Characterization of Carnivorous Plants *Sarracenia Purpurea* L. Transformed with *Agrobacterium Rhizogenes*

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Research Article

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Abstract

Carnivorous plant of *Sarracenia* genus are used by human in folk medicine for centuries. The reason for this phenomenon is biochemical composition of *Sarracenia* plants, which possess many bioactive compounds with anti-inflammatory, antioxidant, antiviral and antibacterial properties.

The subject of this research was genetic transformation of *Sarracenia purpurea* L. with *Agrobacterium rhizogenes* strain 15834, 9402 and A4 by using two alternatively methods of bacterial injection or co-culture the bacteria with plants explants. These studies confirmed the possibility of hairy roots induction in *S. purpurea* using the strain of *A. rhizogenes* 15834 and the injection method. Seven lines of transformed plants, exhibiting the integration of *rolB* gene, were obtained. The formed hairy roots 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 transformed plants showed biochemical changes i.e., the increase in the accumulation of total polyphenols and triterpenes in comparison to untransformed plants, especially when induced roots were analyzed. HPLC analysis showed increase in the level of betulinic acid in some transformed *Sarracenia* lines. Betulinic acid remains pentacyclic triterpenoid compound with high pharmacological significance. The further work connected with isolation and identification of the other bioactive compounds will be done in the nearest future.

Key Message

1. Genetic transformation of *purpurea* was successfully performed by agroinfection with *Agrobacterium rhizogenes* 15834 strain.
2. The accumulation of polyphenols and triterpenes in transformed roots was noticed.
3. Increased level of betulinic acid was observed for five transformed lines in leaves and two lines in induced roots.
4. The values of bioactive compounds determined experimentally and predicted in the mathematical model were similar.

Introduction

Sarracenia purpurea L. belongs to the carnivorous plants, which remain interesting as the ornamental plants, but what is more important they possess many valuable compounds. *S. purpurea* L. is well known as Cree medicinal plant (Cieniak et al., 2015). The plants from *Sarracenia* genus were found to possess antioxidant, antidiabetic, antiviral and antibacterial constituents (Kannan et al., 2020; Muhammad et al., 2013, Cieniak et al., 2015), among them sarracenin (Hu et al., 2009), betulinic acid, ursolic acid (Cieniak et al., 2015), hyperoside (quercetin-3-O-galactoside), morroniside (Harris et al., 2012) and many others were detected and confirmed (Table 1).

Table 1
Potential bioactive properties of compounds found in plants of the *Sarracenia* genus.

Lp	Effect	Compound	Reference
1.	Analgesic therapy	Sarapin	(Harris et al., 2012; Campos et al., 2013)
2.	Anticancer properties	Betulinic acid Betulinaldehyde Ursolic acid Plumagin Ramentaceon Quercetin	(Miles et al., 1974)
3.	Antidiabetic properties (T2D)	Quercetin Morroniside Quercetin-3-O-glucoside Rutin	(Harris et al., 2010; Harris et al. 2012)
4.	Antiglycation activity	Catechin Myricetin Quercetin-3-O-galactoside Rutin	(Beaulieu et al., 2009; Harris et al. 2010, Harris et al. 2012)
5.	Antimycobacterial properties (*Mycobacterium tuberculosis*)	Betulinic acid, Ursolic acid	(Moerman, 1998; Morrison et al., 2016)
6.	Antipoxivirus properties (*monkeypox*- MPXV, *poxvirus bovis*- VACV, *variola virus*- VARV)	Quercetin	(Arndt et al, 2012; Miles, 1862)
7.	Dyspepsia and constipation (laxative properties)	Betulinic acid Ursolic acid	(Arndt et al, 2012)
8.	Gynecological disorders	Betulinic acid	(Harris et al, 2012)
9.	Neurodegenerative diseases (mainly: Parkinson's disease, Alzheimer's disease)	Luteolin	(Cieniak et al, 2015)

Lp	Effect	Compound	Reference
10..	Stimulated AMPK signaling pathway	Quercetin-3-glucoside Quercetin-3-O-galactoside Rutin	(Eid et al. 2010; Nachare at al. 2013)

Because hairy roots cultures are promising method for the production of interesting secondary metabolites in plants (Tian, 2015) and these cultures can be applied for enhanced synthesis of bioactive compounds, the aim of this study was to establish the tissue culture of *Sarracenia purpurea* L. plants and transformation of them *via* agroinfection method with *Agrobacterium rhizogenes* strain.

