TRANSGENIC ROOT CULTURES OF *GENTIANA PUNCTATA* L.

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ABSTRACT

Shoot cultures of *Gentiana punctata* L. were inoculated with suspension of *Agrobacterium rhizogenes* strain A4 M70GUS. Hairy roots which appeared 2-3 weeks later were cultured on hormone-free, liquid, WPM (Lloyd and McCown 1980) basal medium for more than 5 years (60 subcultures). Growth rate of transformed roots was higher than the growth rate of non-transformed roots. Spontaneous shoot regeneration occurred only in three culture vessels in subcultures No. 40 and 42. Plants had phenotype characteristics typical for *A. rhizogenes* transformed plants including: wrinkled leaves, short internodes, plagiotropic roots and in general their growth rate was reduced. These plants also manifested precocious formation of flower buds without vernalization and flowering under in vitro conditions. Flowers were pale yellow, the same as in the standard phenotype.

KEY WORDS: *Agrobacterium rhizogenes*, *Gentiana punctata* L., excised root cultures, shoot regeneration, in vitro flowering.

INTRODUCTION

Gentiana punctata L. together with *G. lutea* L. is an endangered, nearly extinct species in Yugoslavia. Devastation of both species is caused by destructive harvesting of roots and rhizomes which are used in traditional medicine and for preparation of aperitives.

The annual requirement of *Gentiana lutea* roots needed for industrial purposes in Europe has been estimated at 1500-2500t. Gentians for commercial use can be grown from seeds and cultivated in plantations. However plant biotechnology offers an alternative approach founded on the use of excised root culture and aimed at production of root biomass on commercial basis in bioreactors/ fermentors. Furthermore, it has been shown that hairy root cultures obtained from plants transformed with *Agrobacterium rhizogenes* are characterized by very fast growth which often exceeds growth of untransformed root cultures (Tepfer and Casse-Delbart, 1987).

Inoculation of plants with *Agrobacterium rhizogenes* and first hairy root cultures were obtained and investigated by Tepfer (1983, 1984), later followed by numerous reports of other authors. Transformation of plants with *A. rhizogenes* was reviewed by Hamill et al. 1987; Birot et al. 1987; Mugnier 1988; Tepfer 1990; Porter 1991 and Doran 1997. Procedures for micropropagation of various Gentians were presented by Wesołowska et al. (1985), Lamproye et al. (1987), Viola and Franz (1989), Skrzypczak et al. (1993), Momčilović et al. (1997b) and Vinterhalter and Vinterhalter (1998). Transformation of Gentians and related species with *Agrobacterium rhizogenes* was reported by Ishimaru et al., 1990 (*Swertia japonica*), Handa et al., 1992 (*Eustoma grandiflorum*), Sugiuma and Akihama, 1995 (*Gentiana scabra*) and

Momčilović et al., 1997a (*Gentiana acaulis*, *G. cruciata*, *G. lutea* and *G. purpurea*). In *Gentiana cruciata*, Mugnier (1988) reported only formation of tumor callus cultures following inoculation.

Agrobacterium mediated transformation of *G. punctata* was for the first time reported by Vinterhalter et al. (1993) and the presence of bitter secoiridoid glycosides and xanthenes in transformed root cultures by Vinterhalter et al. (1997).

The goal of this work was to obtain stable root cultures of *G. punctata* transformed with *Agrobacterium rhizogenes* and evaluate its potential use for biomass production.

MATERIAL AND METHODS

Plant material, culture media and culture conditions

Shoot cultures of *Gentiana punctata* L. were obtained from embryos excised from aseptically germinated seeds which were collected on Šara mountain (2300 m). Procedure for establishment and maintenance of cultures was the same as previously published by Vinterhalter and Vinterhalter (1998).

Basal medium (BM) contained WPM (Lloyd and McCown, 1980) macro salts; MS (Murashige and Skoog, 1962), micro-nutrient salts and iron stock solution and LS (Linsmaier and Skoog, 1965) vitamins, 2% sucrose and 0.62% agar. Medium pH was adjusted to 5.8 prior to autoclaving which lasted for 20 minutes at 114°C. Prior to inoculation cultures were subcultured on media with 0.25 mg l⁻¹ BA and 0.1 mg l⁻¹ IAA.

