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ESTABLISHMENT OF HIGHLY EFFICIENT *AGROBACTERIUM RHIZOGENES*-MEDIATED TRANSFORMATION FOR *STEVIA REBAUDIANA* BERTONI EXPLANTS

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Leaves and internodes from *Stevia rebaudiana* Bertoni plants growing in different conditions were used for transformation with two strains of *Agrobacterium rhizogenes*: ATCC 15384 and LBA 9402. Hairy roots formation was observed and the percentage of the transformed explants depended on the type of explant, time of inoculation and inoculum concentration. Inoculation of explants from *ex vitro* and *in vitro* plants with LBA 9402 strain led to higher efficiency of transformation than inoculation with ATCC 15384 strain. Growth rate of hairy roots in liquid culture was assessed under light and dark conditions. It was found that the growth of hairy roots decreased significantly under light conditions. Transformation of hairy roots growing in different culture conditions was confirmed at the molecular level using PCR method with primers constructed against *rolB* and *rolC* genes from *A. rhizogenes*.

Keywords: *Agrobacterium rhizogenes*, transformation, steviol glycosides

INTRODUCTION

Stevia rebaudiana Bertoni is a perennial herb of the Asteraceae family. It is native to certain regions of South America. Leaves of *Stevia rebaudiana* accumulate large quantities of steviol glycosides. These secondary metabolites belong to two groups of

compounds: stevioside which appear 300 times sweeter than sucrose (0.4 % solution) and rebaudioside (especially rebaudioside A) which could be 400 times sweeter than sucrose (Sreedhar et al., 2008). In addition to this, steviol glycosides are non-caloric and clinical studies suggest that they can reduce blood glucose and blood pressure (Hsieh et al., 2003; Gregersen et al., 2004). Because of the mentioned facts, purified steviol glycosides are increasingly used in food industry as sweeteners and food additives. An important feature of steviol glycosides composition in *S. rebaudiana* plants used in industry is the ratio of stevioside to rebaudioside A. This is because rebaudioside A reduces the feeling of bitterness that is the aftertaste of stevioside. Steviol glycosides are accumulated in leaves of *S. rebaudiana* at concentrations as high as 20% of the dry weight (Brandle et al., 1998), depending on the climate in which the plants are grown. Different quantitative compositions of rebaudiosides and steviosides in samples of leaves of *S. rebaudiana* are presented by Kovylyaeva et al., 2007. Clearly, smaller accumulation of these compounds is noted in moderate climate regions. Moreover, in such conditions, the ratio of rebaudioside A to stevioside is less favorable, compared to plants of tropical and subtropical climates. Furthermore, classical plant propagation is limited due to very poor seed germination and, consequently, production of seedlings. For that reason propagation of stevia is usually done by stem cuttings; however, the number of stems present on a single plant also limits the vegetative multiplication through cuttings.

Clonal propagation in *in vitro* conditions is useful for small scale production because it is associated with large economical costs (Singh and Rao, 2005). *Agrobacterium rhizogenes* transformation of plant explants could be an alternative method for obtaining steviol glycosides by hairy roots (HR) cultures. After infection, T-DNA from the Ri plasmid of bacteria is integrated with the plant genome, changing the plant metabolism and thus causing proliferation of hairy roots on the infected explants. Transformed roots are able to produce secondary metabolites at levels that are comparable or greater than in uninfected plants (Giri and Narasu, 2000). Moreover, the possibility to regenerate stevia plants in *in vitro* conditions opens a new perspective concerning the genetic manipulation based on establishment of an efficient transformation method.

The aim of our work was to obtain hairy root cultures of *S. rebaudiana* after transformation of different kinds of explants with two strains of *A. rhizogenes*: ATCC 15834 and LBA 9402 for further manipulation to produce secondary metabolites.

