ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2024. Vol. 40. N 1. P 58–67
http://dx.doi.org/10.7124/bc.000AAE

UDC [577.13+543.645]:581.143.6

Effect of light and temperature on the content of some biologically active substances in *Deschampsia antarctica* tissue culture

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Aim. The aim of the work was to study the influence of growth conditions (darkness/light and temperature 18/26 °C) on the content of phenolic compounds and flavonoids in *D. antarctica* morphogenic tissue culture. **Methods.** *In vitro* tissue culture, Folin-Ciocalteu method, spectrophotometry, HPLC analysis. **Results.** The total content of phenolic compounds and flavonoids in *D. antarctica* morphogenic tissue cultures, obtained from the plants genotypes DAR12 and G/D11-1/3 was determined. It was shown that growth of the calli in light of 6500 lux and at raised temperature of 26 °C led to a decrease in the content of biologically active substances (BAS). When culturing the calli of both genotypes in the darkness, regardless of temperature, the level of phenolic compounds (2 times for DAR12 and 2.8–3.1 times for G/D11-1/3) as well as of flavonoids (2.3–2.4 times for DAR12 and 4.6–5 times for G/D11-1/3) decreased. The antioxidant and antitumor compound tricin was found, the content of which was three times higher in the initial plant DAR12 compared to the G/D11-1/3 plant. The content of tricin in the calli was lower than in the initial plants. **Conclusions.** It was found that the highest level of the BAS accumulation in the morphogenic tissue culture of *D. antarctica* was observed when it was growing in light of 6500 lux and at temperature of 18 °C. The tricin detection in the initial plants of genotypes DAR12 and G/D11-1/3, as well as in the tissue cultures provides a basis for further biochemical study of *D. antarctica in vitro* as a potential source of BAS, which can be used for therapeutic and prophylactic purposes.

Keywords: *Deschampsia antarctica* E. Desv., plant tissue culture, phenolic compounds, flavonoids, tricin.

Introduction

Biologically active substances (BAS) of plants, in particular phenols and flavonoids, ensure plant resistance to extreme growth conditions and usually synthesized in response to abiotic stress, caused by low or high temperature, limited access to water and nutrients, long periods of darkness in winter, and high level of ultraviolet radiation in summer, the presence of heavy metals in the soil, *etc.* [1, 2]. A number of phenolic compounds and flavonoids are widely used in pharmacy. The practical value of these BAS is due to the relatively low toxicity, antioxidant, antimicrobial, anti-inflammatory, photo- and cryoprotective properties, *etc.* [3, 4]. One of the approaches stimulating the increased biosynthesis of secondary metabolites in plants is the use of abiotic stress [5, 6].

The *in vitro* culturing is an alternative approach for obtaining plants and tissue cultures that produce a target BAS, which has a number of advantages. Firstly, it is a possibility to obtain secondary metabolites throughout the year, regardless of geo-climatic conditions and season and the impact on the ecosystem. Second, during the cultivation of isolated tissues and organs, the BAS spectrum changes are often occur due to the synthesis of secondary metabolites that are not characteristic of the wild plants. Third, the *in vitro* culture makes it possible to obtain more target BAS in the biomass by creating highly productive cell lines and selecting optimal conditions for their cultivation, as well as using the various effects on the level of accumulation of secondary metabolites through the action of abiotic factors [7].

Deschampsia antarctica É. Desv. is an extremophile plant, which adapted to the harsh environmental conditions of Antarctica and may be a promising source of phenolic compounds and flavonoids with a wide spectrum of biological activity [8–10]. Phenolic compounds of this species have been shown to inhibit the melanoma cell proliferation, to induce antitumor immunity against the colorectal carcinoma and its metastasis to the liver, and also can be used to develop new drugs for the treatment of colorectal carcinoma [11, 12]; antiproliferative effect of flavonoids was shown to be comparable with the effect of modern anticancer substances [13].

Previously, we introduced *D. antarctica* plants to the *in vitro* culture, obtained tissue culture and regenerated plants, and seed generations of cloned plants. The content of phenolic compounds and flavonoids in intact plants and in some tissue cultures of this species was also determined [14–16].

