

***Agrobacterium rhizogenes*-mediated transformation of *Hypericum sinaicum* L. for the development of hairy roots containing hypericin**

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Hypericum sinaicum L. is an endangered Egyptian medicinal plant of high importance due to the presence of naphthodianthrone (hypericins), which have photodynamic properties and pharmaceutical potential. We sought to assess *H. sinaicum* ability to develop hairy roots that could be cultured in contained conditions *in vitro* and used as a source for hypericin production. We used four *A. rhizogenes* strains differing in their plasmids and chromosomal backgrounds to inoculate excised *H. sinaicum* root, stem and leaf explants to induce hairy root development. Additionally, inoculum was applied to shoots held in Rockwool cubes supporting their stand after removal of the root system. All explant types were susceptible to *A. rhizogenes* although stem explants responded more frequently (over 90%) than other explant types. The A4 and A4T *A. rhizogenes* strains were highly, and equally effective in hairy root induction on 66-72% of explants while the LBA1334 strain was the most effective in transformation of shoots. Sonication applied to explants during inoculation enhanced the frequency of hairy root development, the most effective was 60 s treatment doubling the percentage of explants with hairy roots. However, shoot transformation was the most effective approach as shoots developed hairy roots within 10 days after inoculation. Molecular analyses confirmed that the established hairy root cultures *in vitro* were indeed obtained due to a horizontal gene transfer from bacteria. These cultures grew fast and the hypericin content in hairy roots was about two fold higher than in *H. sinaicum* plants as determined by HPLC.

Keywords: *In vitro* culture. Medicinal plant. Naphthodianthrone. Plant genetic engineering.

INTRODUCTION

The genus *Hypericum* belonging to the *Hypericeae* family comprises approximately 450 species of trees, shrubs and herbs found in different temperate regions in the world (Mártonfióvá *et al.*, 2014). Some *Hypericum* species like *H. perforatum* (St. John's wort) have been recognized as valuable medicinal plants. In particular, *Hypericum* species have attracted attention due to the

content of unique bioactive constituents like hypericin and hyperforin classified to naphthodianthrone and phloroglucinols, respectively. These compounds exhibit a significant range of pharmacological activities and the most important are their wound-healing, anti-inflammatory, diuretic and sedative properties. Hypericin has been also used to treat neurological disorders and traumas. Additionally, its antiviral, antibacterial and antitumor effects are being thoroughly evaluated (Wölflé, Seelinger, Schempp, 2014). Hypericin is a hydroxylated phenanthroperylenequinone, a fluorescent red pigment and one of the strongest known plant derived photosensitizer. It induces photocytotoxic activity *in vivo* and *in vitro* and

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displays potential as a photosensitizer for photodynamic therapy (Karioti, Bilia, 2010). The contents of hypericin and pseudohypericin vary from species to species (0.03-0.3%) and is sometimes also found to vary within the same species, depending on the plant developmental stage, growing location or harvesting time (Karioti, Bilia, 2010). In some taxa *H. hirsutum* and *H. empetrifolium* only hypericin can be found (Kitanov, 2001) while some other species such as *H. boissieri*, *H. barbatum*, *H. rumeliacum* may contain a 2-4 fold higher amount of hypericins than *H. perforatum* (Karioti, Bilia, 2010).

In Egyptian folk medicine, another hypericin containing species i.e., *H. sinaicum* L. has been used. This is the endemic species in Sant Katherin Protectorate South Sinai, Egypt also found in North Saudi Arabia that was included in the IUCN red list of threatened plants in 1994 (Boulos, 2002). *H. sinaicum* is a herbaceous perennial *H. sinaicum* reproduced by seeds and rootstocks and there has been a growing interest to cultivate this plant commercially (Khafagi, Hatab, Omar, 2012). However it is a small plant that grows slowly thus the herb production is low (Omar 2014). Production of valuable secondary metabolites in bioreactors may be considered as an alternative and several successful applications have been demonstrated (Li *et al.*, 2008).

Phytopharmaceutical preparations of *Hypericum* are usually produced from *in vivo* grown plants but the limited area of the plant existence, harvesting season, loss of biodiversity, variability in quality, and contamination issues, trigger to search for alternative methods of hypericin production. In phytopharmaceutical industry, one solution could be the utilization of micropropagated plants derived in aseptic conditions (Omar, 2014). In our previously studies we showed that *H. sinaicum* seeds were able to germinate *in vitro* and the process was more efficient when using the MS medium (Murashige, Skoog, 1962) with full salts strength in comparison to other nutrient media. The ability to micropropagation depended on the treatment with plant growth regulators and nodal or root explants cultured for 3 weeks were the most suitable for callus production and regeneration to shootlets (Khelifa *et al.*, 2016a, b).

Several studies have been carried out to investigate the influence of various factors on the accumulation of secondary metabolites in *Hypericum* cultures carried out *in vitro* (Bivadi *et al.*, 2014). Attempts were made to generate populations producing and characterized by maximum concentrations of these bioactive compounds, either *in vivo* or *in vitro*

cultures. Also *H. sinaicum* has been assessed for its ability to produce hypericin *in vitro*. Chemical analysis confirmed hypericin presence in callus and in shootlets regenerated indirectly from nodal explants. These materials contained more hypericin than root segments (Khelifa *et al.*, 2016a, b).

Modification of a plant genome through genetic transformation has been shown to be an indispensable tool for crop improvement, investigation of plant functional genomics, genome editing, and synthetic biology. (Hwang, Galvin, Lai, 2015). This approach can be also utilized for the production of bioactive compounds either through redirecting or modifying biosynthesis pathways (Komarovská *et al.*, 2009). Hairy roots can accumulate or elicit large quantities of desired compounds as the effect of enhanced biosynthesis of secondary metabolites due to turning on the transcription of plant defense genes (Ono, Tain, 2011). Hairy roots develop as a result of *Agrobacterium rhizogenes* infection of injured plants. *A. rhizogenes* is a well known, soil born, gram negative bacteria possessing a root inducing (Ri) plasmid harboring two transfer (TR and TL) DNA fragments with several sequences of rol and aux loci. These genes are transferred from bacteria to plant cells via a horizontal gene transfer where they are responsible for hairy root morphological and physiological alterations (Komarovská *et al.*, 2009; Koperdakova *et al.*, 2009a). In controlled laboratory conditions, hairy root induction can be stimulated by tissue wounding and subsequent inoculation with *A. rhizogenes*. The developing roots are characterized by the ability to fast growth due to elevated amounts of endogenously synthesized auxins, disturbed geotropism and usually easy to distinguish hairy phenotype (Koperdaková *et al.*, 2009b). Hairy roots excised from the source tissue retain their vigorous growth and exhibit extensive lateral branching when cultured on growth regulator-free media, and can be easily maintained in *in vitro* culture using either a solid or liquid medium (Li *et al.*, 2008). They also show potential for stable production of various bioactive compounds that can be extracted if desired (Tusevski *et al.*, 2013). Thus plant genetic engineering resulting from the transfer of Ri T- DNAs has been found as an effective indirect way of induction and high level accumulation of plant secondary metabolites using contained systems (Abdi *et al.*, 2017). Induction of hairy roots using transgenesis approach is thus crucial, in particularly for medicinal plants as cultured hairy roots may be a convenient source of industrially important metabolites used in high quality medicinal products (Bafana, Lohiya, 2013).

