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TRANSGENIC CALLUS CULTURE ESTABLISHMENT, A TOOL FOR METABOLIC ENGINEERING OF *Rhodiola rosea* L.

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ABBREVIATIONS

MS – Murashige and Skoog
NAA – α-Naphthaleneacetic acid
BAP – 6-benzylaminopurine
DTE – Dithioerythritol
GUS – β-glucuronidase
Polyclar – Polyvinylpyrrolidone
2iP – 6-γ-γ-[Dimethylallylamino]-purine
IBA – Indole-3-butyric acid
IAA – Indole-3-acetic acid

Abstract. *Agrobacterium tumefaciens* EHA101 (pTd33) strain carrying *uidA* (GUS) reporter gene was used in model experiments on roseroot callus transformation. The T-DNA of pTd33 binary vector plasmid harbors *npt*II gene conferring resistance to kanamycin, and a *uidA* reporter gene, encodes the ß-glucuronidase enzyme. Roseroot seeds were sterilized and germinated on half strength MS media of which 70% germinated without any pretreatment. Calli were obtained from leaf segments of the *in vitro* grown seedlings. Calli was grown on solid MS medium supplemented with 1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Different types of calli were obtained of which the green and compact type was chosen for transformation experiments. After co-cultivation with agrobacteria, calli were transferred to the same medium supplemented with 20 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin and 300 mg l^{-1} claforan with antioxidants (Polyclar and DTE) for selection. GUS test using a titron buffer was applied for monitoring the transformation of the calli. DNAs of 20 individual samples was extracted and subjected for PCR analysis proved the stable transformation in all of the taken samples by amplifying the *npt*II gene

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fragment. The method introduced here can be a tool for inserting and over-expressing the genes encoding for hypothesized enzymes to be involved in the biosynthesis of pharmaceutically important bioactive molecules of roseroot and therefore facilitating the applications for callus culture of roseroot in different bioreactor systems for pharmaceutical productions.

Key words: roseroot, transformation, transgenic callus culture, *A. tumefaciens*, *npt*II, GUS

INTRODUCTION

Roseroot (*Rhodiola rosea* L.) is a dioecious, perennial herb from Crassulaceae plant family. The taxon is found in alpine habitats of the boreal zone of Eurasia and Appalachia, as well as in most parts of the Arctic, the Far Eastern coasts of Eurasia and North-Atlantic coast of N. America [Clausen 1975, Ohba 1989]. The pharmaceutical values of *R. rosea* and more notably its bioactive secondary metabolites namely, rosin, rosavin, rosarin and salidroside are extensively investigated [Panossian et al. 2010] in many different aspects by scientists worldwide. There are many published and available results from a tremendous number of experimental research and human clinical studies proving the multipurpose medicinal character of roseroot in respect of adaptogenic, stress-protective, geriatric, anti-fatigue, anti-oxidant, antidepresive, anxiolytic and so forth that constantly reviewed by Panossian et al. [2010] and Hung et al. [2011].

The very slow development of roseroot in its natural sites of alpine climate in one hand, and the growing demand for raw plant materials on the other hand have resulted in a rapid and severe depletion of its populations and has necessitated a legal protection for this species [Weglarz et al. 2008]. Although the cultivation of this species is possible in appropriate climatic conditions, the optimal cultivation time is quite long, at least 5 years [Galambosi 2006] between planting and harvesting the underground parts of the plants and needs a long term investment during this period of time. Another restriction factor for cultivation is the high level of heterozygosity that results in high intraspecific morphological, developmental and chemical variability [Ohba 1981, Kurkin et al. 1988, Kołodziej and Sugier 2013]. These cultivation difficulties encourage the investigation of roseroot *in vitro* cultures to obtain a fast and efficient way of bioactive metabolites production. However, roseroot callus does not produce the pharmaceutically active metabolites similar to the mature plants under *in vitro* culture condition, as was proved in several studies [Kurkin et al. 1991, György et al. 2004]. Hence, one of the most reasonable approaches to enhance the formation of roseroot bioactive compounds in *in vitro* culture is genetic engineering in their biosynthetic pathway by regulating the involved enzymes activities [Ma et al. 2007, Mirmazloum and György 2012]. From the various methods developed to introduce DNA into plant cells so far, most include a transformation step that is mediated by *Agrobacterium tumefaciens* [Gelvin 2000, Zupan et al. 2000] with an antibiotic resistance marker gene which is typically used as an indicator of gene replacement. As a reporter for transformation, GUS is frequently utilized because it offers several advantages, such as a high stability in translational fusion with other proteins and a fine resolution in histochemical staining that allows detection of signals even in single cells [Jefferson et al. 1987, Lindsey et al. 1993].