It is known that abiotic factors (temperature, light, humidity, *etc.*) can change and under certain conditions significantly increase the content of BAS in the cellular biomass of plants [7]. For *D. antarctica*, even the temperatures that are quite common for the plants of the temperate zone can be stressful, since the summer temperature in the Antarctic usually does not exceed 10 °C. Therefore, it is interesting to investigate whether the secondary metabolism of this species change in response to the unusual cultivation temperatures. Accordingly, the aim of this work was to study the influence of growth conditions (darkness/light and temperature 18/26 °C) on the content of phenolic compounds and flavonoids in the morphogenic *D. antarctica* tissue culture.

Materials and Methods

The morphogenic tissue cultures of *D. antarctica* obtained from the *in vitro* plants of two genotypes, Darboux Island (DAR12) and Galindez Island (G/D11-1/3), were investigated in this study. The plants were cultured at 16–18 °C with a 16 h light / 8 h dark photoperiod in light of 6500 lux and relative humidity of 55–65 % on the B_5 medium [17] supplemented with 0.1 mg/L of 1-naphthylacetic acid [16].

The tissue cultures were obtained from the root and shoot growth point segments of the *in vitro* plants DAR12 and G/D11-1/3. They were grown in Petri dishes on Murashige and Skoog medium [18] supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid and 0.1 mg/l of kinetin in darkness at 16–20 °C (Fig. 1).

The influence of two abiotic factors (light and temperature) on the content of BAS in the morphogenic tissue cultures of *D. antarctica* was studied. Four groups with different cultivation conditions were under investigation:

I — in light of 6500 lux and at temperature of 18 °C;

II — in light of 6500 lux and at temperature of 26 °C;

III — in the darkness and at temperature of 18 °C;

IV — in the darkness and at temperature of 26 °C.

The experiment continued throughout three subcultures every four weeks. For each transplant in a fresh nutrient medium, the tissue cultures were taken for biochemical analysis and subjected to lyophilic drying. To determine the total content of phenolic compounds and flavonoids in calli, 96 % ethanolic extracts of lyophilized tissues were used. Five repetitions of each sample were taken.

The total phenolic content in cultured tissues extracts was determined using the Folin and Ciocalteu assay [19]; flavonoids were quantified using spectrophotometric assay, based on the ability of flavonoids to form a coloured complex with aluminium [20]. The optical density of the formed complexes was measured on a spectrofluorometer Fluorate-02-Panorama at a wavelength of 765 nm (phenolic compounds) and 510 nm (flavonoids). The calibration curve was constructed using standard solutions of ferulic acid (phenolic compounds) and rutin (flavonoids). The data were expressed as mg per 1 g of dry weight.

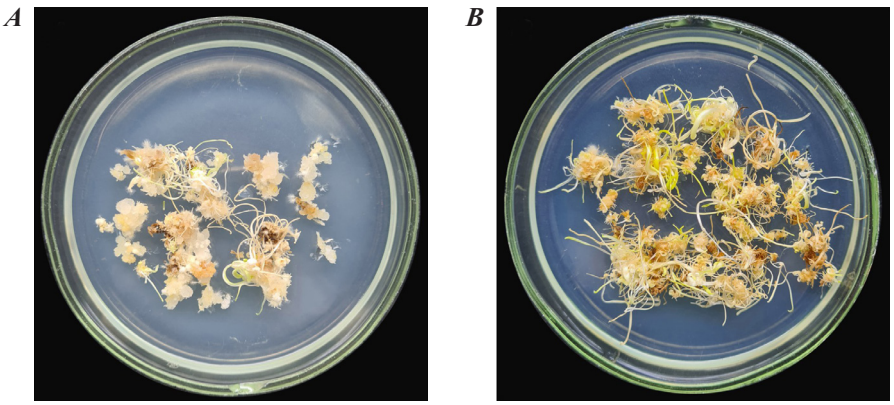

Fig. 1. Morphogenic tissue cultures of *D. antarctica*, obtained from the *in vitro* plants of genotypes DAR12 (***A***) and G/D11-1/3 (***B***).

High performance liquid chromatography (HPLC) analysis was done on a Shimadzu HPLC10Avp system (Japan) using a Zorbax Eclipse column (XDB-C18, 6´250 mm, 5 µm, Agilent) with a Waters Symmetry C8 precolumn. 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. Before analysis, the extracts of tissue cultures were concentrated 10 times in vacuo using the Thermo Scientific Savant SpeedVac system.

Results and Discussion

The data on the content of phenolic compounds and flavonoids in the tissue cultures of *D. antarctica* (genotypes DAR12 and G/D11-1/3) under the influence of studied abiotic factors are presented in the form of diagrams (Figs. 2, 3). As a control, we used the initial *in vitro* plants from which the tissue cultures were obtained and which were cultivated at 18 °C.