Conditions of the growth room were: temperature 25 ± 2°C, photoperiod: 16h light/8 h dark, light intensity 45 μmol m⁻² s⁻¹ as measured by Li Cor 190SA quantum sensor coupled with Li-1000 Datalogger (Lincoln, Ne, USA).

Fig. 1. Appearance of A4M70GUS transformed roots on shoots 20 days after inoculation with bacterial suspension.

Fig. 2. Transformed roots on hormone-free liquid medium.

Fig. 3. Transformed roots on hormone-free agar solidified medium (47-th subculture $\approx$ 4.5 years).

Fig. 4. Shoot cultures regenerated from transformed roots with in vitro formed flowers.

Fig. 5. Expression of GUS gene demonstrated with colour reaction following treatment with X-gluc. Left – staining of terminal bud and adjacent leaflets; right – strong staining of transformed root with regenerated shoots.

Fig. 6. Transection of transformed root stained with X-gluc. Intensive colour develops in epidermal cells and in vascular tissues. Bar = 60 μ m.

Biomass corresponds to fresh weight of the explant at the beginning of the experiment or the weight of the whole culture at the end of subculture calculated per culture vessel. Index of biomass production is calculated as final tissue weight divided by initial weight for a pre-defined vessel and medium volume.

Bacterial strain

In this work we used *Agrobacterium rhizogenes* non disarmed agropine-type strain A4M70GUS constructed by Dr. M. Tepfer (INRA, Versailles, France). This strain is harbouring cointegrative plasmid with GUS construct integrated into TL region of the pRiA4 (Tepfer and Casse-Delbart, 1987). GUS construct contains *uidA* sequence under the 70S promoter (enhancer-doubled 35S CaMV promoter), followed by NOS polyadenylation sequence. Bacterial strains were maintained on agar (1.5%) supplemented YEB medium (Van Larebake et al., 1977) with addition of 100 mg l⁻¹ neomycin sulphate (ICN-Galenika). Plants were inoculated with 24 h old bacterial culture grown at 28°C on a shaker adjusted to 220 rev. min⁻¹. Suspension density was $\approx 10^8$ cells/ml.

Inoculation, hairy-root culture and plant regeneration

Shoots 2 cm in length consisting of 3-4 nodes were inoculated by puncturing nodal regions with a hypodermic needle dipped into bacterial suspension. Explants were placed on hormone-free BM from which 4-5 days later they were transferred to hormone-free BM supplemented with 200 mg l⁻¹ cefotaxime-Na (Jugoremedia, Zrenjanin, Yugoslavia). When roots reached 20 mm in length they were excised and transferred to the cefotaxime supplemented medium in Petri dishes. Concentration of the antibiotic was gradually decreased so the fifth subculture was made on antibiotic-free medium following a thorough test for microorganism contamination.

For root elongation and branching experiments, ten 15 mm long apical root explants were used, and placed in either Petri dishes with 25 ml agar solidified medium or 100 ml wide neck Erlenmeyer flasks with 40 ml liquid medium. Flasks with liquid medium were maintained on shakers.

The effect of 0.1-8% sucrose was also investigated in liquid medium using explants consisting of 400 mg of root biomass.

Shoot regeneration from transformed root cultures was investigated on cytokinin supplemented media containing 0.01-5.0 mg l⁻¹ BA, kinetin, CPPU or TDZ. Regenerated shoots were cultured on medium with 0.5 mg l⁻¹ BA and 0.2 mg l⁻¹ GA₃.

Histochemical GUS assays

Histochemical localization of GUS expression was performed as described by Jefferson (1987). Tissue samples were incubated overnight at 37°C in 0.05% (w/v) solution of X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) in 50 mM

sodium phosphate buffer, pH 7.0. Reaction of X-gluc with substrate gives indigo-blue colour. For histological investigation longitudinal and cross sections were made from material which was fixed in FAA and embedded in paraffin. Sections 5-10 μ thick were photographed on Leica, DMRB microscope.

