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Alterations in the Antioxidant Status of Transgenic Roots of *Artemisia* spp. Representatives after *A. rhizogenes*-Mediated Genetic Transformation

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Abstract—The effect of *A. rhizogenes*-mediated genetic transformation on the antioxidant status of *Artemisia tilesii*, *A. vulgaris*, *A. dracunculus*, and *A. annua* transgenic roots has been studied. Antioxidant activity (AOA) of aqueous extracts was determined using methods based on the ability to reduce $DPPH^+$ and $ABTS^+$-radicals. The level of AOA (DPPH) in 50% of extracts obtained from transgenic roots was higher than the level of activity possessed by extracts from untransformed roots. An increased ability to reduce the $ABTS^+$ radical was observed in 80% of the extracts. Extracts of *A. annua* and *A. tilesii* transgenic roots were the most active, while the lowest antioxidant activity was shown in *A. dracunculus* extracts. Thus, *A. rhizogenes*-mediated transformation has led to a change in the antioxidant status of the "hairy" roots of several *Artemisia* spp. plants (except *A. vulgaris*). It can be used as a method for the enhancement of the natural antiradical properties of plants belonging to the *Artemisia* genus.

Keywords: Artemisia, "hairy" root cultures, $DPPH^+$, $ABTS^+$, antioxidant activity
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INTRODUCTION

One of the most important mechanisms of plant homeostasis is the activation of the antioxidant defense system under the influence of stress factors. The biological role of this system is to protect membranes and genetic material from reactive oxygen species (ROS), free radicals (superoxide anions (O^{2-}), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2). [1] The intensive formation of the latter generates a cascade of unfavorable oxidative reactions, and an excessive amount of these reactions is destructive to plant cells.

The formation and accumulation of ROS accompanies any type of stress (abiotic, biotic). Several mechanisms exist, including enzymatic (increased activity of antioxidant enzymes—superoxide dismutase, peroxidase, catalase, etc.) and nonenzymatic (accumulation of low-molecular weight antioxidants, such as glutathione, ascorbate, proline, carotenoids, tocopherol, etc.) in the plant cell for protection against the negative effects of oxidizing agents [2]. Compounds involved in antioxidant protection are electron donors, stopping oxidative processes by the reduction of free radicals.

Genetic transformation is one of the most popular tools of plant biotechnology. It also may be a stress factor for plants. The genetic transformation method, in particular *Agrobacterium*-mediated, consists of using a soil bacterium as a vector for transferring genes of interest into the genome of recipient plants, allowing the creation of transgenic plants with new properties [3]. In turn, the changes in the morphological, physiological, and biochemical parameters of plants are the result of such gene transfer [4, 5]. For example, after genetic transformation, changes in the growth rate, the protein content, the content of secondary metabolites [6–8], the activity of the antioxidant defense system [9], etc. can occur. The latter can be due to the reaction of the plant to the stress factor.

During the process of *Agrobacterium*-mediated transformation there are damages of membranes, contact with a pathogenic microorganism, the insertion of T-DNA into the genome. These stages as well as in vitro cultivation stage can have a stressful effect on plants [10]. Thus, genetic transformation can induce changes in the antioxidant protection system of plants. Therefore, the study of the effect of *Agrobacterium*-mediated transformation on the antioxidant status of plants obtained by transformation, which carry transferred genes, as well as the study the possibility of creating plant-producers of antioxidant compounds by genetic transformation, is important. Such plants are of interest for the pharmaceutical industry, since plant

antioxidants are also active in human cells, protecting them from oxidative stress. Medicinal plants can serve as raw materials for achieving this goal. Plants of *Artemisia* genus include many species known as therapeutic agents. In particular, *Artemisia* spp. plants exhibit antimicrobial [11], antiviral [12], and antispasmodic activity [13]. Thus, plants of the *Artemisia* genus, synthesizing compounds under natural conditions, are used for the treatment and prevention of a number of diseases, and their use as objects of genetic transformation allows obtaining a complex of biologically active compounds.