As seen from the data obtained, the content of BAS in cultures which grow in the light in almost all cases is very similar to that in the initial plants, despite the fact that these cultures consist of non- and poorly differentiated cells. Cultivation of the tissue cultures in the darkness has a negative effect on the biosynthesis of both phenolic compounds and flavonoids — their amount in the cell biomass was low regardless of the cultivation temperature. For example, the amount of phenolic compounds in the DAR12 calli cultivated in the darkness decreased by half, and flavonoids by 2.3–2.4 times regardless of the growing temperature. The same was observed in the G/D11-1/3 tissue culture: the content of phenolic compounds decreased by 2.8–3.1 times, and flavonoids — by 4.6–5 times. That is, the lack of lighting has a much greater negative effect on the growth of morphogenic tissue cultures and the accumulation of BAS, compared to the effect of temperature (both 26 °C and 18 °C in the control).

When cultivating the calli in light of 6500 lux, it has been found that temperature of 18 °C is favorable for their growth and

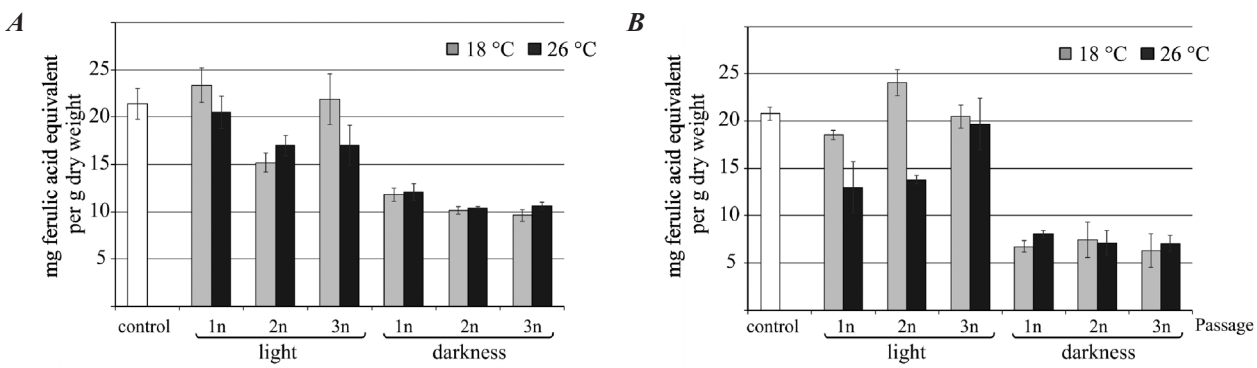

Fig. 2. The total content of phenolic compounds in the *D. antarctica* tissue cultures obtained from the *in vitro* plants DAR12 (***A***) and G/D11-1/3 (***B***) under different growth conditions.

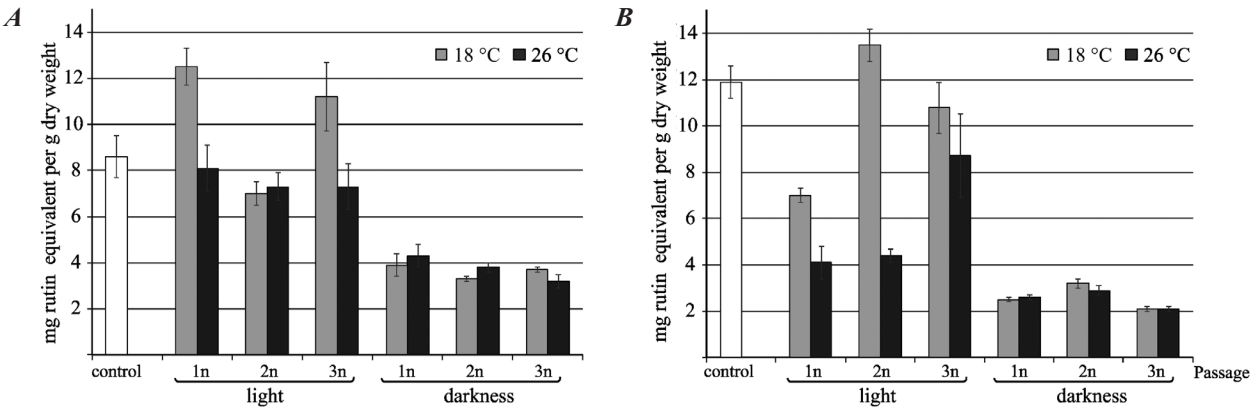

Fig. 3. The total content of flavonoids in the *D. antarctica* tissue cultures obtained from the *in vitro* plants DAR12 (***A***) and G/D11-1/3 (***B***) under different growth conditions.

