Manipulation of root biomass and biosynthetic potential of *Glycyrrhiza glabra* L. plants by *Agrobacterium rhizogenes* mediated transformation

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Abstract

Agrobacterium rhizogenes is gram-negative soil bacteria and the causal agent for the development of the hairy root disease in plants. Hairy roots cultures have been proven as being a natural source for secondary metabolites that are normally biosynthesized in roots of differentiated plants. Here we established an experimental condition for obtaining hairy roots culture of medicinal plant Glycyrrhiza glabra as effect of integration and expression of rolB gene from Ri plasmid of A.rhizogenes. Two Agrobacterium strains, LBR56 and LBA9402 were tested for hairy roots induction but only LBR56 strain show an increase in transformation efficiency relative to wild-type. This high productive root biomass was induced from in vitro old roots and internodes segments by direct infection with A. rhizogenes LBR56 strain. Several independent hairy roots clones were obtained and maintained in vitro and further evaluated for growth, morphology, integration and expression of Ri T-DNA genes. The PCR analysis with specific primers for the rol B gene and nptII marker gene confirmed the integration of T-DNA fragment of Ri plasmid from A.rhizogenes into the genome of G.glabra transgencic plants. RT-PCR analysis confirmed the expression of hairy roots inducible gene. The glycyrrhizic acid (GA) yield has been monitored in the hairy roots biomass by UV spectral analysis.

Keywords: medicinal plants, *Glycyrrhiza glabra*, transformation, hairy roots, *rol A, B, C, D* oncogenes, secondary metabolites

Introduction

Over the past three decades, the advancement in genetic engineering of plants with desired traits relied heavily on the various strains of *Agrobacterium tumefaciens*, strains modified in such of way to transfer different genes situated on Ti plasmid to plant genome followed by selection and characterization of transgenic lines comprising the 'interest" transgene. However, many plants are still recalcitrant to *Agrobacterium* mediated transformation and because of the extensive debate focused on GMO productive technology, an alternative, more environmental and hence bioethical friendly system has been assayed beginning with the 90s. The high potential of *A.rhizogenes* mediated genetic transformation determined the development of a new domain of medicinal plant biotechnology [8]. The current technology is based on the capacity to introduce high biosynthetic rate in secondary metabolites [32; 24; 34; 29]. The induction of hairy roots in medicinal plants represents the main objective of such technologies and has been widely reported in the literature [23; 30; 28]. The hairy roots are thus well established as an experimental system and most

importantly, it has provided many insights into root specific metabolism and its regulation. An advantage of using *A. rhizogenes* for plant transformation is based on a higher growth rate and genetic stability of hairy roots cultures. Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemical unstable and often synthesize very low levels of useful secondary metabolites [21; 16; 10].

The European liquorice (*Glycyrrhiza glabra*) is a very popular medicinal plant, which is reach in isoflavonoid constituents containing glycyrrhizin, a hormone compound used as a natural sweetener as well as a source for anti-inflammatory drugs. There are data suggesting also that glycyrrhizin in combination with methyluracil possesses a radio protective effect [33; 14; 11; 4] and licorice roots, are reported to have antioxidant effects, which can be linked to its hepatoprotective capabilities [14]. Flavonoids from licorice have been reported to provide protection to hepatocytes exposed to carbon tetrachloride, and galactosamine [12]. The research suggests that the anti-lipid peroxidation effect of licorice is the central mechanism contributing to its protective action against carbon tetrachloride-induced hepatotoxicity [9].

The biotechnological potential of *Glycyrrhiza glabra* L. may be improved by its roots cultural conditions. The genetic engineering of licorice roots represents an alternative means for scale production of high-value secondary metabolites from transgenic cell lines. Recently, transgenic hairy roots culture have become of interest because of their continuous and active proliferation on phytohormone-free medium and their capacity to produce valuable materials synthesized and accumulated *in vitro*, at a higher level as compared to their concentration in non transformed plants [3; 11; 31; 13]. Also, the root biomass of medicinal plants may be obtained and maintained by monitoring the culture system in a bioreactor [15].

Hairy roots represent a valuable source of phytochemicals useful as pharmaceuticals, cosmetics, and food additives [27]. Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products. Sevon (2002) [24] as summarized the most important alkaloids produced by hairy roots, including *Atropa belladonna* L., *Catharanthus tricophyllus* L., and *Datura candida* L.

