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Agrobacterium rhizogenes — MEDIATED TRANSFORMATION AS AN APPROACH OF STIMULATING THE SYNTHESIS OF ANTIOXIDANT COMPOUNDS IN *Artemisia absinthium* L.

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Artemisia absinthium L. plants are known as producers of substances with antioxidant properties. Among others, phenols and flavonoids are found in these plants. The synthesis of these bioactive compounds can be activated by genetic transformation. This process can be carried out even without the transfer of specific genes involved in the synthesis of flavonoids. Thus, "hairy" roots, obtained after *Agrobacterium rhizogenes* — mediated transformation, can produce a variety of valuable substances.

The aim of this study was obtaining *A. absinthium* "hairy" roots with high phenolic content.

Methods. "Hairy" roots were obtained by co-cultivation leaves with suspension of *A. rhizogenes* with pCB124 vector. The presence of transferred genes was confirmed by PCR. The reactions with $AlCl_3$ and Folin-Ciocalteu reagent were used to determine the total flavonoids and phenols content. The antioxidant activity of extracts was evaluated by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

Results. PCR analysis detected the presence of bacterial *rol* genes and the absence of pCB124 plasmid genes. Root lines differed in growth rate. "Hairy" roots were characterized by a higher phenolic content, particularly flavonoids (up to 4.784 ± 0.10 mg/g FW) compared to control (3.861±0.13 mg/g FW). Also, extracts from transgenic roots demonstrated higher antioxidant activity in the reaction with DPPH reagent (EC_{50} = 3.657 mg) when compared with extracts from control plants (EC_{50} = 6,716 mg).

Conclusions. *A. rhizogenes*-mediated transformation of *A. absinthium* can be applied for obtaining transgenic root lines with increased phenolic content and higher antioxidant activity.

Key words: *Artemisia absinthium L., Agrobacterium rhizogenes*-mediated transformation, "hairy" roots, flavonoids, phenolic compounds, antioxidant activity.

Artemisia absinthium L. (wormwood) is a perennial shrubby plant of the Compositae family. It is native to Asia, the Middle East, Europe and North America [1].

A. absinthium extract is known to have antioxidant [2], immunomodulatory [3], wound-healing [4], anti-inflammatory, analgesic [5], antitumor [6], antiulcer [7], antibacterial, antifungal [8], antiparasitic [9] activities, as well as neuroprotective [10], hepatoprotective [11], hypoglycemic [12] effects. Such a wide range of properties is related to the *A. absinthium* chemical composition. Such compounds as lactones, terpenoids, essential oils, organic acids, resins, tannins and phenols were previously detected in the extracts of this plant [1]. For example, isolated dimeric guaianolides, that were found in *A. absinthium*, exhibited cytotoxic activity, inhibited cyclooxygenase-2, and had anti-HIV-1 protease activity [13].

Flavonoids, which are important secondary plant metabolites, are also synthesized in *A. absinthium*. Flavonoids are composed of two aromatic rings and one heterocyclic ring with an oxygen atom. Flavonoids are characterized by high antioxidant activity due to such chemical structure [14]. Thus, they can protect cells from oxidative stress, which can cause in human such pathological diseases as cancer,

atherosclerosis and cardiovascular diseases, neurodegenerative diseases, diabetes and inflammation [15]. Therefore, *A. absinthium* can be a valuable source of bioactive compounds, particularly flavonoids.

Plant bioactive compounds are usually obtained from natural raw materials. However, this method has a number of disadvantages. This traditional way of biosynthesis depends on the growing temperature, rainfall, soil contamination, etc. At the same time, "hairy" roots, which are formed after plants contact with phytopathogenic bacteria *Agrobacterium rhizogenes*, are supposed to be a better source of plant chemical compounds [16–18].

