *t*-test at $P<0.05$.

Results and Discussion

Biochemical analysis showed that the content of phenolic compounds and flavonoids in ethanolic extracts of *D. antarctica* tissue cultures ranged from 1.65 to 4.46 mg/g in terms of ferulic acid and from 0.15 to 7.17 mg/g in terms of rutin, respectively.

The data in Fig. 1 show that the largest amount of phenolic compounds among the studied calli was found in the tissue culture of genotype L57, and the largest content of flavonoids was in the tissue culture of genotype G/D12-2a.

Previous study determined the quantitative and qualitative content of phenolic compounds and flavonoids in *D. antarctica* plants growing in nature and cultured *in vitro*, regenerated plants and plants grown in a growth chamber of seven genotypes (DAR12, DAR13, G/D12-2a, R30, Y66, Y67, L57) [14, 20]. Biochemical analysis of *D. antarctica* tissue cultures showed that the content of phenolic compounds in calli of different origins was 2–5 times lower compared to the content of these substances in the explant donor plants. Only in tissue culture of root origin of genotype L57, the amount of phenolic compounds was twice as high as in the roots of the initial *in vitro* plant. The content of

flavonoids in tissue cultures was 1.2–4 times lower compared to aseptic plants, and in the tissue culture of genotype DAR13 it was 18 times lower. In the roots of the plant of genotype R30 the content of flavonoids was the highest (17.4 mg/g), whereas in the tissue culture obtained from it the flavonoid amount was two orders of magnitude lower (only 0.15 mg/g).

There are a number of reports showing that the content of secondary metabolites and their profile depends on the type of tissue of the initial explant. Thus, in particular, a significant difference in the quantitative content of various secondary metabolites was shown in callus cultures of different origins (obtained from cotyledons, nodes, leaves, and roots), including furanocoumarin psoralen in *Psoralea corylifolia* L. [21], podophyllotoxin and other lignanes in *Podophyllum peltatum* L. [22], and isoflavonoids in *Pueraria lobata* (Willd.) Sanjappa & Pradeep [23].

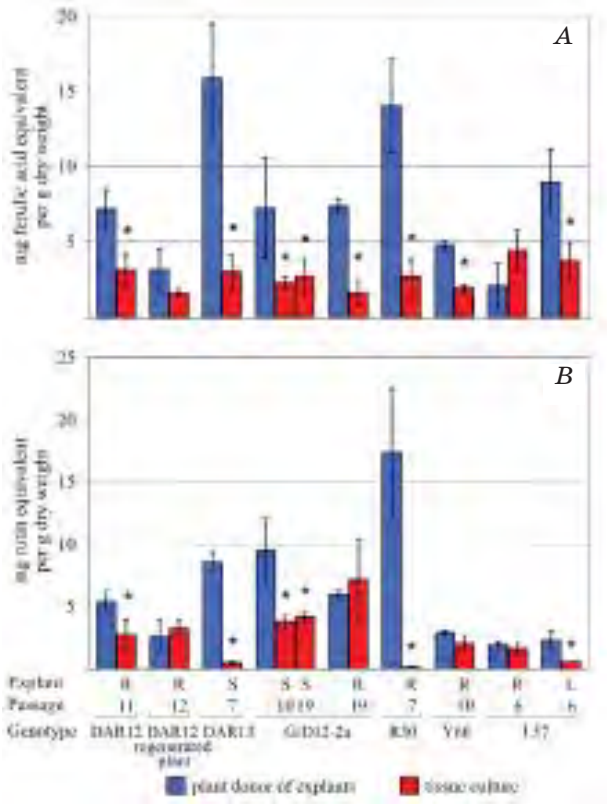

Fig. 1. **The quantitative content of phenolic compounds (*A*) and flavonoids (*B*) in *D. antarctica* tissue cultures of different genotypes obtained from different explants**

Explants, which were used to initiate tissue culture: r. — root, l. — leaf, s. g. p. — shoot growth point.

* — statistically significant differences from the explant donor plant at $P < 0.05$

The results of biochemical analysis showed that *D. antarctica* tissue culture obtained from the shoot growth point explants did not differ significantly from the callus culture of root origin in the content of neither phenolic compounds nor flavonoids.