The manipulation of Ri plasmid of *Agrobacterium rhizogenes* may involve the cloning of marker gene, such as *nptII* (neomycinphosphotransferase II), for the assistance of the *rol A, B, C, D* (root inducing locus) genes transfer into the plant genome. Depending on the *rol* gene types and synergic interactions, variable hairy roots phenotypes have been observed [3; 1; 30]. The hairy roots developed at the site of the infection may serve as an efficient source of the further root cultures. These are characterized by genetic stability, rapid growth in culture media without phytohormones, and a high synthesis rate for metabolites, which are natively synthesised in the no transformed roots and other plant organs [7; 24].

Considering the great interest in the exploitation of such root potential the aim of this study was to establish the experimental condition for an efficient transfer, integration and expression of *A. rhizogenes rolB* gene in *Glycyrrhiza* plants in order to obtain a high root biomass for the pharmaceutical use. The secondary metabolite of interest, GA, was as basic component of the alcoholic extracts commercialized on our pharmaceutical market. Furthermore, a transgenic root system offers tremendous potential for introducing additional genes along with the Ri plasmid, especially with modified genes, into medicinal plant cells with *A. rhizogenes* vector systems. The cultures have turned out to be a valuable tool with which to study the biochemical properties and the gene expression profile of metabolic

pathways. Moreover, the cultures can be used to elucidate the intermediates and key enzymes involved in the biosynthesis of secondary metabolites.

Materials and methods

Plant material and culture conditions for its *in vitro* maintenance

Seeds of *G. glabra* purchased from Iasi Botanical Garden (Romania) were sterilised in 10 ml solution containing 1% sodium hypoclorite and few drops of Tween 20 for 20 min followed by three times rinsing in baths containing sterile deionised water. They were then germinated on basal MS medium [17]. In order to obtain *in vitro G. glabra* plantlets the rooting and calli induction medium have been used. For callus induction we use the MS basal medium supplemented with 0.01-0.03 g/l 2,4 D (dichlorophenoxyacetic acid). After 30 days of proliferation, the *G. glabra* calli have been transferred on MS medium supplemented with 0.03g/l zeatin for shooting induction. For high rooting initiation we use the MS basal medium supplemented with 0.01g/l BAP (benzyl amino purine), 0.0010 g/l AIA (indolyl acetic acid) and 0.0005-0.002 g/l jasmonic acid.

Bacterial strains

Agrobacterium rhizogenes strains LBR56 (pA_4RS) and LBA9402 [19] were used to determine the transformation efficiency. LBR56 strain harbours pA4RS plasmid which contains on his T-DNA fragment the *rolB* oncogene that confer the hairy roots phenotype and the reporter/marker *nptII* gene that confers the kanamycin resistance both under the control of *nos* promoter. LBA9402 strain is a hyper-virulent strain containing on Ri plasmid the wild-type *rolA, rolB, rolC* genes [19; 30; 29].

The *A.rhizogenes* LBR56 culture was grown into 25ml liquid MYA medium (MYA, yeast extract 5 g/l, casamine acids 0.5 g/l, ammonium sulphate 2 g/l, mannitol 5 g/l, natrium chloride 5.0 g/l) supplemented with 0.05 g/l rifampicine and 0.1 g/l kanamycin. The bacterium has been cultured in the dark at 28^0C for 48h until the optical density (OD600) was approximately 0.8. The LBA9402 culture was shaken on YMB medium (YMB, 0.5 g/l K_2HPO4. 7H2O; 0.1 g/l NaCl, 10.0 g/l manitol, 0.4 g/l yeast extract; pH 7.0) supplemented with 0.05 g/l rifampicine.

Genetic transformation and establishment of hairy roots cultures

Young leaf (2 weeks) and mature leaf (6 weeks), root segments and stem segments from 6 weeks in vitro grown plants of *G. glabra* were inoculated with 2 days old bacteria culture. Both bacterial strains were tested and different concentration of bacteria has been used (10^6-10^8 cfu/ml). Hairy roots culture was established by infecting internodes and roots segments with a scalpel containing 2 days old *A.rhizogenes* LBR56 liquid co-culture medium [26]. The infected segments were incubated at 25^0C under 3000 lux light conditions. After 2 days of co-culture, explants were first transferred on fresh MS medium supplemented with 0.5 g/l cefotaxime in order to eliminate bacteria. Three weeks after the bacterial infection, hairy roots emerged from the infected sites. The elongating roots tips were cut off and transferred to growth regulator containing MS agar medium without cefotaxime in order to obtain hairy roots cultures on a solid medium.

G. glabra hairy roots were also cultured in MS liquid medium with 3% sucrose adjusted to pH 6.8. The culture flasks (200 ml) were inoculated with about 1g fresh hairy roots segments and then placed on shaker at 100 rpm at 25 ^{0}C temperature and 3000 lux (16h light/ 8h dark). After 3-4 weeks the growing hairy roots were transferred on fresh medium in order to obtain a liquid medium hairy root culture for obtaining root biomass used for extraction and detection of GA content.

