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Characterization of Carnivorous Plants *Sarracenia purpurea* L. Transformed with *Agrobacterium rhizogenes*

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Abstract: People have used carnivorous plants of the genus *Sarracenia* in folk medicine for centuries due to the biochemical composition of *Sarracenia* plants, which are rich in numerous bioactive compounds with anti-inflammatory, antioxidant, antiviral and antibacterial properties. The subject of this study was the genetic transformation of *Sarracenia purpurea* L. with *Agrobacterium rhizogenes* strains 15834, 9402 and A4 using two different methods: bacterial injection or co-culture of the bacteria with plant explants. This study confirmed the possibility of hairy root induction in *S. purpurea* using *A. rhizogenes* strain 15834 and the injection method. Seven lines of transformed plants, exhibiting the integration of the *rolB* gene, were obtained. The hairy roots formed showed morphological differences in comparison to the roots of unmodified plants. A mathematical model was used to optimize the conditions for the extraction of bioactive compounds. Extracts isolated under optimal conditions from the transformed plants showed biochemical changes, i.e., an increase in the accumulation of total polyphenols (line 7#1 in hairy roots: 71.048 mg GAE g^{-1} DW; in leaves: 9.662 mg GAE g^{-1} DW) and triterpenes (line 7#1 in hairy roots: 1.248 mg BA g^{-1} DW; in leaves: 0.463 mg BA g^{-1} DW) in comparison to untransformed plants (polyphenols in roots: 7.957 mg GAE g^{-1} DW and in leaves: 5.091 mg GAE g^{-1} DW; triterpenes in roots: 0.298 mg BA g^{-1} DW and in leaves: 0.296 mg BA g^{-1} DW), especially when induced roots were analyzed. HPLC analysis showed an increase in the level of betulinic acid in some transformed *Sarracenia* lines. Betulinic acid is a pentacyclic triterpenoid compound with high pharmacological significance.

Keywords: composite plants; genetic engineering; hairy roots; insectivorous plants; secondary metabolites

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1. Introduction

Sarracenia purpurea L. is a carnivorous plant that remains an interesting ornamental species. Importantly, it contains many valuable compounds. *S. purpurea* L. is well known as a Cree medicinal plant [1]. The plants from the genus *Sarracenia* have been found to possess antioxidant, antidiabetic, antiviral and antibacterial constituents [1–3], including sarracenin [4], betulinic acid, ursolic [1], hyperoside (quercetin-3-O-galactoside), morroniside [5] and many others (data as below in Section 3.4).

The problem of the increasing incidence of cancer, bacterial and viral diseases and antibiotic-resistant pathogenic bacteria, as well as the lack of effective treatment methods for cancers entail serious consequences for human life and health. An important area of research focuses on seeking new therapies for diseases and therapeutic compounds that would be safe and easily available. Genetic engineering techniques offer an alternative and increasingly more common solution allowing for the precise modification of organisms' traits, including those of plants.

As hairy root cultures are a promising method for the production of interesting secondary metabolites in plants [6] and can be applied for the enhanced synthesis of bioactive compounds, the aim of this study was to establish the tissue culture of *S. purpurea* L. plants and transform them via the agroinfection method with an *Agrobacterium rhizogenes* strain.

It should be pointed out that hairy root cultures are aseptic and they are characterized by high genetic stability and a high growth rate without the addition of any plant growth regulators [7,8]. Thus, these cultures can be considered tools for plant physiology and interaction analysis, e.g., allelopathy [9], or for the production of medical compounds, e.g., heterologous proteins. An example is a human tissue plasminogen activator produced in the hairy roots of oriental melon (*Cucumis melo* L.) [10] or human gastric lipase produced in *Arabidopsis* hairy roots [11]. Furthermore, the level of metabolites produced in hairy root cultures is often higher than that observed in the mother plant or roots. For example, it has been demonstrated that the total flavonoid content in 24-day-old Isatis tinctoria L. hairy root cultures was about 30% higher than in 2-year-old roots derived from field cultivation [12].

Additionally, the elicitation of hairy root cultures can enhance the production of interesting compounds [7]. Another strategy may be the genetic transformation of *A. rhizogenes* with desired genes. However, as shown in other studies, genetic transformation with the application of this bacterial strain can cause a reduction in the effectiveness of hairy root formation in plants [8]. It should be also pointed out that many plant species that remain recalcitrant to *Agrobacterium tumefaciens* infection and transformation have been successfully transformed with *A. rhizogenes*. These include *Salix* spp. L. [13], *Prunus* spp. [14], *Populus* spp. [15] and many others. Thus, *A. rhizogenes* is regarded as an effective biotechnological tool of genetic engineering.