It should be pointed out that hairy roots cultures are characterized by high genetic stability and high growth rate without addition of any plant growth regulators (Shakeran et al., 2017, Rana et al., 2017). Thus these cultures can be considered as the tool for plant physiology and interaction analysis i.e. allelopathy (Stanišić et al., 2019) or for the production of medical compounds i.e. heterologous proteins. In this case human tissue-plasminogen activator was produced in hairy roots of oriental melon (*Cucumis melo* L.) (Kim et al., 2012) or human gastric lipase in *Arabidopsis* hairy roots (Guerineau et al., 2020). The level of produced metabolites in hairy root cultures is also often higher than in mother plant or root, for example it was exhibited that for hairy root cultures of *Isatis tinctoria* L. total flavonoids content in 24-day-old cultures was about 30% higher than in 2-year-old roots, derived from field cultivation (Gai et al., 2015).

Additionally, the elicitation of the hairy root cultures can enhance the production of interesting compounds (Shakeran et al., 2017), the second strategy may be genetic transformation of *A. rhizogenes* with desired genes. However, as it was shown genetic transformation with application of this bacterium strain can cause reduction in the effectiveness of hairy root formation in plants (Rana et al., 2017). It should be also pointed out that many plant species, which remain recalcitrant *to A. tumefaciens* infection and transformation, were successfully transformed with *A. rhizogenes*. Among them it can be distinguished *Salix* spp. L. (Gomes et al., 2019), *Prunus* spp. (Bosselut et al., 2011), *Populus* spp. (Yoshida et al., 2015) and many others. Thus *A. rhizogenes* is noticed as very effective biotechnological tool of genetic engineering.

In present study wild-type strain of *A. rhizogenes* was used for hairy root induction in *Sarracenia* plants. *A. rhizogenes* (syn. *Rhizobium rhizogenes*) is Gram-negative bacterium, which infects plants and transfers the fragment of Ri plasmid. The transfer and integration of its T-DNA (containing genes encoding enzymes necessary for auxin and cytokinin synthesis) to plant genome causes the growth of hairy roots (Rana et al., 2017).

To our best knowledge this is the first report concerning transformation of plants from *Sarracenia* genus with *Agrobacterium rhizogenes* strain.

Materials And Methods

Plant material

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

Bacterial strain

Agrobacterium rhizogenes ATCC 15834, A4 and LBA 9402 were applied for hairy roots generation. Strains were stored at -80°C in 65% glycerol. The bacteria were grown in YEB medium (yeast extract 1g/l, beef extract 5g/1, peptone 5g/l, sucrose 5g/l, $MgSO_4 \cdot 7H_2O$ 0,49 g/l, pH 7,2) at 26°C for 48 hours in the darkness on plates. Then bacteria were introduced into liquid cultures (YEB medium) and used for plant transformation.

Establishment of aseptic cultures of Sarracenia plants

Before proper sterilization, whole plants were thoroughly washed with distilled water. Then, under sterile conditions, the leaves were cut with sharp- bladed scalpel into squares about 0,5 cm. These fragments were rinsed for 1 minute in 70% of ethanol solution. Then explants were washed in sterile distilled water for 3 minutes and in 10% of hydrogen peroxide solution for 5 minutes. Then explants were rinsed with sterile, distilled water and 10% of bleach and 10% of SDS. Then, these fragments were washed 4 times for 5 minutes with sterile distilled water. The fragments were cut into smaller pieces to remove any damage on the edges caused by the bleach. Explants were rinsed in a 3% PPM (PPM™, Plant Cell Technology) solution for 4 hours and placed in medium containing: 1,46 g/L MS (Murashige and Skoog, 1962), 3% of sucrose, 0.8% agar (Sigma); 750µl/L PPM. Explants were transferred to the fresh medium every week and sterile plant tissue cultures were established.

Plant transformation

The explants of *Sarracenia purpurea* L. were infected with *Agrobacterium rhizogenes* 15834, A4, LBA 9402 (OD_{600} = 0,6) to obtain the hairy roots cultures. In this purpose two methods of transformation were used: by injection and co-culture. In the first method cultures of *A. rhizogenes* were injected with the use of the sterile needle to the plant explants and then the explants were incubated at 26°C for 24h on MS medium without antibiotic. Then explants were rinsed in 45 ml of sterilize water with 200µl carbenicillin (100 mg/L) and were 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/1. After 14 days of incubating these explants in the dark, they were cultivated in medium with 3% of sucrose and PPM (750µl/ 1L). After next two weeks explants were transferred to medium without antibiotics. Explants were transferred to fresh medium every week. After about 6 weeks the first hairy roots were formed at the wound sites. There were only in explants transformed with strain *Agrobacterium rhizogenes* 15834.

