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EFFECT OF PHENYLALANINE AND LIGHT ON THE GROWTH OF HAIRY ROOTS OF *Artemisia tilesii* Ledeb.

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Aim. To analyze the possibility of using phenylalanine of various concentrations and different lighting modes separately and in combination to boost the biomass accumulation and biosynthesis of flavonoids in two lines of *Artemisia tilesii* Ledeb. hairy roots.

Methods. The roots were grown on solidified medium with phenylalanine at high (1 mM) and low concentrations (0.05 and 0.1 mM) with lighting (3000 lx, 16 h) and in darkness. After four weeks cultivation, weight gain, flavonoid content and DPPH-scavenging activity were determined according to the standard tests.

Results. Roots grown in light were greenish in color, more branched and thick, yet the roots were more elongated after maintenance in the dark. Addition of 1 mM phenylalanine has led to inhibition of growth of all samples. The tolerance to lower concentrations varied among the lines. The flavonoid content for all samples of both lines was higher in the light (up to 3.14 times), regardless of the concentration of phenylalanine. The antioxidant activity was as well higher for the roots grown in light and the values of EC_{50} correlated with the flavonoid content.

Conclusions. Illumination boosted the synthesis of flavonoids and antioxidant activity in all samples of both hairy root lines. The effect of phenylalanine addition on biomass accumulation and flavonoid biosynthesis was line-specific.

Key words: *Artemisia tilesii* Ledeb.; hairy roots; phenylalanine; light mode; elicitors; flavonoids; antioxidant activity.

Hairy roots of medicinal plants are a promising source of biologically active compounds as these cultures carry *rol* genes that are secondary metabolism activators. Hairy roots of *Artemisia tilesii* Ledeb. are of special interest. This is a rare plant adapted to the action of extreme factors [1]. *A. tilesii* is rich in polyphenolic compounds, such as flavonoids, with antioxidant, anti-inflammatory and antiviral effects [2–5]. To increase flavonoids content, different precursors [6–14] of their biosynthesis and various elicitors [15–32] can be added. Their presence and concentration in the nutrient medium can affect the synthesis of flavonoids and, accordingly, the bioactivity of plants.

All polyphenolic compounds originate from the shikimate pathway that leads to phenylalanine and tyrosine — aromatic amino acids. This makes phenylalanine one of possible elicitors for the activation of flavonoids biosynthesis. Indeed, Peng et al. [6] showed the beneficial effect of combination of microwave and L-phenylalanine on the specific activities of phenylalanine ammonia-lyase, chalcone isomerase, and *FLS* flavonol synthase in Tartary buckwheat sprouts. Demirci et al. [7] explored the effect of 24-epibrassinolide and L-phenylalanine on the root growth, total phenolics, total flavonoids, and caffeic acid derivatives accumulation in hairy roots of *Echinacea purpurea*. Treatment with 0.5 mg L^{-1} 24-epibrassinolide for 50 days resulted in the highest fresh root weight, dry root weight, and growth index, while L-phenylalanine had no significant influence on root growth. Cao et al. [8] showed the benefit of combination of 10 mM

phenylalanine and 50 mM chitosan on the dry weight, flavonoid and phenolic contents. Arya and Patni [9] reported significant enhancement of quercetin in callus culture of *Pluchea lanceolata* by incorporation of cinnamic acid and precursor feeding with L-phenylalanine. Syk owska-Baranek et al. [10] determined the effect of L-phenylalanine on PAL activity and production of naphthoquinone pigments in suspension cultures of *Arnebia euchroma*. Andi et al. [11] determined the boosting effect of L-phenylalanine on the synthesis of stilbenes and flavonoids in suspension cultures of *Vitis vinifera*.

The changes in certain cultivation conditions (temperature, lighting, agitation, aeration) can result in the alterations of the secondary metabolites biosynthesis as well. Thus, it is important to determine the parameters essential for the best ratio of biomass accumulation and biosynthesis in plant cultures *in vitro*.