Until now, only *A. rhizogenes* (Komarovská *et al.*, 2009) and biolistic-mediated (Franklin, Oliveira, Dias, 2007) transformation procedures for a few *Hypericum* species have been applied. In the first successful transformation of *H. perforatum* wild agropine strain *A. rhizogenes* ATCC 15834 was used (Di Guardo *et al.*, 2003). Also, an efficient transformation protocol of this species was reported with *A. rhizogenes* A4M70GUS (Vinterhalter *et al.*, 2006). Prior reports indicated that *A. rhizogenes* strains and types of explants significantly influenced the induction of hairy roots (Sudha *et al.*, 2012). In addition, *A. rhizogenes* strain A4-mediated transformation system applied to *H. perforatum* led to the development of hairy root cultures amenable for subsequent shoot regeneration. These transgenic shoots produced naphthodianthrone compounds, and the concentration of hypericin and pseudohypericin was about 12-fold higher than in not transgenic control (Bivadi *et al.*, 2014). However, only few studies have been focused on secondary metabolite production in *H. perforatum* hairy roots (Tusevski *et al.*, 2013) and plants regenerated from them (Koperdákóvá *et al.*, 2009). Two other *Hypericum* species (*H. tomentosum* and *H. tetrapterum*) were also successfully transformed with *A. rhizogenes* ATCC 15834 and A4 (Komarovská *et al.*, 2009).

To our best knowledge, there is no reports showing successful induction and culture of hairy roots in *H. sinaicum*. In this work we evaluate *H. sinaicum* ability to develop hairy roots that can be considered as potential source of hypericin. We demonstrate susceptibility of *H. sinaicum* explants to *A. rhizogenes* and compare conditions promoting hairy root induction, including the selection of bacterial strain, sonication time, explant type and method of inoculation. The results presented here indicate for the first time that hypericin-rich hairy roots can be obtained and cultured *in vitro*. The developed method can be helpful in studying regulatory aspects involved in the biosynthesis of important bioactive metabolites in this species.

MATERIAL AND METHODS

Agrobacterium rhizogenes strains

Three *A. rhizogenes* strains A4T (derivative of C58 with pRiA4) (McInnes *et al.*, 1989), A4 (pRiA4) (Moore, Warren, Strobel, 1979), and LBA1334 (derivative of C58 with pRi1855) (Visser *et al.* 1989) were used. All strains possessed the binary plasmid pBIN-m-gfp5-ER (Haseloff *et al.* 1997) with T-DNA containing the neomycin

phosphotransferase (nptII) gene under the nos promoter control. Additionally, strain A4T, hereinafter denoted as A4T-GUS, harboring the pCambia1301 binary plasmid (Hajdukiewicz, Svab, Maliga, 1994) was used. The plasmid T-DNA contained the hygromycin (hptII) resistance gene and the β -glucuronidase (uidA-int) gene with the castor bean catalase intron preventing glucuronidase expression in bacteria, both genes were under the CaMV 35S promoter control. All plasmids possessed kanamycin resistance gene expressed in bacteria.

A. rhizogens inoculum

A. rhizogenes strains were cultured on a solidified lysogeny broth (LB) containing 50 mg/L kanamycin and 50 mg/L rifampicin at 28 °C. Single colonies were used to inoculate 30 mL of liquid LB containing 50 mg/L kanamycin and were incubated at 28 °C using a rotary shaker set to 200 rpm. An overnight culture was centrifuged at 8000 rpm for 10 min. The pellet of *Agrobacterium* cells was re-suspended in 2 mL of liquid Murashige and Skoog macro- and microelement medium with vitamins (Murashige, Skoog, 1962), and 30 g/L sucrose, pH=5.8 (MS). The suspension was diluted to OD600 = 0.5 after the measurement of its optical density using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The final inoculum was enriched with 100 μ M acetosyringone (Sigma, St. Louis, USA).

Plant material

Seeds of *Hypericum sinaicum* were obtained from Sant Katherin Protectorate South Sinai governorate, Egypt. After surface sterilization they were placed on a solidified MS medium and incubated for four weeks essentially as described before (Khlifa *et al.*, 2016a). Then the seedlings were transferred to a fresh MS medium (one seedling per glass jar) and they were grown for the next 6 weeks. Tobacco (*Nicotiana tabacum*) *in vitro* grown plants on the same medium and in the same conditions as *H. sinaicum* was used as the reference material for transformation experiments.

Transformation of explants exposed to a solid mineral medium

Leaves, nodal segments and root sections were cut and transferred to sterile Petri dishes. Leaves were cut into approx. 5 mm sections. Nodal segments and roots were

cut into approx. 15 mm long fragments and were gently wounded with a scalpel. Explant sections were immersed in *A. rhizogenes* inoculum in 50 mL Falcon tubes and exposed to sonication treatments for 15 s, 30 s, or 60 s using an ultrasonic bath (Polsonic, Warsaw, Poland). Then the explants were shortly blotted on a sterile filter paper and transferred to a solidified, growth regulators-free MS medium with 100 μ M acetosyringone and co-cultivated at 25 \pm 2 $^{\circ}$ C in the dark for two days. Then they were transferred to a fresh MS medium with 1mg/L IBA, 400 mg/L cefotaxime to eliminate bacteria and 50 mg/L kanamycin or 25 mg/L hygromycin for the selection of transgenic events, and cultured for four weeks at the same conditions. Explants treated with bacteria-free inoculum were considered as a negative control.

Transformation of shoots held in Rockwool cubes

Sterile Rockwool cubes type AO36/40 (Hummert International, Earth City, USA), commonly used for seed and cutting propagation, were punctured with a pipette tip to its $\frac{3}{4}$ depth, placed in a glass jar and soaked with 4 mL of inoculum. Single shoots were inserted in to the cube wholes after roots were cut-off from plants already grown *in vitro*. The jars were left open allowing dehydration for a few hours until the first wilt symptoms were noticed. Then cubes were saturated with MS medium and the closed jars were placed in a growth chamber at 25 \pm 2 $^{\circ}$ C and 16/8 h photoperiod. Rockwool cubes were checked periodically and MS medium was added when necessary during the culture period. After the appearance of roots, MS medium with addition of 400 mg/L cefotaxime, 50 mg/L kanamycin or 25 mg/L hygromycin, and 1 mg/L IBA was added when necessary to ensure medium excess in the jar, and the plants were

cultured for further two weeks. MS medium without kanamycin and hygromycin was used for control shoots treated with bacteria-free inoculum.

Hairy root culture

Hairy roots were excised from transformed explants or plants, placed in Petri dishes containing MS medium supplemented with 1mg/L IBA and 400 mg/L cefotaxime, 50 mg/L kanamycin or 25 mg/L hygromycin, and incubated at 25 \pm 2 $^{\circ}$ C in the dark. Sub-cultures were done every three weeks by transfer to fresh medium. Control, non-transformed roots excised from Rockwool grown shoots were cultured on the same medium free of kanamycin and hygromycin. Additionally, 0.5 g of hairy root samples were transferred to 100 mL Erlenmeyer flasks containing 30 mL MS liquid medium supplemented as described above. Root cultures were maintained on a rotary shaker set to 200 rpm at 25 \pm 2 $^{\circ}$ C in the dark.

PCR

PhireTM Plant Direct PCR kit (Thermo Fisher Scientific, Waltham, USA) was used to perform direct PCR according to the producer's protocol. Aliquots of 0.5 μ L were used as DNA templates and the PCR reactions were carried out in the final volume of 20 μ L. Three primer pairs were used to detect *A. rhizogenes* rolD, aux1 and aux2 genes in transgenic material (Table I). The reaction conditions were: hot start at 94 $^{\circ}$ C for 4 min followed by 30 cycles of denaturation at 94 $^{\circ}$ C for 4 min, annealing at 58 $^{\circ}$ C for 1min, and extension at 72 $^{\circ}$ C for 1min, and the final extension at 72 $^{\circ}$ C for 5 min. The amplified products were resolved in 1% agarose gel, stained by SimplySafe (EURx, nd) and visualized in UV light.