synthesis of secondary metabolites. In such conditions the influence of elevated temperature (26 °C) was clearly expressed. During the first and second passages, the cultures of both genotypes responded to the temperature stress in the same way, namely, by a decrease in the phenolics and flavonoids biosynthesis. In the DAR12 calli the amount of phenolic compounds decreased from 21.4 to 18.2 mg/g of dry weight, in tissue culture G/D11-1/3 — from 20.8 to 15.5 mg/g of dry weight. The content of flavonoids also decreased: from 8.6 to 7.6 mg/g of dry weight for DAR12 and from 11.9 to 5.7 mg/g of dry weight for G/D11-1/3. However, the genotypes significantly differed further in their ability to adapt to elevated temperatures, that is the content of phenolics and flavonoids in the DAR12 calli remained unchanged, while the G/D11-1/3 tissue cultures increased their biosynthesis to the level, which occurred at 18 °C. So, the tissue culture of genotype G/D11-1/3 was found to be more sensitive to the changes in temperature and light regime compared to the DAR12 calli.

It can be assumed that a sharp decrease in the phenolic compounds and flavonoids biosynthesis in the darkness, regardless of temperature, is caused by the lack of photosynthetic activity of cells in such conditions. It is known that biosynthesis of these BAS requires the presence of aromatic amino acids, the synthesis of which in turn depends on the presence of compounds formed in the photosynthesis process. A sharp decrease of phenolic and flavonoid compounds in the plants cultivated in the light at the 26 °C, can be explained by a decrease in the photosynthesis intensity at this temperature. E.g., it has been shown that the photosynthetic activity of *D. antarctica* sharply decreases at elevated temperatures [21].

The qualitative analysis was performed by the HPLC method and the flavonoid tricin was detected in most of the examined calli, as well as in the *in vitro* initial plants (Fig. 4). Its content in the plants DAR12 was 0.15 mg/g of dry weight, while in the plants G/D11-1/3 it was three times lower — 0.05 mg/g of dry weight.

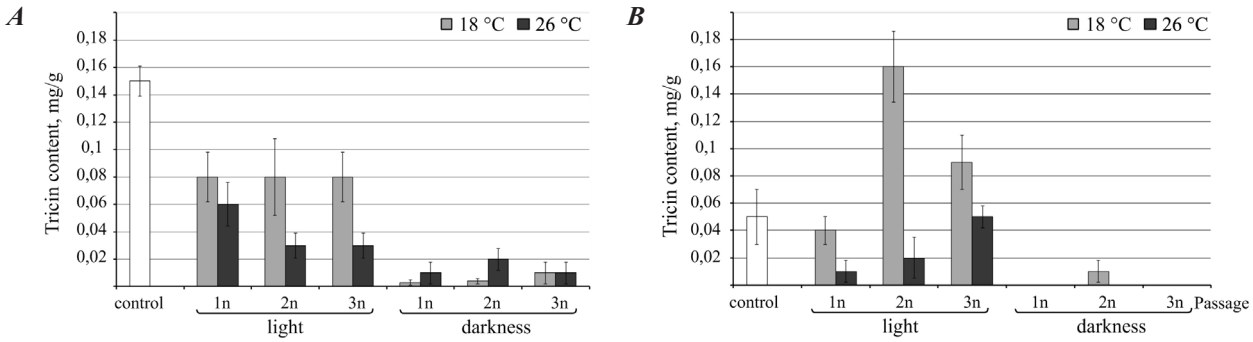

Fig. 4. Tricin content in tissue cultures obtained from *D. antarctica* plants of genotypes DAR 12 (***A***) and G/D11-1/3 (***B***) under exposure to light and temperature.

In the tissue cultures, the amount of this flavonoid was significantly lower compared to the initial plants.

The amount of tricin in the tissue culture of DAR12, which was grown in the darkness, was very low — 0.003–0.02 mg/g of dry weight; in some samples it was present only in trace amounts. The tissue culture of genotype G/D11-1/3, which was grown in the darkness, practically did not contain tricin (Fig. 4). These results correlate well with the low total content of phenolic compounds and flavonoids in the calli which were cultivated in the darkness.

