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Study of Superoxide Dismutase Activity in Long-Term Cultivated *Artemisia* and *Althaea* "hairy" Roots

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Abstract

The aim of the study was to evaluate the long-term effect of *Agrobacterium rhizogenes* genes transfer on plant antioxidant system by the study of superoxide dismutase (SOD) activity in "hairy" roots of *Artemisia* and *Althaea* spp plants. PCR analyses revealed stability of the transformation and presence of bacterial *rol* B and *rol* C genes in the "hairy" roots after 4–6 years from the transformation event. SOD activity in the roots of untransformed in vitro cultivated plants used for the initiation of "hairy" roots growth was in the range of 45.8 ± 8.7 U/μg (*Althaea officinalis*) and 275 ± 97.1 U/μg (*Artemisia ludoviciana*). After a long-term in vitro cultivation more than half of tested "hairy" root lines (54%) showed a significant increase in SOD activity values compared to untransformed roots. The highest SOD activity values of "hairy" root lines (24-fold increase) were founded in *A. officinalis* (1105 ± 174 U/μg) and *A. dracunculus* (1356 ± 402 U/μg). The increase of the activity was found also in "hairy" roots of *A. vulgaris* (up to 375 ± 28.2 U/μg, sevenfold increase), *A. ludoviciana* (1001 ± 191 U/μg, 3.6-fold increase), and *A. tilesii* (438 ± 104 U/μg, 1.6-fold increase). The results of our study indicate that transformation by wild-type *A. rhizogenes* not harboring any foreign genes implementing in SOD activity regulation can often stably activate plant antioxidant enzyme system. This effect, observed in the "hairy" roots of five plant species in 4–6 years of the transformation event, obviously, should be taken into account in works aimed at creating transgenic plants by *Agrobacterium*-mediated transformation.

Introduction

The majority of environmental stress factors of various origins such as high salinity, drought, extreme temperatures, pests, bacterial or viral infections, and soil pollution typically result in oxidative stress of plant cells. It includes formation and rapid accumulation of reactive oxygen species (ROS), free radicals such as superoxide anions (O^{2-}), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) [1], that can significantly reduce crop productivity. As a natural mechanism to maintain cell homeostasis under oxidative stress, plants activate the antioxidant defense system. There are several types of responses such as the production of antioxidant enzymes, different secondary metabolites including low molecular weight antioxidants. Enhancing the activity of antioxidant enzymes: superoxide dismutase (SOD),

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peroxidase, catalase, peroxiredoxins, glutathione peroxidase provides ROS scavenging [2, 3], while low molecular weight antioxidants, such as glutathione, ascorbate, proline, carotenoids, tocopherol, also protect cell components against the negative effects of oxidizing agents [4]. Some secondary metabolites (terpenes, polyphenols, and alkaloids) stabilize cell structures under oxidative stress and provide non-enzymatic ROS scavenging [5]. However, it is considered that SOD plays a crucial role in ROS homeostasis. Numerous studies report that the expression of SOD-coding genes in non-transgenic plants results in the reduction of oxidative stress [6].

Genetic manipulations are successfully implemented to transfer foreign genes of mostly plant origin targeted to enhance SOD activity and create stress-tolerant transgenic varieties [6]. *Agrobacterium*-mediated transformation is one of the common approaches to create transgenic plants. It is based on the use of the natural capacity of soil phytopathogenic *Agrobacterium* spp bacteria to transfer foreign genes into the plant genome. Studies report that natural agrobacterial infection, as well as agrobacterial-based vectors harboring different foreign genes, may affect plant antioxidant defense system [7, 8]. Nevertheless, the effects of *Agrobacterium*-mediated transformation itself, as well as the long-term effect of a "bacteria attack" in the aspect of the interaction of bacteria and plants, are not in focus in such articles. At the same time, the insertion of T-DNA genes into the plant genome during the transformation process without insertion of other specific target genes might cause changes in plant metabolism in general and in the antioxidant defense system in particular. According to the data [9, 10] *A. rhizogenes* infection resulted in ROS levels decrease. The study of the activity of ascorbate peroxidase, catalase and Cu/Zn superoxide dismutase genes using real-time PCR demonstrated enhanced expression of the genes. Authors discussed the role of bacterial *rol* B gene in the activity of genes coding synthesis of ROS-detoxifying enzymes.