TABLE I - Nucleotide sequences of PCR primers

Gene	Primer	Nucleotide sequence (5' – 3')	Expected length of the amplified fragment (bp)
<i>RolD</i>	rolD-for	CTGAGCGTGTGGCTCATG	101
	rolD-rev	GGAGGTAAAGACGAAGGACAGAG	
<i>aux1</i>	aux1-for	CATAGGATCGCCTCACAGGT	198
	aux1-rev	CGTTGCTTGATGTCAGGAGA	

(continuing)

TABLE I - Nucleotide sequences of PCR primers

Gene	Primer	Nucleotide sequence (5' – 3')	Expected length of the amplified fragment (bp)
<i>aux2</i>	aux2-for	AACGATAATAGCCCGCTGTG	217
	aux2-rev	CGTCTTGGGTTTGTGGTTCT	

GUS histochemical staining

Histochemical localization of GUS activity in hairy roots was performed using the 5-bromo-4-chloro-3-indolyl glucuronide (x-Gluc) (Duchefa Biochemie, Haarlem, The Netherlands) substrate essentially according to Jefferson *et al.* (1987) and described in details before (Klimek-Chodacka, Baranski, 2014). Then hairy root pieces were rinsed in 70% ethanol for 5 min and GUS activity was scored based on tissue blue staining.

HPLC detection and quantification of hypericin

Hypericin detection and quantification *in vitro* derived plants (control), root explants and transgenic hairy roots was carried out using HPLC as described before (Khlifa *et al.*, 2016a, b). The reference hypericin standard (Sigma-Aldrich, Munich, Germany) was used to calculate the equation of calibration line ($y = 7020.1x + 15.4$) with coefficient of determination $R^2 = 0.9995$.

Statistical analysis

Data were subjected to a factorial analysis of variance and then the Tukey multiple comparison test was used for testing significance between means at $p=0.05$. The t-test was used for comparison of two means. Frequencies of shoots held in Rockwool cubes and developing hairy roots were compared using a statistical test for significance level between two proportions. Pearson linear correlation coefficients were calculated to assess relationship between hairy root and shoot characters. Experiments were set up in a randomized designed with minimum five replications. Data were processed using CoStat_v6.303 statistical software.

RESULTS

Development of hairy roots on explants exposed to a solid medium

Control *H. sinaicum* explants not incubated with *A. rhizogenes* remained viable on a solid MS medium throughout the culture period. Leaf and stem explants developed mainly new shoots while root explants enlarged and developed callus. In *A. rhizogenes*-mediated explants callogenesis was limited and the development of new shoots delayed. These explants were capable of hairy root formation that were easily distinguished by their characteristic hairy phenotype and disturbed geotropism (Figure 1). Usually several hairy roots developed from one explant but some explants did not respond. In particular, leaf explants showed necrosis that could restrict hairy root development.

We assessed explant susceptibility to four *A. rhizogenes* strains and, on average, 66-72% of explants developed hairy roots independent on whether they were co-cultivated with either A4 or any of the A4T strains ($p = 0.119$). In contrast, hairy roots were observed significantly less frequently ($p < 0.001$) on LBA1334 treated explants (3.3%). However, the LBA1334 strain virulence was confirmed ($p < 0.001$) by comparing reaction of *H. sinaicum* and tobacco explants, the later used as a reference material. Independent on the explant type, on average, 80.6% of tobacco explants produced hairy roots confirming LBA1334 was able to transform plant cells efficiently but was hardly able to transform *H. sinaicum*.

H. sinaicum has never been used for genetic transformation hence we compared three explant types in their susceptibility to three (A4, A4T and A4T-GUS) strains and their suitability for further hairy root production. In general, explants of different types responded to *A. rhizogenes* with various efficiencies ($p < 0.001$) and most stem explants (83.6%) produced hairy roots 1.45-fold more frequently than leaf explants (57.7%). Root explants

responded less frequently (68.3%) than stem explants. This relationship was observed for all three strains although for the A4T strain there was no difference between root and stem explants (Figure 2). Notably, tobacco leaf and stem explants were equally highly susceptible to the LBA1334 strain. Detailed comparison indicated that the highest

percentage of *H. sinaiicum* explants developing hairy roots was obtained after stem segments co-cultivation with the A4 and A4T-GUS strains (88.3% and 91.7%, respectively). The A4 strain and both A4T strains considered together were equally efficient ($p = 0.622$) in transformation of leaf, root and stem explants (Figure 3).

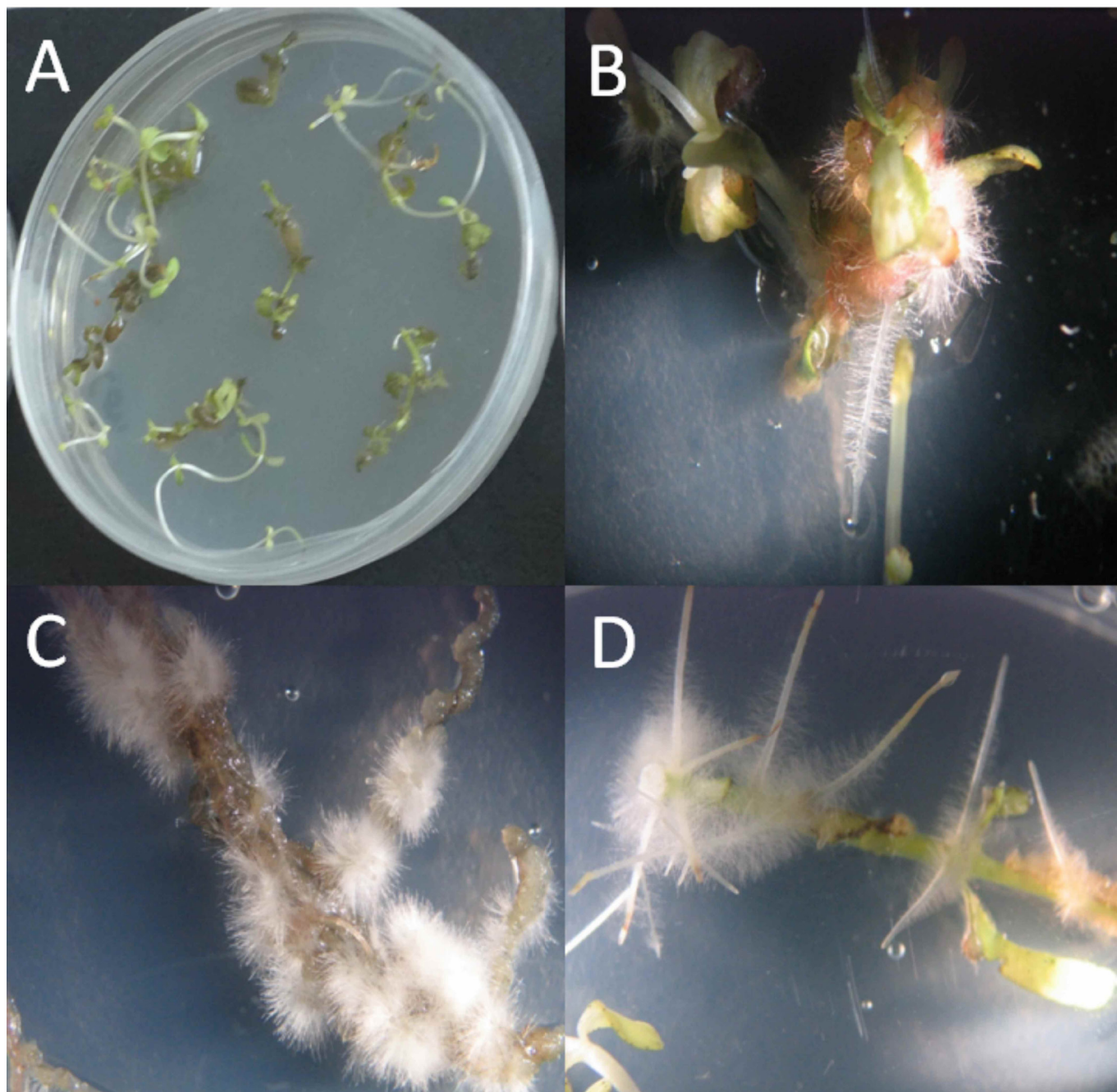


FIGURE 1 - Development of hairy roots on *H. sinaiicum* explants. Control stem explants not inoculated with bacteria (A), leaf (B), root (C) and stem (D) explants inoculated with the A4 *A. rhizogenes* strain and exposed to a solid MS medium.