During transformation a fragment of the bacterial Ri-plasmid (T-DNA) is transferred into the genome of the host plant. It contains plant-expressed genes that control synthesis of phytohormones, such as auxin and cytokinin. T-DNA integration gives transformed roots the ability to hormone-independent growth [19]. The *A. rhizogenes rol*-genes induce synthesis of secondary metabolites, including flavonoids [20]. It allows to select the lines producing valuable compounds. "Hairy" root culture is characterized by genetic stability, the ability to grow in a nutrient medium without costly compounds. That enables to scale-up the root growth process and to obtain bioactive compounds in different bioreactors [21, 22].

Plants of the *Artemisia* genus have previously been used in genetic transformation studies. For example, obtained *Artemisia vulgaris* L. "hairy" roots produced β-caryophyllene [23], *Artemisia annua* and *Artemisia pallens* Wall. transgenic roots synthesized artemisinin [24, 25]. However, there is currently only one publication about *A. absinthium* transformation using *A. rhizogenes*. Nin et al [26] obtained wormwood "hairy" root culture by transformation via 1855 and LBA 9402 *A. rhizogenes* strains. They showed a significant difference in the qualitative and quantitative content of essential oils from transgenic roots compared to the control. Thus, there was absence of β-thujone in "hairy" roots, and linalyl 3-methylbutanoate was about 37% of the transgenic roots essential oil.

The aim of our work was to optimize the method of *A. absinthium* genetic transformation to obtain "hairy" roots. The other purpose was to evaluate their biosynthetic potential as producers of compounds with antioxidant properties.

Materials and Methods

1. A. rhizogenes cultivation.

Bacteria were cultivated 24 hours in liquid LB medium [27] at 28 °C and 180 rpm using rotation shaker SpeedVac Savant AES 2010 (Labconco, USA). Next the bacterial suspension was centrifuged at 3000 *g* for 10 min. Precipitated cells were resuspended in 10 mM $MgSO_4$ solution. Obtained suspension was used for *A. absinthium* transformation.

2. A. rhizogenes-mediated transformation of A. absinthium.

A. rhizogenes A4 agropine strain with pCB124 vector (Fig. 1) was used for genetic transformation. This vector was constructed at Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine. It contained a selective neomycin phosphotransferase II gene (*npt*II) with NOS promoter and terminator regulatory sequences, as well as a human interferon-α2b gene (*ifn*-α2b) under control of 35S promoter.

A. absinthium leaves were used as the explants for genetic transformation. Explants were sliced and co-cultivated during 30 min with an overnight bacterial suspension. Then they were soaked using filter paper and cultivated on 1/2 MS agar-solidified medium (Duchera Biochemie) for 3 days. Next explants were transferred to the 1/2 MS agar-solidified medium with 600 mg/l cefotaxime (Borshchahivskiy CPP). Obtained roots were subcultivated every 10 days on the same medium. Each root was considered as a separate transformation event.

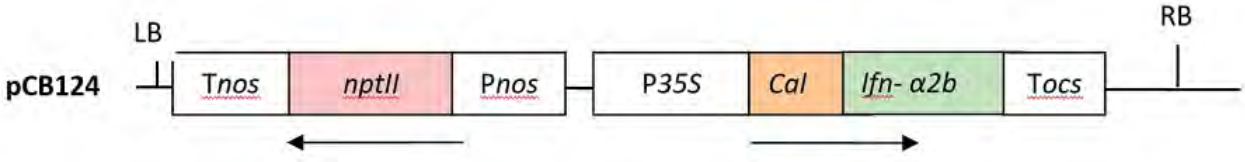

Fig. 1. **Schematic representation of the T-DNA site of pCB124 vector:**

LB i RB — left and right border sequences; *nptII* –*nptII* gene (NOS promoter and terminator); *ifn*-α2b – *ifn*- α2b gene (35S promoter and OCS terminator); *cal* — leader sequence that provides transport of the target protein to the apoplast