Plant DNA isolation and PCR analysis

DNA was isolated from 50 mg fresh tissue with 1 ml DNA-zole™ (Molecular Research Center Inc.; Cincinnati, Oh., USA) according to manufacturer protocol. The success of transformation was confirmed with PCR method in which amplification of part of the *UidA* gen (168bp) was performed. Following primers were used: 5'-ACTCGACGGCTGTGGG-CAT-3' and 5'-CTGATACCAGACGTTGCCCGCATAA-3'. Every PCR reaction mixture was 50 μ l in volume containing 1 x PCR bufer, 10 μ M dNTPs, 20 μ M MgCl₂, 5 μ M of each primer and 0.5 U Taq DNA polymerase (Perkin Elmer). Before amplification, samples were denatured at 94°C for 4 min. Each of 25 PCR cycles consisted of denaturation step at 94°C lasting for 30" followed by annealing at 65°C lasting for 45" and a polymerisation reaction at 72°C lasting for 45". PCR reaction was performed on DNA Thermal Cycler-Perkin Elmer 9600.

RESULTS

Transformation and culture of transgenic roots

Three weeks after the inoculation adventitious roots appeared in 32 out of 188 (17%) of shoots which were treated with bacterial suspension. Roots were tender and fragile and it was better to allow them to elongate for one more subculture prior to excision (Fig. 1). After isolation, roots started to elongate faster whilst branching was poor. There was no apparent difference among individually established root clones therefore all regenerated roots were considered to belong to the same clone. These A4GUS transformed root cultures have been so far maintained in our laboratory for more than 5 years (over 60 subcultures). During this period occasionally some roots formed callus. This callus could not be maintained as a separate culture (clone) since it became necrotic and perished after subculturing on fresh medium.

If the excised roots are cultured on liquid (Fig. 2) instead of agar solidified medium (Fig. 3) then the increase of their biomass expressed as weight of a single root is much higher in liquid medium. This increase results not only from better root elongation but also from root branching which occurs in liquid medium (Table 1). Comparison of A4GUS transformed with non-transformed roots shows that former have higher elongation and biomass production whilst latter have superior branching. It is important to notice here that non-transformed

TABLE 1. Effect of agar solidified versus liquid medium on root elongation and biomass production of non-transformed and A4GUS transformed roots after 35 days.

Culture type	Medium	No. of excised roots	The increase in root length, mm $\pm$ SE	No. of lateral roots per root $\pm$ SE	Fresh weight per root, mg $\pm$ SE
non-transformed	agar	50	0.34 $\pm$ 0.2	3.2 $\pm$ 0.3	4.6 $\pm$ 0.8
	liquid	170	15.7 $\pm$ 1.2	7.8 $\pm$ 0.4	17.9 $\pm$ 2.5
A4GUS transformed	agar	59	38.8 $\pm$ 1.4	0	23.2 $\pm$ 2.2
	liquid	99	45.2 $\pm$ 1.9	1.54 $\pm$ 0.2	36.8 $\pm$ 4.8

TABLE 2. Effect of sucrose concentration on the biomass increase of A4M70 GUS transformed roots.

% sucrose	No. explants	Initial root clump weight, mg $\pm$ SE	Final root clump weight, mg $\pm$ SE	Index of biomass increase
0.1	5	401.6 $\pm$ 59.9	583.6 $\pm$ 52.9	x 1.5
2	53	487.7 $\pm$ 18.4	1660.0 $\pm$ 100.0	x 3.4
4	24	388.8 $\pm$ 11.7	929.7 $\pm$ 55.5	x 2.4
6	23	432.2 $\pm$ 20.7	759.7 $\pm$ 46.2	x 1.7
8	11	400.9 $\pm$ 17.1	691.6 $\pm$ 26.3	x 1.7

root culture medium was supplemented with 0.2 mg l⁻¹ IBA and 0.1 mg l⁻¹ GA₃ which was previously found to support highest root elongation and branching (Vinterhalter et al. 1993).

If explants for subculturing instead of single apical root segments consisted of clumps of A4GUS transformed roots then their growth was equally good on agar solidified and liquid media. Maximum growth of such root cultures in liquid medium was registered at 2% sucrose (Table 2). Higher sucrose concentrations not only decreased the biomass of culture but also induced start of tissue necrosis.

Regeneration of shoots

Attempts to obtain shoot regeneration from transformed roots on cytokinin (0.01-5.0 mg l⁻¹ BA, kinetin, CPPU and TDZ) supplemented media were not successful. However in the 40-th subculture spontaneous shoot regeneration was observed on a single root and in 42-nd subculture on two more roots. These shoots belonged to a phenotype which differed in comparison with the phenotype of shoots from non-transformed plants. They were characterized by short internodes, leaf epinasty, formation of callus on the cut surface of the explant and tendency to become hyperhydric.