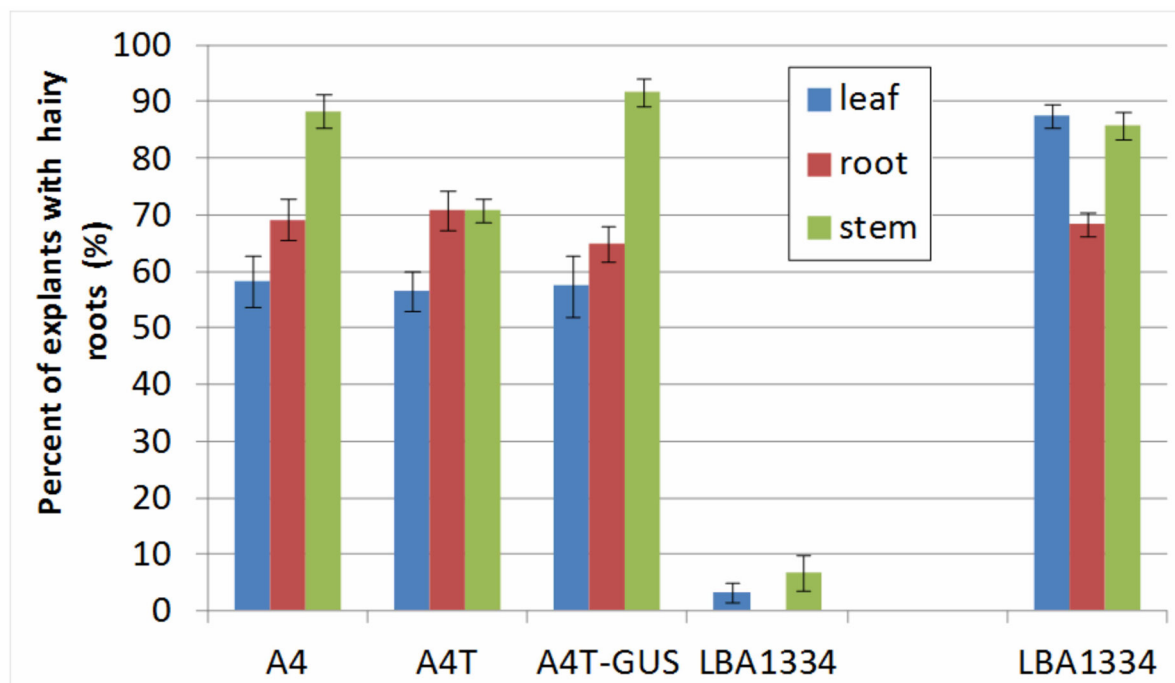


FIGURE 2 - Percentage of *H. sinaicum* explants exposed to a solid MS medium and developing hairy roots four weeks after transformation with four *A. rhizogenes* strains. Additionally, percentage of tobacco explants with hairy roots after inoculation with the LBA1334 strain. Whiskers - standard error.

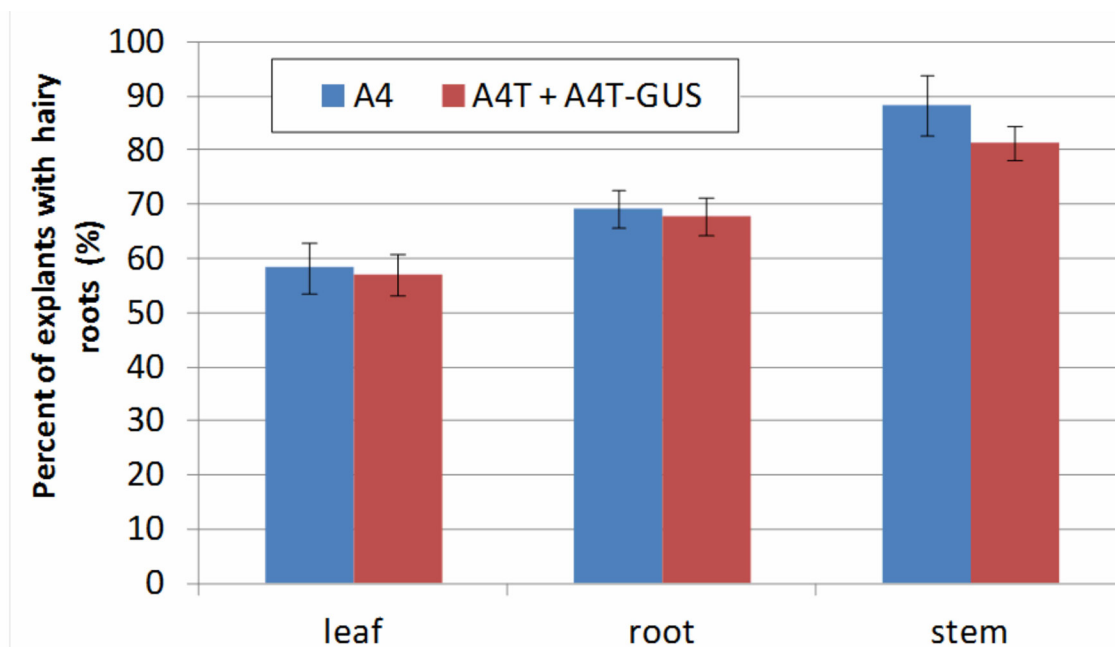


FIGURE 3 - Effectiveness of the A4 and A4T strains in the induction of hairy root development on *H. sinaicum* explants exposed to a solid MS medium. A4T - combined data for explants inoculated with the A4T and A4T-GUS strains. Whiskers - standard error.

The highest response was observed when stem explants were sonicated in *A. rhizogenes* inoculum for 30 s or 60 s (Figure 4). Longer sonication time increased the efficiency of stem transformation ($p = 0.004$) also when A4T strain was used. This treatment stimulated efficient response of leaf and root explants independently on the bacteria strain used. The most

pronounced change was observed for leaf explants. The extension of sonication time from 15 s to 60 s doubled the percentage of responding explants. For root explants, the change from 15 s to 30 s had no significant effect, while after 60 s sonication the percentage of responding explants was 1.46-fold higher than after 15 s treatment.