In the second method of co-culture of leaf explants with *Agrobacterium* strain, 50µL of bacterial suspension (OD600 = 0,6) and plant explants were placed in 25 ml of sterile distilled water and shaken for

48 hours at 26°C. Then the transformed explants were placed on medium described above.

The confirmation of T-DNA integration

The presence of *rolB* gene in plant genome was detected *via* PCR method. Genomic DNA was isolated according to Thermo Scientific Phire Plant Direct PCR kit. The sequence of primers used to select transformants were: F: 5'GCTCTTGCAGTGCTAGATTT3' and R:

5'GAAGGTGCAAGCTACCTCTC3' (for strain ATCC 15 834). The amplified fragment was 423 bp for 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 serve as a negative control and was used as template in PCR method, performed in the same conditions as described above.

Selection of optimal extraction method of bioactive compounds

Ultrasound extraction has been found to be the best method to isolate compounds from *Sarracenia purpurea* plants (unpublished data). Extracts were performed in an ultrasonic bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan, Lativia), equipped with a 150W (Bubalo at all., 2016). The optimization of extraction method of phenolic compounds and triterpenes was performed via Box- Behnken design (Panić at al., 2019). 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 variable, extracted phenolic compounds (TP) and triterpenes (TT) was investigated (Table 2). Based on Fernández et al. (2018), for optimization method fifteen experiments with three center points per block were performed. With a second- order polynomial equation was fitted the responses.

Table 2
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 (% v/v)	X_2	64	80	96
Temp. (°C)	X_3	25	4.5	60

For the analysis of ANOVA Design- Expert software (Version 7.0.0., Suite 48, Minneapolis, MN 55413) was used. Analysis of variance was used to obtain the quadratic polynomial mathematical model. The model was established to describe interaction of process parameters on extraction of total phenolics and

triterpenes (Panić et al, 2019). To predict model capability were used the model p and the value of determination (R^2).

Total polyphenol content

Total polyphenol content of prepared extracts was determined using the Folin-Ciocalteu method. Extracts were prepared by ultrasound extraction of 6 mg of plant tissue with 6 ml of 80% EtOH. Extraction was performed for 10 minutes at 64°C. 80% EtOH was used to dilute the samples. 250 µL of diluted samples and serial standard solutions, which was gallic acid were placed in glass test tubes. 1.25 ml of 10 times diluted Folin-Ciocalteu reagent was placed into the tubes. After 5 min of incubation in the darkness at room temperature, 1 ml of Na_2CO_3 (75 g/L) was added and incubated for 5 min (50°C, in the darkness). 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) (Debetić at all., 2020). All analyses were done in triplicate.

Total triterpenes content

Total triterpenes content of prepared extracts was determined using modified spectrophotometric method using sulphuric acid (Le at all, 2018). 50µL of plant extracts, reagent blank and standards were incubating 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) sulphuric acid. After incubation, the samples were cooled for 5 min and then the absorbance (560 nm) was measured.

HPLC analyses of betulinic acid

HPLC analyses were performed on the system Agilent 1200 series HPLC (Agilent, San Jose, CA, USA) with Phenomenex C18 column (Kinetex, 2.6µm, 100A, 150 x 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. Flow rate was 0.9 ml/ min. Before analysis, samples were filtered through polytetrafluoroethylene filters (0.22 µm). The autosampler temperature was kept at 4°C and the column 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.

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

Results

Transformation of Sarracenia plants

Two different methods were used for plant transformation: co-culture method of plant explants and *Agrobacterium rhizogenes* and alternatively injection of *A. rhizogenes* into plant tissues. Three different strains of *A. rhizogenes* were applied for plant transformation i.e. ATCC 15834, LBA 9402 and A4. The

transformed roots were however achieved only when injection technique was used for transformation and in the case of one strain of *A. rhizogenes* i.e. ATCC 15834, two other strains did not result in hairy roots formation. It should be pointed out that strain of *A. rhizogenes* 15834 was also described as the most effective for other plants e.g. flax (unpublished data), *Trapa natans* (Mikhaylova et al., 2020). Obtained composite *Sarracenia* plants were cultured in tissue cultures conditions and used for selection and characterization.