Shoots and plants regeneration

Root segments were cultured on MS medium supplemented with 0.02 g/l zeatin (S2 medium). The explants were further cultured under 16h light and 8h/dark regime in a clean room, at 22^0C and every two weeks transferred to a fresh S2 medium [25]. When shoots developed and more than two leaves emerged, a transfer to MS hormone-free solid medium was performed. In vitro plants regenerated from roots segments have short internodes and are more branched than untransformed ones. The abnormal growth observed in plants transformation with *A.rhizogenes* can represent an advantage in some instances. These plants had larger root biomass, which can be further used as source of biopharmaceutical important compounds.

Molecular analysis

Genomic DNA isolation and PCR amplification

Hairy roots, plants regenerated from hairy roots and untransformed roots were analyzed both by genotype and phenotype approaches. The specific properties of the rapidly growing and branched roots (hairy roots) as the expression of Ri T-DNA, offered a direct visual possibility to estimate the efficiency of transformation process by the phenotype approach (Fig.1a). The genotype analysis comprises the DNA extraction from hairy roots and identification of rooting locus genes (*rolB*) and *nptII* marker gene by polymerase chain reaction.

DNA was isolated from hairy roots and leaf-regenerated material by CTAB modified method as described by Doyle and Doyle (1990) [6]. The polymerase chain reaction (PCR) was used to confirm the presence of the *rolB gene* in roots and entire plant. The primer used was the 5'-ACTATAGCAAACCCCTCC-3'/5'-TTCAGGTTTACTGCAGCAG-3' yielding a DNA fragment of 635bp from the *rolB* oncogene. Also, the presence of *nptII* marker gene was confirmed using the followed primer pair 5'-GCATACGCTTGATCCGGCTACC-3'/5'-TGATATTCGGCAAGCAGGCAT-3', yielding a DNA fragment of 221bp.

The PCR reaction mixture (25ml total reaction volume) consisted of: 50mM of each dNTP, 0.050 μg of genomic DNA, 1mM of each primer, Red Taq DNA polymerase and 10X standard PCR buffer. The PCR parameters set up on a Biometra Thermal Cycler used for amplification consisted of a denaturation step of 5 min at 94^0C and 35 cycles each comprising: 55s denaturation at 94^0C, 50s annealing at 55^0C for *rolB* and 56^0C for *nptII* marker gene, 2 min extension, followed by a final extension at 72^0C for 7 min. The PCR products were fractioned by electrophoresis on a 2% agarose gel, stained with (0,001g/l) ethidium bromide. The amplicon samples were run comparatively with a DNA weight marker "PCR Low Ladder" provided by Sigma. The electrophoresis gel has been visualized and photographed in a dark chamber UVP Jencons-PLS, at 302 nm.

RNA isolation and RT-PCR analysis

Total RNA was isolated from both hairy roost and non-transformed roots cultures using TRIzol reagent (Life Technologies, CA, USA). To remove contaminating genomic DNA, the total RNA (6μg) was subsequently incubated with 6 units (1U/μl) RQ1 RNase-free DNase (Promega, M6101) for 30 min at 37^0C, extracted with phenol and chlorophorm, and finally precipitated with ammonium acetate and dissolved in DEPC treated water. The total RNA concentration was determined spectrophotometrically at 260nm.

First strand cDNA was synthesized using a Promega (Madison, WI) Reverse Transcription System according to the manufacturer's instructions. The PCR reaction was performed in a 50 ml total volume with primers specific for the *rolB* gene. RT-PCR using primers directed

against actin mRNA was used as internal standard. Primer sequences were as follows: for 5-AATATTGGACAATGGGCGCAAGCC-3' and 5'- ACACAGGATGTCAAGGGCTGGTAA-3' for detection of *rol B* transcripts in *hairy* roots; for actin, 5'-AAGCTGCAGGAATCCACGAGACTA-3' and 5'- AAGGTGCTGAGGGAAGCCAAGATA-3'. Each gene was amplified using the following conditions: 94^{o} C for 5 min, followed by 25-40 cycles of 94^{o}C for 30s, 52^{o}C for 30s, and 72^{o}C for 45s, with a final extension at 72^{o}C for 4 min. PCR products were separated by electrophoresis through 1.0% agarose gels and photographs of the ethidium bromide-stained gel scanned using UVP Jencons-PLS camera.

