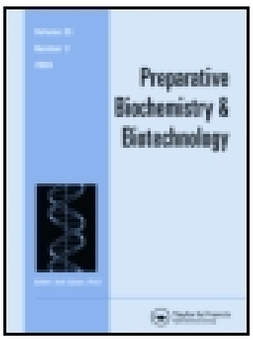

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Study of Artemisinin and Sugars Accumulation in *Artemisia Vulgaris* and *Artemisia Dracunculus* "Hairy" Root Cultures

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We studied the effect of genetic transformation on biologically active compounds (artemisinin and its co-products (ART) as well as sugars) accumulation in *Artemisia vulgaris* and *A.dracunculus* "hairy" root cultures. Glucose, fructose, sucrose and mannitol were accumulated in *A. vulgaris* and *A. dracunculus* "hairy" root lines. Genetic transformation has led in some cases to the sugars content increasing or appearing of non-relevant for the control plants carbohydrates. Sucrose content was 1.6 times higher in *A.vulgaris* "hairy" root lines. Fructose content was found to be 3.4 times higher in *A.dracunculus* "hairy" root cultures than in the control roots. The accumulation of mannitol was a special feature of the leaves of *A.vulgaris* and *A. dracunculus* control roots. *A. vulgaris* "hairy" root lines differed also in ART accumulation level. The increase of ART content up to 1.02 mg/g DW in comparison with the non–transformed roots (up to 0.687 mg/g DW) was observed.

Thus, *A. rhizogenes*-mediated genetic transformation can be used for obtaining of *A.*

vulgaris and *A. dracunculus* “hairy” root cultures produced ART and sugars in a higher amount than mother plants.

KEYWORDS: *Artemisia vulgaris*, *Artemisia dracunculus*, “hairy” root culture, sugars, artemisinin

INTRODUCTION

«Hairy» root culture is a type of plant tissue culture, which is considered to be an alternative way for producing valuable plant–derived compounds (1). It could be obtained *via* transformation by *Agrobacterium rhizogenes*, soil bacteria which naturally cause hairy–root disease in plants (2). *A. rhizogenes* genetically transforms its host by transferring DNA segment (T–DNA) carrying several oncogenes and opine catabolism genes from its root inducing (Ri) plasmid to the host cell genome. Such mechanism leads to the neoplastic growth of the transformed tissue. *Agrobacterium* cells can carry not only the resident *A. rhizogenes* Ri–plasmid containing the root locus *rol* genes responsible for root proliferation but also the wide range of other target genes.

«Hairy» root culture is characterized by unlimited and fast growth without use of exogenous hormones. In addition to above, the technology of *in vitro* cultivation is low cost because “hairy” roots are unpretending to the growth conditions and doesn’t require lighting, so they can be cultivated in bioreactors (3–4). In contrast to cell culture, “hairy” root culture is characterized by high genetic stability. Establishment of transgenic roots in some cases takes only a few weeks. The use of "hairy" roots as a bioreactor enables to

obtain compounds which are naturally produced in the mother plants as well as recombinant compounds for medical purposes. This implies that “hairy” root culture could attract particular attention for study of BAS production possibility.

Agrobacterial T–DNA insertion and corresponding expression of *rol* genes alter morphology and plant host secondary metabolism. This fact was described in experimental articles and discussed in reviews (5–11). *Rol* genes can excessively affect the growth and morphology of “hairy” roots, expression of genes and therefore accumulation of specific secondary metabolites. Thus genetic transformation may also affect plants metabolism resulting in BAS content increase.

Artemisia spp plants are known to synthesize biologically active compounds. These plants are used to treat malaria, they demonstrate antidiabetic, antiprotozoal, antiinflammatory anticoagulating, anticancer, antioxidant properties (12–14). For example, *A. annua* is listed in the Chinese Pharmacopoeia and is used as a remedy for malaria (15). *A. dracunculus* L. plants are used as an antidiabetic agent and anticoagulant (16). *A. vulgaris* plants posses analgesic, hepatoprotective antiinflammatory and spasmolytic activities (17–18).