Analysis of rolB gene integration

The integration of *rolB* gene from Ri plasmid into plant genome was performed via PCR method as described in Materials and methods section. Among 70 analyzed roots the positive results were obtained for 7. Applied in PCR reaction primers amplified fragment of *rolB* gene of 423 bp (Fig. 2). The presence of this gene was confirmed for 7 transgenic lines of transformed roots and it was not detected in the genome of *Sarracenia* root, derived from control, untransformed plants. It should be pointed out that the integration of *rolB* gene is the most important in the transformation process (Sevón and Oksman-Caldentey, 2002).

Optimization of the extraction of bioactive compounds

The most efficient extraction of bioactive compounds (polyphenols) from obtained plants *Sarracenia purpurea* are: 10 minutes, 64% (v/v) EtOH and 60°C. The highest values of the phenylpropanoids and triterpenes were obtained in predicted conditions. Thus, the content of determined compounds under the established conditions were consistent with the assumptions resulting from Box- Behnken design (Table 3). All the extracts for determining the content of individual compounds were prepared under these optimal conditions.

Table 3
Values expected (A) and real (B) of the content of polyphenols and triterpenes from extracts prepared in optimal extraction conditions.

Variant	A [mg/ L]	B [mg/ ml]
Total polyphenols content	121.86	126.0606 ± 5.3569
Total triterpenes content	4.46	4.5737 ± 0.1552

Analysis of total polyphenols content

The increase in the content of total polyphenols was observed for all induced hairy roots when compared to untransformed roots (Fig. 3). The highest increase was noticed for two lines 7#1 and 7#2, for which 9-fold and 7- fold, higher polyphenols content was measured.

The stems of plants did not exhibit this tendency and the total polyphenols amount was not changed when compared to wild type plants.

Analysis of total triterpenes content

The highest level of total triterpenes content was observed for three lines of transformed roots i.e. lines 4#5, 7#2 and 7#1, and the increase was respectively 4, 7 and 9 fold in comparison to wild-type *Sarracenia* roots (Fig. 4). In contrast to the level of polyphenols, the amount of measured triterpenes high increase was determined for most stems and this increase was higher than observed for induced transformed roots. It should be pointed out that reason for this phenomenon is unknown, probably it may be the effect of infection of plants by *A. rhizogenes*. It is known that triterpenes remain compounds intermediating plant–pathogen interactions.It was also exhibited that triterpenes play a crucial role in plant defense system (Cárdenas et al., 2019) and in the allelopathy (Wang et al., 2014). Thus, it can be speculated that transformation of *S. purpurea* with *A. rhizogenes* caused the plant response to the pathogen attack.

Betulinic acid content

All analyzed composites plants were used for isolation and measurements of betulinic acid, the compound of antibacterial, antiviral and anticancer properties. The activity of betulinic acid against *Escherichia coli* and *Staphylococcus aureus* (Taralkar and Chattopadhyay, 2012), as well as anti-HIV and even antimalarial properties were described (Cichewicz and Kouzi, 2004). It should be pointed out that this compound exhibits also cytotoxic effect against tumor cells (melanoma) (Cichewicz and Kouzi, 2004; Weber et al., 2020) Five tested transgenic lines exhibited higher betulinic acid amount in leaves than in control *Sarracenia* plants (Fig. 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. These lines showed 70%, 62% and 66%, respectively, higher level of betulinic acid than wild-type, control plants. Transformed roots did not exactly show the same tendency and for two lines: 4#2 and 4#4 elevated amount of betulinic acid was noticed, for line 4#4 the highest amount was determined and it was 34% higher than in untransformed roots. Obtained results indicated that transformation of *A. rhizogenes* caused increase in the betulinic acid amount, which remains the compound with medicinal significance.

Conclusions

Herein, we described the transformation of *Sarracenia purpurea* L., insectivorous plants to generate composite plants with hairy roots. The obtained composite plants and wild-type differed in the aspect of biochemical composition i.e. the increased accumulation of phenolic compounds and triterpenes in the obtained hairy roots was noted. Transformation of plants with *Agrobacterium rhizogenes* 15834 may have a positive effect on the content of compounds with pharmacological potential in the composite plants. 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.