Regenerated shoots were transferred on hormone-free basal medium but shoot multiplication was absent. Shoot were then transferred to medium with BA 0.5 + GA₃ 0.1 mg l⁻¹ on which multiplication occurred at a low rate for more than a year. After a 12 months in culture, one clone formed three flower buds. All three floweres opened in vitro (Fig. 4), they were yellow in colour and appeared normal except somewhat deformed pistils. In vitro fertilization was performed bud seeds failed to develop since cultures which formed floweres perished.

Histochemical localization of GUS activity in leaf and root of transgenic plants

Activity of the A4M70GUS gene in the transformed tissue was demonstrated with histochemical tests in which x-gluc was used as substrate. Since transformation comprises incorporation of an active β -glucuronidase gene, action of this gene can be monitored through its enzyme reaction during which intensively blue colour develops. This reaction was demonstrated in situ in apical root segments and leaves of transformed plants (Fig. 5). As a control non-transformed plants were used.

Hystological analysis of sections enabled more accurate localization of enzyme reaction. In cross sections of roots, blue colour indicating GUS activity was observed in epidermal cells, trichomes and cells of root cap especially in meristematic and vascular cells (Fig. 6).

PCR analysis

Results of the PCR reaction further supported our suggestion that we established transgenic root culture of *G. punctata*. Appearance of 168 bp amplification product in the lane 2 of the figure 2 confirmed the presence of GUS gene in the genome of cells making up our root cultures. The PCR product of the same size was obtained in the reaction where the DNA template was genomic DNA isolated from the *Arabidopsis* mutant (Orbović et al., 1995) which was previously shown to carry the GUS gene (Fig. 7 lane 5). On the other hand, when the tissue sample from the non-transformed roots was tested under the same conditions, no amplification product was found (Fig. 7 lane 1). To prove that the PCR product from the lane 2 was not an artifact we ran two additional reactions where we omitted DNA (Fig. 7 lane 3) or Taq enzyme (Fig. 7 lane 4) and they yielded no product.

DISCUSSION

Plants transformed with *A. rhizogenes* exhibit a distinct set of features which according to Suganuma and Akihama (1995) and literature cited therein include: wrinkled leaves, reduced apical dominance, short internodes, plagiotropic roots and in some species early flowering and formation of flower buds without vernalization. We observed all mentioned features in *G. punctata* plants which spontaneously regenerated from hairy root cultures. This observations together with results obtained with GUS reaction and PCR analysis provide sufficient evidence that hairy root cultures of *G. punctata* contain functional foreign genes.

Fig. 7. PCR analysis: 0 – standard DNA marker, 1 – non-transformed tissue, 2 – A4 M70 GUS transformed roots, 3 and 4 –negative controls, 5 – positive control.

The growth rate of *G. punctata* hairy root cultures was much higher than in excised root cultures of non-transformed plants. Since we previously showed (Vinterhalter et al. 1997) that hairy root cultures produce much more gentiopicroin than non-transformed root culture (0.57% vs. 0.02%) we believe that the idea to use hairy root cultures of *G. punctata* for commercial production of secondary metabolites in bioreactors has so far been justified. Next step in this research would be to investigate which of the several bioreactor types (Whitney, 1992) would be the best choice for hairy root cultures of Gentians.

It is important to note that hairy root cultures of *G. punctata* did not contain amarogentin found as 0.02% in non-transformed root cultures (Vinterhalter et al., 1997). Similarly, Ishimaru et al., (1990) analysing secondary metabolites in hairy root cultures of *Swertia japonica* (*Gentianaceae*) found two bitter compounds amarogentin and amaroswerin but could not detect all xanones which were present in *in vitro* cultured plants.