In the present study, a wild-type strain of *A. rhizogenes* was used for hairy root induction in *Sarracenia* plants. *A. rhizogenes* (syn. *Rhizobium rhizogenes*) is a Gram-negative bacterium that infects plants and transfers a fragment of the *Ri* plasmid to the plant genome. The transfer and integration of T-DNA (containing genes encoding enzymes necessary for auxin and cytokinin synthesis) to the plant genome cause the growth of hairy roots [8].

The study showed that it is justified to introduce genetic modifications in *Sarracenia purpurea* L. to obtain hairy roots, as the plants display an increased accumulation of valuable compounds with pharmacological potential. To our best knowledge, this is the first report concerning the transformation of plants from the genus *Sarracenia* with the *A. rhizogenes* strain.

2. Materials and Methods

2.1. Plant Material

The plant material used in this study was a carnivorous plant *Sarracenia purpurea* L. The plants were obtained thanks to the Botanical Garden of the University of Wroclaw.

2.2. Bacterial Strain

Agrobacterium rhizogenes strains ATCC 15834, A4 and LBA 9402 were used for hairy root induction. The strains were stored at −80 °C in 65% glycerol ($C_3H_8O_3$). The bacteria were dark-grown on YEB medium plates (yeast extract 1 g/L, beef extract 5 g/L, peptone 5 g/L, sucrose [$C_{12}H_{22}O_{11}$] 5 g/L, magnesium sulphate heptahydrate [$MgSO_4 \cdot 7H_2O$] 0.49 g/L, pH 7.2) at 26 °C for 48 h. Then, a single colony of bacteria was introduced into liquid cultures (YEB medium) and used for plant transformation.

2.3. Establishment of Aseptic Cultures of Sarracenia Plants

Before proper sterilization, the whole plants were thoroughly washed with distilled water. Then, under sterile conditions, the leaves were cut with a sharp-bladed scalpel into ~0.5 cm fragments. These fragments were rinsed for 1 min in 70% ethanol solution. Then, explants were washed in sterile distilled water for 3 min and in a 10% hydrogen peroxide solution for 5 min. Then, the explants were rinsed with sterile distilled water, 10% bleach and 10% sodium dodecyl sulfate (SDS). Next, these fragments were washed 4 times for 5 min with sterile distilled water. The fragments were cut into smaller pieces to remove any damage on the edges caused by the bleach. The explants were rinsed in a 3% PPM (PPM™, Plant Cell Technology) solution for 4 hours and placed in a medium containing 1.46 g/L MS [16], 3% sucrose, 0.8% agar (Sigma) and 750 µl/L plant preservative mixture

(PPM). The explants were transferred to fresh medium every week, and sterile plant tissue cultures were established.

2.4. Plant Transformation

The explants of *Sarracenia purpurea* L. were infected with *A. rhizogenes* strains 15834, A4 and LBA 9402 (OD600 = 0.6) to obtain hairy root cultures as described in Swain et al., 2012 [17]. For this purpose, two methods of transformation were used: injection and co-culture. In the first method, cultures of *A. rhizogenes* were injected with the use of a sterile needle to the plant explants, and then the explants were incubated at 26 °C for 24 h on MS medium without antibiotics [18]. Then, the explants were rinsed in 45 mL of sterile water with 200 µL carbenicillin (100 mg/L) and placed on solid medium with claforan (400 mg/L) and carbenicillin (100 mg/L). After one week, the concentration of claforan was reduced to 200 mg/L. After 14 days of incubating these explants in the dark, they were cultivated in medium with 3% sucrose and PPM (750 µL/L). After another two weeks, the explants were transferred to the medium without antibiotics. The explants were transferred to a fresh medium every week. After about 6 weeks, the first hairy roots were formed at the wound sites. They were present only in explants transformed with strain *A. rhizogenes* 15834.

In the second method involving a co-culture of leaf explants with an *Agrobacterium* strain, 50 µL of bacterial suspension (OD600 = 0.6) and plant explants were placed in 25 mL of sterile distilled water and shaken for 48 h at 26 °C [8]. Then, the transformed explants were placed on the medium described above.