The overall objective of the current research was to develop a protocol for callus induction and *in vitro* multiplication of *R. rosea* calli for the purpose of gene transformation. The reliable experiment presented here provides a consistent method for callus induction and gene transformation, which can be used as a tool to enhance the content of the pharmaceutically important metabolites in bioengineering projects by over expression or suppression of the involved genes in the plant genome and justifying bioprocessing of *Rhodiola rosea* cell culture for mass production of desired compounds.

MATERIAL AND METHODS

Plant material and culture establishment. Seeds of *Rhodiola rosea* L. were kindly assured by the University of Oulu (Finland) Botanical garden from an Austrian originated population. Seeds were surface sterilized by being washed in running tap water, immersed in 70% ethanol for 3 min followed by submerging in 50% sodium hypochlorite for 4 min and then rinsed four times in sterile distilled water. For germinating medium, half-strength MS [Murashige and Skoog 1962] salts and 30 g l^{-1} sucrose solidified with 4.5 g l^{-1} agar. PH was adjusted to 5.8 prior to sterilizing the medium by autoclaving at 121°C for 18 min. 25 ml from medium was distributed in glass baby food jars in which 40 seeds were placed on medium (fig. 1 a). Seeds were germinated and grown aseptically at 22 ±2°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 µmol m^{-2} s^{-1} at culture level which provided by cool-white fluorescent lamps. Having seed germination (fig. 1 b) within 8–10 days of culture, the seedlings were sub-cultured in the same medium after 6 weeks (appearance of the 5th leaf). For callus induction, three individual seedlings were selected based on good vigor under *in vitro* condition on 16th week after germination (fig. 1 c). The leaves of 4–6 mm in diameter were cut from the seedlings and scratched at the edges by using a sharp sterile scalpel blade. The leaves were put on the surface of new MS medium (fig. 1 d) enriched with 30 g l^{-1} sucrose and gelled with 4.5 g l^{-1} agar at the same PH, supplemented with NAA, BAP, IAA, IBA and 2iP phytohormones with different concentration and combinations (tab. 1) in glass Petri dishes. Callus formation occurred (fig. 1 e) under the same light and temperature conditions during the following 6 weeks. Callus pieces of 0.5–1 cm in diameter were selected for transformation based on their vigor, friability and green color in further sub-cultures in the same medium (fig. 1 f).

Transformation of the callus. 12 pieces of the most vigorous callus masses (6–8 mm in diameter) were placed on MS solid medium without any growth regulators in each Petri dish, 2 days before the transformation. The callus was transformed using an *Agrobacterium tumefaciens* strain EHA101 (pTd33) gene construction [Szegedi et al. 2001]. The T-DNA of pTd33 binary vector plasmid harbors a neomycin phosphotransferase II (*npt*II) gene conferring resistance to aminoglycoside antibiotics such as kanamycin, and a *uidA* (GUS) reporter gene, encoding ß-glucuronidase enzyme [Tinland et al. 1995]. Both the reporter and selection marker genes are under the control of cauliflower mosaic virus (CaMV) 35S promoter. The bacterium containing the gene construction was placed on solid AB medium [Lichtenstein and Draper 1986] 48 h before the transformation to obtain fresh growing bacterial clone. For the co-cultivation, small