Tusevski et al. [15] showed that dark-grown and photoperiod-exposed hairy root cultures of *Hypericum perforatum* differed in phenolic acids, flavonols, flavan-3-ols, and xanthones accumulation. Light served as the elicitor for the quinic acid, kaempferol, and seven identified xanthones production. On the other hand, dark-grown cultures had a higher content of flavan-3-ols (catechin, epicatechin, and proanthocyanidin dimers). The beneficial effect of light on cyanidin 3-*O*-rutinoside accumulation was also observed in Tartary buckwheat hairy roots [16]. Marsh et al. showed that the American skullcap hairy roots cultivated under continuous illumination in cobination with β-cyclodextrin accumulated flavone glycosides better, while the cultures maintained in the dark with the same treatment had maximum contents of baicalein and wogonin (5.4 mg/g DW and 0.71 mg/g DW, respectively) [17]. Wongshaya et al. showed a better response to elicitation of the *Arachis hypogaea* hairy roots maintained in the dark than those grown in the light [18]. Other researchers [19–25] showed the beneficial effect of different light waves, photoperiod optimization and ultraviolet radiation on the enhancement of flavonoids accumulation.

All these findings suggest that the addition of precursors and changes in cultivation parameters can have different effects on the growth of *in vitro* plant cultures and activity of their biosynthesis. Thus, the research was focused on the possibility of using phenylalanine of various concentrations and different lighting modes separately and in combination to boost the biosynthesis of flavonoids in two lines of *A. tilesii* hairy roots.

Materials and Methods

Plant material

Two *A. tilesii* hairy roots lines from the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used as the plant material for the study [33]. Both of the lines were obtained by the transformation using *Agrobacterium rhizogenes* A4 wild strain.

Phenylalanine elicitation

The roots were grown for four weeks on the solidified nutrient Murashige and Skoog medium (Duchefa Biochemie, The Netherlands) with halved macrosalt content (½ MS) and the addition of sucrose at a concentration of 20 g/l. Phenylalanine at high (1mM) and lower concentrations (0.05 and 0.1 mM) was added to the nutrient medium. Roots were grown at a temperature of +24 °C with lighting (3000 lx, 16 h) and in darkness. After cultivation, weight gain, flavonoid content and antioxidant activity were determined in both experiments.

Weight gain

After four weeks of cultivation, the grown hairy roots were separated from the medium, washed with distilled water, dried using filter paper and weighed on a Sartorius balance with a standard deviation of ± 0.005 g. Weight gain was determined as the difference between the final and initial weights in terms of one growth point.

Flavonoids content

Determination of the content of flavonoids was carried out according to the method described in [34] with modifications. To prepare the extracts, the roots were separated from the medium, washed with deionized water, dried using filter paper, weighed 0.3 g each and homogenized in 3 ml of 70% ethanol. The homogenate was centrifuged in an Eppendorf Centrifuge 5415 C at 15 000 g for 10 min. The reaction mixture contained 0.25 ml of extract supernatant, 1 ml of deionized water, 0.075 ml of 5% $NaNO_2$ (Merck, Germany) solution. After standing for 5 min, 0.075 ml of 10% $AlCl_3$ (Merck, Germany) solution was added and held for another 5 min. Then 0.5 ml of 1 M NaOH (Merck, Germany) and 0.6 ml of deionized water were added. Absorption was determined

at λ = 510 nm on a Fluorate-02-Panorama spectrofluorimeter. The calculation of the content of flavonoids was carried out in the rutin equivalent (RE) according to the following formula (after calibration graph was plotted), and converted to grams of fresh weight (FW):

$$C = (1.4497 \cdot OD) \cdot V / m, (R^2 = 0.9633)$$

where C — concentration of flavonoids in 1.0 g of fresh weight of plant material, mg RE/g FW; OD – optical density of the investigated solution, U; V — volume of ethanol used to prepare the extract; m — mass of plant material used for research. The total content of flavonoids (in all biomass) was calculated as the multiplication of the concentration of flavonoids and the weight gain per one growth point.

Antioxidant activity (AOA)

The antioxidant activity of ethanol extracts of hairy roots was studied using the DPPH (2,2-diphenyl-1-picrylhydrazyl, Merck, Germany) test according to the method described in [35]. The optical density of the solutions was measured at a wavelength of λ = 515 nm on a Fluorate-02-Panorama spectrofluorimeter. The percentage of inhibition was calculated according to the following formula:

$$\%\ inhibition = (OD_1 - OD_2) / OD_1 \cdot 100,$$

where OD_1 — optical density of the DPPH solution, U; OD_2 — optical density of the of the reaction mixture after carrying out the reaction with DPPH, U.