Studies dedicated to the clonal propagation of 5 Gentian species performed in our laboratory (*G. acaulis*, *G. cruciata*, *G. lutea*, *G. purpurea* – Momčilović et al. 1987b) and *G. punctata* – Vinterhalter and Vinterhalter (1998) showed a marked similarity between *G. punctata* and *G. lutea* specially in the rooting stage since both species required auxins for root initiation. Following transformation with A4M70 GUS hairy root cultures of all 5 Gentian species grew in absence of exogenous auxin. According to (Momčilović et al. 1997a) spontaneous shoot regeneration was observed only in *G. cruciata* whilst in *G. purpurea* it could be induced indirectly via regeneration of organogenic callus which developed in 5% cultures cultivated on media with 0.1-4.0 mg l⁻¹ Kin. The other two species, *G. acaulis* and *G. lutea* failed to regenerate shoots either spontaneously or upon cultivation on cytokinin supplemented media same as *G. punctata*. Although we obtained shoot regeneration of *G. punctata* the low frequency of this event can be attributed to chance alone and not to the regeneration ability of this species. Among other Gentians regeneration ability was demonstrated in *G. scabra* (Suginuma and Akihama, 1995) and in *Eustoma grandiflorum* (*Gentianaceae*) Handa (1992).

LITERATURE CITED

- BIROT A.M., BOUCHEZ D., CASSE-DELBART F., DURAND-TARDIF M., JOUANIN L., PAUTOT V., ROBAGLIA C., TEPFER D., TEPFER M., TOURNEUR J., VILAINE F. 1987. Studies and uses of the Ri plasmids of *Agrobacterium rhizogenes*. *Plant Physiol. Biochem.* 25: 323-335.
- CHRISTEY, M.C. 1997. Transgenic crop plants using *Agrobacterium rhizogenes*- mediated transformation. In: Hairy roots: culture and applications. Edit Doran, P.M., Harwood Acad. Pub., Amsterdam, 99-111.
- HANDA, T. 1992. Regeneration and characterization of prairie Gentian (*Eustoma grandiflorum*) plants transformed by *Agrobacterium rhizogenes*. *Plant Tissue Culture Letters* 9: 10-14.
- HAMILL J.D., PARR A.J., RHODES M.J.C., ROBINS R.J., WALTON N.J. 1987. New routes to plant secondary products. *Bio/Technology* 5: 800-804.
- ISHIMARU K., SUDO H., SATAKE M., MATSUNAGA Y., HASEGAWA Y., TAKEMOTO S., SHIMOMURA K. 1990. Amarogentin, amaroswerin and four xanones from hairy root cultures of *Swertia japonica*. *Phytochemistry* 29: 1563-1565.
- JEFFERSON R.A., KAVANAGH T.A., BEVAN M.W. 1987. GUS fusion: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907.
- LAMPROYE A., CREVECOEUR M., KEVERS C., GASPAR Th. 1987. Multiplication vegetative *in vitro* de *Gentiana lutea* et de *Gentiana pneumonanthe*. *Med. Fac. Landbouww. Rijksuniv. Gent.* 52: 1255-1257.
- LINSMAIER E.M., SKOOG D. 1965. Organic growth factor requirements for tobacco tissue cultures. *Physiol. Plant.* 18: 100-128.
- LLOYD G., McCOWN B. 1981. Commercially-feasible micropropagation of Mountain Laurel, *Kalmia latifolia* by use of shoot tip culture. *Comb. Proc. Int. Plant Prop. Soc.* 30: 421-427.
- MOMČILOVIĆ I., GRUBIŠIĆ D., KOJIĆ M., NEŠKOVIĆ M. 1997a. *Agrobacterium rhizogenes* – mediated transformation and plant regeneration of four *Gentiana* species. *Plant Cell, Tissue and Organ Culture* 50: 1-6.
- MOMČILOVIĆ I., GRUBIŠIĆ D., NEŠKOVIĆ M. 1997b. Micropropagation of four *Gentiana* species (*G. lutea*, *G. cruciata*, *G. purpurea* and *G. acaulis*). *Plant Cell, Tissue and Organ Culture* 49: 141-144.
- MUGNIER J. 1988. Establishment of new axenic hairy root lines by inoculation with *Agrobacterium rhizogenes*. *Plant Cell Reports* 7: 9-12.
- MURASHIGE T., SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- ORBOVIĆ V., TRAAS J., CABOCHE M., Hofte H. 1995. Phenotypic and molecular analysis of mutant with an altered meristem function. 6th International Congress on Arabidopsis research, Madison, USA. Book of abstracts – 613.
- PORTER J.R. 1991. Host range and implications of plant infection by *Agrobacterium rhizogenes*. *Critical Rev. Plant Sci.* 10: 387-421.
- SKRZYPCZAK L., WESOŁOWSKA M., SKRZYPCZAK E. 1993. *Gentiana* species: *in vitro* culture, regeneration and production of secoiridoid glucosides. In: *Biotechnology in Agriculture and Forestry*, Ed. Bajaj YPS. Vol. 21: 172-186.
- SUGINUMA C., AKIHAMA T. 1995. Transformation of gentian with *Agrobacterium rhizogenes*. *Acta Horticulturae* 392: 153-160.
- TEPFER D. 1983. The biology of genetic transformation of higher plants by *Agrobacterium rhizogenes*. In: *Molecular genetics of the bacteria-plant interaction*. (Edit. Pühler, A.). Springer. Berlin-Heidelberg, 248-258.
- TEPFER D. 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. *Cell* 37: 959-967.
- TEPFER D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. *Physiol. Plant.* 79: 140-146.
- TEPFER M., CASSE-DELBART F. 1987. *Agrobacterium rhizogenes* as a vector for transforming higher plants. *Microbiological Sciences* 4: 24-28.
- VAN LAREBEKE N., GENETELLO C.H., HERNALSTEENS J.P., DE PICKER A., ZAENEN I., MESSENS E., VAN MONTAGU M., SCHELL J. 1977. Transfer of Ti plasmids between *Agrobacterium* strains by mobilization with the conjugative plasmid RP4. *Molec. Gen. Genet.* 152: 1119-1124.
- VINTERHALTER B., VINTERHALTER D. 1998. *In vitro* propagation of spotted gentian *Gentiana punctata* L. *Arch. Biol. Sci., Belgrade*, 50 (3): 177-162.
- VINTERHALTER B., SUBOTIĆ A., VINTERHALTER D. 1993. Transformation of *Gentiana punctata* by *Agrobacterium rhizogenes*. 10-th Symposium of Yugoslav Assoc. Plant Physiol., Zemun Polje, 153.
- VINTERHALTER B., MENKOVIĆ N., ŠAVIKIN-FODULOVIĆ K., VINTERHALTER D. 1997. Quantification of glycosides in shoot and A4 GUS transformed root cultures of *Gentiana punctata* L. *Archiv of Pharmacy, Belgrade* 47: 544-545.
- VINTERHALTER B., VINTERHALTER D. 1998. *In vitro* propagation of spotted gentian *Gentiana punctata* L. *Arch. Biol. Sci., Belgrade*, 50 (3): 177-162.
- VIOLA U., FRANZ, CH. 1989. *In vitro* propagation of *Gentiana lutea*. *Planta Medica* 55: 690.
- WESOŁOWSKA M., SKRZYPCZAK L., DUDZIŃSKA R. 1985. Rodzaj *Gentiana* L. w kulturze *in vitro*. *Acta Polon. Pharm.* 42: 79-83.
- WHITNEY P.J. 1992. Novel bioreactors for the growth of roots transformed by *Agrobacterium rhizogenes*. *Enzyme Microb. Technol.* 14: 13-17.