Considering the economic importance of transgenic plants, it is essential to understand the impact of genetic transformation itself, find out how transformed plants differ from wild-type. It is important also to study the duration and stability of potential metabolic changes initiated by the transformation. Additionally, recent studies report that the insertion of agrobacterial genes into the host plant is a widely occurred natural phenomenon. Agrobacterial genes become a permanent component of the plant genome, stably expressed in generations [11, 12]. This allows hypothesizing that transformation by wild-type *Agrobacterium* spp. as a biotechnology tool also can produce transgenic lines possessing new useful properties including higher tolerance to oxidative stress induced by different factors.

Detection of *rol* genes in the plant genome, which are the essential components of *Agrobacterium* T-DNA, is the basic criterion of stable *Agrobacterium*-mediated transformation. Therefore, the aim of this study was to assess a long-term effect of *A. rhizogenes*-mediated transformation and bacterial *rol* genes transfer on SOD activity in "hairy" root lines of *Artemisia* and *Althaea* species after their in vitro cultivation during 4–6 years. We used plants indigenous to different regions: *Artemisia vulgaris* (Asia, Northern Africa, and North America, Europe), *A. dracunculus* and *A. ludoviciana* (Asia, central Europe, North America), *Althaea officinalis* (Europe, North Africa, and Asia), and also cold-resistant plants *A. tilesii* (Alaska, Northern territory of Canada and Russia).

Materials and Methods

Plant Material

"Hairy" root cultures of *Artemisia vulgaris, A. ludoviciana, A. tilesii, A. dracunculus, Althaea officinalis* were obtained by *Agrobacterium rhizogenes*-mediated transformation (A4 wild strain) in 2013–2015 [13–15]. The roots and mother plants were cultivated in in vitro conditions on the solidified half-strength Murasige and Skoog basal medium (Duchefa Biochemie) at 24 °C in standardized conditions without any stress factors (Fig. 1). These materials were used for the study of the activity of the superoxide dismutase enzyme and also for the PCR analyses of the presence of bacterial *rol* genes after a long time in vitro subcultivation of "hairy" root clones.

PCR Analysis

DNA extraction was carried out according to CTAB-method. The presence of *rol B* and *rol C* genes was determined in multiplex reaction using Mastercycle personal 5332 amplifier (Eppendorf) by PCR analysis. DNA amplification was carried out in a total volume of 20 µl. The reaction mixture contained 80–100 ng DNA, 1 × DreamTaq reaction buffer (Thermo Scientific), contains 2 mM $MgCl_2$), 0.5 U DreamTaq DNA Polymerase (Thermo Scientific), 0.2 mM deoxynucleotide triphosphates, 0.25 µM of each primers.

The conditions of amplification were as followed: primary denaturation – 94 °C, 3 min, 30 cycles of amplification (94 °C, 30 s – 60 °C, 30 s –72 °C, 30 s), final polymerization –72 °C, 5 min. Products of reaction were separated in 1.0% agarose gel. O`GeneRuler 1 kb Plus DNA Ladder #1163 (Thermo Scientific) was used for the sizing of *rol* B, *rol* C, and *vir* D genes. Primers (our design, Table 1) were used to confirm the presence of *rol B* and *rol C* gene and to study the presence of *vir* D gene in "hairy" root clones.