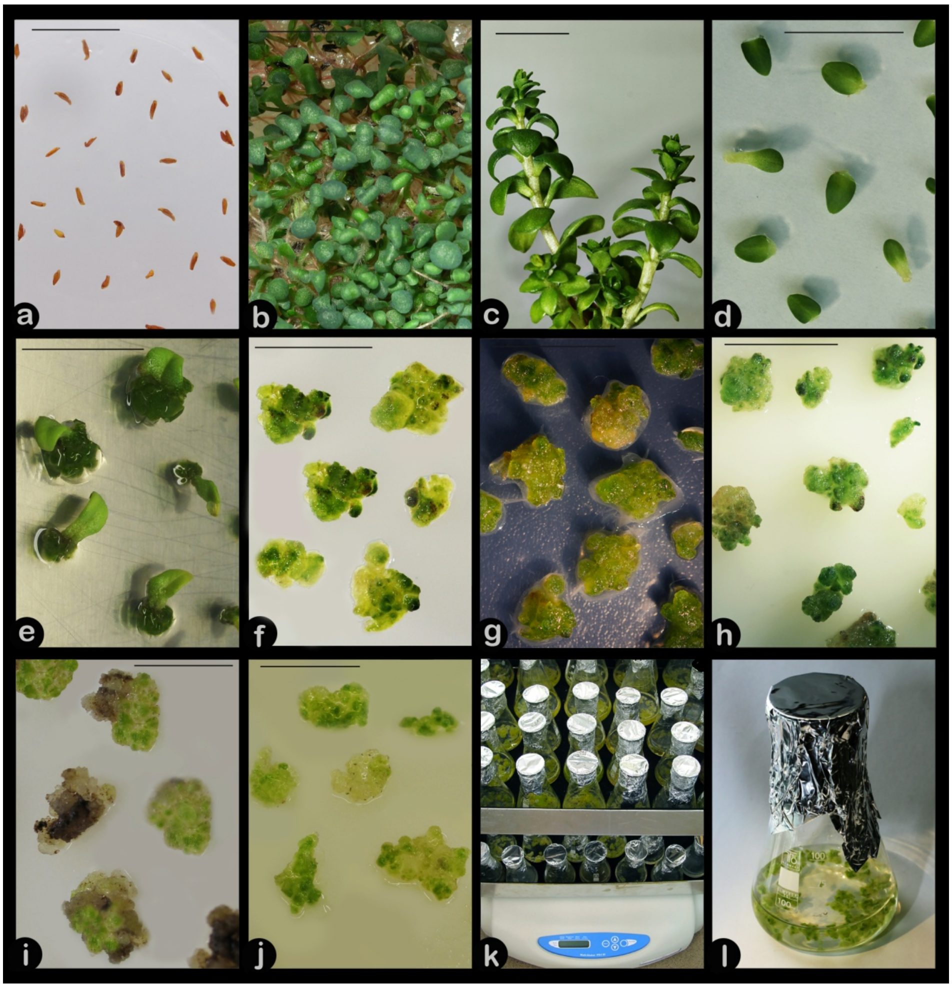

Fig. 1. Callus induction and Agrobacterium-mediated transformation of *Rhodiola rosea* calli on MS solid medium: a – decontaminated roseroot seeds on germination medium; b – 4 week old roseroot seedlings; c – 4 month old roseroot seedlings ready for callus induction from the leaves; d – leaves of roseroot on callus-inducing solid MS medium enriched with 30 g l^{-1} sucrose and solidified with 4.5 g l^{-1} agar supplemented with 1 mgl^{-1} NAA and 0.5 mgl^{-1} BAP in glass Petri dishes; e – roseroot callus formation after 6 weeks; f – sub-culturing the vigorous roseroot callus on the fresh medium of the same composition; g – co-cultivation of roseroot calli with agrobacteria on phytohormone free MS medium in dark (48 h); h – callus on the selection medium containing kanamycin, carbenicillin, claforan and anti-oxidants; i – callus on selection medium after 1 month**.** The green parts survived the agorobacteria co-culture and contain the transgenic cells; j –further selection of transgenic sells by sub-culturing on the same fresh medium; k – the final selection procedure by liquid culture of transgenic calli in kanamycin containing medium; l –100% transformed calli ready for mass production or any further application (Bars = 1 cm)

Table 1. Effects of different phytohormones combination on callus induction of *R. Rosea* leaves

No	Culture medium type	Callus induction rate (%)*	Callus colour and quality
1	MS + 1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	80	Green and Compact
2	MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP	60	Light Green and Compact
3	MS + 0.5 mg l^{-1} NAA + 1.5 mg l^{-1} BAP	50	Yellowish Green and Compact
4	MS + 3 mg l^{-1} 2iP + 0.3 mg l^{-1} IAA	80	Green and Friable
5	MS + 0.6 mg l^{-1} NAA + 3 mg l^{-1} 2-iP	80	Yellowish Green and compact
6	MS + 0.1 mg l^{-1} BAP + 1 mg l^{-1} NAA	36	Green and Friable
7	MS + 1 mg l^{-1} BAP + 0.1 mg l^{-1} IBA	4	Green and Friable
8	MS + 1 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	20	Green and Compact