3. PCR analysis.

The presence of *rol*B, *rol*C, *vir*D, *ifn*-α2b, *nptII* genes in obtained "hairy" roots, leaves and roots of control plants was studied by PCR analysis. DNA extraction was carried out according to CTAB-method [28]. The presence of genes was determined in multiplex reaction using Mastercycle personal 5332 amplifier (Eppendorf). DNA amplification was carried out in a total volume of 20 μl. The reaction mixture contained 80–100 ng DNA, 1x DreamTaq reaction buffer (Thermo Scientific, proprietary composition, contains 20 mM $MgCl_2$), 0.5 U DreamTaq DNA Polymerase (Thermo Scientific), 0.2 mM deoxynucleotide triphosphates, 0.25 mM of each primers.

The conditions of amplification for all primers were as followed: primary denaturation — 94 °C, 3 min; 30 cycles of amplification (94 °C, 30 sec → 60 °C, 30 sec → 72 °C, 30 sec); final polymerization — 72 °C, 5 min. Reaction products were separated in 1.0% agarose gel with 0.005% (V/V) ethidium bromide (Sigma). O`GeneRuler 1 kb Plus DNA Ladder was used for the sizing of amplified fragments.

Primers 5′-ctc act cca gca tgg agc ca-3′ and 5′-att gtg tgg tgc cgc aag cta-3′ (592 bp — size of the amplified fragment), 5′-atg tcg caa ggc agt aag ccc a -3′ and 5′-gga gtc ttt cag cat gga gca a-3′ (432 bp), 5′-tgg agg atg tga caa gca gc-3′ i 5′-atg cct cac caa ctc acc agg-3′ (473 bp) were used to study the presence/absence of *rol*B, *vir*D, *rol*C genes in "hairy" root clones and control plants.

We used primers 5′-cct gaa tga act cca gga cga ggc a-3′ and 5′-gct cta gat cca gag tcc cgc tca gaa g-3′ for amplification of *nptII* (622 bp) and *ifn*-α2b (396 bp) gene fragment respectively.

The presence/absence of *rol*B, *vir*D, *ifn*-α2b, *nptII* genes in *A. rhizogenes* strain was studied similarly. 0.2 μl of overnight bacterial suspension was added to the reaction mixture.

4. The study of "hairy" roots growth rate.

Explants of the apical part of "hairy" roots (1cm long, each one considered as one growth point) were cultivated on 1/2 MS agar-solidified medium (Duchera Biochemie) at +25 °C during 34 days. Roots of untransformed wormwood plants were used as a control. Then we determined the weight gain from one growth point for the entire cultivation period.

5. Determination of total flavonoids and phenolic content.

Fresh plants material of two "hairy" root lines (No 3 and No 4), leaves and roots of control plants were homogenized in mortar with the appropriate amount of 70% ethanol. The ratio of the fresh plants weight (g) to the solvent volume (mL) was 1:10. Extract was centrifuged (Eppendorf Centrifuge 5415 C) at 16 000 g for 14 min. Supernatant was collected and used for analyses.

Total flavonoids content in obtained extracts was estimated by modified $AlCl_3$ method [29]. Therefore, 1 ml of deionized water and 0.075 ml of 5% $NaNO_2$ (Sigma) were added to 0.25 ml of extract. The mixture was resuspended and incubated for 5 min. Then 0.075 ml of 10% $AlCl_3$ (Sigma) was added. Next we added 0.5 ml of 1 M NaOH (Sigma) and 0.6 ml of deionized water. Optical density (OD) was measured at 510 nm on the Panorama Fluorate-2. Rutin solution in 70% ethanol was used to create the calibration plot. Total flavonoids content (C, mg/ml) was determined according to the calibration graph (C = 0.8842*OD, $R^2 = 0.9926$).