2.5. The Confirmation of T-DNA Integration

The presence of the *rolB* gene in the plant genome was detected via the PCR method. Genomic DNA was isolated according to the Thermo Scientific Phire Plant Direct PCR kit as described in Wróbel-Kwiatkowska, 2019 [19]. The sequence of primers used to select transformants was as follows: F: 5'GCTCTTGCAGTGCTAGATTT3' and R: 5'GAAGGTGCAAGCTACCTCTC3' (for strain ATCC 15834). The amplified fragment was 423 bp for the plasmid from this strain. The PCR reaction was carried out in a volume of 20 µL; PCR conditions were in accordance with the Thermo Scientific Phire Plant Direct PCR Master Mix reagent kit protocol. PCR products were separated with electrophoresis (1% agarose in the presence of Midori Green Advance DNA stain).

The genomic DNA isolated from the untransformed *Sarracenia* roots served as a negative control and was used as a template in PCR, performed in the same conditions as described above.

2.6. Selection of Optimal Extraction Method of Bioactive Compounds

Extraction was performed using an ultrasonic bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan, Latvia) with a heating capacity of 150 W [20]. The optimization of the extraction method of phenolic compounds and triterpenes was performed via a Box–Behnken design [21]. The influence of the independent variables, extraction time (X1, 10–60 min), ethanol concentration (X2, 64–96% v/v) and extraction temperature (X3, 25–60 °C), on the dependent variables, extracted phenolic compounds (TP) and triterpenes (TT), was investigated (Table 1). Based on a study by Fernández et al. [22], fifteen experiments with three center points per block were performed for method optimization. A second-order polynomial equation was fitted to the data.

ANOVA Design-Expert software (Version 7.0.0., Suite 48, Minneapolis, MN 55413) was used for the analysis. The analysis of variance was used to obtain the quadratic polynomial mathematical model. The model was established to describe the influence of process parameters on the extraction of total phenolics and triterpenes [21]. The model p-value and the value of determination (R2) were used to predict model capability.

Table 1. Independent variables for the experimental design.

Independent Variable	Variable Levels			
	Symbol	Low (−1)	Center (0)	High (+1)
Time (min)	X_1	10	35	60
EtOH (%)	X_2	64	80	96
Temperature (°C)	X_3	25	4.5	60

2.7. Total Polyphenol Content

The total polyphenol content of the prepared extracts was determined using the Folin–Ciocalteu method. The extracts were prepared by ultrasound extraction of 6 mg of plant tissue with 6 mL of 64% EtOH. Extraction was performed for 10 min at 60 °C. First, 64% EtOH was used to dilute the samples. Then, 250 µL of diluted samples and serial standard solutions of gallic acid were placed in glass test tubes, and 1.25 mL of Folin–Ciocalteu reagent diluted 10 times was placed into the tubes. After 5 min of incubation in the dark at room temperature, 1 mL of Na_2CO_3 (75 g/L) was added and incubated for 5 min (50 °C, in the dark). After incubation, the tubes were cooled to 4 °C and absorbance was measured at 760 nm. The results were expressed as mg gallic acid equivalents (GAE) per gram of dry weight (mg GAE g^{-1} DW) [23]. All analyses were performed in triplicate.

2.8. Total Triterpene Content

The total triterpene content of the prepared extracts was determined with a modified spectrophotometric method using sulfuric acid [24]. First, 50 µL of plant extracts, reagent blank and standards were incubated at 60 °C for 15 min in a shaker bath, with 50 µL of 8% (*w*/*v*) vanillin in ethanol and 0.5 mL of 72% (*v*/*v*) sulfuric acid. After incubation, the samples were cooled for 5 min and the absorbance (560 nm) was measured. All analyses were performed in triplicate.