The effective concentration (EC_{50}) was calculated as the fresh weight of the root (mg FW) required to scavenge 50% of DPPH in the reaction with the radical.

Data analysis

All analyzes were performed in triplicate. Results were calculated in Microsoft Excel and presented as mean ± confidence interval. The data were analyzed for statistical significance using ANOVA followed by Tukey HSD test using R software version 4.0.4. The differences between mean values were considered statistically significant at $P < 0.05$.

Results and Discussion

For both lines cultivated without phenylalanine, hairy roots accumulated more biomass while growing in the dark. Such roots were white, elongated and less branched. Weight gain of the roots No.1 and No. 2 grown in darkness exceeded this parameter of the roots of these lines grown with lighting (2.33 and 3.94 fold, respectively) (Fig. 1, *A*). Therewith, flavonoid content was higher in the roots grown in light. Such roots were greenish in color and highly branched. Flavonoid content in ethanolic extracts obtained from roots No.1 and No.2 grown in light (Fig.1, *B*) was 2.53 ± 0.28 and 5.45 ± 0.37 mg RE/g of FW, respectively. For the samples grown in dark, the values were much lower: 2.3 and 4.74 mg RE/g of FW, respectively. Thus, high increase of biomass correlated with low biosynthesis of flavonoids and vice verse. This result may be explained by the fact that plant cells have limited quantity of all needed precursors and energy both for the root growth and secondary metabolites accumulation. Consequently, as the roots biomass was higher in the dark, their rate of growth was rapid and the flavonoid biosynthesis level was low. Accordingly, roots grown in light had higher biosynthetic activity and flavonoids accumulation, and their growth rate was slower.

All samples grown on the medium with addition of 1 mM phenylalanine (PHE) had drastic inhibition of growth. Line No. 1 tolerated the addition of PHE better and the weight gain was 0.022 ± 0.014 and 0.008 ± 0.003 g per one growth point while grown in light and dark, respectively. Flavonoid content in the ethanolic extracts of these samples was comparable with that of control sample: 2.36 ± 0.17 (grown in light) and 1.84 ± 0.06 (grown in dark) mg RE/g of FW. In addition, total flavonoid content in the whole mass of the hairy root samples was the following: 0 mM PHE light (0.10 mg RE) > 0 mM PHE dark (0.09 mg RE) > 1 mM PHE light (0.05 mg RE) > 1 mM PHE dark (0.01 mg RE). Such result once again confirmed the inhibiting effect of phenylalanine on the growth of *A. tilesii* hairy roots and their biosynthetic activity. Moreover, it was shown that it is better to grow wormwood hairy roots with lighting.

Line No. 2 was more sensitive to the addition of PHE than root line No. 1 (Fig.1, *A*). The inhibition was so drastic, that it was not possible to obtain enough plant material to prepare the extracts for the study of flavonoids accumulation and antioxidant activity.

As phenylalanine at 1 mM concentration was shown to be inhibiting for these *A. tilesii* hairy roots, for the next part of the study lower concentrations were chosen, namely 0.05 and 0.1 mM.