*Induction rate is the mean of 10 replicates in each, 20 leaf explant were cultured

volumes (20–30 μl) of bacterial suspension (10^8 cells ml^{-1}) were placed onto the surface of callus on MS phytohormone-free solid medium. The co-cultivation with agrobacteria was conducted in dark and for 24, 36 or 48 h at 26–28ºC, then the calli were transferred onto solid medium containing 20 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin, 300 mg l^{-1} claforan, 4 g l^{-1} insoluble polyvinylpyrrolidone [Perl et al. 1996, Mozsár et al. 1998] and 0.1 g l^{-1} dithioerythritol [Bornhoff and Harst 2000]. Calli were transferred to fresh selection medium of the same composition in each 20 days for selecting the transgenic cells. Final selection was conducted by culturing the calli in shaking liquid medium to obtain 100% transgenic cells in response to direct contact with kanamycin. The transformation stages can be seen in Fig. 1 g–l.

DNA extraction and polymerase chain reaction (PCR). To determine the foreign gene insertion in the callus, genomic DNA was individually extracted from 20 different transgenic callus pieces and 1 from non-transformed callus from the 5th sub-cultures by using a SP Plant mini kit (Omega, VWR International Kft.). PCR was performed in 25 μl reaction volume containing 20–80 ng DNA, 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.02 mM dNTP mix, 1 μmol of each forward (5'GAGGCGAGGCGGCTATGACTG3') and reverse (5'ATCGGGAGCGGCGATACCGTA3') primers [Hoffmann et al. 1997], 1 unit of Taq DNA polymerase (Fermentas, Szeged, Hungary) and sterile distilled water. Primer pairs of *npt*II gene were used for the DNA fragment amplification. The reactions were carried out in a Swift MaxPro thermocycler (Esco Healthcare, Csertex Kft. Hungary). For amplification of the transgene fragment the following program was used: initial denaturation at 94°C for 4 min; followed by 30 cycles of 94°C for 60 s, 54°C for 60 s, 72°C for 90 s; and a final synthesis at 72°C for 3 min. For positive control, pTd33 plasmid DNA was used as a template in the reaction mix and to control the PCR performance, one reaction mix without any DNA template was also included. The PCR products were applied on a 1% (w/v) ethidium bromide-stained agarose gel in 1 × TBE buffer with xylencyanol loading buffer to verify the occurrence of the amplification. 10 μl of the PCR products stained with 2 μl of loading dye were run for 1 h at 80 V.

Amplicons were scored visually for presence (1–20 and P) or absence (N-C) of nucleotide bands with 700 base pair length. DNA samples were also tested with VCF (5'-ATCATTTGTAGCGACT-3') and VCR (5'-AGCTCAAACCTGCTTC-3') primers [Sawada et al. 1995] to verify the absence of any *Agrobacterium* in the medium or on the calli.

Histochemical GUS assay. For verification of the transformation, callus samples were transferred to 1ml of assay solution [Jefferson et al. 1987, Oláh 2005] in 1.5 ml Eppendorf tubes. The solution contained 150 µl of 100 mM Na-Phosphate buffer (pH 7.0, 50 mM Na_2HPO_4 and 50 mM KH_2PO_4), 100 µl of 50 mM Na-EDTA, 25 µl of 5 mM K-ferricyanide, 25 µl of 5 mM K-ferro-cyanide, 100 µl of 0.005% Triton X-100, 25 µl of 0.3% X-Gluc and 575 µl of distilled water. The test Eppendorfs were kept in a shaker-incubator for 1h at 37ºC. Transient GUS expression has been tested for 20 pieces of calli from 10 co-cultivation Petri dishes (2 out of 12 pieces from each Petri dish).