2.9. HPLC Analyses of Betulinic Acid

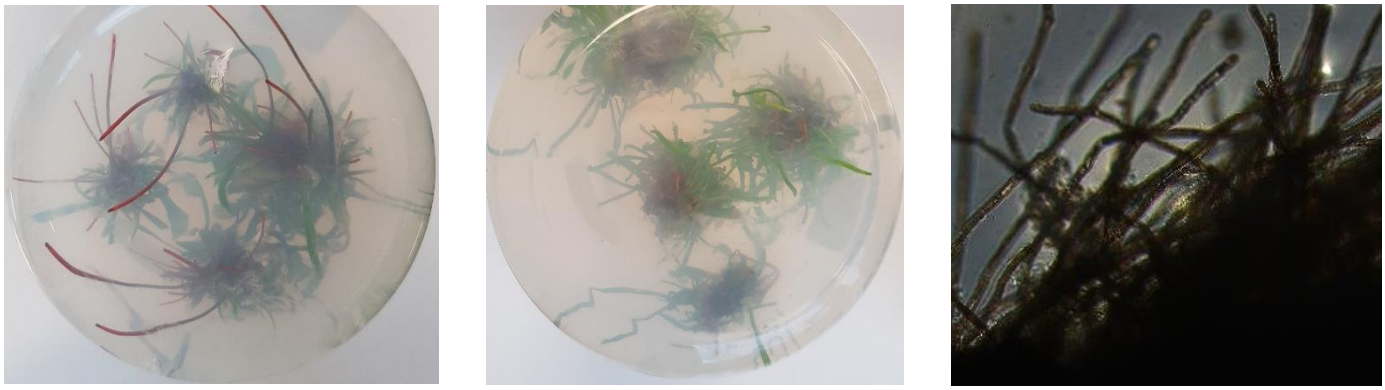
HPLC analyses were performed using the Agilent 1200 series HPLC system (Agilent, San Jose, CA, USA) with a Phenomenex C18 column (Kinetex, 2.6 µm, 100 A, 150 × 4.6 nm) and a diode array detector (DAD). The mobile phases water/formic acid (99.9:0.1 *v*/*v*) (solvent A) and acetonitrile/water (99.9:0.1 *v*/*v*) (solvent B) were used for betulinic acid analysis. The flow rate was 0.9 mL/min. Before analysis, the samples were filtered through polytetrafluoroethylene filters (0.22 µm). The autosampler temperature was kept at 4 °C and the column temperature was 30 °C. Standard of betulinic acid (Sigma) was used for identification. Betulinic acid in biomass was quantified with an external standard of betulinic acid (1 mg L^{-1}) at 210 nm. All analyses were performed in triplicate.

Samples for HPLC analysis were prepared in triplicate. The content of polyphenols was expressed as mg of compound per g of dry weight (DW).

3. Results and Discussion

3.1. Transformation of Sarracenia Plants

Two different methods were used for plant transformation: co-culture of plant explants and *A. rhizogenes*, and injection of *A. rhizogenes* into plant tissues. Three different strains of *A. rhizogenes* were applied for plant transformation, namely ATCC 15834, LBA 9402 and A4. However, transformed roots (Figure 1) were obtained only when the injection technique was used for transformation and only in the case of one strain of *A. rhizogenes*, ATCC 15834. The other two strains did not cause hairy root formation. It should be pointed out that *A. rhizogenes* strain 15834 has also been described as the most effective for other plants such as flax and *Trapa natans* [25]. The obtained composite *Sarracenia* plants were cultured in tissue culture conditions and used for selection and characterization.



(**a**) (**b**) (**c**)

Figure 1. Morphology of transformed roots of *S. purpurea* obtained after infection of *A. rhizogenes* strain ATCC 15834. *S. purpurea* in vitro plantlets exhibiting transformed roots (**a**) in comparison to wild-type, untransformed roots (**b**). Microscopic analysis of transformed *Sarracenia* roots (**c**).

3.2. Analysis of rolB Gene Integration

The integration of the *rolB* gene from the *Ri* plasmid into the plant genome was performed via the PCR method as described in Section 2. Of seventy analyzed roots, positive results were obtained for seven. PCR reaction primers amplified a 423 bp fragment of the *rolB* gene (Figure 2). The presence of this gene was confirmed for seven transgenic lines of transformed roots and it was not detected in the genome of the *Sarracenia* roots derived from untransformed control plants. It should be noted that the integration of the *rolB* gene is the most important part of the transformation process [26].