TRANSGENICZNE KULTURY KORZENI *GENTIANA PUNCTATA* L.

STRESZCZENIE

Z kultur tkankowych pędów *Gentiana punctata* L. inokulowanych zawiesiną szczepu A4M70GUS *Agrobacterium rhizogenes*, po 2-3 tygodniach wzrostu izolowano włośniki korzeniowe, które następnie hodowano w płynnym podstawowym roztworze WPM (Lloyd i McCown 1980) pozbawionym hormonów, przez ponad 5 lat (60 subkultur). Szybkość wzrostu transformowanych korzeni przewyższała szybkość wzrostu korzeni nie transformowanych. Spontaniczna regeneracja pędów wystąpiła tylko w trzech naczyniach hodowlanych, w subkulturach nr 40 i 42, a otrzymane rośliny wykazywały cechy fenotypowe charakterystyczne dla roślin transformowanych *A. Rhizogenes*, takie jak: pomarszczone liście, krótkie międzywęźla, plagiotropowe korzenie i obniżone tempo wzrostu. Rośliny te charakteryzowało także przedwczesne zawiązywanie pączków kwiatowych nie poprzedzone wernalizacją i zdolność do zakwitania w kulturach in vitro. Podobnie jak w standardowym fenotypie, kwiaty roślin transformowanych były bladożółte.

SŁOWA KLUCZOWE: *Agrobacterium rhizogenes*, *Gentiana punctata* L., izolowane kultury korzeniowe, regeneracja pędów, zakwitanie in vitro.