RESULTS AND DISCUSSION

Our work provided a reliable method for transformation and selection of *Rhodiola rosea* at callus level. Different steps of the experiment from germination until transgenic callus culture establishment are shown in Figure 1.70% of germination capacity was obtained after 8 days without any pretreatment. Callus was obtained from leaves and stems of the explants that were grown in MS medium supplemented with different combination of plant growth regulators with the highest rate (80%) being on the MS medium supplemented with (1.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP), (3 mg l^{-1} 2iP + 0.3 mg l^{-1} IAA) and (0.6 mg l^{-1} NAA + 3 mg l^{-1} 2iP) but their morphologies and induction rates were different in subsequent cultures (tab. 1). The effects of different phytohormones on callogenesis rate and quality are given in Table 1. The calli grown on the medium with 2iP and IAA were very friable and sensitive to sub-culturing, ending with getting colorless and finally brown after 2 weeks. The calli grown on the medium with 2iP and NAA were compact but mostly yellowish. Whereas the calli from the medium with NAA and BAP were more compact and fleshy, making them easy to sub-culture. Callus initiation was noticeably faster from the leaves (10 days after culture) comparing to stems (20 days) and their subsequent growth was also much faster. The leaf explants gave the best rate and high quality callus which was used in further sub-cultures and transformation. In contrast, colorless and watery callus was obtained from stem explants. *In vitro* cultures are dependent on endogenous levels of plant growth regulators and are enhanced by exogenously applied ones. Growth and morphogenesis of plant tissues under in vitro conditions are indeed influenced by the culture medium composition.

Different morphogenesis pattern have been reported from different *Rhodiola* species *in vitro* culture [Liu et al. 2006, Debnath 2009, Tasheva and Kosturkova 2012a] and even different responses to plant growth regulators have been observed in different *Rhodiola* ecotype in our earlier experiments. Callogenesis of 55% have been obtained by Tasheva and Kosturkova [2010] form a Bulgarian originated *Rhodiola* explants in a MS media containing three mg l^{-1} 2iP, 6 mg l^{-1} NAA and 150 mg l^{-1} glutamic acid in contrast with 3 of our supplementation which led to 80% of callus induction. It can be concluded that the *in vitro* culture and micro-propagation of roseroot plants is not complicated and can be optimized for the purpose of the experiment in regard with explants origination readily. During the callus sub-culturing in our experiment, two types of leaf originated calli were distinguishable. Type 1, was mostly opaque with white to yellowish color and the type 2, with more compact and sharp light green color which was chosen for sub-culturing and transformation in our experiment. Different callus type formation has been also reported by Furmanowa et al. [1995] in Roseroot *in vitro* cultures. It should be noticed that the mentioned characteristics which led to choosing the second calli type for transformation was in accordance to bioreactor culture circumstances like high stability in liquid culture and high growth rate. The biomass of the calli almost doubled in every 2 weeks. After co-cultivation with Agrobacteria, more than 50% of the calli survived the infection after 2 weeks of culture on selection medium for the first time. In the second and further selection sub-cultures in each 2 weeks of interval, the cell death rate decreased exponentially until the 100% stable antibiotic resistant callus has been obtained after 5 sub-cultures on solid medium and 2 sub-cultures in liquid medium. Liquid culture in selection medium was essential to eliminate the non-transformed cells that were growing on the upper parts of the callus which were not in direct contact with the medium. The transgenic callus has been growing normally in both solid and liquid culture containing 20–50 mg l^{-1} kanamycin. No morphological changes have been observed during sub-culturing. There was no significant difference in transformation efficiency according to different times of co-cultivation (24, 36 or 48 h) with agrobacteria (data are not shown). Positive growth regulating effect of claforan and carbenicillin in the liquid sub-cultures supplemented with 300–550 mg l^{-1} of them (with 4time higher growth rate, fresh and dry weight for the calli grown in the medium supplemented with 450 mg l^{-1} of both claforan and carbenicillin comparing to the control in one month) was also observed which were originally used for agrobacteria elimination (data are not shown).

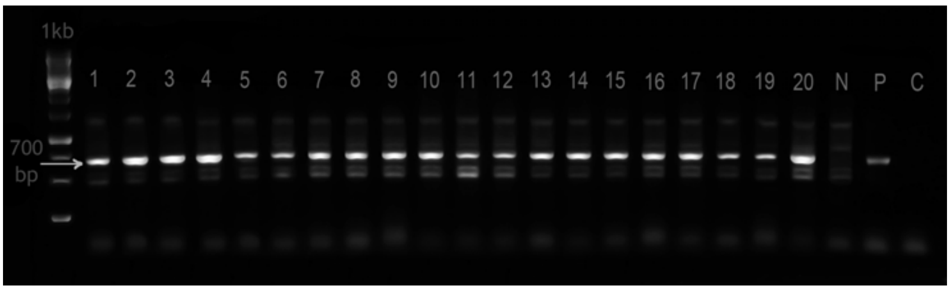

Fig. 2. Molecular analysis of transgenic roseroot callus using primers for a 700 bp fragment of neomycin phosphotransferase II (*npt*II) gene, 1–20: PCR amplicons from individual lines of transgenic callus; N Negative control representative of Non-transgenic callus; P positive control amplified from agrobacteria plasmid; C PCR reaction mix without DNA template