UV spectral analysis of GA content in roots biomass

The hairy roots were subculture on growth-regulator-free MS liquid medium for 20-40 days, then harvested and dried to constant weight. The hydro-alcoholic root extract (250 mg dry hairy roots were grounded and extracted with 70% ethanol at 105^{0}C for 30 min; the extract was then filtered and the extraction procedure was repeated for two times till 10 ml total volume extract.) have been obtained and measured comparatively with the standard GA (provided by Sigma Aldrich, St. Louis MO, USA). The GA working solutions of1 x10^{-5}, 10^{-5}, 7.5 x10^{-5}, 10^{-4}mol/l was obtained by dissolving it in methanol and 0.1% trifluoracetic acid, and performing the appropriate dilution. The alkaloid content was addressed as mg GA/g root biomass by spectophotometric quantification.

Results

Establishment of *Glycyrrhiza glabra* hairy roots cultures

In the present study we established an experimental model for transformation of *Glycyrrhiza glabra* plants in which the foreign genes (*rol B*) expressed quite efficiently (Fig. 1a, b). Two *Agrobacterium* strains were tested for transformation efficiency but LBA9402 strain does not show any hairy root formation after infection for any of tissue explants tested. This strain is hyper-virulent and after infection we observed a necrosis on tissue at the infection sites (data not shown). In our experiment all tissue explants tested were not competent for transformation with LBA9402 strain. The highest efficiency in transformation evaluated by hairy roots phenotype was obtained with LBR56 strain (Table 1). Old roots segments (3 weeks plant material maintained *in vitro* culture) represent the suitable material competent for transformation.

Table1. Transgenic *G.glabra* explants that show hairy root phenotype after infection with *Agrobacterium rhizogenes* strains at different bacteria concentration Nd: not determined (no root formation)
10^{6}-10^{8} bacterium concentration

***A. rhizogenes* strain**	**% of *G. glabra* segments showing hairy roots**	
	10^{8}	10^{6}
LBR56	80% (1300)	60%(1300)
LBA9402	0.8% (1200)	nd

We observed an increase in percentage of segments (80%) showing hairy roots for LBR56 strain for both bacteria concentration tested in comparison with LBA9402 strain. Further we have tested the condition for regeneration of *Glycyrrhiza* transgenic plants from hairy roots. The hairy roots and plants regenerated from hairy roots were analysed by PCR in order to demonstrate the integration of *rol* genes in plant genome. Kovalenko et al. 2003, have previously mentioned studies regarding transformation of *Glycyrrhiza* species [11]. The authors demonstrated that

Glycyrrhiza species is highly sensitive especially to *Agrobacterium rhizogenes* super virulent strains and needed an additional fungal elicitor for better root biomass development. It has very well documented that additional exogenous hormones added to culture media have positive influence in increasing the transformation efficiency [22].

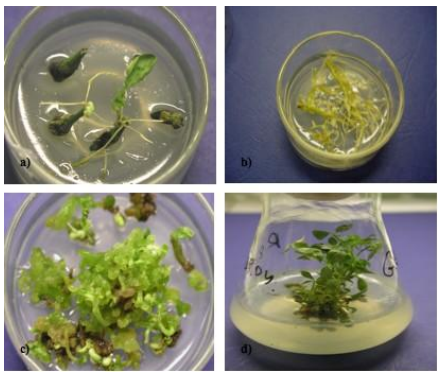

Figure 1. Development of hairy roots lines and regeneration of *Glycyrrhiza* plantles. a) Induced roots emergent from root and internode segments; b) Hairy roots development on the solid surface medium (on hormone-free MS medium after 3 weeks); c) calli induction from roots sectors on MS medium with hormones; d) varied phenotype of transgenic licorice plants.

The presented results, confirmed that *Glycyrrhiza glabra* is susceptible for transformation with *Agrobacterium rhizogenes* LBR56 strain and the roots explants respond quite efficient to transformations without any additional hormones added to culture media. We speculate that the susceptibility of *Glycyrrhiza* plants for transformation depends on concentration of *Agrobacterium* used, on type of explants but depend also on agrobacteria strain used. In our experiment we observed that the roots explants are suitable for infection in comparison with leaf explants at low concentration of bacteria (10^{-6}cfu/ml). The hairy roots emerged from the sites of infection and they develop quit faster *in vitro* without any additional hormones. It was hypothesized that the cells, which have a higher concentration of sugars and auxin, are the ideal targets for hairy roots induction [18]. Our experiment showed a slow process of hairy root development as compared with our other previous studies in which we have used the same transformation system for the hairy roots induction in *A.belladonna* plants [30].