Also, as other Asteraceae representatives, *Artemisia* plants produce fructans and inulin, used for diabetes treatment. However, recent biotechnological studies of *Artemisia* plants were mostly devoted to plant micropropagation (19–21) and artemisinin synthesis stimulation (22–24).

Study of the effect of transformation on biologically active compounds accumulation is of special interest because transgenic plants or "hairy" root culture can be used as a source of valuable substances for medical application. *A. rhizogenes*–mediated genetic transformation was conducted earlier for obtaining of "hairy" root cultures of some *Artemisia* genus representatives: *A.annua* (23, 25), *A.dubia and A.indica* (26), *A.aucheri* (27), *A.vulgaris* (28), *A. absinthium* (29). The aim of these investigations was to study the effect of different genes transfer on cell metabolism and artemisinin accumulation in the "hairy" root cultures. Nevertheless, there are no publications regarding fructans and monosaccharides content in *in vitro* cultivated *Artemisia* plants and "hairy" roots.

Thus, the aim of this work was to study artemisinin accumulation as well as sugars content in *in vitro* cultivated *Artemisia vulgaris* and *Artemisia dracunculus* plants and to compare the content of these compounds in "hairy" roots obtained after *A.rhizogenes*–mediated transformation.

EXPERIMENTAL

Plant Material

A. vulgaris and *A. dracunculus* "hairy" root cultures were used for experiments (Figure 1). They were obtained earlier (30, 31) and were cultured on hormone free half strength Murashige and Skoog (½ MS) (32) basal medium. One month cultivated "hairy" roots were lyophilized and mashed into powder. Lyophilized leaves and roots of non-

transformed plants cultivated in *in vitro* conditions on the 1/2MS medium were used as a control.

Preparation Of The Extracts

Dried milled samples were used to make extracts for carbohydrates and artemisinin content measurement.

The samples for analysis of carbohydrates content were prepared according to the method as described by Guerrant and Moss (1984) (33).

The samples for analysis of artemisinin content were prepared according to Lapkin et al. (2009) (34). The plant material was macerated using ethyl acetate solvent in 1/40 ratio. The duration of extraction was 20 minutes. The mixture was filtered using vacuum pump. Solution was evaporated using SpeedVac Savant AES 2010 concentrator (Labconco, USA). Precipitate was dissolved in 2 ml of acetonitrile. This solution was used to determine content of artemisinin using HPLC method.

Determination Of Carbohydrates Content

Determination of carbohydrates content was performed by gas liquid chromatography using "Chrom–5" chromatograph (Czech Republic) with flame ionization detector. Temperature of column was in range 180–235°C with 3°C min^{-1} temperature gradient. Helium was chosen as a carrier gas, the flow rate was 20 ml min^{-1}, glass column with a diameter of 3 mm, length 120 cm, phase 3% of GP 100/200 Supelcoport (manufacturer

Supelco, USA) was used. Ethyl acetate was chosen as a solvent. The identification was performed by retention time of samples' relative to the standard's retention time (mixture of monosaccharides).

Determination Of Artemisinin Content

Artemisinin and its co-products (ART) content analysis was performed using HPLC–MS system. Chromatographic analysis was performed using equipment of Centre of collective usage of Zabolotny Institute of Microbiology and Virology NAS of Ukraine (High–efficiency liquid chromatograph Agilent 1200, Agilent Technologies, USA). The separation was carried out by isocratic method using ZORBAX SB–C18 2,1 mm × 150 mm, 3,5 µm (Agilent Technologies, USA) column with a 30°C. Water with methanol and acetonitrile (30/20/50 v/v) was used as a mobile phase with 0,4 ml min^{-1} flow–rate. Diode array detector (DAD) wavelength used was set to 210, 280 and 216 nm respectively. The samples were analyzed separately according to their retention times. The spectrum of samples was compared with the spectrum of artemisinin standard (Sigma–Aldrich, catalog number 63968–64–9).