Declarations

Author Contribution Statement

KMP and MWK designed research and wrote ms. KP performed plant transformation, selection of hairy roots, performing compounds determination.MP and KMP analysis of polyphenol and triterpenes content and HPLC results. All the authors approved the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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Figures

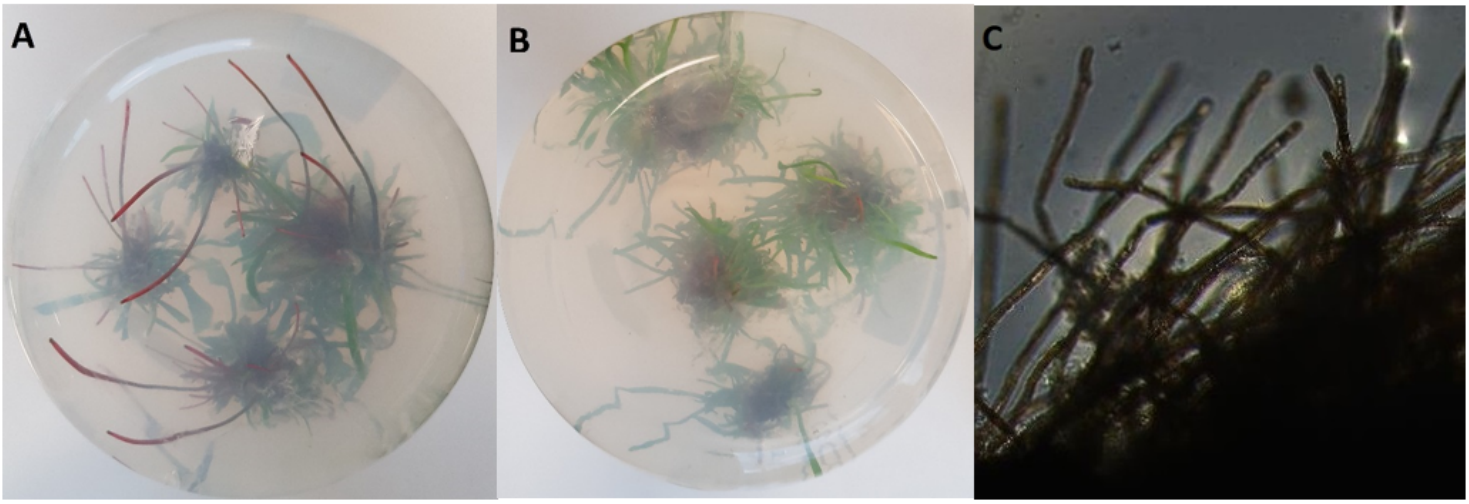

Figure 1

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). C. Microscopic analysis of Sarracenia transformed roots.