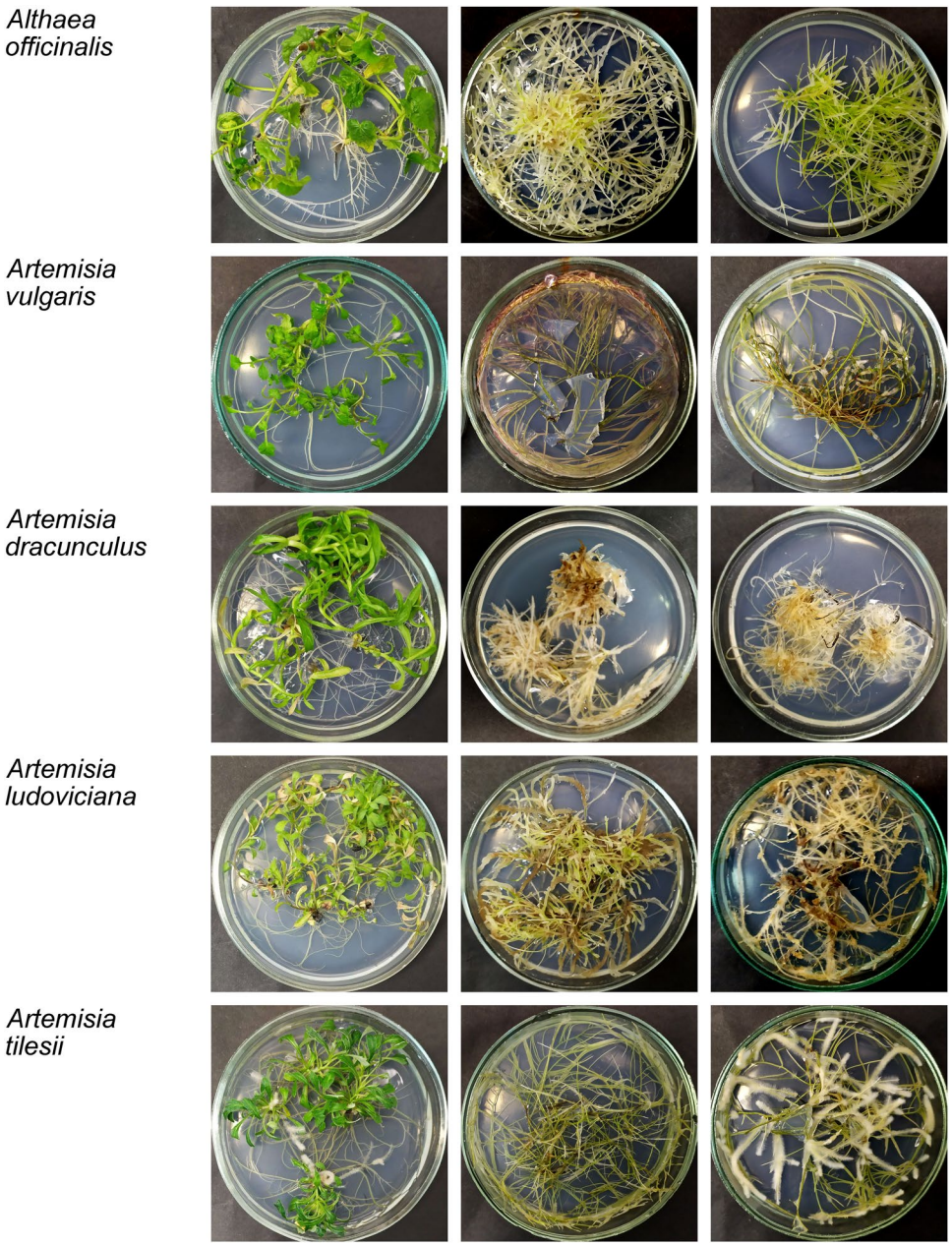

Fig. 1 Morphology of in vitro cultivated untransformed control plants (left column) and "hairy" root lines (center and right columns) used in the experiments

Table 1 Primer sequences used for PCR amplification

Gene name	Primer sequences, 5'-3'	Size of the amplified fragment, bp
rol B	Forward: ctcactccagcatggagcca Reverse: attgtgtggtgccgcaagcta	592
rol C	Forward: tggaggatgtgacaagcagc Reverse: atgcctcaccaactcaccagg	473
vir D	Forward: atgtcgcaaggcagtaagccca Reverse: ggagtctttcagcatggagcaa	432