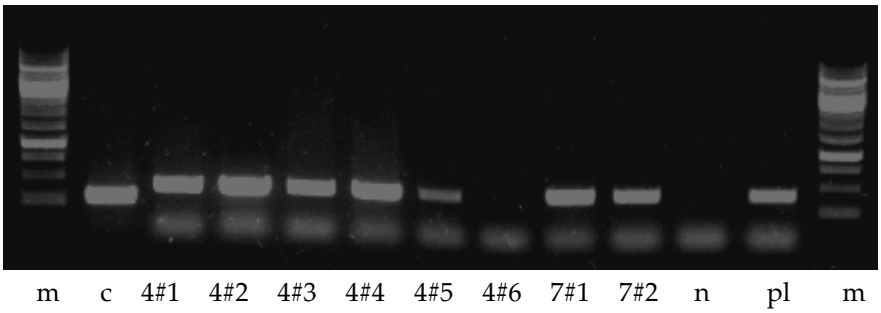


Figure 2. Selection of hairy roots performed via the PCR method. The 423 bp fragment of the *rolB* gene was amplified and detected in genomic DNA isolated from transformed roots (lines are numbered); n—negative control, genomic DNA was isolated from wild-type root and used as template in PCR; pl—positive control, plasmid Ri from *A. rhizogenes* ATCC 15834 was applied as template in PCR; c—control of kit, fragment of conserved region of DNA (297 bp) amplified with specific primers provided by kit producer; m—marker 1 kb ladder.

3.3. Optimization of the Extraction of Bioactive Compounds

The most efficient extraction conditions of bioactive compounds (polyphenols) from the obtained *Sarracenia purpurea* plants are 10 min, 64% (*v*/*v*) EtOH and 60 °C. The highest levels of the phenylpropanoids and triterpenes were obtained in the predicted conditions. Thus, the content of these compounds under the established conditions was consistent with the assumptions resulting from the Box–Behnken design (Table 2). All the extracts used for determining the content of individual compounds were prepared under these optimal conditions.

Table 2. Expected (A) and real (B) values of the content of polyphenols and triterpenes from extracts prepared in optimal extraction conditions.

Variant	A (mg/L)	B (mg/mL)
Total polyphenol content	121.86	126.0606 ± 5.3569
Total triterpene content	4.46	4.5737 ± 0.1552

3.4. Analysis of Total Polyphenol Content

S. purpurea L. remains a source of many valuable compounds, among them polyphenols and triterpenes (Table 3). Thus the aim of this study was to determine the level of total polyphenol content in modified *Sarracenia* plants. The increase in the content of total polyphenols was observed for all induced hairy roots as compared to untransformed roots (Figure 3). The highest increase was noticed for two lines, 7#1 and 7#2, for which 9-fold and 7-fold higher polyphenol content was measured, respectively.

Table 3. Potential bioactive properties of compounds found in plants of the genus *Sarracenia*.

Effect	Compound	Reference
Analgesic therapy	Sarapin	[5,27]
Anticancer properties	Betulinic acid Betulinaldehyde Ursolic acid Plumagin Ramentaceon Quercetin	[28]
Antidiabetic properties (T2D)	Quercetin Morroniside Quercetin-3-O-glucoside Rutin	[5,29]
Antiglycation activity	Catechin Myricetin Quercetin-3-O-galactoside Rutin	[5,29,30]
Antimycobacterial properties (Mycobacterium tuberculosis)	Betulinic acid Ursolic acid	[31]
Antipoxivirus properties (monkeypox-MPXV, poxvirus bovis-VACV, variola virus-VARV)	Quercetin	[32,33]
Dyspepsia and constipation (laxative properties)	Quercetin	[32]
Gynecological disorders	Betulinic acid	[5]
Neurodegenerative diseases (mainly Parkinson's disease, Alzheimer's disease)	Luteolin	[1]
Stimulated AMPK signaling pathway	Quercetin-3-glucoside Quercetin-3-O-galactoside Rutin	[34,35]