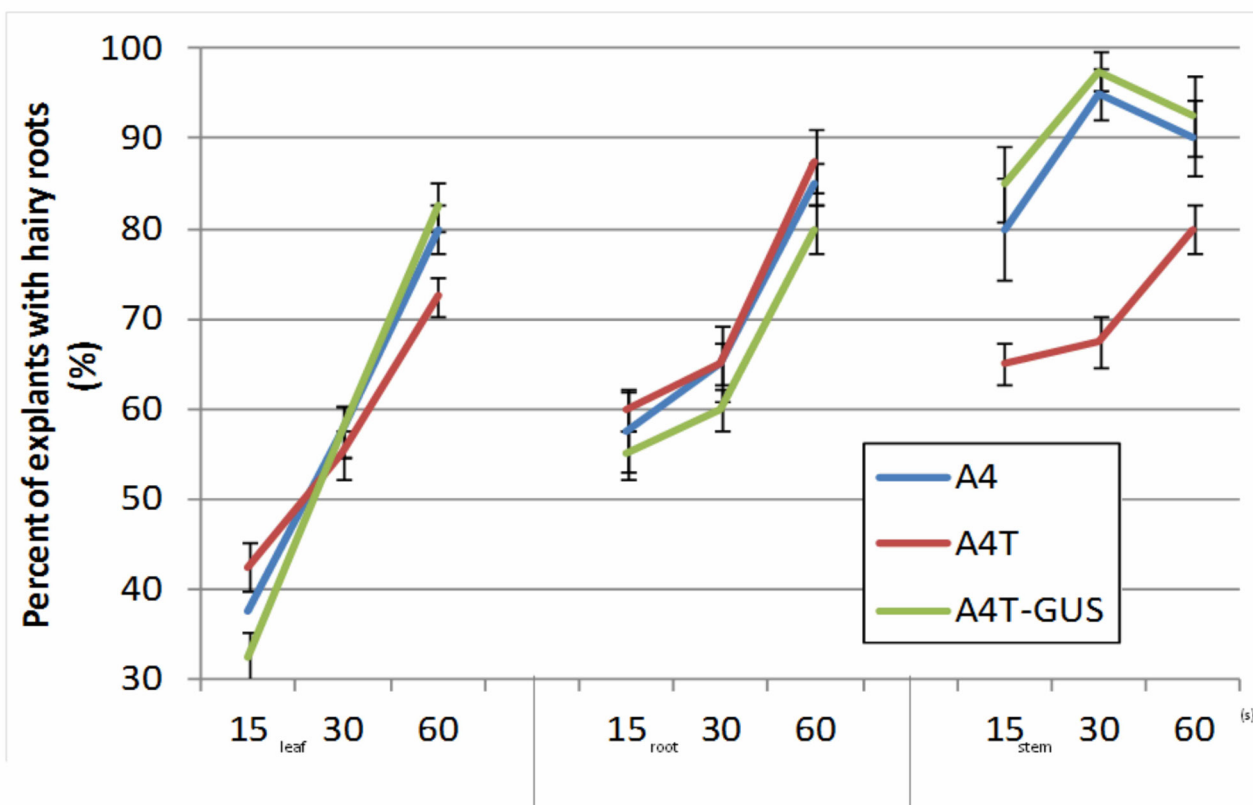


FIGURE 4 - Effect of sonication time on the percentage of *H. sinaiicum* explants developing hairy roots depending on the explant type and *A. rhizogenes* strain used. Whiskers - standard error.

Development of hairy roots from shoots inoculated in Rockwool cubes

Rockwool cubes were used to hold shoots upright after their root system was cut-off and to enable inoculum transfer to basal shoot part. The shoots inserted in cubes soaked with inoculum remained vigorous while being closed in sterile jars and they continued their growth developing new leaves and shoots from axillary buds. After ten days, first hairy roots emerged on Rockwool cube sides (Figure 5). They elongated fast and showed typical hairy phenotype. Four-week-old hairy roots were

branched and overgrew the cubes. They were produced by shoots co-cultured with all four *A. rhizogenes* strains used although with various efficiencies. All shoots incubated with LBA1334 produced hairy roots (Table II), unlike in transformation experiments with explants inoculated using this strain and exposed to a solid medium. Only a half or less shoots that were incubated with both A4T and A4T-GUS strains developed hairy roots. The response may remain underestimated as hairy roots might develop inside the cube and did not reach its surface making the observation of hairy roots not possible.

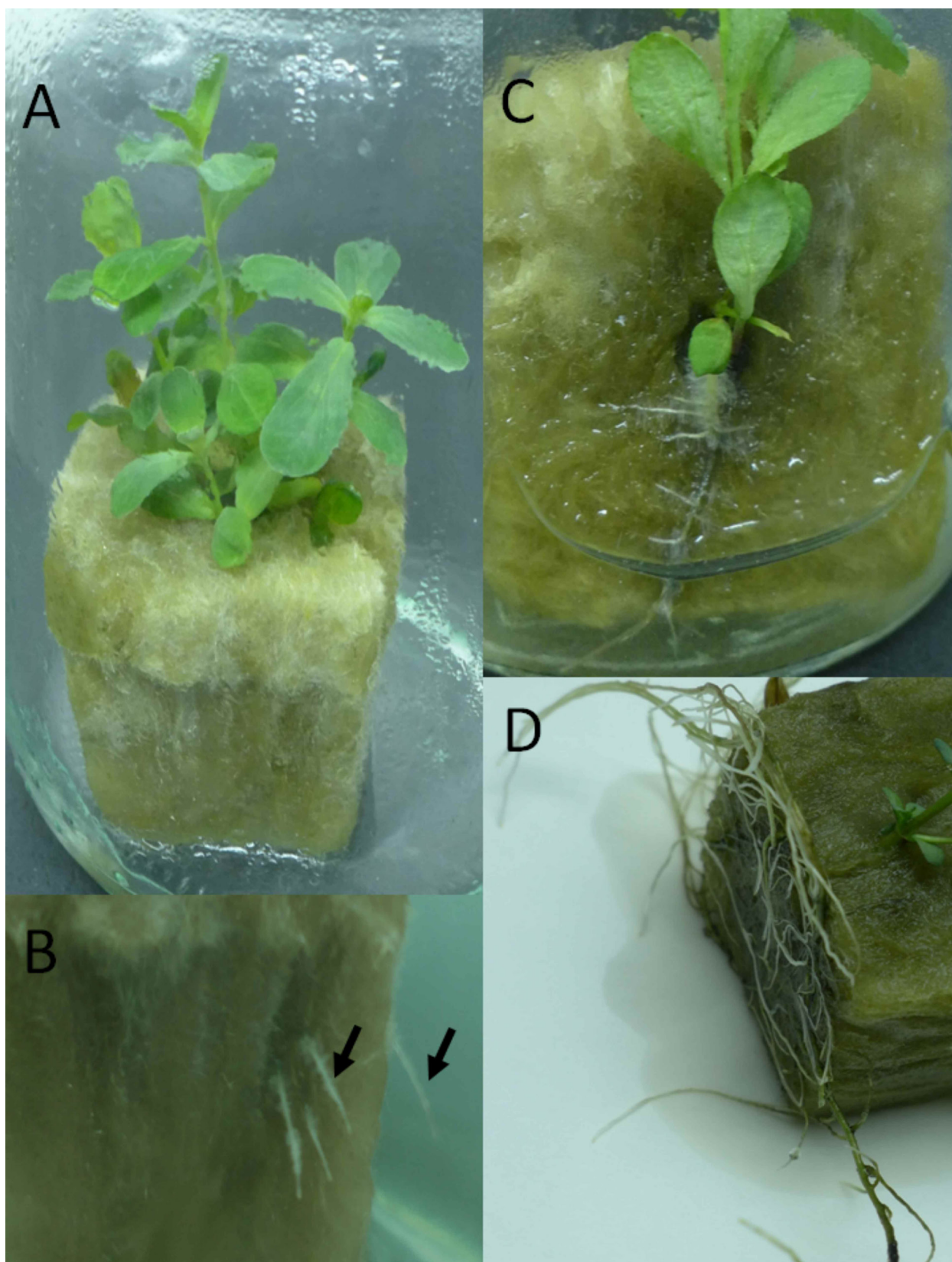


FIGURE 5 - Hairy root development on *H. sinaicum* shoots held in Rockwool cubes *in vitro* and inoculated with the A4 *A. rhizogenes* strain. Control shoots after a six-week-culture (A), hairy roots appearing at Rockwool cube sides 10 days after inoculation (B), branched hairy root (C), Rockwool cube overgrown by hairy roots three weeks after shoot inoculation (D).

TABLE II - Efficiency of hairy root development by shoots inoculated in Rockwool cubes, and the performance of hairy roots and responding shoots after three-week culture (mean values with standard errors)

Strain	Proportion of shoots with hairy roots		Hairy roots						Shoots with hairy roots					
			elongation (cm)			no. of branches			elongation (cm)			no. of branches		
LBA1334	1.0	a*	6.6	± 0.2	a	10.9	± 0.3	a	8.8	± 0.3	a	2.5	± 0.2	a
A4	0.7	ab	6.5	± 0.1	a	9.7	± 0.5	ab	7.3	± 0.2	b	2.1	± 0.1	ab
A4T-GUS	0.5	bc	4.0	± 0.5	b	8.0	± 1.4	bc	6.7	± 0.3	bc	1.6	± 0.2	bc
A4T	0.3	c	4.5	± 0.2	b	6.7	± 0.3	c	6.2	± 0.1	c	1.3	± 0.3	c
Control	0.0	-	0.5	± 0.2	**	0.0	-		2.8	± 0.2	**	1.6	± 0.2	**

* Means followed by the same letter do not differ significantly at $p = 0.05$

** Non-transgenic roots and control shoots

Control shoots did not produce hairy roots although some of them developed hair-free roots after prolonged culture. The root elongation in control plants was slow, 0.5 cm (± 0.2 cm std. err.) in three weeks, and no root branching was observed (Table II). In contrast, hairy roots elongated on average by 5.8 ± 0.3 cm in three weeks and they produced many branch roots. Hairy roots produced by shoots co-cultivated with LBA1334 and A4 strains grew faster, and developed 8 to 12 branches while those co-cultivated with A4T and A4T-GUS strains grew slower and had lower tendency for branching (3 to 11 branches). Thus LBA1334 and A4 treated plants had finally better developed system of hairy roots, and they were more vigorous having tendency for the development of more lateral shoots that also elongated faster (Table II). The A4T and A4T-GUS treated shoots showed similar tendency for branching as the control. Correlations between the percentage of shoots responding to *Agrobacterium* inoculation, root branching, shoot elongation and shoot branching were very high ($r > 0.9$). Correlation coefficients between these variables and hairy root elongation were slightly lower and ranged between 0.79 and 0.87.