Determination of the Activity of the Superoxide Dismutase (SOD) Enzyme

The activity of the superoxide dismutase was studied using nitro blue tetrazolium chloride [16]. Root material (100 mg) was placed in an Eppendorf tube (1.5) and triturated with 1 ml 50 mM Tris–HCl buffer (pH = 8.0), and then centrifuged at 13,000 g (4 °C) for 15 min. The supernatant was used for analysis. The reaction was carried out in Eppendorf tubes (1.5 ml). The reaction mixture consisted of 10 µl of plant extract, 540 µl of 50 µM Tris–HCl buffer, 130 µl of 65 mM methionine, 47 µl of 630 µM of nitro blue tetrazolium chloride, 12.5 µl of 1 mM of riboflavin. One tube for

each specimen was left in the dark, another was held under the influence of a light white lamp (fluorescent lamp T5 / G5 model ELI—230A—T5-8 W) for 5 min in a thermostat at 26 °C. Adsorption of the combined reaction mixture against the unleaded reaction mixture was measured at 550 nm using BioPhotometer (Eppendorf) v.1.35. The zero-sample contained all of these components with the exception of plant extract.

Statistical Analysis

SOD activities for control and experimental groups were measured in triplicates. All calculations were carried out in R software (version 3.6.1.). Data of SOD activity were checked for normality (Shapiro–Wilk test) and homogeneity of variance (Bartlett's test). If data met requirements of both tests significance of differences between groups of transformed clones and untransformed control samples were assessed by one-way analysis of variance (ANOVA). Statistical significance of differences in SOD activity in pairs of control/clone and clone/clone roots was assessed by Tukey's honestly significant difference (HSD) multiple comparison test. Otherwise, the Kruskal–Wallis rank sum test followed by Kruskal–Wallis multiple comparison test for non-parametric data was applied. Results of the calculation of SOD activities were performed as mean values ± 95% confidence intervals. For each comparison, a *P*-value < 0.05 was considered statistically significant, and p-value < 0.01 – extremely significant.

Results

PCR Analysis of "hairy" Root Clones

The collection of transgenic roots used in the experiments was established in 2013–2015. All "hairy" root clones were characterized by the specific phenotype and grown on the hormone free medium (Fig. 1).

The stable transformation was confirmed again by PCR analysis by the detection of bacterial *rol* genes in "hairy" root clones. The last PCR analysis was performed in January 2020. According to this study, all clones carried *rol B* and *rol C* genes after 4–6 years of in vitro cultivation (Fig. 2).

Although we studied the roots that were cultivated in vitro for a long time after the transformation event, an analysis was performed to confirm of the absence of bacterial contamination. PCR analysis with primers specific for *vir*D gene confirmed the absence of bacteria in all "hairy" root clones (Fig. 3 presents the data for some clones).

Superoxide Dismutase Activity Assay

Superoxide dismutase activity in the roots of the control plants significantly differed with the lower mean values for *A. officinalis* (45.8 ± 8.7 U/μg), *A. dracunculus* (55.4 ± 8.8 U/μg) and *A. vulgaris* (56.3 ± 3.5 U/μg) and higher mean values for *A. tilesii* (263 ± 42 U/μg) and *A. ludoviciana* (275 ± 97.1 U/μg) (Fig. 4).

According to results of Tukey and Kruskal–Wallis multiple comparison tests the two patterns were observed for