Statistical analysis was performed according to Student multiple range test. Differences from control values were significant at $p \leq 0.05$. Experiment was conducted in three replications. Statistical analysis (ANOVA, Tukey's test etc) was performed using built-in functions of R software environment (35).

RESULTS AND DISCUSSION

Carbohydrates And ART Accumulation In The Non–Transformed Plants Cultivated In Vitro

Leaves of *A.vulgaris* and *A. dracunculus* control plants were characterized by the accumulation of glucose, fructose and sucrose (Figure 2). Fructose content was higher in leaves of *A. dracunculus* control plants – up to 61.7±0.21 mg/g DW, while roots of control plants accumulated fructose in amount only up to 14.235± 0.24 mg/g DW. The high glucose content was also found in leaves of *A. dracunculus* control plants – 31.29±0.45 mg/g DW (as compared with 13.35±0.12 mg/g DW in roots). Contrasting to written above fructose, glucose and sucrose content was higher in roots of *A.vulgaris* than in leaves. These compounds accumulated in roots of *in vitro* cultivated plants in 20.93±0.31 mg/g, 17.37±0.26 mg/g and 25.48±1.14mg/g DW concentration respectively. At the same time fructose, glucose and sucrose content in *A.vulgaris* leaves was 14.71±0.28 mg/g, 10.15±0.1 mg/g and 12.27±1.39 mg/g DW respectively.

The accumulation of mannitol was found to be the special feature of the leaves of *A.vulgaris* and also *A. dracunculus* control roots. Mannitol content varied from 0.63±0.007 mg/g DW to 0.70± 0.01mg/g DW.

Inulin was found out both in the roots and leaves of *A.vulgaris* and *A. dracunculus* (Figure 3). The amount of this compound was up to 114.5±2.72 mg/g and 44.8±0.59 mg/g DW in roots and leaves of *A.vulgaris.* However, the higher content was surprisingly found in *A. dracunculus* leaves as compared with the content of this compound in roots.

This feature is peculiar because inulin is known to be accumulated mostly in the roots of the plants (36, 37). However, this difference wasn't statistically proved ($p \geq 0,3$).

Thus, roots and leaves of *in vitro* cultivated non–transformed *A. vulgaris* and *A. dracunculus* plants differed in carbohydrates content. Carbohydrate content varied depending on plant type, growing location and weather conditions. The content differs also in roots and leaves because carbohydrates synthesis starts in leaves and frequently these sugars accumulate just in plant roots as a storage compounds (38).

Also, *A. vulgaris* and *A. dracunculus* i*n vitro* cultivated plants were found to accumulate ART both in leaves and roots of the control plants. However, despite of the fact that HPLC-MS method offers great resolution, accurately qualifying and quantitating substances still can be difficult if multiple components elute approximately at the same time, such as artermisinin and deoxyartemisinin. Thus, our investigation may overestimate artemisinin content due to the co-elution of deoxyartemisinin and other artermisinin co-products. It should be noted, that in our work we determined artermisinin and its co-products (such as deoxyartemisinin), but not artemisinin itself.

ART content in the leaves of *A.vulgaris* was much higher than in the roots – up to 1.53 and 0.69 mg/g DW respectively. This compound accumulated in small amount up to 0.14 mg/g DW also in *A. dracunculus* leaves. At the same time the content of the one in the roots of these plants was much more and reached 2.47 mg/g DW. So, ART accumulation

in roots of *in vitro* cultivated *A. dracunculus* was surprisingly higher than in the leaves of this plant ($p \leq 0{,}02$).