Transgenic ("hairy") root culture can be obtained by *Agrobacterium rhizogenes*-mediated transformation. Such roots are capable to isolate growth on a hormone-free medium and are a promising source of valuable biologically active compounds [14]. The culture of transgenic roots has a number of advantages over the suspension culture: higher genetic stability; the possibility of cultivation under controlled conditions of bioreactors with a relatively small amount of initial plant material; economic profitability (no need to use additional growth regulators, simple cultivation, large biomass yield, possibility of cultivation without light, additional heating, use of expensive compounds in nutrient medium) [15, 16]. In addition, the cultivation of "hairy" roots in bioreactors is environmentally friendly and does not contradict the rules of biosafety. The listed features of the "hairy" root culture make it a potential system for the production of a number of compounds, in particular, exhibiting antioxidant properties, especially if such roots are capable of synthesizing a complex of biologically active compounds.

One of the most common methods for studying antioxidant activity are methods based on the reduction of free radicals, including $DPPH^+$ [17] and $ABTS^+$ [18] by antioxidant compounds. The principle of the methods using $DPPH^+$ and $ABTS^+$ radicals is based on the measurement of the color intensity of the corresponding radicals' alcohol solution before and after adding the plant extract. In the presence of antioxidant (radical-absorbing) properties of the extract, the radical is reduced, which leads to a discoloration of the solution. These methods allow spectrophotometric determination of the degree of antiradical activity of plant extracts containing both water- and fat-soluble antioxidants [19].

The investigation of the effect of genetic transformation on the antioxidant status of plants *Artemisia tilesii*, *A. ludoviciana*, *A. dracunculus*, *A. annua*, and *A. absinthium* by determining the antiradical activity of extracts from untransformed and transgenic roots of these species was the goal of this study.

MATERIALS AND METHODS

The culture of the transgenic roots was obtained earlier [20–22] by *A. rhizogenes*-mediated transformation by pCB124 [23] and pCB1b1 [24] vectors carrying the human interferon-α2b gene (*ifn-a2b*) and neomycin phosphotransferase II (*nptII*) gene as well as by using of wild *A. rhizogenes* A4 strain. Roots were cultured in vitro for 30 d at a temperature of 24 ± 2°C on Murashige and Skoog nutrient medium [25] with twice reduced content of macrosalts.

For preparation of the extracts, the plant material was weighed, homogenized with distilled water (100 mg of dry root weight/4 mL of water), and centrifuged at 10000 *g* for 10 min. The resulting supernatant was used to determine the antioxidant activity.

Determination of Antioxidant Activity of Extracts in Reaction with $DPPH^+$ Radical

For determination of the antioxidant activity, $DPPH^+$ solution in 96% ethyl alcohol (concentration of the radical was 10^{-4} M) was used. The reaction was carried out in 96-well microtiter plates according to a conventional procedure [17].

The reaction mixture was kept at 24°C in the dark for 30 min. After this procedure the optical density of the mixture and the intrinsic optical density of the extract solutions of the corresponding concentrations in alcohol (without the addition of $DPPH^+$) were measured. The aqueous solution of ascorbic acid with a concentration of 1 mg/mL was used as a positive control. The absorption of the free radical (antioxidant activity, AOA) was expressed quantitatively as the percent inhibition and was calculated according to the formula

$$AOA_{(DPPH)} = \left[(A_k - A_e)/A_k\right] \times 100,$$

where A_k is the optical density of the DPPH solution and A_e is the optical density of the studied extract solution with DPPH solution.

The optical density of the samples was measured using an Eppendorf BioPhotometer Plus spectrophotometer at a wavelength of 550 nm.

The experiment was carried out in triplicate. For statistical analysis of data, standard functions of the software environment for the programming language R version 3.3.2 [26] were used. The logistic transformation was used for the data expressed as percentage [27].

Determination of Antioxidant Activity of Extracts in Reaction with ABTS Cation-Radicals

A solution of $ABTS^+$ radicals for the determination of the antioxidant activity was obtained by mixing 5 mL of 7×10^{-3} M aqueous ABTS solution and 88 μL of 140×10^{-3} M $K_2S_2O_8$ aqueous solution. After a 16-h exposure in the dark, the solution of the cation radical was dispersed in water [28].