The morphology and growth characteristics of the hairy roots depended

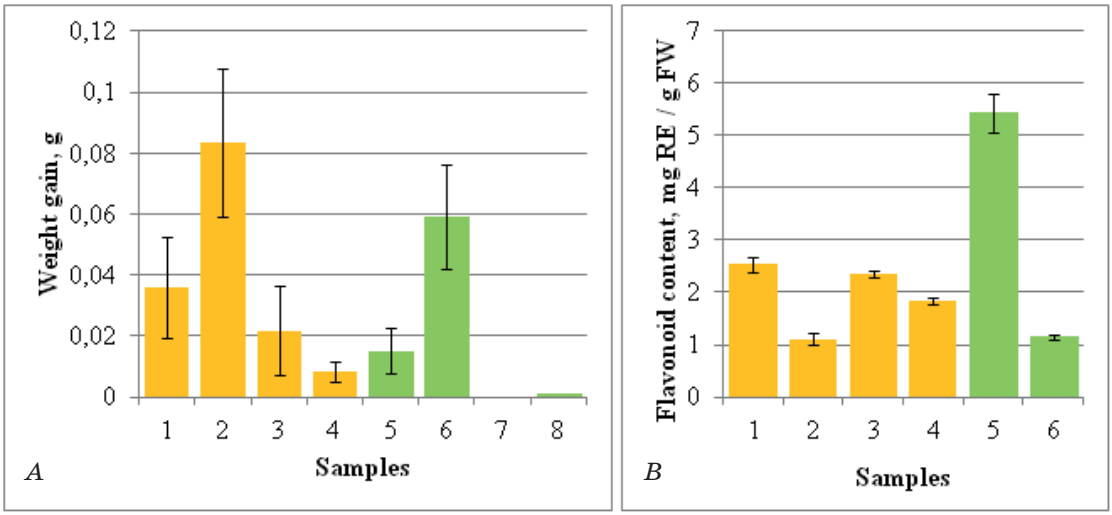

Fig. 1. **Weight gain (*A*) and flavonoid content (*B*) of the *Artemisia tilesii* hairy roots grown on two lighting modes without and with addition of high concentration of phenylalanine:**
orange columns — line No. 1, green columns — line No. 2. Growth conditions: columns 1 and 5 — light, without phenylalanine (controls); 2 and 6 – dark, without phenylalanine; 3 and 7 — light, 1 mM phenylalanine; 4 and 8 — dark, 1 mM phenylalanine

on the presence of lighting (Fig. 2) the same way as in the first experiment. Illumination acted in favor of the color of hairy roots, their branching and thickness. All samples of hairy roots were slightly greenish in the light and white in the dark.

It was established that in this part of experiment for both lines and in all concentrations of phenylalanine the weight gain of hairy roots was higher in the samples grown with lighting (Fig. 3, *A*) that contradicts the data in the previous experiment. Therefore the difference in the effect of lighting on the weight gain may be explained by the thickness of the roots: all root samples grown in light this time were much thicker (up to 1.5 mm), than roots grown in dark. This thickness contributed to the increased weight gain. Weight gain of the roots No.1 and No. 2 grown in light was 1.16–2.91 and 1.26–1.59 times higher respectively than the same parameter of the roots cultivated in darkness. Addition of phenylalanine at any concentrations (0.05 or 0.1 mM) inhibited the growth of the roots. This inhibition varied among the lines in the same manner as in the previous experiment: line No. 1 tolerated phenylalanine better than line No. 2. The weight increase in the roots No. 1, grown under the same lighting conditions, was similar when PHE was added in different concentrations. At the same time, increasing the concentration of PHE led to the inhibition of growth of roots No. 2.

The content of flavonoids (Fig. 3, *B*) in all the samples of both lines was higher in the light, which may prove the possible effect of lighting as flavonoids biosynthesis activator for the wormwood hairy roots that was suggested in the result of the previous experiment. Indeed, regardless of the presence and concentration of phenylalanine in the solid nutrient medium, the flavonoid content was 1.48–2.69 times higher in the roots No. 1 and 1.02–3.14 times higher in the roots No. 2 grown in light. As in the first experiment, even though the biomass accumulation of roots No. 1 was vividly higher than in the line No. 2, the flavonoid content was higher in all the samples when comparing the variants grown at the same conditions. This once more suggests the explanation proposed in the first experiment that biomass accumulation and secondary metabolites biosynthesis often don't occur at the same time and can vary greatly depending on the line of hairy roots.

Similarly, the antioxidant activity in all the samples was higher in the roots grown under lighting conditions. Values of EC_{50} (Fig. 4) correlated with the flavonoid content in the samples. Lower values of EC_{50} and thus higher antioxidant activity was interrelated with higher flavonoid biosynthesis.