Total phenolic content was determined by photoelectric calorimetric method [30]. Therefore, 0.2 ml of ethanol plant extract was added to 1 ml of 10% Folin-Ciocalteu reagent (Sigma). This mixture was resuspended and incubated for 6 min. Then 0.8 ml of 7.5% Na_2CO_3 solution (Sigma) was added. The mixture was incubated for 2 h. The optical density was measured at 740 nm. Total phenolic content (C) was determined according to the rutin calibration graph (C = 427.41*OD, $R^2 = 0.9286$). We used rutin solution in 70% ethanol at concentrations of 0.05-1 mg/ml to make a calibration plot.

6. Determination of antioxidant activity.

Antioxidant activity of the samples was studied by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [28]. Three dilutions in 0.004% DPPH solution (Sigma) were prepared for each extract. The total volume of extract with DPPH solution was 2 ml. The optical density of the mixture was determined at 515 nm on the Panorama Fluorate-2 spectrophotometer. The percentage inhibition of DPPH extract was determined by the formula:

$$\%_{\text{inhibition}} = \frac{OD_0 - OD_1}{OD_0} \times 100\%,$$

where OD_0 — optical density of DPPH solution, OD_1 — optical density of DPPH solution after adding extract.

The graph of the percentage inhibition versus the fresh weight concentration was plotted for each sample. EC_{50} was determined from this graph. EC_{50} is the weight of fresh plant material (mg), which reduces the DPPH solution by 50%.

7. Data analysis.

All analyses were performed in triplicate. All data, except antioxidant activity results, are presented as the mean value accompanied by standard deviation. Obtained data were analyzed by one-way ANOVA to identify significant differences between the samples. Multiple comparisons were performed using the Bonferroni post-hoc test ($P < 0.05$). Correlation analysis was performed by determining the Pearson correlation coefficient. The calculations were performed using Microsoft Office Excel and OriginLab OriginPro 2021b software.

Results and Discussion

Bacteria were grown according to the above-mentioned conditions and used for transformation. Almost all explants survived after cocultivation with bacteria for a short time. Roots on leaf explants started forming in 7–14 days after transformation (Fig. 2). The frequency of transformation was 100%. The transformation efficiency was 5 roots per one explant. Twelve "hairy" root lines were subcultivated for more than a year. Two lines (No 3 and No 4) were selected for further research, which were visually characterized by the highest growth rate and had a characteristic "hairy" roots phenotype (significant branching, negative geotropism, the growth on hormone-free medium). However, the selected lines differed slightly in morphology. Thus, the "hairy" roots of the line No 3 were thinner, had a light-green color. The transgenic roots No 4 were more watered, had a light-brown color (Fig. 2).

PCR analysis of *A. rhizogenes* strain, which was used for transformation, indicated the presence of *rol*B, *vir*D, *ifn*-α2b, *nptII* genes in bacterial DNA (Fig. 3). Also, analysis showed the presence of *rol*B and *rol*C genes in both "hairy" root lines. But these genes were not detected in control leaves and roots (Fig. 4, *A*, *B*). The absence of *vir*D (Fig. 4, *C*) and the presence of *rol*B and *rol*C genes confirm that the studied samples were really transformed.

However, the unexpected absence of *ifn*-α2b and *nptII* genes of pCB124 plasmid may be the outcome of growing roots without the use of a selective medium in the absence of kanamycin. Such selection is usually used to screen transgenic samples carrying *nptII* gene, which determines the resistance of plants to specific antibiotic. Therefore, according to the obtained results, it can be concluded that selection in the presence of kanamycin is necessary to select *A. absinthium* root lines that carry not only agrobacterial genes (*rol*B and *rol*C), but also selective *nptII* gene.