MATERIAL AND METHODS

PLANT MATERIAL

Experiments were performed on *S. rebaudiana* plants which originated from:

1/ *in vitro* conditions: *in vitro* plants were micropropagated from nodes of explants cultured on MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg/L NAA (1-naphthaleneacetic acid), 0.5 mg/L BAP (6-benzylaminopurine), 30.0 g/L sucrose and 9 g/L agar,

2/ *ex vitro* conditions: *ex vitro* plants were micropropagated from *in vitro* micropropagation and then transferred to greenhouse conditions,

3/ *in vivo* conditions: *in vivo* plants originated from standard vegetative propagation and were cultured in field conditions.

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Two types of explants: leaves and internodes, collected from each group of plants, were used for transformation. Explants from *in vivo* and *ex vitro* plants were sterilized using two different protocols:
a/ Explants from *ex vitro* plants were surface sterilized with 70% (v/v) ETOH for 30 seconds, then rinsed 3 times with distilled, sterile water and transferred to solution of 10% (v/v) NaClO for 5 minutes. After this time they were rinsed carefully with sterile distilled water.
b/ Explants from *in vivo* plants were first shaken for 20 minutes in 0.1 % (v/v) Tween 20 solution, then they were rinsed 3 times with distilled, sterile water. Later, they were surface sterilized with 70% ETOH for 30 seconds, rinsed 3 times with distilled, sterile water and then kept in the solution of 10% NaClO for 5 minutes followed rinsing with sterile distilled water.

BACTERIA STRAINS
Two different strains of *A. rhizogenes* were used for transformation:
1/ the wild-type hyper-virulent strain LBA 9402 (a gift from Dr. David Tepfer, INRA Versailles, France)
2/ agropine strain ATCC 15384 (American Type Culture Collection).

A single colony of each strain was grown for 48 h at 28°C on the following media: YMB medium for LBA 9402 strain (Hooykaas et al., 1977), and Nutrient Broth medium for ATCC 15834 strain (Downes and Ito, 2001).

TRANSFORMATION AND ESTABLISHMENT OF HAIRY ROOTS CULTURES
Fragments of leaves (10-20 mm²) and internodes (5-10 mm) were co-cultured with freshly grown *A. rhizogenes* strains with optical density (OD) as follows: A_{600} = 0.1 for 10 min, A_{600} 0,3 for 30 min, or A_{600} 0,5 for 10 min. Control explants were soaked in sterile distilled water for the same time.

After co-culture the explants were placed on hormone-free MS medium (MS) for three days and then they were transferred to MS medium supplemented with cefotaxime (100 mg/L – MSCX medium). Inoculated explants were kept in darkness at 26°C throughout the experimental period.

The transformation frequency expressed as the percentage of inoculated explants producing roots and the number of roots per explant were studied 30 days after inoculation. The hairy roots length was measured at 10 and 30 days after appearance.

Roots (about 1 cm long) formed of the infected explants were excised and transferred on fresh MSCX and incubated in the dark. After four passages, the concentration of cefotaxime was reduced to 50 mg and after next four weeks cefotaxime was omitted from the medium. Some of new lines, which showed good growth, were selected and used for establishment of a liquid culture.

CONFIRMATION OF TRANSFORMATION
Transformation was confirmed by PCR analysis. RNA was extracted according to Chomczyński (1987) from two putative hairy roots lines (100 mg) and from leaf tissues of *ex vitro* plants (100 mg, control). RNA purity was determined by measuring the ratio of absorbance at two wavelengths: $OD_{260\ nm}$ and $OD_{280\ nm}$. A given sample of RNA was considered pure (free from proteins and DNA) when the value A (260) / A (280) ranged between 1.6-2.0. Then, cDNA was obtained with the use of iScript cDNA Synthesis Kit (BioRad, USA) and reverse transcription reaction. Each PCR reaction was performed in a