Artemisia spp. plants are well known as the artemisinin synthesizing plants. Accumulation of this compound was studied in the leaves of *in vivo* grown *A. annua* (1.4% DW) (39), (0.03–1.5%) (40). Artemisinin was detected in *Artemisia apiacea* and *Artemisia lancea* (41), *Artemisia cina* (42), *Artemisia absinthium* (callus tissue) (43), *Artemisia dubia* and *Artemisia indica* (26) and in aerial parts of *Artemisia sieberi* (44). In the investigations of Mannan et al. (2010) (45) the artemisinin content in 17 *Artemisia* app was compared. Authors demonstrated that the highest artemisinin concentration was detected in the leaves (0.44 ± 0.03%) and flowers (0.42 ± 0.03%) of *A. annua*, followed by the flowers (0.34 ± .02%) of *A. bushriences* and leaves (0.27 ± 0%) of *A. dracunculus var dracunculus*. According to the authors, the average concentration of artemisinin varied in the order of flowers > leaves > stems > roots. Several studies have shown that artemisinin synthesis occurs in the glandular trichomes (GLTs) present on flowers, floral buds and leaves (46). That is the reason why roots of *A.vulgaris* plants store less artemisinin. Our research has come in agreement with previous researches and has proved that aerial part of *A. vulgaris in vitro* cultivated plants accumulated more artemisinin than the roots. The difference in artemisinin content between aerial parts and roots was twofold in our study. As for *A. dracunculus*, accumulation of artemisinin in roots isn't usual for *Artemisia* species. Our results haven't come in agreement with other investigations devoted to artemisinin accumulation in *A. dracunculus* plants. The fact that recent studies have shown that *A. dracunculus* leaves accumulate artemisinin in greater

amount than roots can be explained by the fact that all previous experiments (26) were applied to *in vivo* plant material, while in our study we used *in vitro* cultivated plants. So, it may be a peculiarity of *in vitro* cultivated *A. dracunculus* plants.

Content Of Carbohydrates And ART In A.Vulgaris And *A. Dracunculus "Hairy" Roots*

"Hairy" root cultures both *A.vulgaris* and *A. dracunculus* were characterized by the accumulation of glucose, fructose, sucrose and mannitol. The content of the compounds varied in a wide range. Variability of carbohydrates content was observed in *A.vulgaris* transformed lines. Particularly, glucose and fructose content varied in the range 3.8±0.17 – 10.5±0.1 mg/g DW and 3.1±0.07 – 12.08±0.07 mg/g DW respectively. The content of glucose and fructose in transgenic roots of *A.vulgaris* was lover than in the control plants (both leaves and roots). At the same time the content of sucrose in 40% of "hairy" root lines exceeded the one in the control (Figure 2). *A.vulgaris* "hairy" root lines were characterized by mannitol accumulation varied in a wide range in transformed lines: from 0.65±0.04 mg/g DW up to 18.72±0.20 mg/g DW which was almost 30 times higher than in leaves of *in vitro* cultivated *A.vulgaris* non–transformed plants (0.68±0.024 mg/g DW). This substance was not found in the roots of the control plants.

A. dracunculus transgenic roots were found to accumulate glucose, fructose, sucrose and mannitol. Glucose content in "hairy" root lines was 12.45± 0,09 – 12.93± 0.09 mg/g DW, which is similar to the content in control roots (13.35±0.124 mg/g DW). Fructose content was found to be 3.4 times higher than in the control roots. Galactose in a small amount,

0.69±0.06 – 0.9±0.07 mg/g DW, was also detected in "hairy" roots but it was not detected in *A. dracunculus* control roots and leaves. Statistical differences in mannitol content in control and transformed roots (0,63±0.007 and 0,68±0.05 mg/g DW respectively) weren't found.