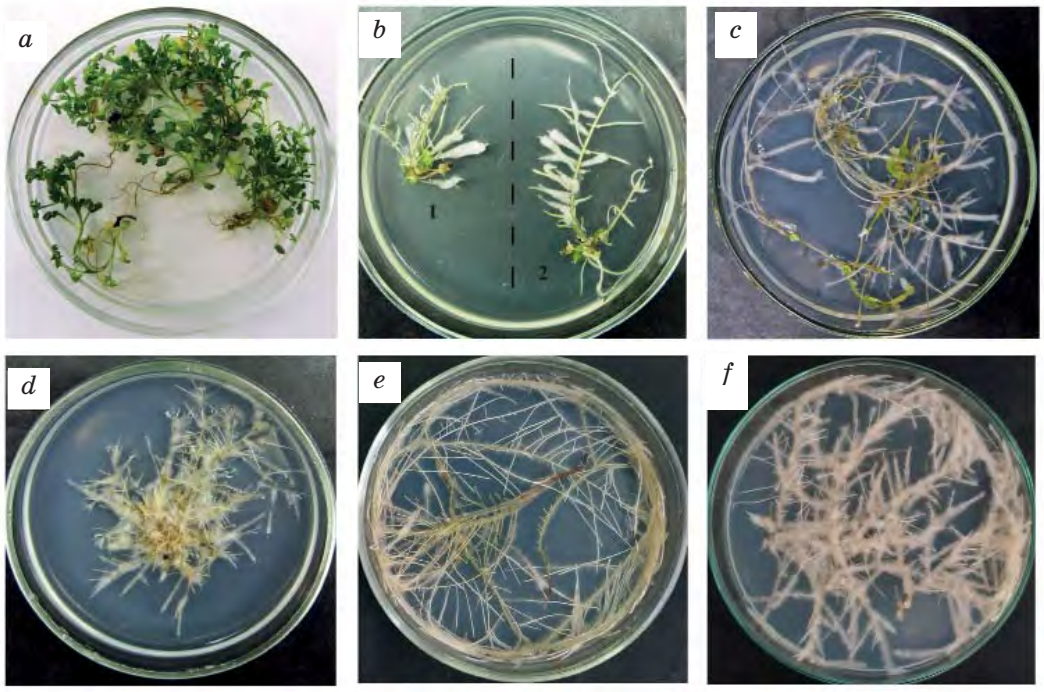

Fig. 2. A. absinthium "hairy" roots induction:
a — control plant; *b* — the beginning of root formation (1 — line No 3, 2 — line No 4); *c* — transgenic roots No 3 after 6 months of cultivation; *d* — "hairy" roots No 4 after 6 months of cultivation; *e* — roots of line No 3 after a year of cultivation, *f* — line No 4 after a year of cultivation

Fig. 3. **Electrophoregram of DNA amplification products** with gene-specific primers to *ifn*-α2b and *nptII* (*A. rhizogenes* DNA, 1); control DNA with *ifn*-α2b and *nptII* (2); *rol*B and *vir*D (4); control DNA with *rol*B and *vir*D genes (5, 6); without DNA (3, 7); M — O`GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) marker

The weight gain per one growth point of the "hairy" roots No4 was 0.39 ± 0.07 g after 34 days of cultivation. The growth rate of transgenic roots No 3 was 0.22 ± 0.06 g per one growth point (Fig. 5). The growth of isolated control roots was almost absent.

The "hairy" roots of the line No 3 accumulated 1.2 and 1.3 times more flavonoids than roots and leaves of control plants cultured *in vitro* (4.784 ± 0.10 mg/g, 3.861 ± 0.13 and 3.752 ± 0.12 mg/g FW respectively) (Fig. 6). However, the total flavonoids content in the line No4 was 1.5 times lower than in the control (2.620 ± 0.21 mg/g FW). These results indicate that *A. rhizogenes*-mediated transformation can differently influence not only the growth rate but also the flavonoids synthesis in "hairy" roots. As we can see, genetic transformation can lead to both an increase and a decrease in the total flavonoids content compared to untransformed control. Such features of the influence of genetic transformation can be explained by the fact that in the transformation of plants using agrobacteria, the incorporation of bacterial *rol* genes is indeterminated. In this case, each "hairy" root line is an independent transformational event with its own characteristics of the influence of transferred genes on the functioning of own plant genes and biosynthesis of different compounds including flavonoids.