A water-alcohol solution with a volume fraction of ethanol of 70% was used to extract the samples. A double extraction of the dry homogenized samples was

carried out with a raw material : extractant ratio of 1 : 40, followed by filtration and combining the extracts [29]. For the determination of the antioxidant activity, 10–200 μL of the solution of the studied extract was added to 2.0 mL of ABTS cation radical solution in a glass cuvette. The optical density of the samples was measured on an Agilent 8453 spectrophotometer after 60 and 280 s, and also after mixing at a wavelength of 734 nm. The optical density was measured at a temperature of 25°C and constant stirring. The activity of extracts and fractions in the reaction with the $ABTS^+$ radicals was determined against water-alcohol Trolox solution.

A comparative evaluation of the antioxidant activity was carried out based on the antiradical parameter (AP), which was calculated as the slope of the direct dependences $D_0 - D$ from the concentration of the extract and antiradical activity (ARA). ARA is the value showing the number of moles of a standard antioxidant Trolox possessing the same effect as 1 mL of the extract

$$ARA_{(ABTS)} = AP/AP_{trolox},$$

where AP is the antiradical parameter (slope of the direct dependences $D_0 - D$ from the concentration, mL/L); AP_{trolox} is the slope of the direct dependences $D_0 - D$ from Troloxconcentration, M/L; D_0 is the optical density of $ABTS^+$radical solution at the initial moment of time; D is the optical density of the $ABTS^+$ solution at time t, s.

RESULTS AND DISCUSSION

A comparison of antioxidant (antiradical) activity of extracts from untransformed and transgenic roots of *Artemisia tilesii*, *A. vulgaris*, *A. dracunculus*, and *A. annua* showed that genetic transformation led to a change in the antioxidant status of transgenic roots. Extracts obtained from "hairy" roots of various *Artemisia* species were different by ability to reduce $DPPH^+$ and $ABTS^+$ radicals.

Antiradical ($DPPH^+$) Activity of Extracts from Untransformed and Transgenic Roots of Genus Artemisia Representatives

The level of $AOA_{(DPPH)}$ in the extracts obtained from the transformed roots of various species varied from 40 ± 6.4 to 73 ± 4.7%. The lowest activity was exhibited by extracts obtained from the untransformed roots of *A. annua*, the highest activity was detected in extracts of *A. tilesii* and *A. dracunculus* control roots. Extracts from untransformed *A. vulgaris* roots exhibited activity: up to 66 ± 2%.

In extracts obtained from transgenic roots, the ability to reduce $DPPH^+$ radical varied within a wide range: from 22 ± 4.2 to 93 ± 5% (Fig. 1). The lowest antiradical activity was found in extracts obtained from two transgenic *A. dracunculus* root lines, while the highest antiradical activity was found in some extracts of *A. annua* and *A. tilesii* root lines. In 50% of the extracts obtained from transgenic lines, the activity level was higher than the activity of the extracts from the control untransformed roots of the corresponding species.

Anti-radical properties of extracts from *A. tilesii* transgenic roots in 75% of samples were higher than in extracts obtained from control roots. For 25% of transgenic root lines of this species, no significant difference between activity of transgenic and control roots was found. The maximal level of anti-radical activity of extracts from one of the transgenic lines exceeded that in extracts from untransformed roots by 1.3 times (93% and 73%, respectively).

The level of activity of extracts obtained from transgenic roots of all *A. vulgaris* lines was not statistically different from $AOA_{(DPPH)}$ of extracts from untransformed roots and ranged from 55 ± 1.6 to 70 ± 3.7%, whereas it was 66 ± 1.9% in the control. Thus, *A. rhizogenes*-mediated transformation did not lead to significant changes in the antioxidant activity of *A. vulgaris*. Data obtained previously are in agreement with these results [30].

The experiments showed that the extracts obtained from 50% of the studied transgenic *A. dracunculus* lines were not statistically different by the level of antiradical activity from those derived from untransformed roots of this species. In the extracts of the two *A. dracunculus* lines (nos. 1/2 and 1/4), the ability to reduce the $DPPH^+$ radical was significantly lower than in the untransformed roots (1.8 and 3.1 times, respectively).