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final volume of 50 µL using DreamTaq Green PCR Master Mix (ThermoScientific) containing: DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM MgCl2. For each reaction we used 25 µL DreamTaq Green Master Mix, 0,5 µM each forward and reverse primer and 1 µg template DNA, all complemented with water nuclease-free to 50 µL. PCR conditions for *rol*C and *rolB* gene (*rolB* 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3'; *rolC* 5'-CTCCTGACATCAAACTCGTC-3' and 5'-TGCTTCGAGTTATGGGTACA-3', Królicka et al. 2001) were as follows: 40 cycles, initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, primer annealing at 53°C for 30 s, elongation at 72°C for 1 min, followed by final elongation for 5min (C1000 Thermal Cycler, BIO RAD). Amplification products were resolved by electrophoresis on 1% agarose gel in TE buffer, stained with ethidium bromide and observed under UV light.

GROWTH STUDIES

For determination of the growth rate root lines called SRY (internodes) and SRL (leaves) transformed by *A. rhizogenes* LBA9402 were chosen among the *rol*C gene positives. Hairy roots (0.5 g of fresh weight) were cultured in 50 ml of MS liquid medium in 300-ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker (80 rpm) in a growth chamber at 26°C in the dark or under light from cool white fluorescent lamps (16 h light/8h dark period; 40 µm m^{-2} s^{-1}). The cultures were grown for 30 days and fresh weight was recorded at 0, 10, 20, 30 day of culture. Each result was based on 3 replicates and the experiment was repeated three times.

RESULTS AND DISCUSSION

Hairy roots production was achieved after infection of different explants from *S. rebaudiana* with two strains of *A. rhizogenes*: ATCC 15384 and LBA 9402. The efficiency of transformation was influenced by the type of the bacteria strain, concentration of inoculum, the time of inoculation and the type of explant (Tab. 1.). The explants inoculated with ATTCC 15834 strain (OD 0,1) for 10 min, exhibited hairy roots formation after 30 days only on the infected internodes from *in vitro* plants. The percentage of transformed explants was poor (only 2%). On the other hand, the inoculation with LBA 9402 strain led to efficient transformation of both *S. rebaudiana* leaves and internodes (Fig. 1A). Hairy roots were formed 13 days after inoculation of leaves explants from *ex vitro* plants for 30 min with LBA9402 strain with OD 0,3 (Fig. 1B). The percentage of transformed explants was 40%. Even greater efficiency of transformation (50%) was demonstrated for the same explants but inoculated for a shorter time (10 min) with more concentrated bacterial strain OD_{600} 0,5 (Tab.1, Fig. 1C)

Hairy roots were also formed from internodes of explants from *ex vitro* and *in vitro* plants inoculated with LBA9402 strain (Fig. 1 D); however, the efficiency of their transformation was significantly lower than in the case of leaves (Tab. 1).

Bacterial cells were removed from the hairy root cultures by the use of cefotaxime in the MS medium (Fig. 1 E). After a few passages hairy roots free of bacteria were transferred in liquid medium (Fig. 1 F) without antibiotics and plant growth regulators and incubated in light and dark conditions. Previously, it had been observed that hairy roots cultured in light exhibited the potential to produce certain secondary metabolites

that were mainly found in aerial organs and never in hairy root cultures normally incubated in the dark. These hairy root cultures also show development of chlorophyll, causing greening of these cultures; therefore, the roots are called green hairy roots (Sharma et al., 2013). A similar effect was found in our experiment where greenish hairy roots were produced when cultured in light conditions. Fresh weight of the cultured hairy roots was recorded after 0, 10, 20, 30 days of culture. The increase of fresh weight was noted for both types of the culture but these from the dark conditions increased their weight seven times while the fresh weight of hairy roots cultured in the light increased only three times (Tab. 2).