Total phenolic content analysis showed that the highest concentrations of these compounds were in the extract of transgenic roots No 3 and No 4 (5.605 ± 0.26 and 4.385 ± 0.25 mg/g FW respectively) (Fig. 6). The quantity of phenolic compounds in plant extracts No 3 was 4.1 and 2.8 times higher than in roots and leaves extracts of control plants (1.355 ± 0.15 and 2.023 ± 0.10 mg/g FW respectively). The phenolic content in the "hairy" roots No 4 was 3.2 and 2.2 times higher than in the control roots and leaves. Comparative analysis of the two transgenic lines showed that the total phenolic content in transgenic line No3 was 1.3 times higher than in line No4. Thus, *Agrobacterium*-mediated transformation of *A. absinthium* has led to increased production of phenolic compounds in both "hairy" root lines.

Evaluation of free radical scavenging activity showed that the highest antioxidant activity was demonstrated by the extracts from the "hairy" roots No 3 (EC_{50} = 3.657mg). This activity was 1.8 and 3.6 times higher than roots and leaves extracts from control plants (EC_{50} = 6.716 and 13.226 mg respectively) (Fig. 7). The antioxidant activity of the line No4 extract (EC_{50} = 6.958 mg) was 1.9 times higher than the scavenging activity of the leaf extracts of control plants. Comparative analysis of the two transgenic lines by this parameter indicated that the free radical

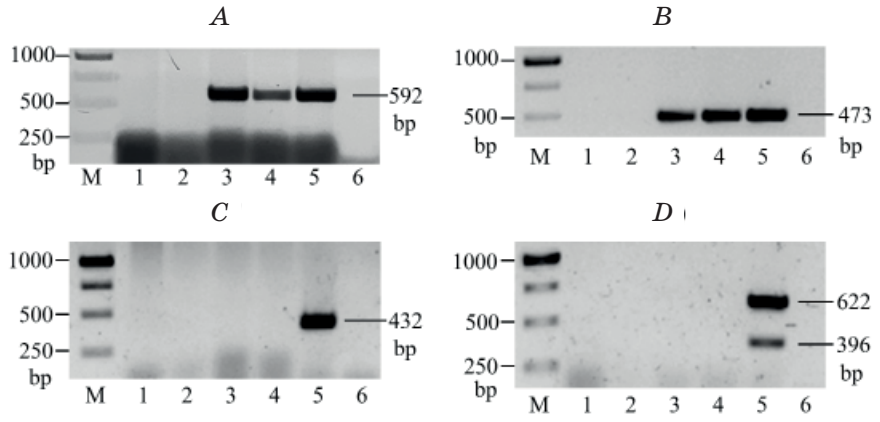

Fig. 4. **Electrophoregram of amplification products DNA of leaves (track 1), roots (track 2), No 3 (3) and No 4 (4) "hairy" roots** using specific primers to *rol*B (*A*), *rol*C (*B*), *vir*D (*C*), *ifn*-α2b and *nptII* (*D*) genes; 5 — plasmid DNA, 6 — without DNA; M — O`GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) marker

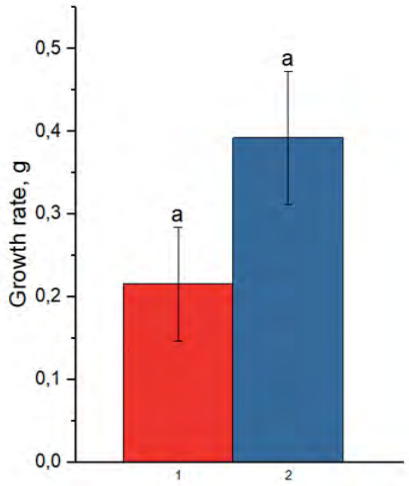

Fig. 5. **Growth of "hairy" roots No 3 (1) and No 4 (2) for 34 days**
Bars "a" letter denote absence of significant differences in growth rate between two values at $P < 0.05$