We conducted qualitative analysis of phenolic compounds and flavonoids in extracts from *D. antarctica* tissue cultures using HPLC. The obtained chromatographic profiles were compared with those of plants of this species. HPLC chromatogram of phenolic compounds contained in ethanolic extracts of *in vitro* plant and tissue culture are presented in Fig. 2.

Previously we found that the main part of flavonoids and phenolic compounds in extracts of *D. antarctica* are five most abundant metabolites, that were present in similar ratios in the samples of wild and *in vitro* grown plants [20]. The most abundant was unidentified substance 3 corresponding to the peak which had the largest area in all analysed samples. One of the five most abundant substances from the extracts of plants was identified as orientin or luteolin-8-C-glucoside (peak 2 in Fig. 2, *A*).

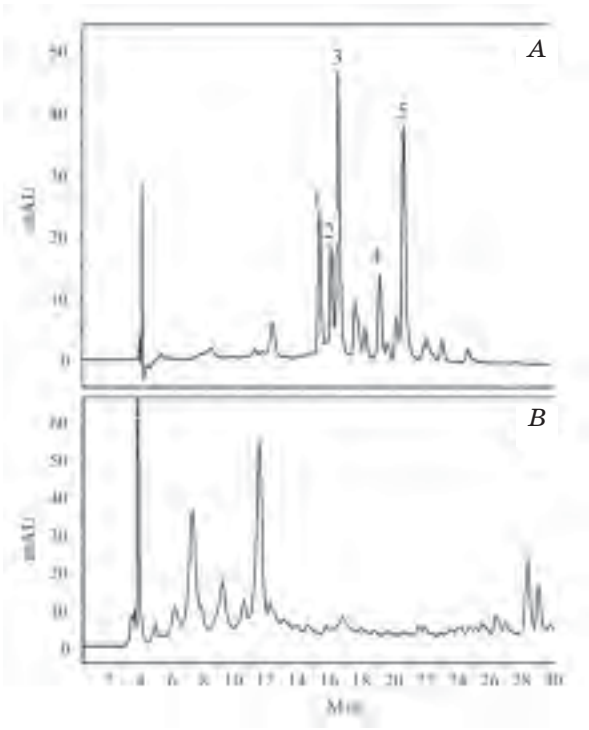

Fig. 2. **HPLC chromatogram of phenolic compounds from the leaf extracts of *D. antarctica* *in vitro* plant (*A*) and tissue culture (*B*)**
Detection wavelength of 318 nm

HPLC analysis showed that *D. antarctica* tissue cultures differed from the initial plants in chromatographic profiles (Fig. 2, *B*). As can be seen from HPLC chromatogram, in tissue cultures, the biosynthesis of BACs is shifted towards the formation of more polar metabolites.

None of the analysed tissue cultures contained any of the five main metabolites (including the orientin we identified) that are present in *D. antarctica* plants. Instead, three other more polar compounds were found, one of which was present in smaller quantities in plants (Fig. 2). Tissue cultures of different origins contained mostly the same set of substances, but the ratios of these metabolites varied to a large extent and were not associated with type of explant used for callus initiation.

Thus, it is shown that the transition of cells to an undifferentiated state in the process of callus formation and subsequent growth of tissue cultures *in vitro* is accompanied with a decrease in the content of BACs and a significant alteration in their qualitative composition.

It is known from the literature that the content of BACs in tissue cultures of some plant species is also low, like *D. antarctica*. For example, the amount of phenolic compounds in callus cultures was 7.69 mg/g in *Salvia officinalis* L. and 4.83 mg/g in *Trigonella foenum-graecum* L. callus; and the content of flavonoids in callus cultures was 4.3 mg/g in *Vinca minor* L. and 6.82 mg/g in *Catharantus roseus* (L.) G. Don [24]. It has been suggested that the low yield of secondary metabolites in tissue cultures may be due to the lack of cells differentiation [2].

