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BRIEF COMMUNICATION

Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS

B. VINTERHALTER[1], S. NINKOVIĆ, A. CINGEL and D. VINTERHALTER

Institute for Biological Research "S. Stanković", Despota Stefana 142, Belgrade, Serbia and Montenegro

Abstract

Hairy root cultures of *Hypericum perforatum* were obtained following inoculation of aseptically germinated seedlings with *A. rhizogenes* strain A4M70GUS. Effect of sucrose on the growth and biomass production of hairy root cultures was investigated. Hairy root cultures spontaneously regenerated shoots buds from which a number of shoot culture clones was established. Transformed shoot cultures exhibited good shoot multiplication, elongation and rooting on a hormone-free woody plant medium. Plants regenerated from hairy roots were similar in appearance to the normal, non-transformed plants.

Additional key words: GUS activity, hairy roots, shoot regeneration, St. John's wort, sucrose.

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Hypericum perforatum L. (St. John's wort) is an important medicinal plant rich in secondary metabolites considered as an interesting drug source for the pharmaceutical industry. Most of the *in vitro* culture studies on this species are usually dedicated to cell and tissue cultures and their ability to produce various secondary metabolites.

The aim of our investigation was to obtain and study hairy root cultures of *H. perforatum.* For this purpose we selected *A. rhizogenes* strain A4M70GUS, previously used in our laboratory to obtain hairy root cultures in several species of *Gentiana* (Momčilović *et al.* 1997, Vinterhalter *et al.* 1999), *Lotus corniculatus* (Nikolić *et al.* 2003/04), *Centaurium erythrea* (Subotić *et al.* 2003/04), *Aesculus hippocastanum* (Zdravković-Korać *et al.* 2004) and *Blackstonia perfoliata* (Bjelović *et al.* 2004). So far there are no papers published on *Agrobacterium* mediated transformation of *H. perforatum.*

Hypericum perforatum L. seeds collected on locations around Belgrade were washed with 70 % ethanol for 1 min and than surface sterilized in 15 % commercial bleach (4 - 5 % NaOCl) for 20 min. Seeds were rinsed 3 × 5 min in autoclaved water and aseptically germinated. Basal media contained woody plant medium (Lloyd and McCown 1981; WPM) macrosalts and Murashige and Skoog (1962; MS) microsalts, iron and vitamins. Media were supplemented with 0.62 % agar and 2 % sucrose. Media pH was adjusted to 5.8 prior to autoclaving performed 20 min at 114 °C. Conditions in the growth room were: temperature of 25 ± 2 °C, 16-h photoperiod and irradiance 46.5 μmol m^{-2} s^{-1}.

Epicotyls were excised from seedling and cultured on basal media supplemented with 0.2 - 0.5 mg dm^{-3} kinetin. Shoot cultures derived from epicotyls were subculture in 5 - 6 weeks intervals.

A. rhizogenes strain A4M70GUS contains a GUS construct integrated into the TL region of the cointegrative plasmid pRiA4 (Tepfer and Casse-Delbart 1987). GUS construct contains *uidA* sequence under the 70S promoter (enhancer-doubled 35S Ca MV promoter), followed by NOS polyadenylation sequence. Bacterial strains were maintained according to Van Larebake *et al.* (1977).

Shoots comprising 3 - 4 internodes were inoculated by wounding with a needle dipped in bacterial suspension. Wounding was done at the first node above

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Abbreviations: GUS - β-glucuronidase; HR - hairy roots; HRRS - hairy root regenerated shoots; Kin - 6-phurphuryl amino purin (kinetin); MS medium - Murashige and Skoog medium; NTS - non-transformed shoots; PCR - polymerase chain reaction; WPM - woody plant medium

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[1] Corresponding author, fax: (+381) 11 2761 433, e-mail: horvat@ibiss.bg.ac.yu

the culture medium. Inoculated shoots were first cultured for 7 - 10 d on the basal, hormone- free medium and then transferred to medium supplemented with 200 mg dm^{-3} cefotaxime-Na (*Jugoremedia*, Zrenjanin, Serbia and Montenegro). Concentration of the antibiotic was gradually decreased in the next three subcultures down to the antibiotic-free medium in the fourth subculture and later. Cultures were then thoroughly tested for presence of bacteria. β-glucuronidase enzyme activity was determined histochemically (GUS assay) after an overnight incubation at 37 °C using X-gluc as a substrate, at pH 7.0 (Jefferson *et al.* 1987). PCR reaction was performed as previously described by Mitić *et al*. (2004).

Cultures comprising excised A4M70GUS transformed roots were cultured on liquid and agar solidified hormone-free medium. Effect of sucrose (0.5 - 8 %) was studied in relation to shoot bud regeneration and biomass production which was calculated as percentage of fresh mass increase.