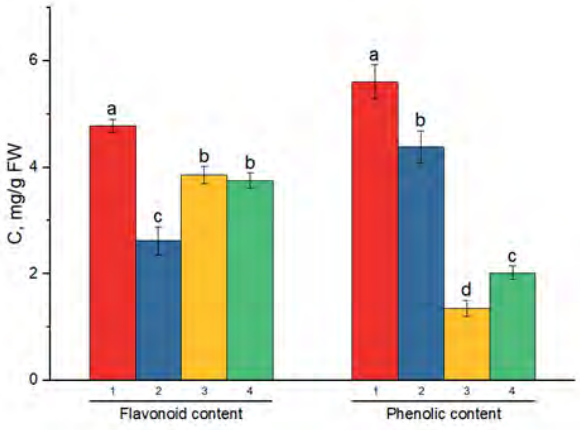

Fig. 6. **Total flavonoids and phenolic content** in ethanol extracts of transgenic roots No 3 (1) and No 4 (2), and roots (3) and leaves (4) of control plants. Bars with different small letters denote significant differences in values among four extracts at $P < 0.05$

scavenging activity of "hairy" roots No 3 extract is 1.9 times higher than the transgenic roots No 4 extract.

As it can be seen from the above-mentioned data, there is a correlation between the flavonoids content and antioxidant activity of transgenic roots No 3 extract. Thus, the extract of these "hairy" roots had the highest flavonoids concentration and the highest antioxidant activity. However, the correlation between these two parameters for other

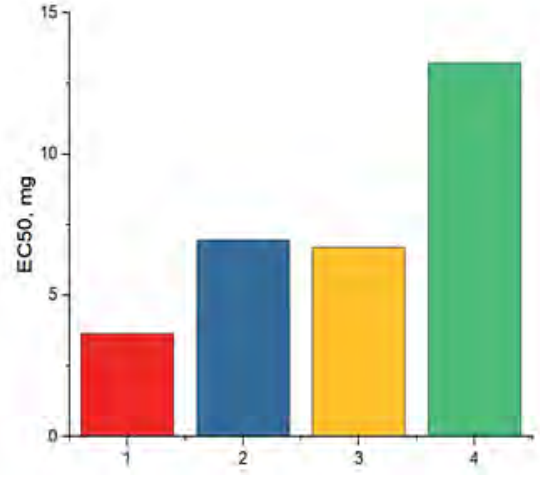

Fig. 7. **Antioxidant activity of water-ethanol extracts of "hairy" roots No 3 (1) and No 4 (2), roots (3) and leaves (4) of control plants**

samples is weak (Pearson's coefficient is 0.4). For example, although the line No4 extract had the lowest concentration of flavonoids, its free radical scavenging was not the lowest. This may be related to the accumulation of other antioxidant compounds, such as phenols. Its content in "hairy" roots No4 were higher than in the control (Fig. 6), and the correlation between this parameter and antioxidant activity was average (Pearson's coefficient was 0.63).

Thus, *Agrobacterium*-mediated transformation of *A. absinthium* plants has led to changes in "hairy" roots secondary metabolism. Particularly, it has resulted in significant increase in the phenolic content. Also, an increased flavonoids content in transgenic roots No 3 correlated with enhanced antioxidant activity. Similar influence of genetic transformation on the biosynthetic activity of "hairy" roots cells has been described in plants of other species. For example, the total flavonoids and phenolic content in the *Althaea officinalis* L. transgenic roots was higher than in control plants [36]. *Cucumis anguria* L. "hairy" roots had a higher concentration of phenolic compounds and flavonoids as well as higher antioxidant activity compared to untransformed plants [37].