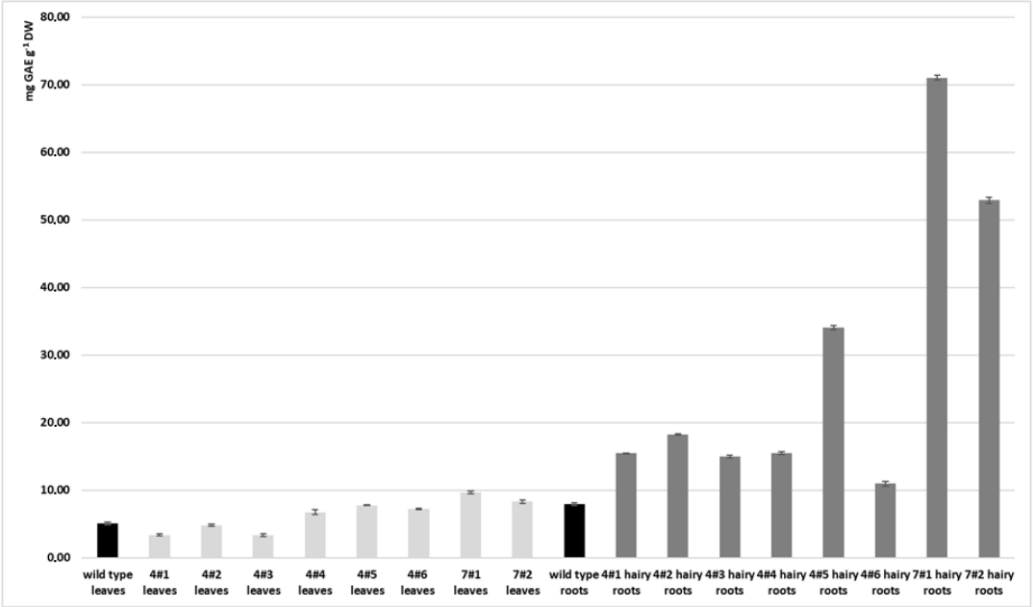


Figure 3. Total polyphenol content measured in hairy roots and composite plants of *S. purpurea*. The levels of polyphenols were analyzed as described in Section 2. The data (±SD) were obtained from three samples per line.

The leaves of plants did not exhibit this tendency, and the total polyphenol content was not changed when compared to that in the wild-type plant leaves.

3.5. Analysis of Total Triterpene Content

The highest total triterpene content was observed for three lines of transformed roots, i.e., lines 4#5, 7#2 and 7#1, and the increase was about 3-fold in the first two lines and 4-fold in line 7#1 in comparison to the wild-type *Sarracenia* roots (Figure 4). In contrast to the level of polyphenols, a high increase in the triterpene content was determined for most stems, and this increase was higher than that observed in the induced transformed roots. It should be pointed out that the reason for this phenomenon is unknown. It might be the effect of infection of plants by *A. rhizogenes*. It is known that triterpenes are compounds intermediating plant–pathogen interactions [36]. It has also been demonstrated that triterpenes play a crucial role in the plant defense system [37] and in allelopathy [38]. Thus, it can be speculated that the transformation of *S. purpurea* with *A. rhizogenes* caused the plant response to the pathogen attack.

3.6. Betulinic Acid Content

All the analyzed composite plants were used for isolation and measurements of betulinic acid, a compound exhibiting antibacterial, antiviral and anticancer properties. The activity of betulinic acid against *Escherichia coli* and *Staphylococcus aureus* [39] as well as its anti-HIV and even antimalarial properties have been described [40]. It should also be mentioned that this compound exhibits a cytotoxic effect against tumor cells (melanoma) [40,41]. Five tested transgenic lines exhibited higher betulinic acid content in leaves than the control *Sarracenia* plants (Figure 5). The best results were obtained for lines 7#1, 7#2 and 4#4, for which the highest amount of betulinic acid was measured. The levels of betulinic acid observed in these lines were 70%, 62% and 66% higher, respectively, than in the wild-type control plants. The transformed roots did not exactly show the same tendency, and for two lines, 4#2 and 4#4, an elevated amount of betulinic acid was recorded, with line 4#4 presenting the highest amount, which was 34% higher than in the untransformed roots. The obtained results indicate that *A. rhizogenes*-mediated transformation caused an increase in the betulinic acid amount, which is a compound of medicinal significance.