Genetic transformation led to an increase in the level of anti-radical activity of extracts in 100% of *A. annua* lines. Thus, the extract of the transgenic line no. 1 obtained with the wild strain A4 showed activity 2.4 times higher than the extract of the untransformed roots of this species and was 93.2 ± 5% (39.4 ± 6.4% in the extract of untransformed *A. annua* roots).

Antioxidant Activity (ABTS) of Extracts from Untransformed and Transgenic Roots of Representatives of the Genus Artemisia

In the model system with ABTS cation radicals, the extracts of the transformed *A. annua* roots exhibited the highest activity (Fig. 2).

A high level of antiradical activity of the transformed *A. annua* roots was detected. The antiradical activity of samples A4 no. 1 and A4 no. 2 were the highest among all studied samples.

For *Artemisia tilesii*, the highest activity was found in the extract of transformed roots 124 no. 1. The antiradical activity of the extract of this line exceeded the antiradical activity of the control roots by almost 1.5 times, but it did not reach the activity level detected in *A. annua* samples.

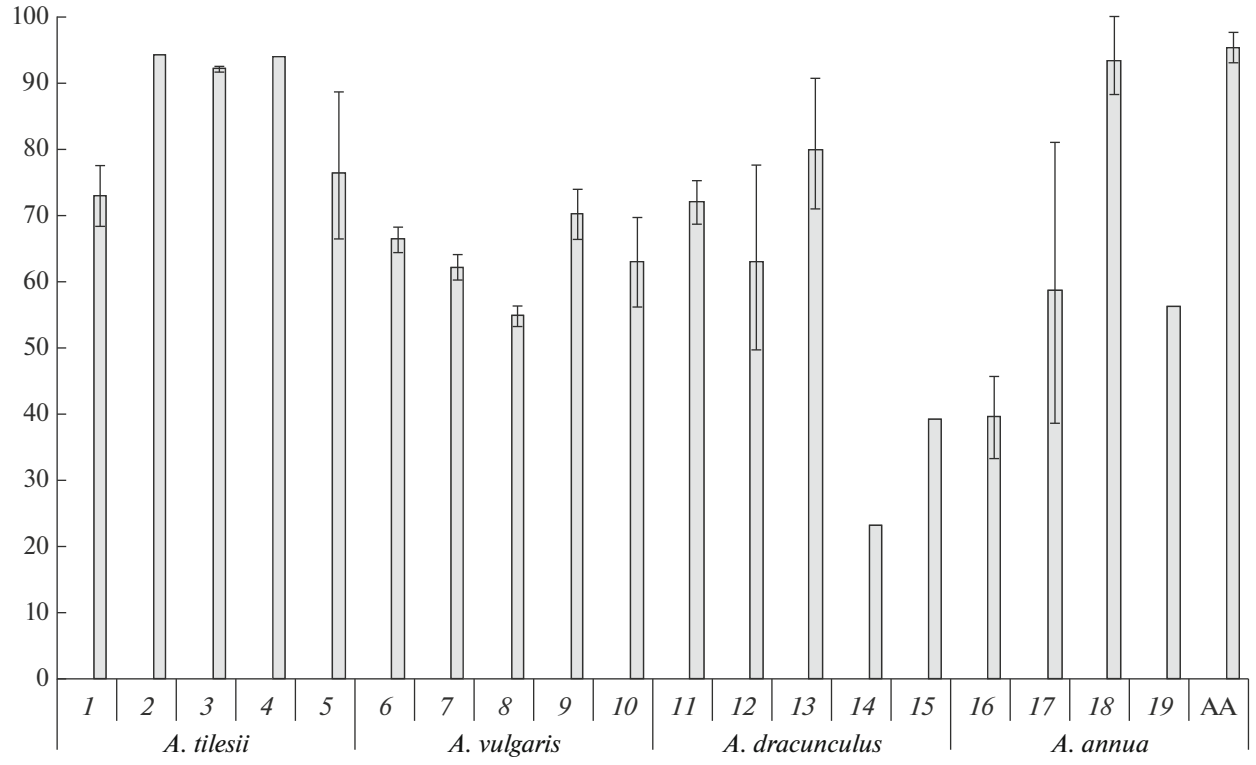

Fig. 1. Antioxidant activity of extracts (vertical is AOA_{DPPH}, %), obtained from the *Artemisia* roots: (*1*) extract from control *A. tilesii* roots; (*2–5*) extracts from transgenic *A. tilesii* roots (pCB124); (*6*) extract from control *A. vulgaris* roots; (*7*, *8*) extracts from *A. vulgaris* "hairy" roots (pCB124); (*9*, *10*) extracts from *A. vulgaris* "hairy" roots (A4); (*11*) extract from control *A. dracunculus* roots; (*12–15*) extracts from *A. dracunculus* "hairy" roots (A4); (*16*) extract from control *A. annua* roots; (*17*, *18*) extracts from *A. annua* "hairy" roots (A4); (*19*) extracts from *A. annua* "hairy" roots (pCB124); (AA) ascorbic acid.