Culture of excised hairy roots

Hairy roots developing on leaf, stem and root explants, and on shoots held in Rockwool cubes were excised 3-4 weeks after inoculation and their fragments

of approx. 1-2 cm in length were placed on the selection media. Hairy roots continued their growth on antibiotic enriched media confirming they acquired resistance to either kanamycin or hygromycin after *A. rhizogenes*-mediated transformation (Fig. 6). Independently on the bacteria strain used for inoculation, the developed clones showed similar ability for root elongation. Clones obtained after transformation with the A4 and LBA1334 strains maintained their high ability for branching (10.8 ± 0.4 and 11.8 ± 0.2 root branches, respectively) only slightly higher than clones after transformation with both A4T and A4T-GUS strains (9.2 ± 0.4 and 9.4 ± 0.2 , respectively). Not transformed roots from control plants did not grow, bleached and finally died on the selection media.

Hairy roots transferred to a liquid medium containing selection antibiotics exhibited rapid elongation and pronounced lateral branching. The most efficient biomass production was observed for clones derived after *H. sinaicum* inoculation with the LBA1334 strain. The mean biomass (4.7 ± 0.3 g) obtained after one-week culture was two-fold higher than the mean biomass of clones derived after using other strains that ranged from 1.8 to 2.9 g.

Molecular confirmation of hairy root phenotype

Hairy root phenotype was expected as a result of *A. rhizogenes* T-DNAs transfer to *H. sinaicum*. Hairy root clones were thus verified for the presence of rolD and two

aux, aux1 and aux2, genes localized on TL-DNA and TR-DNA of *A. rhizogenes* Ri plasmid, respectively. PCR products of approx. 100, 200 and 220 bp were identified after electrophoresis in agarose that corresponded to the expected lengths for amplified fragments of rolD (101 bp), aux1 (198 bp) and aux2 (217 bp) genes, respectively (Fig. 7). The same product lengths were obtained using control DNA from transgenic tobacco while no products were detected for the non-transformed roots.

The use of the A4T-GUS strain containing uidA gene additionally enabled validation of functional gene expression in hairy roots thus successful gene transfer from bacteria to *H. sinaicum*. Hairy root samples collected from clones derived after inoculation with this strain were used for histochemical staining to detect GUS activity. The expected blue staining was observed only in transformed roots whereas control roots did not show

altered pigmentation (Fig. 7). The root apical region and central cylinder were stained the most intensely although the intensity of blue color varied from faint to strong.

Hypericin content in hairy roots

The presence of main naphthodianthrone (hypericins) in *H. sinaicum* was determined in hairy roots. Peak positions in chromatogram obtained from HPLC analysis corresponded to those obtained for the reference compound confirming hypericin presence in hairy roots (Figure 8). The signal intensity for hairy root sample was high and hypericin contents estimated based on the reference standard line was 39.430 $\mu\text{g/g}$ dry weight. Hypericin content estimated for the intact plant and non-transformed root explants were lower, 17.096 $\mu\text{g/g}$ and 2.538 $\mu\text{g/g}$ dry weight, respectively.

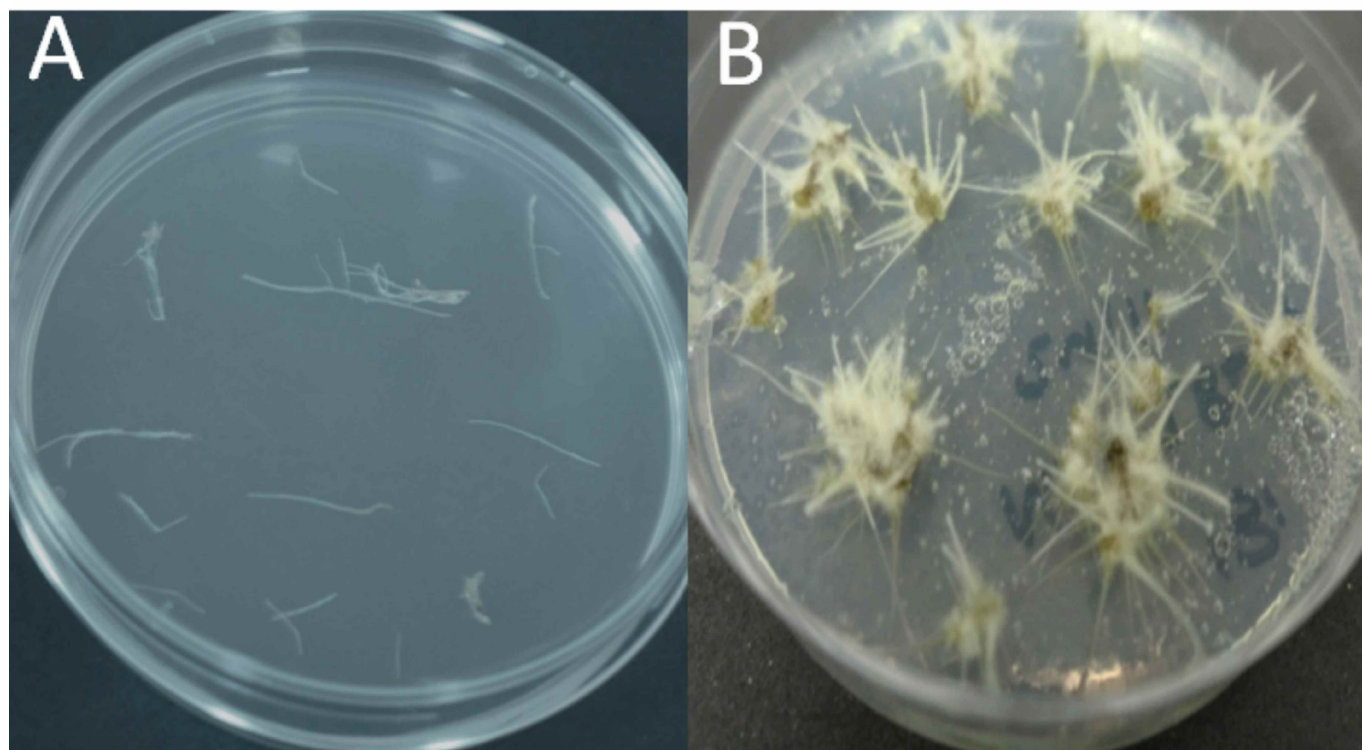


FIGURE 6 - Four-week-old root clones on the kanamycin selection medium. Not developing root fragments taken from the control, not transformed plant (A), intensively growing hairy root clones obtained from explants transformed with the A4 *A. rhizogenes* strain (B).