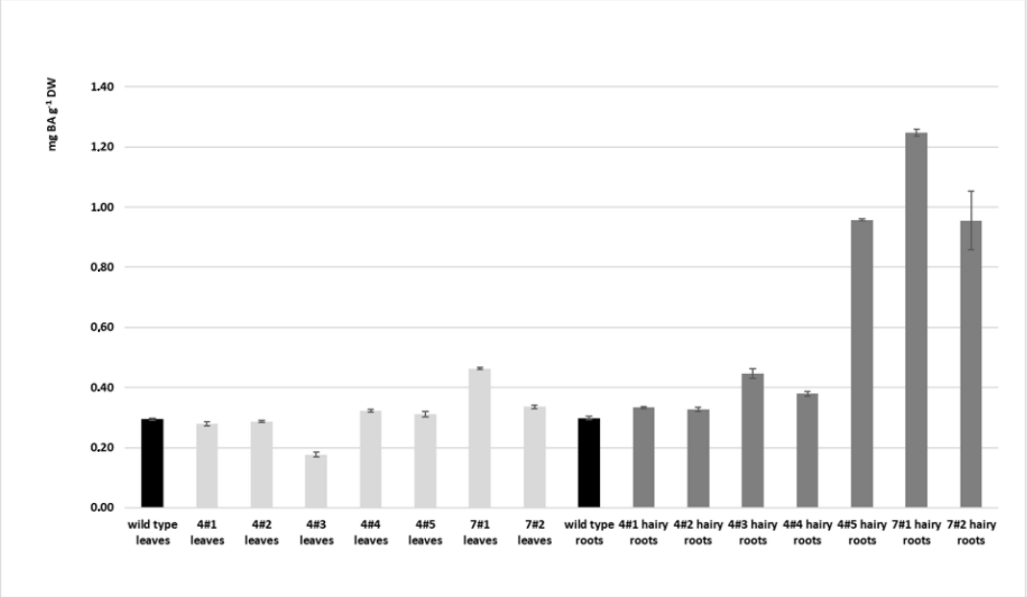


Figure 4. Total triterpene content determined in the investigated hairy roots and composite plants of *S. purpurea*. The analyses were performed as described in Section 2. The data (±SD) resulted from three samples per line.

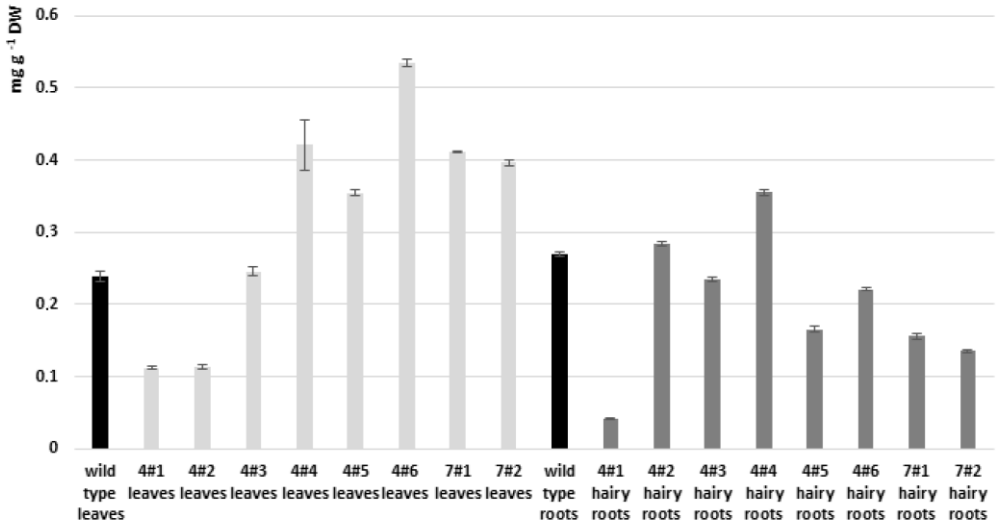


Figure 5. Betulinic acid content measured in the composite plants *S. purpurea* and in the investigated hairy roots by HPLC as described in Section 2. The data (±SD) were obtained from three samples per line.

4. Conclusions

In the present study, we described the transformation of *Sarracenia purpurea* L. insectivorous plants to generate composite plants with hairy roots. Firstly, we observed that only the *A. rhizogenes* 15834 strain was efficient for the transformation of S. *purpurea* L. The only effective method of transformation was the injection technique. Furthermore, as shown in other studies, genetic engineering of *S. purpurea* L. roots is a potent method for the improvement of their biochemical composition. The obtained modified roots of line 7#1 exhibited an almost 9-fold increase in the level of phenolic compounds and a 4-fold increase in the amount of triterpenes when compared to the wild-type roots. The leaves of the modified plants also exhibited a higher level of the analyzed compounds, i.e., a 1.8-fold higher level of phenolics and a 1.5-fold increase in triterpenes in the most effective line 7#1

compared to non-transformed leaves. The transformation also resulted in an increased level of betulinic acid in the leaves of two other lines, 4#6 and 4#4, with the latter line showing an increased amount of this compound also in the generated roots. Thus, the transformation of plants with *A. rhizogenes* 15834 had a positive effect on the content of compounds with pharmacological potential in the composite plants. Our further experiments will focus on studying the effect of plant extracts derived from transgenic *Sarracenia* hairy roots on the proliferation of cancer cells and pathogenic microorganisms.

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