Such changes may be caused by transferring bacterial *rol*-genes to the plant genome and its activity. In particular, the results of a study of *Agrobacterium*-mediated transformation of *Lactuca serriola* L. roots indicate that the *rol*B genes integration into the plant genome increased the expression of *CHI, PAL, FLS* and *CHS* genes, which are responsible for

flavonoid biosynthesis. According to the authors, these changes in plant genome led to increase in phenolic and flavonoids content in "hairy" roots extract and increased antioxidant activity compared to the control [35].

Conclusions

This research is the first study of the effect of *Agrobacterium rhizogenes*-mediated transformation on flavonoids and phenolic compounds synthesis and antioxidant activity of *A. absinthium* "hairy" roots. It showed that wormwood transformation using *A. rhizogenes* agropin strain A4 was possible. The transformation frequency was 100%. The transfer of bacterial *rol*B and *rol*C genes has been confirmed. However, the expected transfer of neomycin phosphotransferase II and human interferon-α2b genes did not occur. Probably, it happened due to the lack of a selection step on kanamycin medium.

The analyzed root lines differed in growth rate, flavonoids and phenolic content, and antioxidant activity. Moreover, the high flavonoids content (4.784 ± 0.10 mg/g FW) in transgenic roots No 3 correlated with increased antioxidant activity rate (EC_{50} = 3.657mg compared to 6.716 and 13.226 mg for control roots and leaves). Therefore, the obtained results indicate that the *A. absinthium* transformation using *A. rhizogenes* can be applied to obtain lines with a high phenolic content and a high antioxidant activity.

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Agrobacterium rhizogenes — ОПОСЕРЕДКОВАНА ТРАНСФОРМАЦІЯ ЯК СПОСІБ СТИМУЛЮВАННЯ СИНТЕЗУ АНТИОКСИДАНТНИХ СПОЛУК У *Artemisia absinthium* L.

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Рослини *Artemisia absinthium* L. відомі як продуценти речовин з антиоксидантними властивостями. Зокрема, в них виявлено поліфеноли та флавоноїди. Активізувати синтез цих сполук можна шляхом генетичної трансформації навіть без перенесення специфічних генів, які беруть участь у біосинтезі. Так, «бородаті» корені, одержані після *Agrobacterium rhizogenes*-опосередкованої трансформації, можуть бути продуцентами комплексу цінних метаболітів.

Метою роботи було одержати «бородаті» корені *A. absinthium* як продуценти поліфенольних сполук.

Методи. «Бородаті» корені одержували шляхом культивування листків з суспензією *A. rhizogenes* з вектором *pCB124*. Наявність перенесених генів підтверджували методом ПЛР. Для визначення вмісту флавоноїдів та поліфенолів використовували реакції з $AlCl_3$ та реактивом Фоліна-Чокальте. Антиоксидантну активність оцінювали за здатністю екстрактів відновлювати DPPH радикал.

Результати. ПЛР аналіз виявив наявність бактеріальних *rol* генів та відсутність генів плазміди pCB124. Лінії коренів відрізнялися між собою за швидкістю росту. «Бородаті» корені характеризувалися більшим вмістом поліфенолів, зокрема, флавоноїдів (до 4.784±0.10 мг/г ВМ) та вищим рівнем антиоксидантної активності (EC_{50} = 3.657 мг) у порівнянні з контролем (3.861±0,13 мг/г СМ та EC_{50} = 6.716 мг відповідно).

Висновки. Трансформацію *A. absinthium* із застосуванням *A. rhizogenes* може бути використано для одержання ліній з підвищеним вмістом поліфенольних сполук та більшою антиоксидантною активністю.

Ключові слова: Artemisia absinthium L., *Agrobacterium rhizogenes*-опосередкована трансформація, «бородаті» корені, флавоноїди, поліфенольні сполуки, антиоксидантна активність.