As it can be seen from the graph (Fig. 3, *B*), lower concentrations of PHE didn't inhibit the flavonoids biosynthesis, although inhibition was observed when PHE was added at a higher concentration (1 mM, Fig. 1). Nonetheless, such value as the total content of flavonoids calculated per whole biomass gain showed that the inhibition of growth that

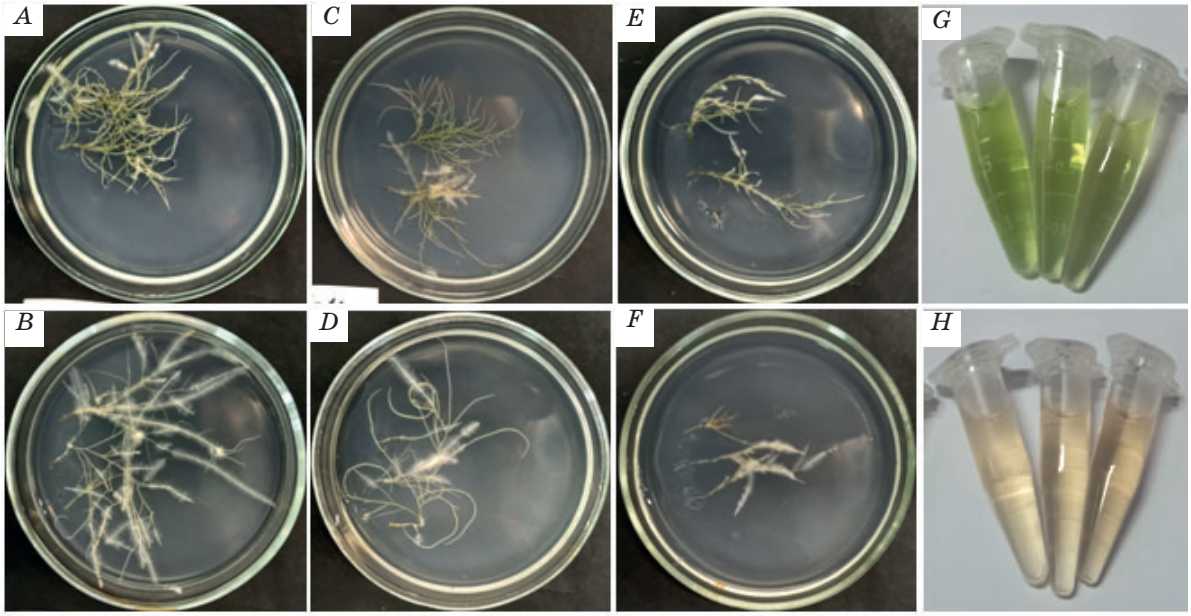

Fig. 2. **Differences in morphology of line No. 2 of *A. tilesii* hairy roots grown in different lighting conditions and phenylalanine addition:**
A — light without phenylalanine addition (control); *B* — dark without phenylalanine; *C* — light, 0.05 mM phenylalanine; *D* — dark, 0.05 mM phenylalanine; *E* — light, 0.1 mM phenylalanine; *F* — dark, 0.1 mM phenylalanine; *G* — ethanolic extracts prepared from the sample of hairy roots grown in light; *H* — ethanolic extracts prepared from the sample of hairy roots grown in dark