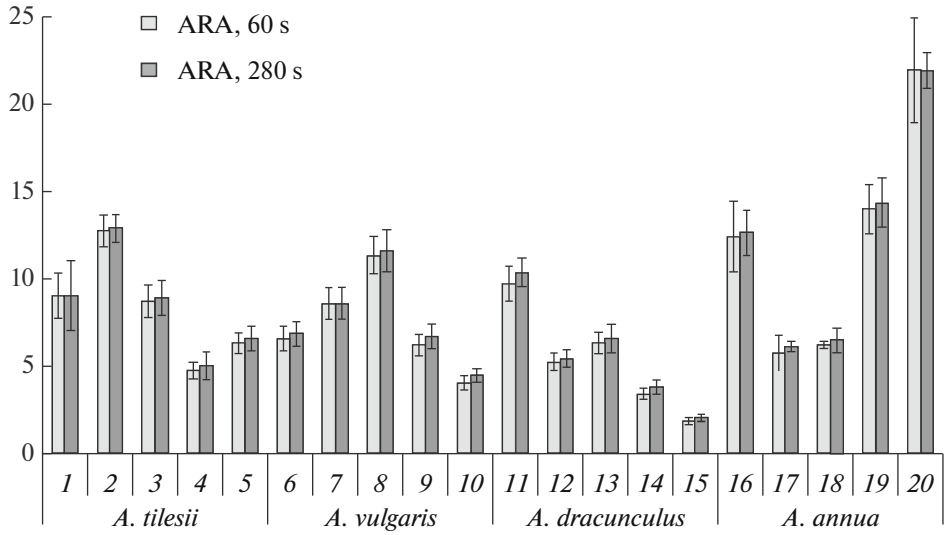

Fig. 2. Antioxidant activity of extracts (vertical is ABTS, μM of Trolox per 1 mL of extract) obtained from *Artemisia* roots: (*1*) extract of untransformed *A. tilesii* roots; (*2*, *3*) extracts from *A. tilesii* "hairy" roots (pCB124); (*4*) extract from untransformed *A. vulgaris* roots; (*5–7*) extracts from *A. vulgaris* "hairy" roots (pCB1b1); (*8*, *9*) extracts from *A. vulgaris* "hairy" roots (pCB124); (*10*, *11*) extracts from *A. vulgaris* "hairy" roots (A4); (*12*) extract from untransformed *A. dracunculus* roots; (*13–15*) extracts from *A. dracunculus* "hairy" roots (A4); (*16*) extract from untransformed *A. annua* roots; (*17*, *18*) extracts from *A. annua* "hairy" roots (pCB124); (*19*, *20*) extracts from *A. annua* "hairy" roots (A4).

In *A. vulgaris*, the antiradical activity of the leaves and roots was not high, whereas the genetically transformed roots of these plants showed a much significant effect in the model system with the $ABTS^+$ cation radicals. This phenomenon was observed for individual lines transformed with strain A4 as well as for lines additionally modified with the pCB124 vector.

A. dracunculus appeared to be the least active in this model system, and although an increased activity was observed in the extracts of the line transformed with strain A4, it did not exceed 20–21% of the control values.