All of the samples from transformed calli subjected for DNA isolation and polymerase chain reaction (PCR) experiment, showed the 700 bp amplified fragment of the inserted neomycin phosphotransferase II (*npt*II) gene (Fig. 2, 1–20) whereas, no such an amplicon was synthesized during PCR cycles in the case of non-transgenic callus (fig. 2 N). The PCR reaction for positive control by using the plasmid DNA as template showed the exact size of synthesized amplicone (fig. 2 P) in comparison with transgenic calli. As a more quantitative expression indicator, GUS test was performed once 5 days after transformation and cultivation on selection solid medium (fig. 3 b), and once later in selection liquid cultures 4 months after transformation (fig. 3 c). The latter test clearly showed positive results by expressing blue color indicating the inserted model gene which was visible in all parts of the callus. After 4 weeks of liquid culture hardly any green callus part was visible in course of the test and the blue color was dominant. The same result has been obtained after 6 months and several sub-culturing. To verify the absence of *Agrobacterium* in putatively transformed calli which may lead to a false PCR amplification product, DNA samples were also tested with *virC* gene primers which amplify a 730 bp virulence region located on the separate helper Ti plasmid. No PCR product was observed on the agarose gel using the above mentioned primers indicating the absence of any *Agrobacterium* in the medium or on the calli.

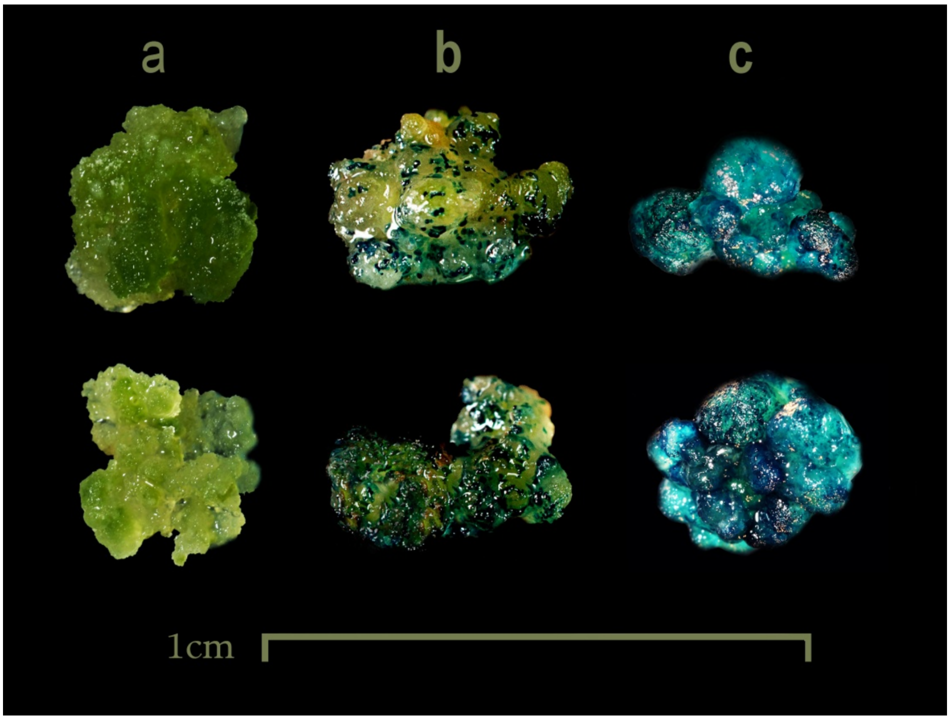

Fig. 3. GUS reporting expression pattern in transgenic *Rhodiola rosea* callus: a – before transformation; b – calli 5 days after transformation on solid medium; c – GUS expression in transgenic calluses cultured in liquid selection medium after 2 weeks