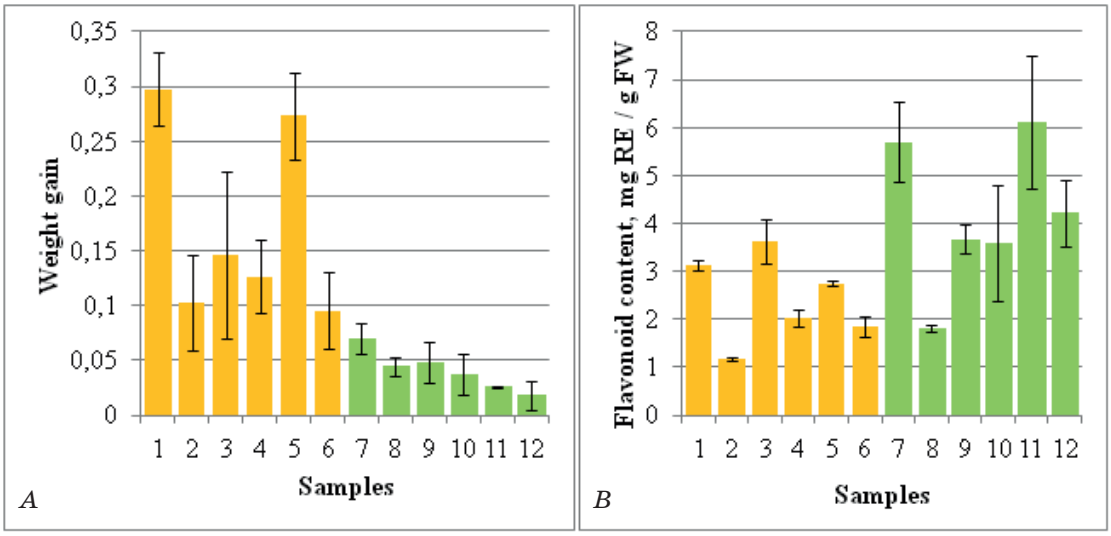

Fig. 3. **Weight gain (*A*) and flavonoid content (*B*) of the *A. tilesii* hairy roots grown on two lighting modes without and with addition of low concentrations of phenylalanine:**
orange columns — line No. 1, green columns — line No. 2. Growth conditions: columns 1 and 7 — light, without addition of phenylalanine (controls); 2 and 8 — dark, without phenylalanine; 3 and 9 — light, 0.05 mM phenylalanine; 4 and 10 — dark, 0.05 mM phenylalanine; 5 and 11 — light, 0.1 mM phenylalanine; 6 and 12 — dark, 0.1 mM phenylalanine

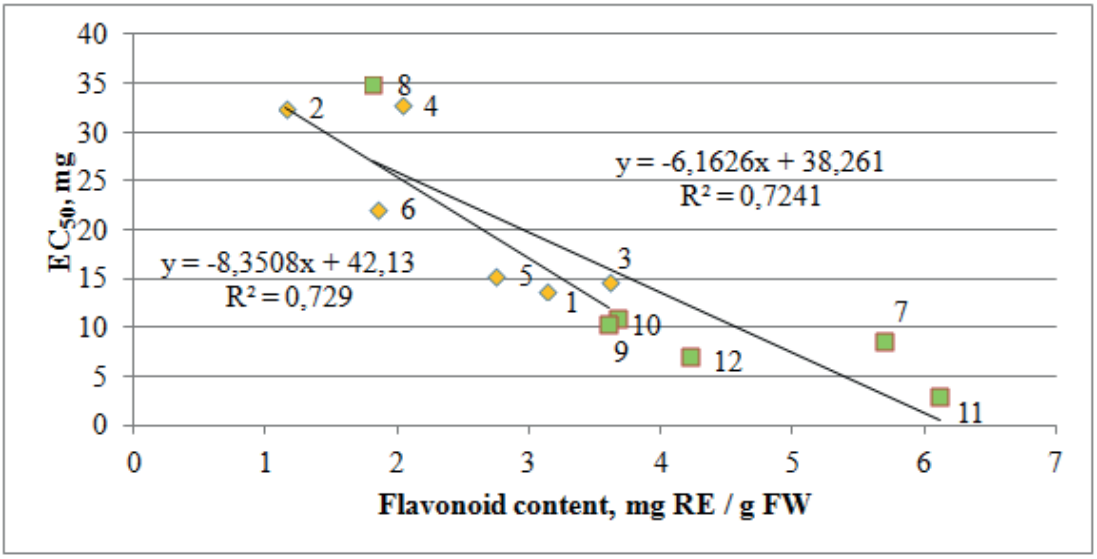

Fig. 4. **Correlation graph of antioxidant activity (EC_{50}) and flavonoid content of the *A. tilesii* hairy roots grown on two lighting modes without and with addition of low concentrations of phenylalanine:** orange markers — line No. 1, green markers — line No. 2. Markers and corresponding conditions: 1 and 7 — light, without addition of phenylalanine (controls); 2 and 8 — dark, without phenylalanine; 3 and 9 — light, 0.05 mM phenylalanine; 4 and 10 — dark, 0.05 mM phenylalanine; 5 and 11 — light, 0.1 mM phenylalanine; 6 and 12 — dark, 0.1 mM phenylalanine

resulted after the addition of phenylalanine was more prominent that the increase in the concentration of flavonoids in the extracts. In none of the samples the value of the total content of flavonoids per whole biomass was higher than in the control root samples (roots grown in light without addition of phenylalanine). This result shows that for both of the *A. tilesii* hairy root lines phenylalanine is not a suitable elicitor, regardless of the chosen concentration. The possibility of stimulating the biosynthesis of metabolites in plants using phenylalanine was previously studied [7, 10]. Obviously, such an effect can be species-specific.