Integration of the T-DNA into *S. rebaudiana* genome was confirmed at the molecular level by the PCR with cDNA obtained after reverse transcription on the base of RNA isolated from hairy roots from light and dark conditions and primers constructed on the sequences of *rolB* and *rolC* genes of *A. rhizogenes*. The PCR reaction was also conducted for control explants, incubated in water instead of bacteria strain and grown in the same conditions as hairy roots. PCR products were absent in the non-transformed tissue. However, transcripts of *rolB* and *rolC* were presented in both light (Fig. 2A) and dark culture (Fig. 2 B) of hairy roots. PCR product of 383 bp DNA for *rolB* gene as well as PCR product of 586 bp DNA for *rolC* gene were obtained from RNA isolated from hairy root tissues.

In literature there is only one publication concerning an attempt at *S. rebaudiana* transformation where the authors showed only 2% efficiency of transformation after application of leaf explants and *A. rhizogenes* (Yamazaki et al., 1991). Lately, the information concerning *A. tumefaciens* mediated infiltration of *S. rebaudiana* leaves as a gene silencing approach was published (Guleria and Yadav, 2013). However, there is still lack of studies carried out on transformed stevia plants (Singh et al., 2009). It is probably due not only to lack of good plant regeneration system but also to lack of an efficient transformation protocol. In our studies we achieved, for the first time, highly effective transformation of different explants from *S. rebaudiana* plants with *A. rhizogenes* strains. It is worth mentioning that all the investigated explants from *in vivo* plants did not respond positively to the infection with both *A. rhizogenes* strains. Plants which originate in the natural environment are exposed to many stress factors, including pathogens. In order to survive, they are equipped with more efficient defense system than plants which grow in controlled, sterile conditions and, therefore, are more resistant to the infection. Thus, for future experiments we recommend use of plants under controlled conditions. Moreover, we found that the best strain for transformation of *Stevia rebaudiana* is the wild-type hyper virulent strain LBA 9402.

Liquid cultures of hairy roots achieved after transformation of *S. rebaudiana* explants allow us to continue the studies concerning the influence of different factors on steviol glycosides biosynthesis.

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AUTHORS' CONTRIBUTIONS

Ż. Michalec-Warzecha and F. D'Angiolillo performed the experiments, analyzed the achieved data and participated in writing the paper. M. Libik-Konieczny and L. Pistelli designed the experiments and wrote the paper. M. Libik-Konieczny has primary responsibility for the final content. All authors read and approved the final manuscript. The authors declare that they have no conflict of interest.

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TABLE 1. Parameters of *S. rebaudiana* explants transformation.

Agrobacterium rhizogenes strain ATCC 15384

	Percentage of transformed explants (%)			Mean no. of HR[1] per dish/per explant 10 days after root appearance			Mean HR length after 10 days (mm)			Mean HR length after 30 days (cm)			HR appearance-days after inoculation		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
Leaves *in vivo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaves *in vitro*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaves *ex vitro*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Internodes *in vivo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Internodes *in vitro*	2	-	-	10/2	-	-	4	-	-	7	-	-	30	-	-
Internodes *ex vitro*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Agrobacterium rhizogenes strain LBA 9402

	Percentage of transformed explants (%)			Mean no. of HR per dish/per explant 10 days after root appearance			Mean HR length after 10 days (mm)			Mean HR length after 30 days (cm)			HR appearance-days after inoculation		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
Leaves *in vivo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaves *in vitro*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaves *ex vitro*	-	40	50	-	30/3	99/16	-	4	4	-	5	6	-	13	26
Internodes *in vivo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Internodes *in vitro*	30	-	-	25/2	-	-	3	-	-	6	-	-	20	-	-
Internodes *ex vitro*	-	20	-	-	30/2	-	-	4	-	-	5	-	-	20	-

[1] HR: Hairy roots, **T1**: optical density OD_{600} 0,1; inoculation time 10 min., **T2**: optical density OD_{600} 0,3; inoculation time 30 min., **T3**: optical density OD_{600} 0,5; inoculation time 10 min.