Characterization of transformed hairy roots culture: phenotype and genotype analysis

Hairy roots emerged at the infection sites during 4-5 weeks after inoculation (Fig. 1a). Bacterial cells were eliminated after growing the root explants on medium supplemented with cefotaxime. After few passages, the hairy roots on MS medium without hormones were more vigorous and branched (Fig. 1b). The roots were more uniform and displayed a typical phenotype characterized by plagiotropic growth, without high incidence of lateral branching. The variation in the characteristics of hairy roots may be ascribed to differences in the copy number and/or position of integrated T-DNA(s) into the plant genome. Several hairy roots clones shown a higher root biomass on solid medium and the weight of these roots biomass was about 8 fold higher than non-transformed roots. Also, these hairy roots clone were more branched than non-transformed roots (Fig. 2c, d).

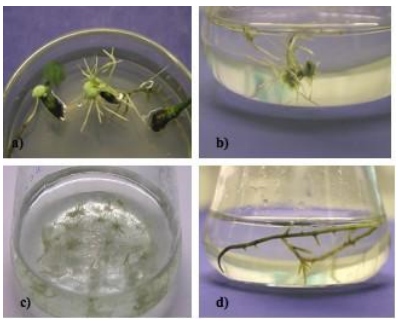

Figure 2. Steps in the hairy root culture development on liquid medium corresponding to different periods of time: a) hairy roots 2 weeks after transformation; b) hairy roots transferred on liquid media; c) 30 days old hairy root culture; d) wild-type root (d).

Different explants were used for transformation (leave, roots and internodal segments) but a succesful infection and hairy roots development was obtained using root explants provided from *G.glabra* plants that have been mantained for more than three month in *in vitro* condition. Previous study demonstrate the importance of age of explants for increasing transformation efficiency. For exemple, Mehrotra et al. 2008 [15] show that leave explants of *Glycyrrhiza glabra* older than tree weeks are the suitable tissue for increasing transformation and the over-expression of squalene synthase gene in *Glycyrrhiza uralensis* increases the production of glycyrrhizin. In the present study we obtained a higher root biomass formation when *A.r* LBR56 was used for transformation (Fig. 1a,b). Figure 1c shows the regeneration of shoots from the root segments through calli formation. The whole plants were further regenerated after separation and growing separately the shoots (Fig. 1d). The initiation of a hairy root culture on liquid medium has been also assayed (Fig. 2a, b). Different phenotype chracteristics for the transformed state (more elongated, vigurous, but branched) have been observed (Fig.2b, c) as compared with the non-transformant one (Fig. 2d).

Integration of T-DNA into *G.glabra* genome was confirmed by the PCR reaction with specific primers for *rolB* oncogenes and *nptII* marker gene, respectively (Fig. 3a, b). The electrophoresis behaviour of the amplified DNA fragment, as compared with the DNA weight marker, showed an amplicon of 635bp specific for *rolB* and a PCR product yielding 221bp for *nptII* gene. These results indicated that the *rolB* and *nptII* genes from the Ri plasmid of *A.rhizogenes* LBR56 were integrated into the genome of *G. glabra.* The PCR product was absent in non-transformed tissue (control)

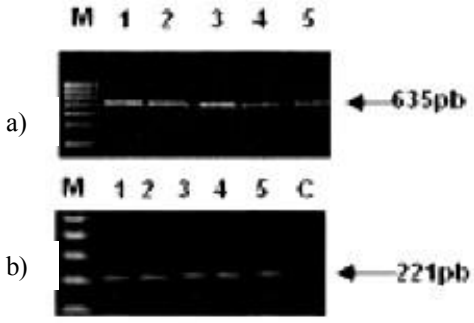

Figure 3. Molecular analysis of transgenic hairy roots and transgenic plants of *Glycyrrhiza glabra.* PCR analysis for the presence of: a) 635bp for *rolB* gene and b) 221bp for the presence of *nptII* marker genes. (M-DNA molecular weight marker, 1-3- DNA from hairy roots; 4-5-DNA form transgenic plants; C-negative control (DNA from untransformed plant);

The expression of *rolB* in *Glycyrrhiza* transgenic roots was observed by RT-PCR analysis. However, results obtained by RT-PCR analysis confirmed the expression of *rolB* genes. No transcript was observed for non-transformed root (Fig. 4). Any amplification was observed in all samples without revers-transcriptase, which confirmed that the total RNA was free of DNA. As an internal standard we used the actin gene and in all samples assayed the specific amplification was obtained (Fig. 4). The *rolB* gene is known as been the most important oncogene involved in the root development and we correlate the increases in root biomass with integration and expression of *rolB* in *G.glabra* genome. We suggest that accumulation of *rolB* transcript in *Glycyrrhiza* plants increases the root formation and transformation efficiency.