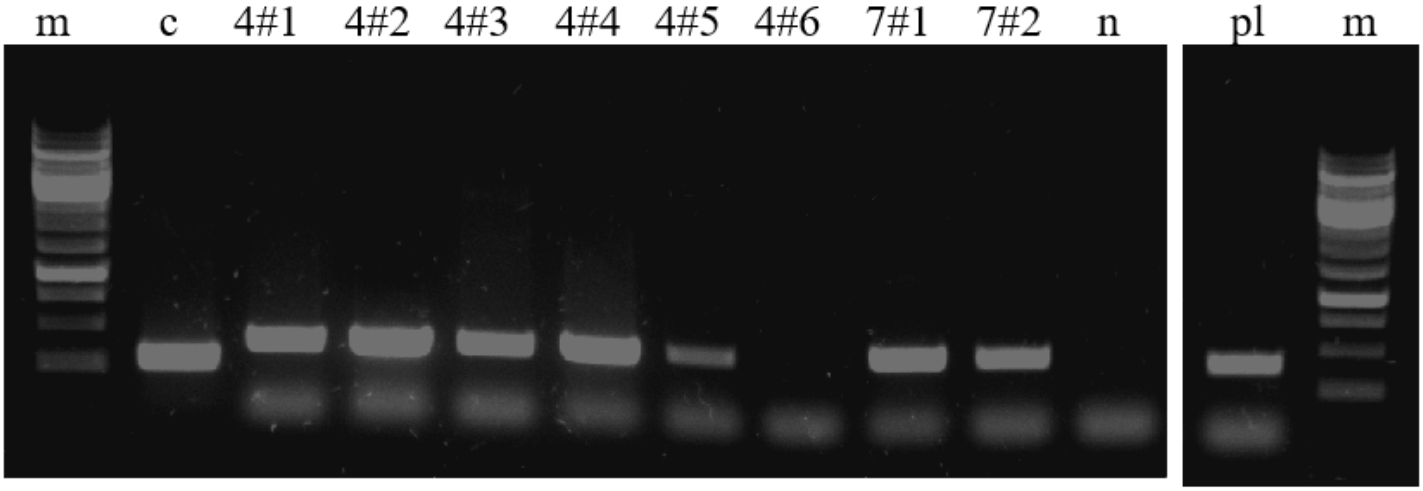

Figure 2

Selection of hairy roots performed via PCR method. The fragment of 423 bp of 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 plastid DNA (297 bp) amplified with specific primers provided by kit producer; m- marker 1 kb ladder.

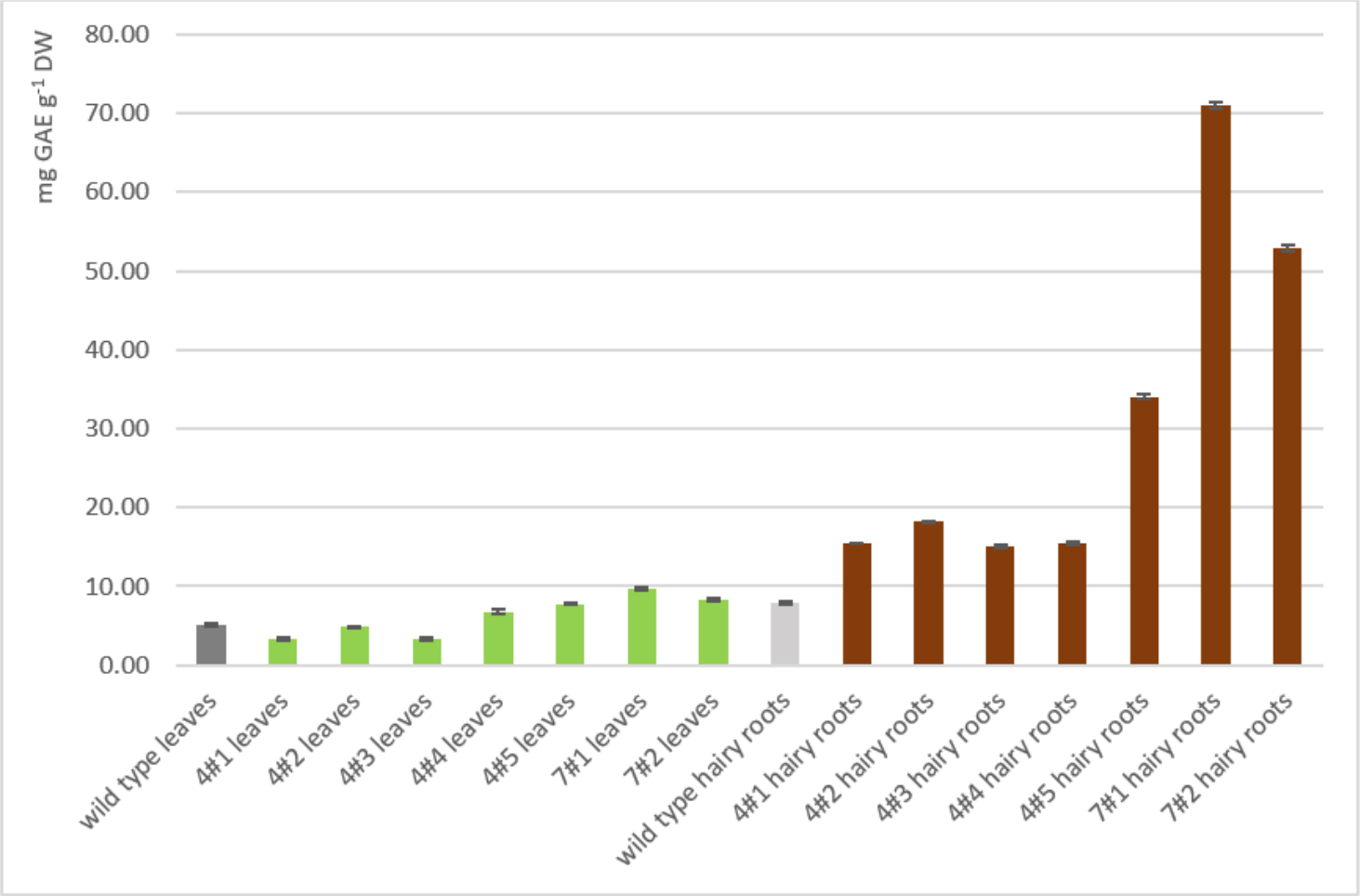

Figure 3

Total polyphenols content measured in hairy roots and composite plants of S. purpurea. The level of polyphenols were analysed as described in “Materials and methods” section. The data (±SD) was obtained from three samples per line.