It is known that the synthesis of secondary metabolites in *in vitro* culture is influenced by such factors as the initial plant genotype, culture duration, mineral and carbohydrate composition of the nutrient medium, the concentration of phytohormones, etc. [4, 25, 26]. It should be noted that all the studied tissue cultures of G/D12-2a genotype were cultured on the B_5 medium supplemented with 2 mg/L 2,4-D and 1 mg/L BAP, whereas the others were cultured on the MS medium supplemented with 1 mg/L 2,4-D and 1 mg/L Kin. Therefore, it can be assumed that the highest content of flavonoids (3.78–7.17 mg/g) in the calli G/D12-2a among all tissue cultures may be associated both with the composition of the nutrient medium and with the influence of the genotype. No significant changes in the content of phenolic compounds and flavonoids were observed in cultures of this cell line with different period of subculturing (10 and 19 passages).

In tissue culture under isolated growth conditions, the range of synthesized compounds may expand and substances that are not typical of an intact plant may appear [27]. The results of HPLC analysis of tissue cultures *D. antarctica* indicate a similar phenomenon occurring.

Loss of systemic control of metabolic processes, which occur in a whole plant, due to transition of plant cells, tissues, and organs to isolated growth conditions can lead to the appearance of metabolites typical either of the juvenile stage of plant development or of some primitive ancestral species. The biosynthesis of many compounds in undifferentiated tissues is reduced, and the appearance of new substances in tissue culture may be associated with the regeneration of roots, shoots, and other morphological structures, that is, with the process of tissue differentiation [4, 27]. For some plant species, several reports showed the appearance in *in vitro* culture of valuable secondary metabolites, which are not accumulated in intact plants. For example, *Dioscorea deltoidea* Wall. Ex Griseb tissue culture was demonstrated to synthesize 26-S isomers of protodioscin and deltoside, which were not observed in intact plant [28], in *Rauwolfia serpentina* Benth. ex Kurz tissue culture, a number of new indole alkaloids was identified [4, 29], in *Catharantus roseus* tissue culture, a serpentine was detected [24]; whereas *Celosia cristata* L. tissue culture produced a new betalain pigment celoscristatin, which was not found in intact plants, etc. [30].

There are a limited number of studies of *in vitro* cultivation of *D. antarctica*. It was reported about a convenient method of micropropagation of this species through tissue culture, which can increase four to five times the amount of plant material produced within three months, because natural vegetative propagation of *D. antarctica* is very slow [31], and about a method for the culture and mass micropropagation of *D. antarctica in vitro* using a photo-thermo-bioreactor, the advantage of which is the creation of conditions to increase the growth of biomass of this species and increase the synthesis of secondary metabolites with valuable medicinal properties [32]. The use of a specific temporary immersion photobioreactor has been proposed to increase both *D. antarctica* biomass production and phenolic metabolites accumulation by UV-B elicitation during cultivation [33].

Our previous study showed that the quantitative content of phenolic compounds and flavonoids in wild and *in vitro* grown

D. antarctica plants of different genotypes can vary within a certain range [20]. It is known that the biochemical properties of plants producing BACs depend both on genetic characteristics and on growth conditions (cultivation), so the variation within the species at the population level is preserved in *in vitro* culture. Such variation is primarily manifested in the peculiarities of *in vitro* morphogenesis and the ability to synthesize secondary metabolites [4, 34]. The data of biochemical analysis showed a lower content of BACs in the studied tissue cultures of *D. antarctica* compared to explant donor plants. Furthermore, it was found that the reversal of cells to an undifferentiated state in the process of callus formation resulted in the alterations of qualitative composition of phenolic compounds and flavonoids, in particular in increased synthesis of more polar metabolites compared to explant donor plants. The findings of this study provide a basis for further biochemical studies of *D. antarctica* tissue cultures, as well as indicate the necessity for a careful selection of tissue cultures to be used as a potential source of BACs.

Conclusions. The content of BACs in *D. antarctica* tissue cultures obtained from plants of six different genotypes was determined. The highest content of phenolic compounds (4.46 and 3.75 mg/g) was found in tissue culture of genotype L57 obtained from root and leaf explants; and the highest amount of flavonoids (7.17 mg/g) was found in G/D12-2a tissue culture of root origin. No significant changes in the content of these BACs were observed in cultures of this genotype with different period of subculturing (10 and 19 passages). HPLC analysis showed that quantitative and qualitative changes in the profiles of phenolic compounds occur in *D. antarctica* tissue cultures, in particular virtually no compounds typical of wild and *in vitro* grown plants were found. The changes in HPLC profiles of phenolic compounds in tissue cultures were found to occur due to a shift towards the accumulation of more polar metabolites.