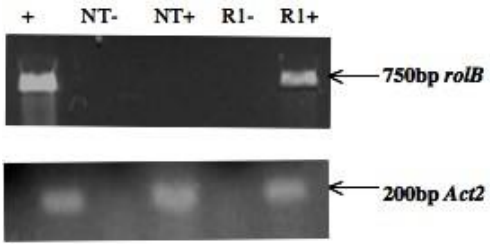

Figure 4. Representative RT-PCR for the expression of *rolB* gene *G.glabra* hairy roots. NT: non-transformed roots; R1+: hairy roots clone; + positive control for rolB gene (plasmid DNA); R1-: RNA isolated from hairy roots without RT-transcriptase (show that RNA was free of DNA). β-actin was used as internal control.

GA yield

The spectral measurement at 244nm confirmed the presence of GA peak in root extracts (Fig. 5a, b) as compared with the standard GA peak obtained in the same solvent. The results showed a slight variation when comparing the GA peaks registered for different root biomass extracts and the control untransformed root extracts. However, when considering the growth rate of the rhizosphere biomass in the same nutritive medium and the same vessel volume, we observed a 2 fold increase during the same period of time.

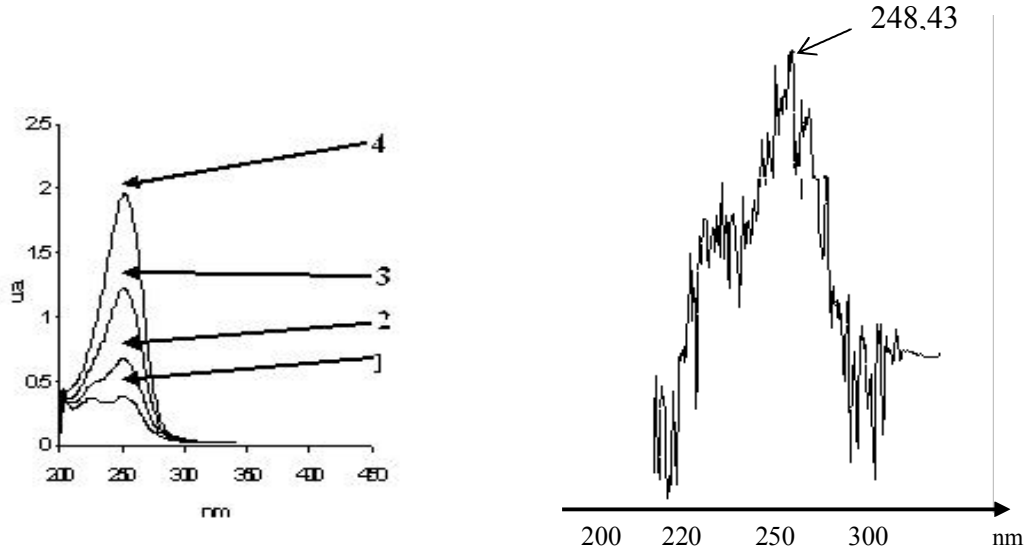

Figure 5. Spectra of GA in a) standard alcoholic solution (1-1x10^{-5}, 2-10^{-5}, 3-7,5x10^{-5}, 4-10^{-4}mol L^{-1} and b) alcoholic extract of hairy roots.

Figures 6a, b, shows the variation in this essential parameter, which we consider to be referred to the estimation of the biosynthetic rate potential of the transformed roots. It may be motivated also by a greater stability of root culture, as compared with that of untransformed roots, which showed necrosis and therefore a significant active biomass loss starting after 30 days. Accordingly, the GA concentration is increased or decreased with a coefficient linked with the biomass weight variation in the same medium volume and the same period of time.

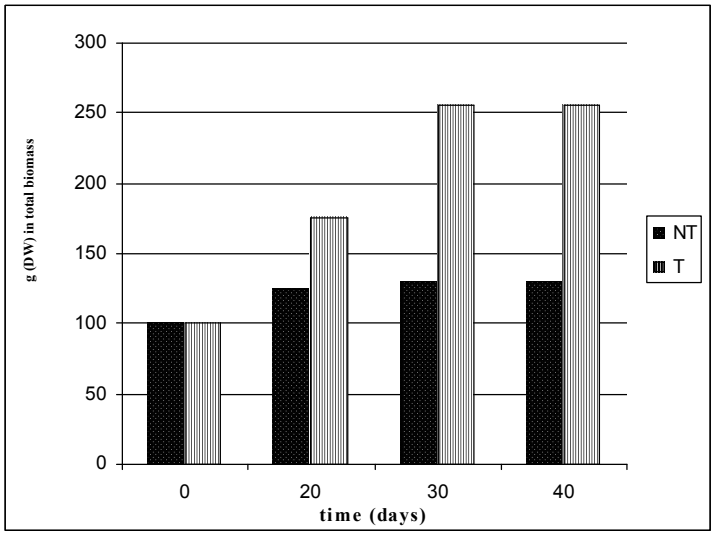

Figure 6. The GA content in T (hairy roots) compared with NT (non transformed roots) corresponding at different moments of root biomass development (0-20-30-40 days).