TABLE 2. Growth rate of hairy roots in liquid MS medium. The values are means of three experiments ± SD. Values within the rows followed by a different letter are statistically different at P≤0.05 (Duncan's test)

Type of culture	Hairy roots weight (g of FW±SD)			
	0	10 days	20 days	30 days
Light culture	0,55 ± 0,06d	0,68 ±0,06c	1,49 ±0,12b	1,79 ± 0,16a
Dark culture	0,62 ± 0,08d	0,83 ± 0,11c	2,20 ± 0,36b	4,43 ± 0,87a

FIGURES

Fig. 1. Transformation of *S. rebaudiana*. A/ Different explants on MS medium after inoculation with *A.rhizogenes* LBA9402; B/ Formation of hairy roots on *ex vitro* leaf explant; C/ Formation of hairy roots on *ex vitro* internode explant; D/ Growth of hairy root culture on *ex vitro* leaf explant. E/ Growth of hairy roots separated from the explant on MS solid medium. F/ Growth of hairy roots in MS liquid medium.

Fig. 2. Identification of *rolB* and *rolC* gene fragments in hairy roots grown after *S. rebaudiana* transformation with *A. rhizogenes* LBA9402 strain. PCR reactions was performed using as a target DNA isolated from: A/ dark conditions; B/ light conditions. Actin (Act) was used as a reference gene. Gene ruler DNA Ladder Mix (Life Technologies) was applied for quantification of visualized bands.

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Figure 1

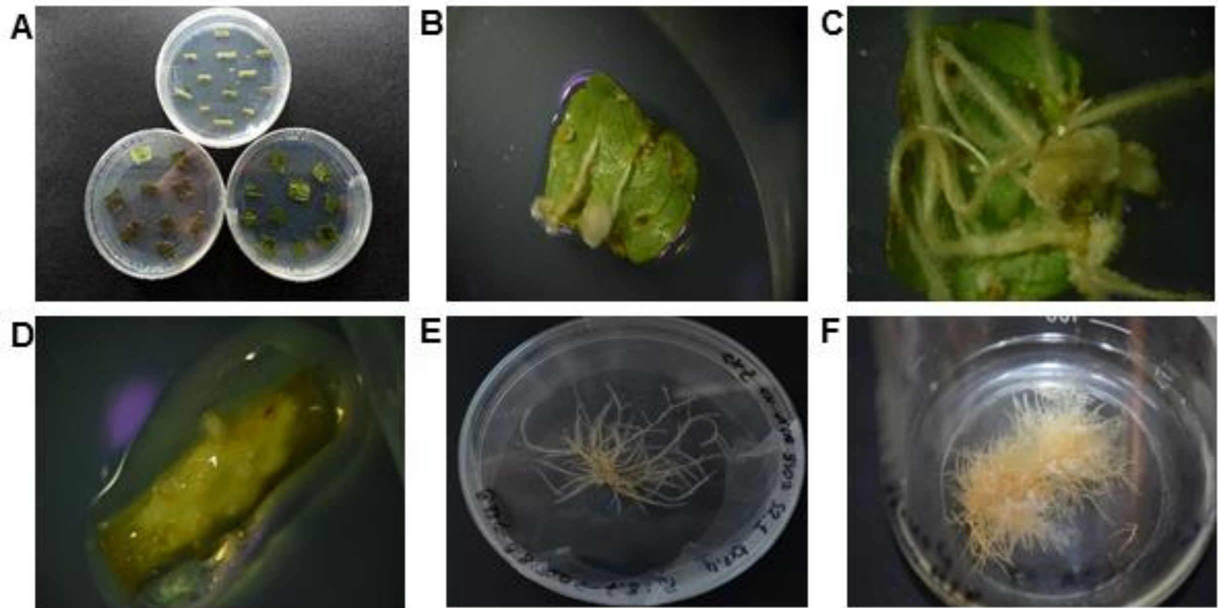

Figure 2