A few reports from genetic transformation of *R. rosea* are available in the scientific literature and all of them were dealing with hairy root induced by *Agrobacterium* strains [Tasheva and Kosturkova 2012b] to obtain higher quantity of roseroot pharmaceuticals. In case of *R. sachalinensis* the *Agrobacterium tumefaciens* mediated transformation have been reported from China by overexpressing the functional genes in salidroside biosynthesis pathway [Ma et al. 2007, Ma et al. 2008, Yu et al. 2011, Zhang et al. 2011] but to the best of our knowledge no report for transgenic *R. rosea* plant is ever published. Although plant regeneration from *R. rosea* callus has been reported [Tasheva and Kosturkova 2013] but the frequency of regenerated plants from callus cultures in their study was very low, not exceeding 5–6%. Even though plants should be used as starting material for *in vitro* cultures to assure the genetic identity of the developing callus, but the transformation in callus level is much easier to conduct in a much shorter period of time with higher expression level as we showed in this experiment. While many genes are involved in the formation of roseroot secondary metabolites but none of them are still proved to be the most key player in the pathway, the transformation in the callus level is the best tool to monitor the results of bioengineering with respect to roseroot liquid callus culture.

CONCLUSION

Rhodiola rosea L. leaf explants cultured in the MS medium supplemented with 1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP resulted in the best rate (80%) and high quality callus (green and compact) and used for further sub-cultures and transformation. Genetic transformation method for *Rhodiola rosea* with *Agrobacterium tumefaciens* was optimized to the co-cultivation of small volumes (20–30 µl) of bacterial suspension in dark and for 24 at 26–28ºC. The optimized selection medium turned out to be of 20 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin, 300 mg l^{-1} claforan, 4 g l^{-1} insoluble polyvinylpyrrolidone and 0.1 g l^{-1} dithioerythritol. The method for establishing transgenic roseroot callus lines as we presented in this work, is fast, stable and didn't affect the normal growth rate and morphology of the calli in liquid culture. This gives the possibility for bioengineering the glycosides pathway of *Rhodiola rosea* by inserting the functional genes and hence facilitating an effective production system in large scale bioreactors for the production of the most valuable pharmaceutically important secondary metabolites of this adaptogenic plant.

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ZAKŁADANIE HODOWLI KALUSOWEJ JAKO NARZĘDZIE W INŻYNIERII METABOLICZNEJ *Rhodiola rosea* L.

Streszczenie. Szczep *Agrobacterium tumefaciens* EHA101 (pTd33) przenoszący gen reporterowy *uidA* (GUS) został użyty w modelowych doświadczeniach nad transformacją kalusa różeńca górskiego. T-DNA z plazmidy wektora binarnego pTd33 mieści w sobie gen *npt*II przekazujący odorność kanamycynie, natomiast gen reporterowy *uidA* koduje enzym β-glukuronidazy. Nasiona korzenia różeńca gorskiego wysterylizowano i poddano kiełkowaniu na połowie zestawu pożywki MS. Spośród nich 70% wykiełkowało bez żadnego wcześniejszego zabiegu. Kalusy otrzymano z segmentów liści sadzonek wyrosłych *in vitro*. Kalusy wyhodowano na stałej pożywce MS z dodatkiem 1 mg l^{-1} NAA oraz 0,5 mg l^{-1} BAP. Uzyskano różne typy kalusa, z których typ zielony i zwarty został wybrany do doświadczeń dotyczących transformacji. Po wspólnej hodowli z agrobakteriami kalusy były przeniesione to tej samej pożywki uzupełnionej 20 mg l^{-1} kanamycyny, 200 mg l^{-1} karbenicyliny oraz 300 mg l^{-1} klaforanu z przeciwutleniaczami (Polyclar i DTE) do selekcji. Zastosowano test GUS przy użyciu bufora trytronowego do monitorowania kalusów. Wyodrębniono 20 indywidualnych próbek DNA i poddano je analizie PCR, która wykazała stabilną transformację we wszystkich próbkach poprzez amplifikowanie fragmentu genu *npt*II. Metoda przedstawiona tutaj może być narzędziem insercji i ekspresji kodowania genów do hipotetycznych enzymów, które mają brać udział w biosyntezie ważnych z punku widzenia farmaceutycznego molekuł różeńca górskiego, co ułatwi zastosowanie hodowli kalusów różeńca górskiego w różnych systemach bioreaktorów w produkcji farmaceutycznej.

Słowa kluczowe: różeniec górski, transformacja, transgeniczna hodowla kalusa, *A. tumefaciens*, *npt*II, GUS

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