Discussion

An efficient transformation system was developed for medicinal plant, *G. glabra*. It enabled the introduction of the *rol* genes into the plant genome leading to the production of *G. glabra* hairy-root cultures in a hormone-free medium. The integration and expression of Ri-T-DNA were confirmed by molecular analysis. Our experiment is using the same genetic transformation system involving *A.rhizogenes* mediation, which had been previously approached with different species (*Solanum tuberosum, Lycopersicon esculentum, Atropa belladonna*) [20; 3; 30]. In the present study, certain peculiarities have been noticed: the hairy roots presented significant lignifications as compared with the cases mentioned above. We suspect, according with the literature reports [5] that an interesting interference may be due to the high yield of phenol compounds characteristic to the *G.glabra* tissues.

In accordance with several reports we demonstrated also an increase in the biosynthetic rate of GA in the transformed hairy roots of *G.glabra*, which we propose to be correlated with the biomass increase due to the *rol* genes effect on cell division [32]. However, the literature highlights also an important influence of the same *rol* genes upon the cell metabolism and hence a higher biosynthetic potential. Ri T-DNA increases dramatic the GA productivity mainly due to the increase in root biomass rather than alkaloids content. The higher growth rate in hairy roots clones has positive effect on secondary metabolite regulation and might leads to higher accumulation of metabolite compounds. Our previous findings indicated a link between methylation process at the level of DNA and polyamines and the secondary metabolites in transformed and untransformed plants, which are influenced by the *rol* genes [2;31]. The manipulation of root biomass and metabolic potential of *G.glabra* offer exciting possibility for large-scale production of root biomass by bioreactors and stable higher production of GA and other metabolites for pharmaceutical use. The root biomass system is

less expensive, less laborious and "eco-friendly" too. A more rigorous study of the direct link between the GA detected in roots and such processes has to be envisaged for a correct estimation of the transformation system effect.

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References

1. ALTAMURA MM (2004) *Agrobacterium rhizogenes rolB* and *rolD* genes: regulation and involvement in plant development. Plant Cell Tissue Organ Cult 77:89–101.
2. CUCU N (2001) Epigenomics: Unifying genomics and proteomics for an efficient functional genomic approach of the current genetic analysis. Rom. Biotechnol. Letters. 6 (6): 453-468.
3. CUCU N, TENEA G, GAVRILA L (2003) Genetically modified organisms III. *Agrobacterium rhizogenes* mediated transfer of *rolB* oncogene and marker *nptII* gene *Atropa belladonna* plants. Rom. Biotechnol. Letters. 8 (3): 259-1262.
4. CHOI PS, KIM YD, CHOI KM, CHUNG HJ, CHOI DW, LIU JR (2004) Plant regeneration from hairy root cultures transformed by infection with *Agrobacterium rhizogenes* in *Catharantus roseus*. Plant Cell Report. Springer-Verlag, 1-8.
5. DAMIANO C, MONTICELLI S (1998) *In vitro* fruits tree rooting by *Agrobacterium rhizogenes* wild-type infection. Journal of Biotechnology. 1(2): 189-195.
6. DOYLE JJ, DOYLE JL (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13-15.
7. FLORES H (1987) Secondary metabolites from root cultures. Trends in Biotechnology. 64-69.
8. HU SB, DU M (2006) Hairy root and its application in plant genetic engineering. Journal of Integrative Plant Biology. 48 (2): 121−127.
9. KISO Y (1984) Mechanism of antihepatotoxic activity of *Glycyrrhizin*. I: Effect on free radical generation and lipid peroxidation. Planta Med. 50 (4): 298-302.
10. KITTIPONGPATANA N, HOCK RS, PORTER JR (1998) Production of solasodine by hairy root, callus, and cell suspension cultures of *Solanum aviculare* Forst. Plant Cell Tissue Org. Cult. 52: 133–143.
11. KOVALENKO PG, ANTONJUK VP, MALUITA SS (2004) Secondary metabolites synthesis in transformed cells of *Glycyrrhiza glabra* L., and *Potentilla alba* L., as producents of radioprotective compounds. Ukrainica Bioorganica Acta. 1(2): 13-22.
12. LI W, ASADA Y, YOSHIKAWA T (1998) Antimicrobial flavonoids from *Glycyrrhiza glabra* hairy root cultures. Planta Med. 64 (8): 746-757.
13. LU H-Y, LIN J-M, ZHANG H-C, YIN T, GAO S-L (2008) Ri- mediated transformation of *Glycyrrhiza uralensis* with Squalene Synthase gene (GuSQS1) for production of glycyrrhizin. Plant Mol Biol Rep. 26:1-11.
14. LUPER S (1999) A review of plants used in the treatment of liver disease: part two. Altern Med Rev. 4 (3): 178-188.
15. MEHROTRA S, KUKREJA A-K, KUMAR A, KHANUJA S. P. S, MISHRA B.N (2008) Genetic transformation studies and scale up of hairy root culture of *Glycyrrhiza glabra* in bioreactor. Electronic Journal of Biotechnology. 11(2): 15.
16. MERKLI A, CHRISTEN P, KAPETANIDIS I (1997) Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. Plant Cell Rep. 16: 632–636.
17. MURASHIGE T, SKOOG F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-475.
18. NILSON O, OLSSON O (1997) Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol. Plant 100: 463-473.