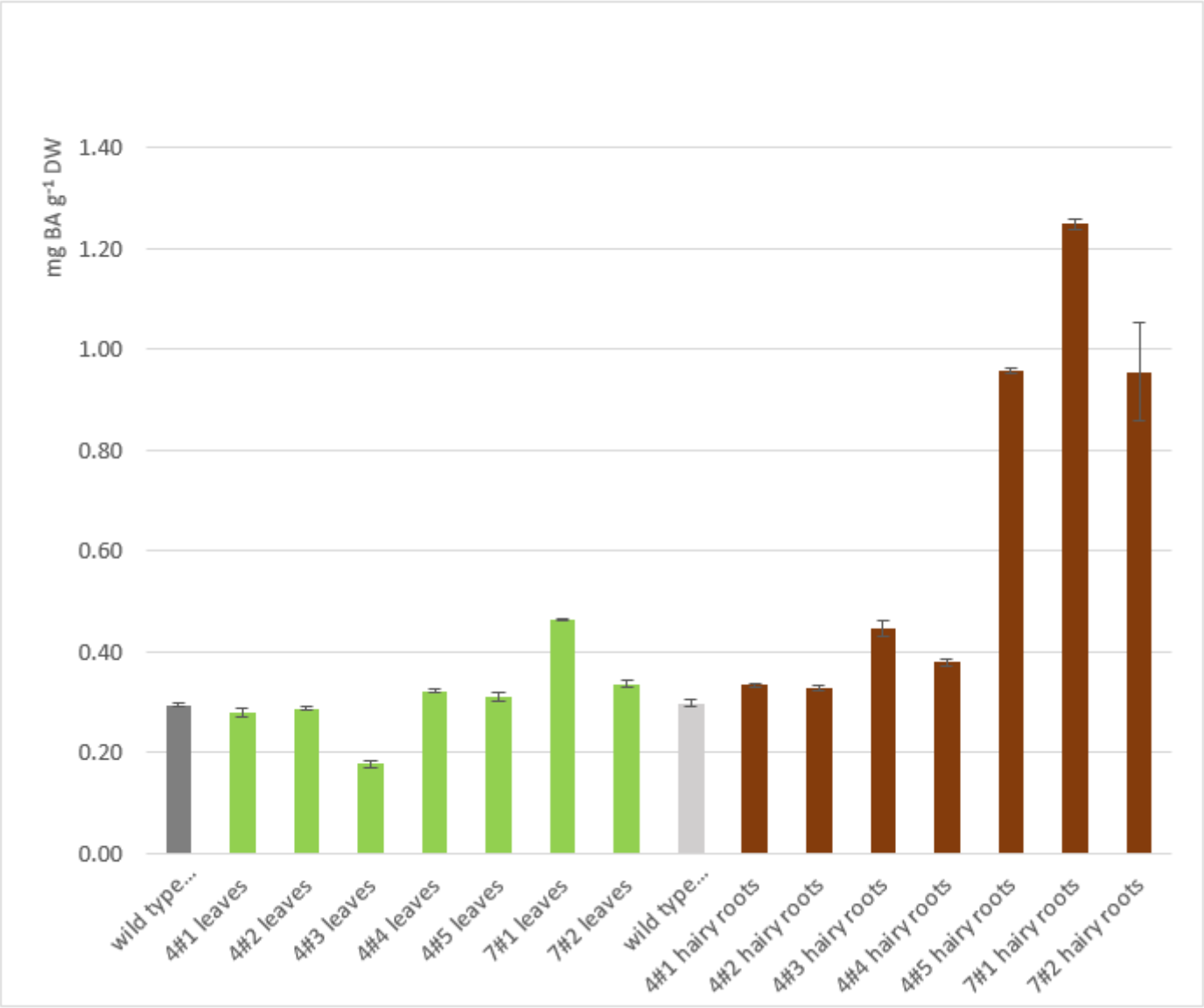

Figure 4

Total triterpenes content determined in the investigated hairy roots and composite plants of S. purpurea. The analyses were performed as described in “Materials and methods” section. The data (±SD) resulted from three samples per line.