Light is known as an important factor affecting plants including their growth and metabolism of plant cells. It can stimulate secondary metabolism but such an effect may be species-specific and varied in the study of different metabolites. Briefly, greening of *Solanum khasianum* roots under light irradiation and stimulation of biosynthesis was studied [36]. Lighing also increased the concentration of polyphenols in *Echinacea purpurea* [37], *Fagopyrum tataricum* [38], *Scutellaria lateriflora* [17] and *Fagopyrum tataricum* [16]. The results of our study demonstrated the positive effect of lighting on *A. tilesii* hairy roots growth and flavonoid accumulation. The similar stimulating effect was founded in the experiments with other plants of *Artemisia* genus — *A. annua* hairy roots [39]. In that experiment the growth rate and artemisinin accumulation increased in case of cultivation of hairy roots at 3000 lx light irradiation for 16 h.

Conclusions

It was shown that illumination has an impact on *A. tilesii* hairy roots morphology, color, branching, biomass accumulation and flavonoids biosynthesis. For both studied root lines it was found that the presence of lighting is more beneficial than constant maintenance in the dark, as light activated the synthesis of flavonoids and increased antioxidant activity.

In this study, no concentrations of the added precursor were found to be beneficial for the total content of flavonoids in the whole biomass of *A. tilesii* hairy roots. Addition of phenylalanine at a concentration of 1 mM inhibited both the growth of hairy roots of *Artemisia tilesii* and the synthesis of flavonoids. Such effect was observed during cultivation with and without illumination. The degree of inhibition varied in the hairy root lines. One of the lines appeared to be more tolerant to PHE at any concentrations than the other. In addition the line that had worse biomass increase at the same time had higher flavonoid accumulation while cultivating on the medium with the addition

of low concentrations of phenylalanine. This can be the result of the dependence of the ratio of rate of growth and secondary metabolites biosynthesis limited by the scarce resources of the plant cells.

Hereby, the addition of phenylalanine in various concentrations to the nutrient medium during the cultivation can have different effects on the hairy root lines of *A. tilesii*. This can be caused not only by cultivation parameters (different lighting regimes), but also by differences in the genotypes of the obtained lines of the same species.

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ВПЛИВ ФЕНІЛАЛАНІНУ ТА ОСВІТЛЕННЯ НА РІСТ «БОРОДАТИХ» КОРЕНІВ *Artemisia tilesii* Ledeb.

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Мета. Проаналізувати вплив фенілаланіну у різних концентраціях та освітлення (окремо та в комбінації) для стимулювання росту біосинтезу флавоноїдів у двох лініях «бородатих» коренів *Artemisia tilesii* Ledeb.

Методи. Корені вирощували на агаризованому середовищі з фенілаланіном високої (1 мМ) і низької (0,05 і 0,1 мМ) концентрацій при освітленні (3000 лк, 16 год) і в темряві. Після чотирьох тижнів культивування приріст ваги, вміст флавоноїдів і активність проти DPPH-радикалу визначали згідно зі стандартними методиками.

Результати. Корені, вирощені на світлі, мали зеленуватий колір, були більш розгалужені та товщі, а після росту в темряві були більш видовженими. Додавання фенілаланіну (1 мМ) пригнічувало ріст усіх зразків. Дві лінії коренів відрізнялися за чутливістю до фенілаланіну у менших концентраціях. Вміст флавоноїдів у всіх зразках обох ліній був вищим на світлі (до 3,14 рази), незалежно від концентрації фенілаланіну. Антиоксидантна активність також була вищою у коренях, вирощених на світлі, причому значення EC_{50} корелювали з вмістом флавоноїдів.

Висновки. Освітлення стимулювало синтез флавоноїдів і підвищувало антиоксидантну активність всіх зразків. Ефект додавання фенілаланіну на накопичення біомаси та біосинтез флавоноїдів залежав від лінії «бородатих» коренів.

Ключові слова: *Artemisia tilesii* Ledeb.; «бородаті» корені; фенілаланін; освітлення; еліситори; флавоноїди; антиоксидантна активність.