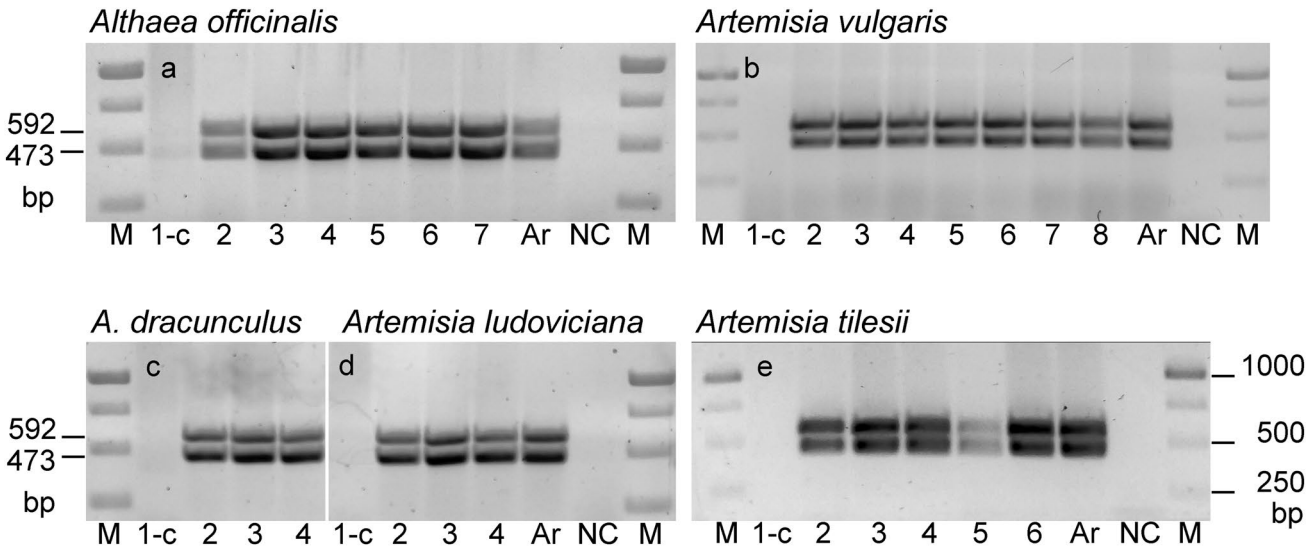

Fig. 2 Electrophoregram of the products of multiplex PCR analysis of "hairy" root clones using primers specific for *rol B* (592 bp) and *rol C* (473 bp) genes. O`GeneRuler 1 kb Plus DNA Ladder #1163 marker for sizing DNA fragments (*M*): 1-c –DNA of the control plants, 1–7 – DNA of "hairy" root clones, Ar – *Agrobacterium rhizogenes* DNA (positive control), -NC – negative control (DNA)

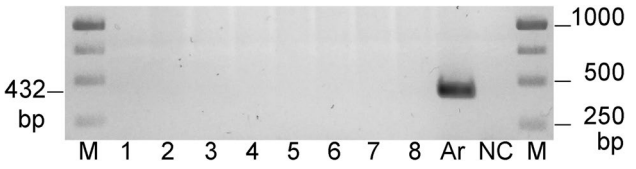

Fig. 3 Electrophoregram of the products of PCR analysis of "hairy" root clones of *A. officinalis* (1, 2), *A. vulgaris* (3, 4), *A. tilesii* (5, 6), *A. ludoviciana* (7), and *A. dracunculus* (8) using primers specific for *vir D* gene. O`GeneRuler 1 kb Plus DNA Ladder #1163 marker for sizing DNA fragments (*M*); Ar – *Agrobacterium rhizogenes* DNA (positive control);—NC – negative control (DNA)

SOD activity in control and transgenic samples: (1) Absence of statistically significant differences in activity values in the control and "hairy" roots – 46% of total clone number (11 of 24 clones); (2) Higher activity values in "hairy" roots compared the control –54% of total clone number (13 of 24 clones). Significant differences in group values of SOD activity between transformed and non-transformed roots were found for all five studied species. Differences were assessed as extremely significant with the following p-values: *A. tilesii* < 0.001, *A. officinalis* $< 5.8 \times 10^{-8}$, *A. ludoviciana* $< 7 \times 10^{-4}$, *A. dracunculus* < 0.015, *A. vulgaris* $< 5.16 \times 10^{-11}$.

Four of six clones of *A. officinalis* revealed a high level of SOD activity (Fig. 2a). Clone 4 and clone 7 demonstrated