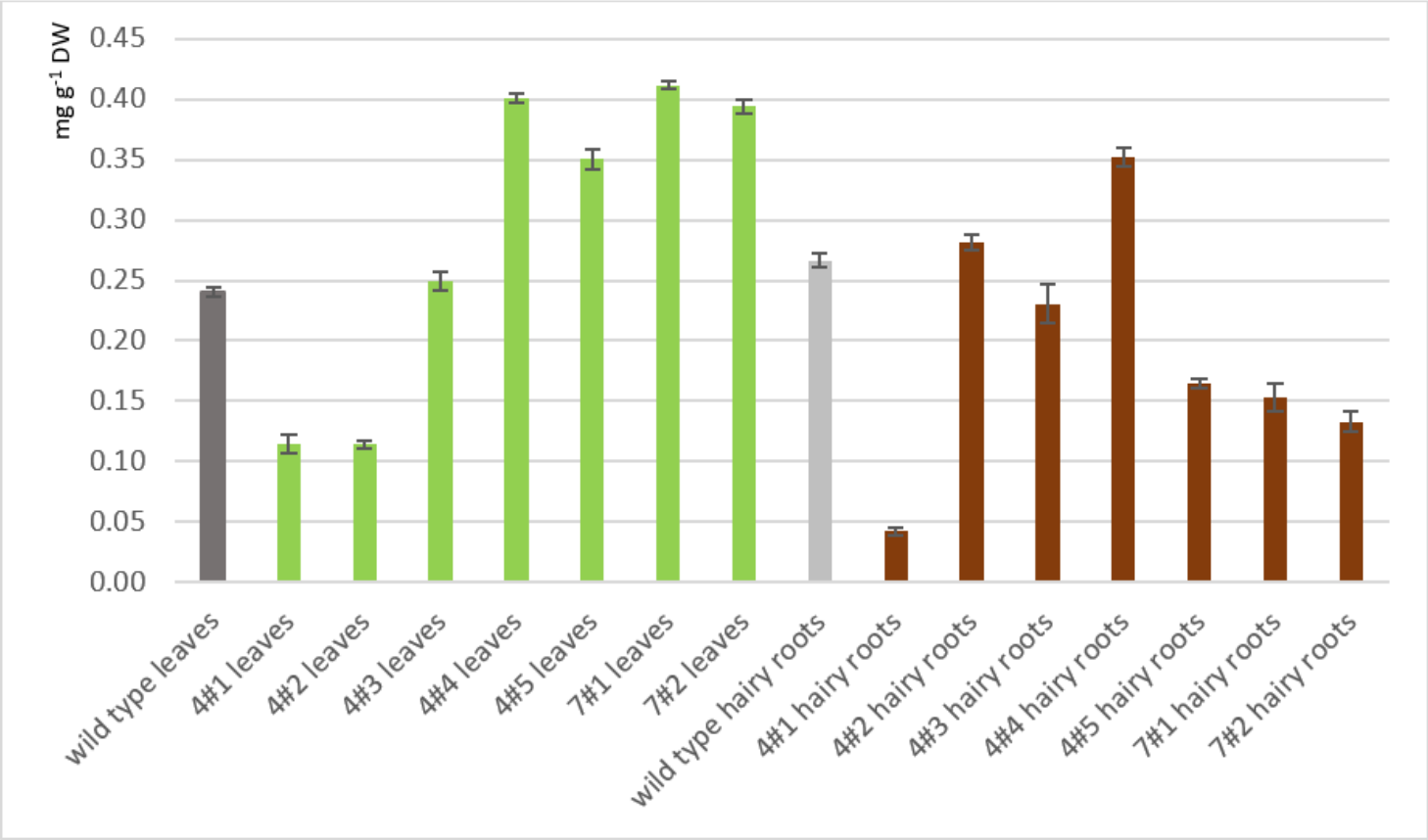

Figure 5

Betulinic acid content measured in the composite plants S. purpurea and in the investigated hairy roots by HPLC as described in section “Materials and methods”. The data (±SD) was obtained from three samples per line.