Inulin content varied in *A.vulgaris* "hairy" roots from 12.9±0.203 to 106.39±4.22 mg/g DW. Transgenic roots accumulated inulin in amount lower than the roots of the control plants ($p \leq 0.001$). So, the genetic transformation has led to decrease of inulin content in *A.vulgaris* "hairy" root lines compared to the control roots. At the same time inulin was accumulated in some *A. dracunculus* transgenic root lines in higher amount than in the control roots, 73.91±1.91 mg/g DW and 27.74±0.54 mg/g DW respectively ($p \leq 0,02$).

We studied earlier artemisinin content in *A. vulgaris* plants and "hairy" root culture (30). We studied now the accumulation of artemisinin and its co-products in *A. dracunculus* plants and "hairy" roots and compared the results with the data obtained in previous experiments. ART in transgenic *A. dracunculus* roots varied in range 0,554 mg/g DW up to 1,056 mg/g DW and was lover than in the control roots but higher than in the leaves of *in vitro* grown control plants ($p \leq 0,05$) (Figure 4).

In case of both species changes in ART content in transgenic root lines didn't depend on the vector used. It is known that *Agrobacterium*–mediated genetic transformation and *rol* genes transfer can be resulted in variation in accumulation of different compounds natural for the plants. For example Dilshad E et al. (2015) has shown that *A. vulgaris*

transformants harboring the *rol B* and *rol C* genes were tend to accumulate more artemisinin (up to 10–fold) compared to the non–transformed plants. Similar findings were reported by another group who demonstrated *rol* genes induced ginsenoside overproduction in transformed cell cultures of *P. ginseng*. *Rol C* transformants were found to accumulate 1.8–3–fold more ginsenoside than control plants, although *rol B* lines were less productive. In contrast, in another study, *rol B* gene transgenics of *Rubia cardifolia* showed enhanced production of anthraquinones (47).

In our study genetic transformation have led to reducing of ART content in some cases as well as to increasing of the latter one. We cannot reconcile the results of our current study with other reports because of the lack of studies of artemisinin content in *A. vulgaris* and *A. dracunculus* "hairy" roots.

So the genetic transformation of *A. vulgaris* and *A. dracunculus* has led to the alteration of the content of sugars and artemisinin in transgenic roots. This alteration resided in the appearance of non–relevant compounds for example synthesis of mannitol in transgenic roots of *A.vulgaris* and synthesis of galactose in *A. dracunculus* "hairy" roots. The discovered changes were also demonstrated in the amount of compounds accumulated in "hairy roots" comparatively to their amount in the non–transformed roots. Increase of artemisinin and carbohydrates content was observed in some "hairy" root lines. For example the amount of sucrose in some *A.vulgaris* "hairy" root lines was 1.3–1.6 higher than in the control. Thus, *A. rhizogenes*-mediated genetic transformation can be used for

obtaining of *A. vulgaris* and *A. dracunculus* "hairy" root cultures produced ART and sugars in a higher amount than mother plants.

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Figure 1. *A. vulgaris* (a, c) and *A. dracunculus* (b, d) *in vitro* cultivated plants and "hairy" root cultures.