The values of antiradical activity calculated for wormwood extracts after 60 and 280 s from the beginning of the reaction did not significantly differ, indicating the presence of mainly strong antiradical agents reacting with the cation radicals within the first minute after addition of the extract in the model system.

Thus, the differences in antioxidant activity of extracts of untransformed and transgenic roots of plants of the genus *Artemisia* in the model system with $ABTS^+$ are shown. In some cases, as a result of genetic transformation, antiradical activity increased two times or more, which may indicate a change in the biosynthesis of secondary metabolites in "hairy" roots and also the prospects of these cultures for further use as a convenient source for the production of biologically active substances with antiradical activity.

It should be noted that the level of antioxidant activity did not depend on whether only bacterial genes (*rol*, transformation with wild *A. rhizogenes* A4 strain) or *ifn*-α2*b* and *nptII* (transformation of *A. rhizogenes* A4 with PCB124, pCB161 vectors) genes nonspecific for agrobacteria were transferred as the result of transformation. For example, the AOA of extracts of two "hairy" root lines obtained by transformation with a wild agrobacteria strain (Fig. 1, *13* and *14*), differed more than threefold. At the same time, there were no significant differences in AOA of extracts obtained from roots transformed with wild strain (for example, Fig. 1, *17*) and agrobacteria with vector pCB124 (Fig. 1, *19*). This indicates that changes in antioxidant activity in this case are most likely not a specific response to the transfer of a specific gene but a consequence of the uncontrolled (not determined by flanking sequencing) incorporation of alien genes and their influence on the functioning of a plant's own genes.

The influence of genetic transformation using soil bacteria of *Agrobacterium* genus on the cellular metabolism of plants was investigated in a number of studies. Thus, in 2016, Dilshad et al. [9] showed that *rol* genes of agrobacteria transferred by *A. tumefaciens* can lead to changes in the accumulation of antioxidant compounds in *A. carvifolia* plants. Shkryl et al. [31], using *Rubia cordifolia*, demonstrated that *rol* genes are potent inducers of metabolism and can lead in many cases to the accumulation of secondary metabolites. An increase in antioxidant activity by 31–50% was also shown for the transgenic *Lactuca serriola* roots [32]. Our studies revealed a significant variability in the antioxidant activity of extracts from the "hairy" root cultures of four species of the *Artemisia genus*. This variability did not correlate with usage of wild A4 strain or agrobacteria, carrying vectors with interferon α2B and neomycin phosphotransferase II. The changes in antioxidant activity of extracts were probably not related to the presence of these genes and they were determined by the transfer of *rol* genes of agrobacteria, which were detected in all lines of "hairy" roots without exception. This conclusion is consistent, in particular, with the results of the studies presented by Dilshad et al. [9], demonstrating that the transfer of *rol* genes leads to an increase in the content of phenolic compounds, flavonoids, and antioxidant activity of *Artemisia dubia*. The authors also demonstrated the effect of the transferred genes on the synthesis of secondary metabolites with antioxidant properties in *A. carvifolia*.

Untransformed plants of the genus *Atemisia* have already been considered as potential sources of compounds with antioxidant properties in a number of studies [33–35]. The possibility of using *A. vulgaris* extracts for the increase in the activity of antioxidant defense enzymes in rats was also demonstrated [33]. It can be concluded that representatives of the genus *Artemisia* can be a potential source of pharmacological compounds.

CONCLUSIONS

A. rhizogenes-mediated transformation has led to a change in the antioxidant activity of the extracts from "hairy" root cultures of *A. tilesii*, *A. vulgaris*, *A. dracunculus*, and *A. annua*. The highest activity was detected in extracts of the transgenic roots of *A. annua* and *A. tilesii*; the lowest activity was shown in *A. dracunculus* extracts. Genetic transformation can be used as a method enhancing the natural antiradical properties of the plants of the *Artemisia* genus, since the level of $DPPH^+$ activity was higher in 50% of the extracts obtained from transgenic lines in comparison with the activity of extracts from untransformed roots and, an increased ability to reduce $ABTS^+$ radical was observed in 80% of extracts.

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