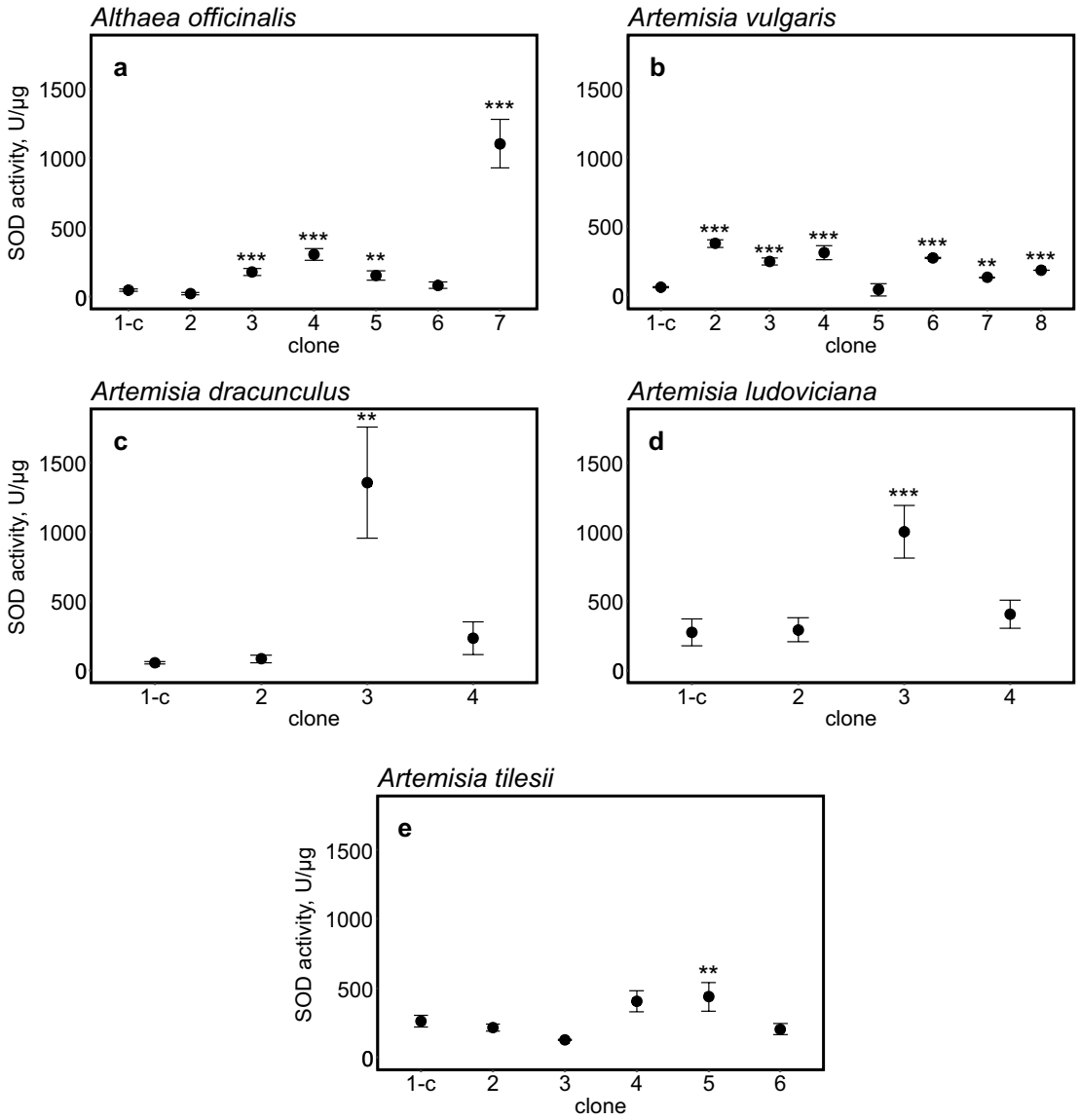

Fig. 4 Superoxide dismutase activity in root clones (mean value, error bars – 95% confidence interval, **—*P*-value < 0.05, ***—*P*-value < 0.01, *P*-value calculated from multiple comparison tests, indicates significance of differences of SOD activity in control and clone samples)

sevenfold (304.0 ± 43 U/μg) and 24-fold (1105 ± 174 U/μg) increase of the activity respectively compared the control (roots of the mother plants). SOD activity in six "hairy" root clones of *A. vulgaris* was greater (129.0 ± 2.1 U/μg—375 ± 28.2 U/μg) than in the control with the highest sevenfold increase for clone 2 (Fig. 2b). There was a minimal difference between the clones of *A. tilesii* and the control samples. SOD activity was 1.6 times higher only in one (438 ± 104 U/μg, clone 5) of five clones (Fig. 2e). SOD activity in most "hairy" root samples of *A. ludoviciana* and *A. dracunculus* did not differ from the control. One of the clones for each species of *A. ludoviciana* and *A. dracunculus* exhibited higher SOD activity than the control samples. *A. ludoviciana* clone 3 and *A. dracunculus* clone 3 demonstrated 3.6-fold (1001 ± 191 U/μg) and 24-fold (1356 ± 402 U/μg) increase in SOD activity, respectively (Fig. 2d, c).