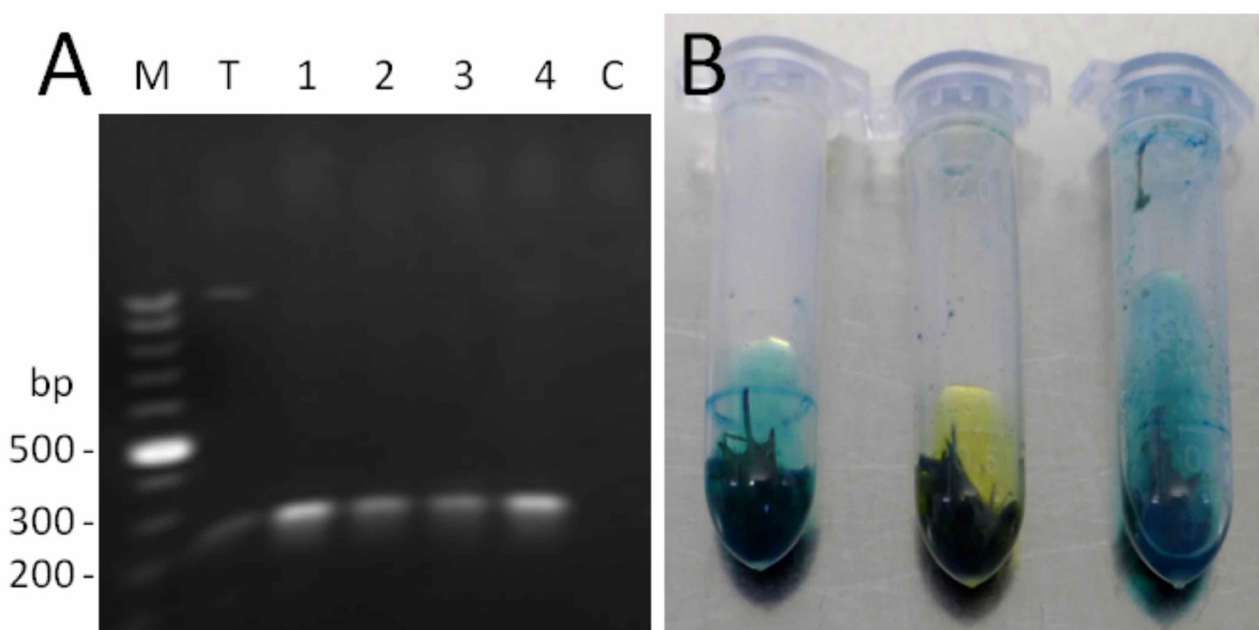


FIGURE 7 - Verification of horizontal gene transfer from *A. rhizogenes* to *H. sinaicum* hairy roots. Presence of the expected 198 bp PCR amplified fragment of *aux1* gene in hairy roots after transformation with *A. rhizogenes* strains. Lane M - GeneRuler DNA Ladder Mix, lane T - reference DNA from tobacco transformed with the LBA1334, lanes 1-4 - DNA from *H. sinaicum* hairy roots after transformation with A4T, A4, LBA1334, and A4T-GUS, lane C - DNA from control, not transformed *H. sinaicum* plant (A). Blue staining resulting from the *uidA* gene transfer from the A4T-GUS *A. rhizogenes* strain to hairy roots and consecutive GUS activity. Unstained roots from the control, not transformed *H. sinaicum* plant are in the middle tube (B).

DISCUSSION

Genetic transformation of *H. sinaicum* has not been performed till now. Several methods were applied to modify *H. perforatum* including particle bombardment and *Agrobacterium*-mediated transformation with various success but the possibility of induction of hairy roots was shown (Franklin, Oliveira, Dias, 2007; Vinterhalter *et al.*, 2006). In this work we show for the first time the successful *A. rhizogenes*-mediated transformation of *H. sinaicum* and the ability of the obtained hairy roots to biosynthesize hypericin, a plant metabolite of pharmaceutical significance. The results obtained indicate that different bacterial strains can be used for hairy root induction in *H. sinaicum* although with various effectiveness depending on the applied method and explant type. All *A. rhizogenes* strains used led to hairy root induction and hairy roots were observed in 10 to 30 days after inoculation that is a common response time observed in other species. However, we have found that shoots held in the Rockwool cubes developed hairy roots much faster

than excised explants. The major advantage of the method relying on the use of Rockwool cubes is thus its rapidity and technical simplicity. In consequence, the production of transgenic roots takes only a few weeks instead of a few months when excised explants exposed to a mineral solid medium are inoculated. The major limitation of this approach is that, since shoots remain not transformed, the developing plants are composite and their maintenance either by micropropagation or self-fertilization is aimless. Thus hairy root cultures have to be established by excising transgenic root segments independent on whether the shoots or explants were inoculated. We have demonstrated that the excised transgenic root segments may be successfully cultured for biomass production. It is also known that hairy roots may exhibit ability to organogenesis that circumvent limitations rising from the use of hairy root culture if plant production is required (Crane *et al.*, 2006).

The choice of bacterial strain for efficient DNA horizontal transfer is important and has been demonstrated before (Gilbert *et al.*, 1996). We have used strains differing in their Ri plasmids but also in

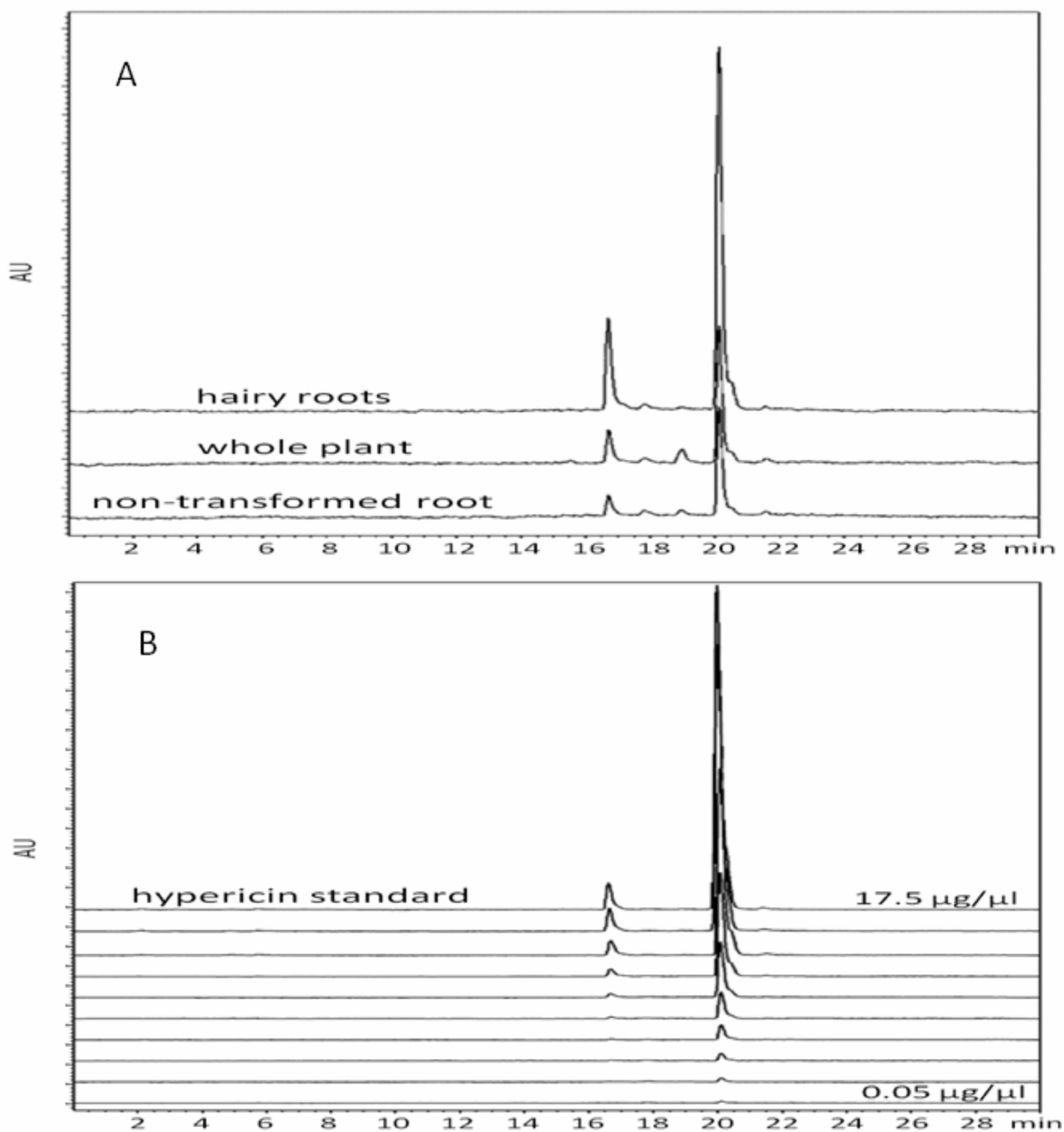


FIGURE 8 - HPLC detection of hypericin in *H. sinaicum* hairy roots and plant. Hypericin characteristic peaks in chromatograms for samples of hairy roots and control, not transformed whole plant and its roots (A). Quantitative analysis of hypericin standard applied in the range 0.05-17.5 µg/µL (B).