19. POMPONI M, SPANO L, SABBADINI MG, CONSTANTINO P (1983) Restriction endonuclease mapping of the root inducing plasmid of *Agrobacterium rhizogenes* 1855. Plasmid 10: 119-129.
20. POPA O, CALIN A, TENEA G, CUCU N, GAVRILA L (2002) *Agrobacterium*-mediated transformation of some Romanian cultivars of tomato (*Lycopersicon esculentum*). Rev. Roum. de Biologie. 1 (2): 13-17.
21. RHODES MJC, ROBINS RJ, HAMILL JD, PARR AJ, HILTON MH, WALTON NJ (1990) Properties of transformed root culture. In: Charlwood BV, Rhodes MJC, eds. *Proceedings of the Phytochemical Society of Europe Secondary Product from Plant Tissue Culture*. Clarendon Press. Oxford. pp. 201–225.
22. RHODES MJC, PARR AJ, GIULIETTI A, AIRD ELH (1994) Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. Plant Cell Tissue Org. Cult. 38: 143–151.
23. SATO F, HASHIMOTO T, HACIYA A, CHOI KB, MORISHIGE T, FUJIMOTO H, YAMADA Y (2001) Metabolic engineering of plant alkaloids biosynthesis. Proc. Natl. Acad Sci. USA 98: 367-372.
24. SEVON N, OKSMAN C, KIRSI M (2002) *Agrobacterium rhizogenes* mediated transformation: Root cultures as a source of alkaloids. Planta Med 68: 859–868.
25. SKOOG F, MILLER CO (1957) Chemical regulation of growth and organ formation in plant tissues culture *in vitro*. Symp Soc Exp Biol. 11:118-231.
26. SPENA A, SCHMIILLING T, KONCZ C, SCHELL J (1987) Independent and synergistic activities of the *rol A, B* and *C* loci in stimulating abnormal growth in plants. Embo J. 6: 3891-3899.
27. SRIVASTAVA, S.,SRIVASTAVA, A.K (2007) Hairy root culture for mass-production of high-value secondary metabolites. Critical Reviews in Biotechnology. 27(1): 29-43.
28. STENTZEL O, TEUBER M, DRAGER B (2006) Putresceine N-methyltransferase in *Solanum tuberosum* L., a calystegine-forming plant. Planta 223:200-212.
29. TIWARI RK, TRIVEDI M, GUANG ZG, GUO GQ, ZHENG GC (2007) Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy roots cultures. Plant Cell Rep 26:199-210.
30. TENEA G, CUCU N, CALIN A, LITESCU S, GAVRILA L (2005a) Genetic transformation of *A.belladonna* using the Ri T-DNA of *Agrobacterium rhizogenes* LBA9402. Romanian Journal of Genetics. 1 (1): 36-43.
31. TENEA G, CUCU N, CALIN A, LITESCU S, GAVRILA L (2005b) A comparative study of transformation belladonna plants using two different *Agrobacterium rhizogenes* strains. Roumanian Biotechnological Letters. 10 (2): 2135-2142.
32. TEPFER D (1987) Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having application in rhizosphere biology, ecology and evolution. Plant Microbe interaction. 3. ISBN 0-07-046281-x.
33. TOLSTIKOW GA, MYTKIN VA (1991) Complex of glycerrhetinic acid with methyluracyl - a new class of any dotes and antiradicals drugs. In: Investigation and Using of Licorice Roots in Medicine. Alma_Ata (Kz): Talym Press. 196.
34. ZHANG L, YANG B, LU B, KAI G, WANG Z, XIA Y, DING R, ZHANG H, SUN X, CHEN W, TANG K (2007) Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing putresceine N-methyltransferase in methyl jasmonate-dependent. Planta 225: 887-896.