Discussion

Noticeable increase in SOD activity in the transformed roots of all five species was observed. The presence of *rol B* and *rol C* genes was confirmed in all 24 clones. Since *A. rhizogenes* without specific foreign genes that affect the regulation of SOD activity were used for the transformation, changes in the level of this activity might be caused by agrobacterial genes transfer. It is known that genes transferred to plant cells after *Agrobacterium*-mediated transformation can be incorporated in different sites. The root lines used in our experiments are the transgenic line resulted from independent transformation events. We would like to emphasize that all studied root lines were characterized by the typical "hairy" phenotype and the possibility to grow on the phytohormone-free medium. At the same time, they differed in phenotype (Fig. 1), growth rate [13, 17], flavonoids accumulation, antioxidant activity [13, 17], and also in their resistance to the cold, heat [18] and metal-induced stress [19]. So, the differences in SOD activity can be explained by the influence of the position of genes transferred to the plant genome after the transformation, their activity and the effect on the function of other plant genes. This hypothesis can be supported by the data published by the authors [20] studied that *rol* B and *rol* C transcript levels positively correlated with the amount of plant secondary metabolites.

The phenomenon of integration of agrobacterial *rol* genes into the plant genome and its influence on plant metabolism is currently considered within two approaches. The first approach studies plant transformation by wild-type *A. rhizogenes* and insertion of *rol* genes in the context of an increase in the production of secondary metabolites such as polyphenols and alkaloids [21]. Enhanced synthesis of valuable compounds in the plant cells by *rol* genes was shown for different species of medicinal plants. At the moment, mechanisms of this activation remain unclear. However, it is known that products of *rol* genes influence the gene silencing process and affect transcription factors responsible for the regulation of plant secondary metabolites synthesis [20]. Recent studies report that variations in metabolite yield can be caused by various patterns of T-DNA integration into plant genome as well as high specificity of *rol* gene to the particular transcription factor [22].

The second approach considers agrobacterial *rol* genes and their effect on SOD activity in plant tissues in the context of the infectious process and plant-bacteria interaction similar to the action of abiotic stress factors. Attack of phytopathogenic microorganisms causes the damage of cell structures followed by electrolyte leakage from chloroplast and mitochondria which results in the formation of reactive oxygen species. In turn, oxidative stress resulted from sharp ROS increase, may cause cell death or production of antioxidant ROS-scavenging enzymes including superoxide dismutase. Some studies report a significant level of cell death as a problem of agrobacterial transformation. ROS accumulation and tissue necrosis were shown for tomato cultivars [23]. At the same time, other studies reported either high antioxidant enzyme activity or enhanced ROS-scavenging in plants and tissues transformed by agrobacteria. "Hairy" roots of *Linum usitatissimum* showed higher free radical scavenging activity [24]. Cherry cultivar infected with *A. tumefaciens* also revealed an increase in expression of genes encoding antioxidant enzymes including SOD [25]. Unlike secondary metabolites, infection-mediated activation of antioxidant enzyme genes has a temporary effect, which appears in increase activity of enzymes only during the first day after infection. For example, patchouli plants showed an increase in SOD activity within 24 h after *Ralstonia solanacearum* infection, followed by a decrease to control levels [8]. SOD activity in wounded shots of *Impatiens walleriana L.* infected with *A. rhizogenes* achieved maximal value after 10 h and then decreased. Moreover, SOD activity correlated with the level of expression of *rol* genes [26]. *Arabidopsis thaliana* and *Rubia cordifolia* cells expressing *rol* B gene also possessed enhanced expression of antioxidant genes and reduced ROS levels. Authors hypothesize that *rol* B protein can affect ROS level and indirectly activate plant antioxidant system [9].