When the cultures were cultivated in the light, the content of tricin was significantly higher than in the darkness. In the calli DAR12, which was cultivated at 18 °C, the tricin content was 0.08 mg/g of dry weight, while in the tissue culture obtained from the plants of genotype G/D11-1/3, grown in the light at 18 °C, the average value of tricin content was 0.1 mg/g of dry weight, which was twice as high as in the initial plants. Noteworthy, the content of tricin in the DAR12 callus remained unchanged during all three passages, while in the callus G/D11-1/3 it changed during cultivation, reaching a maximum (0.16 mg/g of dry weight) at the end of the second passage.

An increase in the cultivation temperature to 26 °C was a stress for the plants, that also affected the tricin content. Thus, in the first passage, its content decreased from 0.08 to 0.06 mg/g of dry weight for calli DAR12, and from 0.04 to 0.01 mg/g — for calli G/D11-1/3. During the following passages, the trends of changes in the content of tricin clearly depended on the genotype. In the tissue culture DAR12, the content of this flavonoid decreased in the second passage and then remained stably low, while the calli G/D11-1/3 showed better stress resistance and adaptability, so the tricin content increased in 2–3 passages reaching the entry level. These data also correlate well with the above-described data regarding the different response of genotypes to the elevated temperature, which resulted in different trends in changes in the total content of phenolic compounds and flavonoids in the tissue cultures of *D. antarctica*.

It can be assumed that the difference in tricin content in the plants of different genotypes *D. antarctica*, diploid G/D11-1/3

(2n = 26) and diploid with B-chromosomes DAR12 (2n = 26+0-3B), as well as in the tissue cultures obtained from them, may be caused by the presence/absence of B-chromosomes: it is known that the level of accumulation of secondary metabolites increases with the presence of B-chromosomes in the karyotype of plants [22].

Tricin, as well as other flavonoids (orientin, luteolin and its derivatives), phenolic compounds, hydroxybenzoic and hydroxycinnamic acids were also detected by other researchers in the *D. antarctica* plants grown *in situ* and *in vitro* [23].

Tricin is a flavonoid (Fig. 5), a metabolite that provides the plants stress resistance, and participates in protective reactions against diseases, weeds and microorganisms [24]. It is present in many types of plants and in various tissues [24, 25]. The whole grains such as rice, barley, oats and wheat are the food sources of tricin [26–28]. Tricin occurs in plants in free or conjugated forms, such as tricin glycosides, lignans, and lignan glycosides [24]. Leaves of pest-resistant rice contain more tricin than sensitive control groups. The content of this flavonoid in frost-resistant wheat was also higher compared to non-resistant one [24].

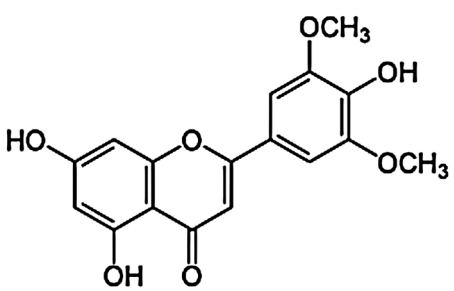

Fig. 5. Structural formula of tricin.

Tricin and its derivatives have been shown to exhibit the antioxidative effect, antiproliferative activity against several human cancer cell lines, and chemoprotective effect reducing the incidence of intestinal and colon cancer in mice [29–31]. The tricin residue is contained in Antartina (tricin 7-O-*β*-D-glucopyranoside), an antitumor compound isolated from *D. antarctica* [12]. Due to the lack of sufficient amount of plant material, the researchers obtained Antartina synthetically. This compound has been shown to be able to induce the antitumor immunity against colorectal carcinoma; its toxicological profile has been characterized in mice, and all tested animals remained healthy and tolerated repeated doses without signs of toxicity. Thus, Antartina has powerful immunostimulatory properties and can be used as a potential antitumor substance to develop new tools for the treatment of colorectal carcinoma [12].