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ВМІСТ ФЕНОЛЬНИХ СПОЛУК ТА ФЛАВОНОЇДІВ У КУЛЬТУРІ ТКАНИН *Deschampsia antarctica*

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Мета. Визначити кількісний та якісний вміст фенольних сполук і флавоноїдів у культурах тканин *Deschampsia antarctica* E. Desv., отриманих від рослин, що походили з різних островів Морської Антарктики.

Методи. Культура *in vitro*, метод Фоліна-Чокальтеу, спектрофотометричний аналіз, ВЕРХ-аналіз.

Результати. Визначено кількісний вміст фенольних сполук та флавоноїдів у культурах тканин *D. antarctica*, отриманих від рослин шести генотипів (DAR12, DAR13, G/D12-2a, Y66, R30 та L57). Найбільшу кількість фенольних сполук (4,46 та 3,75 мг/г) виявлено у культурі тканин, отриманій з кореневих та листкових експлантів рослини генотипу L57. Найбільшим вмістом флавоноїдів (7,17 мг/г) характеризувалася культура тканин G/D12-2a кореневого походження. Кількість досліджених біологічно активних сполук (БАС) практично не змінювалась зі збільшенням тривалості культивування (від 10 до 19 пасажу). Методом ВЕРХ-аналізу показано, що у культурах тканин *D. antarctica* відбувається зсув біосинтезу БАС у бік утворення більш полярних метаболітів, порівняно із рослинами-донорами експлантів.

Висновки. Встановлено, що перехід клітин до недиференційованого росту *in vitro* впливає на вміст БАС, кількість яких зменшується у 2–5 разів одночасно зі значною зміною їхнього якісного складу. Це дає підстави для подальших біохімічних досліджень, а також показує необхідність ретельного добору культур тканин *D. antarctica* для використання їх як потенційного джерела БАС.

Ключові слова: культура тканин рослин, *Deschampsia antarctica* E. Desv., фенольні сполуки, флавоноїди, ВЕРХ-аналіз.

СОДЕРЖАНИЕ ФЕНОЛЬНЫХ СОЕДИНЕНИЙ И ФЛАВОНОИДОВ В КУЛЬТУРЕ ТКАНЕЙ *Deschampsia antarctica*

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Цель. Определить количественное и качественное содержание фенольных соединений и флавоноидов в культурах тканей *Deschampsia antarctica* E. Desv., полученных от растений с разных островов Морской Антарктики.

Методы. Культура *in vitro*, метод Фолина-Чокальтеу, спектрофотометрический анализ, ВЭЖХ-анализ.

Результаты. Проведен анализ количественного содержания фенольных соединений и флавоноидов в культурах тканей *D. antarctica*, полученных от растений шести генотипов (DAR12, DAR13, G/D12-2a, Y66, R30 и L57). Наибольшее количество фенольных соединений (4,46 и 3,75 мг/г) обнаружено в культуре тканей, полученой из корневых и листовых эксплантов растения генотипа L57. Наибольшим содержанием флавоноидов (7,17 мг/г) характеризовалась культура тканей G/D12-2a корневого происхождения. Количество исследованных биологически активных веществ (БАВ) практически не менялось с увеличением продолжительности культивирования (с 10 до 19 пассажа). Методом ВЭЖХ-анализа показано, что по сравнению с растениями-донорами эксплантов в культурах тканей *D. antarctica* происходит смещение биосинтеза БАВ в сторону образования более полярных метаболитов.

Выводы. Установлено, что переход клеток к недифференцированому росту *in vitro* влияет на содержание БАВ, количество которых уменьшается в 2–5 раз одновременно со значительным изменением их качественного состава. Это дает основание для дальнейших биохимических исследований, а также свидетельствует о необходимости тщательного отбора культур тканей *D. antarctica* для их использования в качестве потенциального источника БАВ.

Ключевые слова: культура тканей растений, *Deschampsia antarctica* E. Desv., фенольные соединения, флавоноиды, ВЭЖХ-анализ.