chromosomal background. The A4T and LBA1334 strains are derivatives of the C58 strain thus they both possess *A. tumefaciens* chromosome in contrast to the A4 strain with *A. rhizogenes* chromosome. Transgenesis is a process requiring interaction of virulence genes located in the plasmid and chromosomal genes thus genetic background may be essential (Hardegger, Sturm, 1998). It was demonstrated that in carrot the A4T and LBA1334 strains stimulated hairy root production with higher frequency than the A4 strain that indicates advantageous role of the C58 chromosomal background (Baranski, Klocke, Schumann, 2006). Our results indicate that strains with any of the used chromosomal context can successfully induce hairy roots in *H. sinaicum*.

The transformation efficiency is also species specific as demonstrated for two *Hypericum* species using *A. rhizogenes* wild type strains. The inoculation of *H. tomentosum* with the 15834 strain resulted in 44% efficiency while the use of A4 strain was less effective (35%). Much more pronounced differences were observed for *H. tetrapterum* i.e., 73% and 13%, respectively (Komarovská *et al.*, 2009). Also, higher susceptibility of *H. perforatum* explants was reported when they were transformed with the 15834 strain than with the A4 strain (Bivadi *et al.*, 2014; Di Guardo *et al.*, 2003). However, we have not observed differences between strains used for *H. sinaicum* explant transformation except of LBA1334 was not able to transform either shoot, leaf or root explants. To ensure the strain did not lost its virulence we performed routine screening using tobacco explants and confirmed that LBA1334 remained highly virulent. Thus *H. sinaicum* explants were not susceptible to this strain despite LBA1334 was able to transform another species. Notably, the same strain was highly effective in hairy root induction when shoots held in Rockwool cubes were inoculated. Both A4 and A4T strains harbored pRiA4 plasmid while the LBA1334 strain had pRi1855 plasmid. Previous reports in carrot showed their similar efficiency in DNA transfer when they were provided in the C58 chromosomal context (Baranski, Klocke, Schumann, 2006) but in *H. sinaicum* transformation bacteria with the pRi1855 plasmid seemed more effective when it was co-cultivated with injured shoot base rather than with excised explants. This phenomenon may be due to a longer co-cultivation of shoots with bacteria as no antibiotics were added until hairy roots emerged but also tissue type may be significant. The excised small fragments of leaves, stem or roots are highly injured in contrast to the whole stem that remains vigor and easily

commence further growth despite the root system is removed. The differences in the secondary metabolite release affecting bacterial virulence is thus highly probable but requires further confirmation.

We found also that shoots and the excised stem explants were more sensitive to *A. rhizogenes* than leaf and root explants. These results indicate that *H. sinaicum* plant organs may contain different compounds, or their contents, affecting the transformation process. Various responses of different plant tissues to *A. rhizogenes* were reported by (Abdi *et al.*, 2017). Root and leaf segments of both *H. tomentosum* and *H. tetrapterum* species were used for transformation experiments with two *A. rhizogenes* wild-type, ATCC15834 and A4, agropine strains, but only the root segments were capable of transgenic hairy root formation (Komarovská *et al.*, 2009). Also the *Agrobacterium* viability decreased during co-cultivation with *H. perforatum* flowers and leaves containing hyperforin and hypericin of antibacterial activity (Franklin, Oliveira, Dias, 2007). Stems containing usually less bioactive compounds may thus be less toxic to *Agrobacterium* and more suitable material for agroinfection. Despite that, not only shoots but also leaves, although with a lower efficiency, can be still prone to agroinfection as shown by us for *H. sinaicum* supporting earlier observations for *H. perforatum* by Vinterhalter *et al.* (2006) and Di Guardo *et al.* (2003).

The inoculation and co-cultivation time considerably affect transformation success thus they should be adjusted taking into account bacteria virulence and the explant type used. Longer co-cultivation time is recommended to ensure bacteria attachment to plant cell and T-DNA transfer. The comparison of three inoculation (15, 30 and 45 min) and two co-cultivation (24 and 72 h) times applied to *H. perforatum* showed that the percentage of hairy roots declined with shorter inoculation and co-cultivation time (Bivadi *et al.*, 2014). However, a prolonged co-culture often results in bacteria overgrowth that destroys plant material thus is avoided. To promote agroinfection in shorter co-cultivation time an ultrasound treatment can be applied to explants. During sonication-assisted *Agrobacterium*-mediated transformation (SAAT) plant tissue is partially disrupted that help bacteria intracellular penetration, enlarge contact surface and enable their direct contact with larger number of cells. Moreover, micro wounding of cells enables secretion of phenolic compounds enhancing agroinfection (Pathak, Hamzah,

2008). It was also demonstrated that short ultrasound treatment is the most efficient when applied to explants already immersed in the inoculum (Klimek-Chodacka, Baranski, 2014). We therefore compared three sonication times and have shown that SAAT stimulates hairy root induction independent on the explant type and bacteria strain used. Actually, 60 s sonication doubled the transformation efficiency of leaf explants while for stem explants 30 s treatment was also effective.

A. rhizogenes-mediated transformation results in the hairy roots phenotype accompanied by altered plant morphology and changes in biosynthetic ability (Koperdáková *et al.*, 2009a, b). The transgenic status of the obtained hairy roots was confirmed by PCR using two sets of pRi genes, which are involved in the root induction process; aux genes located in the TR region of the pRi T-DNA and rol (root loci) genes of the TL region (Jouanin, 1984). Aux genes provide transformed cells with an additional source of auxins but they do not seem essential for developing hairy root disease (Chriqui *et al.*, 1996). However, rol genes have functions that are most likely other than that of producing mere alterations in plant hormone concentrations (Nilsson, Olsson, 1997). Finally, Trovato *et al.* (2001) found that, in cells transformed by agropine plasmids, the RolD protein may provoke a stress response through increased proline production, which may further influence cell division and appears to specifically induce flowering. Proline may also increase the biosynthesis of glycoproteins (rich in hydroxyproline), the cell wall structural components involved in the regulation of cell division. Thus hairy roots often have modified metabolism that may be advantageous when altered biosynthesis of the secondary metabolites is expected. Hairy root cultures have been shown to be an efficient mean of producing secondary metabolites that are normally biosynthesized in roots of growing plants. *A. rhizogenes* strains exhibit various stimulating effects on the induction and growth of hairy roots, and on the production of secondary metabolites as demonstrated for example in case of lignan biosynthesis in *Linum tauricum* ssp. (Ionkova, Fussm, 2009). Hairy root system developed from root segments of *in vitro* grown *H. perforatum* seedlings after co-cultivation with the *A. rhizogenes* A4 strain was utilized to identification and quantification of the secondary metabolite xanthone (Tusevski *et al.*, 2013). Our research led to the development of hairy root culture that was characterized by high hypericin content. The amounts identified were higher than in plant organs making the system an

attractive alternative for hypericin production. That may be of particular importance as production in contained conditions like bioreactors will not require cultivation and utilization of endangered *H. sinaicum*.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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