Thus, insertion of agrobacterial *rol* genes into a host plant genome may activate the antioxidant defense system; in particular, provide a sustainable enhanced synthesis of secondary metabolites with antioxidant activity. It correlates with the level of *rol* genes expression or temporary increase in the activity of antioxidant enzymes as a response to the infectious process. "Moderate" expression of *rol* B gene may successfully increase the activity of the antioxidant system to maintain sufficient redox balance, while *rol* B "overexpression" can result in cell necrosis. Sustainable resistance to oxidative

stress is usually associated with transformation by agrobacteria harboring foreign SOD-regulated genes [9, 27]. At the same time, transformed cells may show an increased SOD activity also due to stable expression of *rol* genes over the years after the transformation. These data suggest that the effect of the activity of *rol* genes, in particular, an increase in the activity of enzymes of the antioxidant defense system, may be similar to the effect of acclimatization under the action of stress factors of moderate activity.

Recent studies show that plants that carry agrobacterial genes are common in nature. Agrobacterial genes can be permanently active components of the plant genome and act as an evolutionary factor providing advantages over plants that do not carry these genes. These data support the assumption about the adaptive action of the role of genes for the plants. For the first time expressing T-DNA genes of *Agrobacterium* species was found for sweet potato cultivars [11]. Authors supposed that transformation event occurred centuries ago, during the domestication of sweet potato, and plants harboring *rol* B gene were selected due to advanced root parameters. The most recent study [12] demonstrates the presence of 20 agrobacterial genes including intact, truncated, or containing stop-codons *rol* B- and *rol* C-like genes with 36 – 66% of identity to *A. rhizogenes* genes in natural transformants of 17 plant genera.

Results of our experiment indicate that *A. rhizogenes*-mediated transformation in the case of transfer of *rol* B and *rol* C genes in the plant genome can lead to a long-term increase of SOD activity. At the same time, in several lines in our study the SOD activity did not differ from the control. It can be assumed that this effect is due to the physiological characteristics of the plants themselves and their natural ability to adaptation. In particular, *A. vulgaris, A. dracunculus, A. ludoviciana* and *A. officinalis* plants have a very wide habitat (Europe, Asia, and America) and can grow at different conditions. *A. tilesii* differ significantly from the named species, as they grow only in the northern territories of Canada, Russia and Alaska and are obviously adapted to low temperatures. At the same time, the different activities of the *rol* B and *rol* C genes also can result in the variation of ROS accumulation and the activity of antioxidant ferments in the transformed cells. The suggestion was confirmed in the study of ROS levels in *rol* B and *rol* C transformed cells [9, 10]. High expression of these genes in transgenic *Rubia cordifolia* sell suspension and callus cultures correlated with the low ROS levels. This effect was studied also in *Panax ginseng* and *Arabidopsis thaliana* transformed cell cultures.

Conclusion

In conclusion, results of the current study reveal that 54% of the total number of "hairy" root clones of five plant species obtained after *Agrobacterium rhizogenes*-mediated transformation showed a significant increase in superoxide dismutase activity compared to roots of untransformed samples, while other clones didn't show a reduction in SOD activity values. Results of our experiment indicate the diversity of SOD activity in obtained "hairy" roots lines in spite of presence of *A. rhizogenes rol* B and *rol* C genes in all tested lines. Apparently *A. rhizogenes*-mediated transformation in some cases can activate plant antioxidant enzyme system and maintain a stable high level of SOD activity in the "hairy" roots of five plant species during 4–6 years after the transformation event. This allows us to hypothesize that wild-type agrobacteria potentially can be used for obtaining transgenic clones highly resistant to oxidative stress without insertion of other specific foreign genes into the plant genome, and also contributes to an idea of developing safe and sustainable transgenic crops using *rol* genes transfer to plant genome.

Author Contributions NM, HT, VD, and MK conceived the project and designed the study. NM, AS, YR, and TB performed most of the experiments. HT, NM, VD, and all the authors contributed to data analysis. NM, HT, and VD wrote the manuscript with contributions of MK and the other authors.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval This article does not contain any investigations carried out by any of the authors with the participation of animals or humans.

Consent to participate Not applicable.

Consent for publication Not applicable.

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