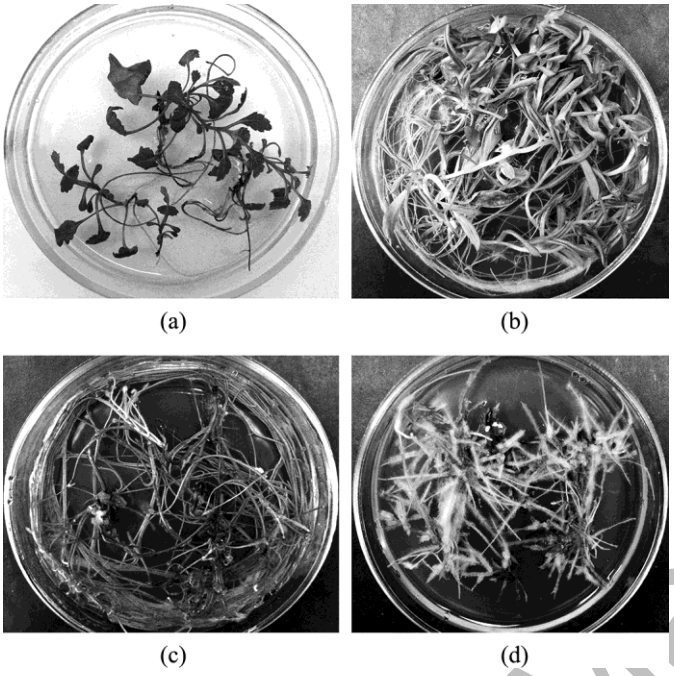

Figure 2. Sugars content (Glu - glucose, Gal - galactose, Mannitol, Sacch - sucrose and Fru - fructose) in *A.vulgaris* non-transformed plants (1 – control roots, 2 – leaves) and *A. dracunculus* plants (3 – control roots, 4 - leaves) and "hairy" root transgenic lines (5-11 *A. vulgaris* "hairy" roots; 12-13 *A. dracunculus* "hairy" roots).

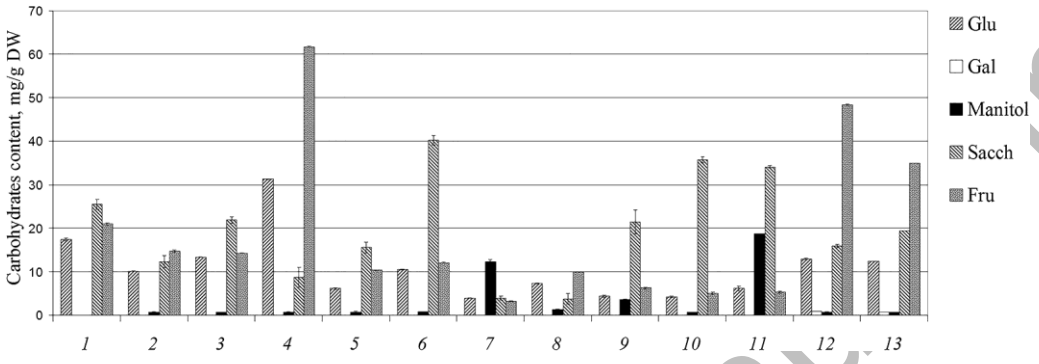

Figure 3. Inulin content in *A.vulgaris* non-transformed plants (1 – roots, 2 – leaves) and *A. dracunculus* plants (3 – roots, 4 – leaves) and “hairy” root lines (5-11 – *A. vulgaris* “hairy” roots; 12-13 – *A. dracunculus* “hairy” roots).

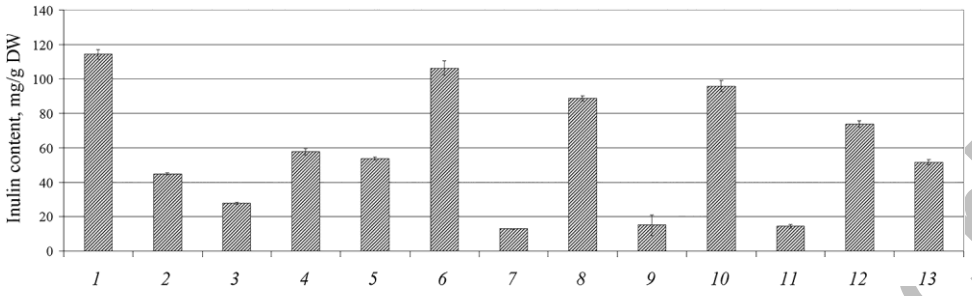

Figure 4. HPLC traces of artemisinin content obtained for A – non-transformed roots of *A.vulgaris* plants; B – *A.vulgaris* “hairy” root culture ; C – non-transformed roots *A. dracunculus* plants ; D – *A. dracunculus* “hairy” root culture.