Our results indicate that the content of BAS in the morphogenic tissue cultures of *D. antarctica* was lower compared to the initial plants. Accumulation of secondary metabolites occurs more intensively in the plants grown under *in vitro* conditions, which have a longer life cycle and, accordingly, the period of synthesis of these compounds [32]. The previously determined content of phenolic compounds and flavonoids in the *D. antarctica* plants of genotypes DAR12, DAR13, G/D12-2a, Y66, R30, and L57 slightly exceeded the similar data in the dedifferentiated tissue cultures [15]. This can be explained by the fact that the content of BAS increases due to the activation of the protective mechanisms, including those during the transition of cells from dedifferentiation to redifferentiation at morphogenesis, as well as

in response to the abiotic or biotic stresses, and as a result of stimulation of the accumulation of secondary metabolites in biomass [7, 33].

Our finding of tricin in the *D. antarctica* samples, particularly in the morphogenic tissue cultures, provides a basis for further detailed biochemical studies of this species, as well as a careful selection of the samples for application as a potential source of BAS for their possible applying in pharmacy as the antioxidant and antitumor agents.

Conclusions

The influence of light and temperature on the total content of phenolic compounds and flavonoids in the morphogenic tissues cultures of *D. antarctica*, obtained from the *in vitro* plants of different genotypes, diploid G/D11-1/3 (2n = 26) and diploid with B-chromosomes DAR12 (2n = 26+0-3B), was studied. Diverse responses to stress conditions of these genotypes are demonstrated. It is shown that more acceptable conditions for the growth of tissue culture and the accumulation of BAS are the presence in light of 6500 lux and temperature of 18 °C. When culturing the calli at temperature of 26 °C, the amount of flavonoids decreased by 1.3–2.1 times, compared to the initial plants. Cultivation of the calli in the darkness leads to a significant decrease in the level of secondary metabolites regardless of the cultivation temperature. In the studied samples, tricin was found, the content of which was higher in both the initial plant *in vitro* and the tissue culture of genotype DAR12. We assume that the detected difference in the content of this flavonoid in the DAR 12 and G/D11-1/3 plants, as well as in the tissue cultures obtained from them, may be related to the influence of the original genotype, in particular the presence/absence of B-chromosomes in the karyotype. The tricin found in the samples of *D. antarctica* provides a basis for the further biochemical studies as a potential source of BAS, which can be used for therapeutic and preventive purposes.

Acknowledgement

The work was conducted as a part of the research project "Genetic, physiological, and biochemical mechanisms for plant adaptation to extreme environmental conditions" (2021–2025) #0120U105249 funded by the National Academy of Sciences of Ukraine.

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Вплив світла та температури на вміст деяких біологічно активних сполук у культурі тканин *Deschampsia antarctica*

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Мета. Метою роботи було дослідження впливу умов вирощування (світло/темрява та температури 18/26 °С) на вміст фенольних сполук та флавоноїдів у морфогенній культурі тканин *D. antarctica*. **Методи.** Культура *in vitro*, метод Фоліна-Чокальтеу, спектрофотометричний аналіз, ВЕРХ-аналіз. **Результати.** Досліджено сумарний вміст фенольних сполук та флавоноїдів у біомасі морфогенної культури тканин *D. antarctica*, отриманої від рослин генотипів DAR12 та G/D11-1/3. Показано, що вирощування культур тканин на світлі за інтенсивності 6500 люкс та підвищеній температурі 26 °С призводить до зниження у них вмісту біологічно активних сполук (БАС). При культивуванні калюсів обох генотипів у темряві, незалежно від температури, знижується рівень як фенольних сполук (у 2 рази для DAR 12 і в 2,8–3,1 рази для G/D11-1/3), так і флавоноїдів (у 2,3–2,4 рази для DAR 12 і в 4,6–5 разів для G/D11-1/3). Знайдено антиоксидантну та протипухлинну сполуку трицин, вміст якої був втричі вищим у вихідній рослині DAR12, порівняно з рослиною G/D11-1/3. Кількість трицину в калюсах була меншою, ніж у вихідних рослинах. **Висновки.** Встановлено, що найвищий рівень накопичення БАС у морфогенній культурі тканин *D. antarctica* спостерігається при її вирощуванні на світлі за інтенсивності 6500 люкс та за температури 18 °С. Знайдений трицин у рослинах DAR 12 та G/D11-1/3, а також в отриманих від них культурах тканин, дає підставу для подальших біохімічних досліджень *D. antarctica in vitro* як потенційного джерела БАС, які можна використовувати з лікувальною та профілактичною метою.

Ключові слова: *Deschampsia antarctica* E. Desv., культура тканин, фенольні сполуки, флавоноїди, трицин.

Received 07.02.2024