Hairy root formation was registered in 20.8 % innoculated shoots (37 out of 178). First hairy roots (HR) were visible two weeks after the inoculation. They were excised and further cultured as 37 separate clones on hormone-free medium on which they all showed good growth and branching. Clones were designed with letters HR followed by a number. HR cultures manifested a high potential for spontaneous shoot bud regeneration (Fig. 1*A*,*B*). The percentage of HR clones, which regenerated shoots, increased from 40.5 % (15) in the first subculture to 62 % (23) in the second subculture.

Shoot buds regenerated by HR cultures were excised and established as 23 distinct shoot cultures (HRRS clones) with numbers corresponding to the HR clone from which they originated. HRRS clones were maintained on hormone-free medium on which they manifested very good shoot elongation and multiplication and 100 % rooting. Five of these clones (HRRS 2, 14, 15, 27 and 29) were later investigated in detail (Table 1).

Table 1. Growth parameters of HRRS shoot cultures on hormone-free medium after 30 d. Means ± SE, n = 50 - 80. Within each column means followed by the same letter were not significantly different according to Duncan's multiple range test at $P \leq 0.05$. Number of glands per leaf of the control (non-transformed shoots) on kinetin 0.5 mg dm^{-3} was 13.38 ± 0.28.

HRRS clone	Shoot length [mm]	Multiplication index	Shoots with axillary buds [%]	Length of axillary shoot [mm]	Number of roots [$plant^{-1}$]	Length of the longest root [mm]	Number of glands [$leaf^{-1}$]
2	66.4 ± 2.3 a	2.8 ± 0.3 a	55.1	10.6 ± 1.3 a	4.8 ± 0.3 a	28.1 ± 2.0 a	13.2 ± 0.3 c
14	67.3 ± 2.0 a	4.0 ± 0.4 b	77.9	11.5 ± 1.4 a	6.2 ± 0.2 c	40.7 ± 2.6 b	14.9 ± 0.3 d
15	59.5 ± 2.1 a	4.1 ± 0.4 b	88.8	15.6 ± 1.6 ab	6.0 ± 0.3 bc	51.5 ± 2.8 c	10.3 ± 0.2 a
27	66.1 ± 3.5 a	2.4 ± 0.4 a	71.1	18.3 ± 1.9 b	5.3 ± 0.2 ab	24.8 ± 2.3 a	10.4 ± 0.3 a
29	80.3 ± 3.3 b	5.2 ± 0.6 b	89.2	17.7 ± 1.8 b	7.3 ± 0.4 d	29.9 ± 2.4 a	11.5 ± 0.2 b

Shoot cultures of normal, non-transformed shoots (NTS), were established and maintained on kinetin supplemented media (Fig. 1*C*) since their growth on hormone-free medium was poor. In comparison to HRRS clones NTS shoots were short, shoot multiplication was minimal and rooting percentage was low (14.8 %).

Table 2. Effect of sucrose on the biomass production and shoot regeneration in the HR clone 14 cultured on liquid hormone-free medium after 35 d. Initial fresh mass of root clumps 256 ± 6 mg. Means ± SE, n = 15 - 20. Within each column means followed by the same letter were not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Sucrose [%]	Final root clump mass [mg]	Biomass increase [%]	Regenerated shoot buds [$explant^{-1}$]
0.5	1180.5 ± 204.8 b	475,6	36.7 ± 5.9 bcd
1	1679.7 ± 166.6 b	673.2	58.0 ± 11.5 d
2	2328.3 ± 228.1 c	927.2	53.3 ± 12.5 cd
4	1671.7 ± 193.8 b	654.2	30.8 ± 5.7 abc
6	1226.7 ± 151.9 b	476.5	16.2 ± 2.6 ab
8	602.9 ± 66.7 a	230.4	9.6 ± 1.4 a

Addition of kinetin increased shoot multiplication and length but further inhibited root formation and elongation.

There were marked differences in the growth parameters between HRRS clones (Table 1) which were stable and reappeared in successive subcultures. Thus clone HRRS 29 had the highest values for all growth parameters (shoot elongation, multiplication and root formation) except for root length. Clones HRRS 14 and HRRS 15 had lower shoot multiplication values which corresponded to the shoot multiplication of NTS shoots on medium with 0.5 mg dm^{-3} kinetin. They also produced the longest roots which often developed on internodes high above medium surface (Fig. 1 *E*). Clones HRRS 2 and HRRS 27 were less vigorous both in axilary bud formation and rooting. Characteristic feature of all HRRS clones was that they produced only plants of normal phenotype. They were also characterized by frequent formation of flower buds which was most prominent in clone HRRS 2 (Fig. 1*F*).

HR clones grew well on agar solidified and liquid hormone-free media. The maximum growth rate and biomass production of all clones was at 2 % sucrose. In the absence of plant growth regulators sucrose was

apparently the major factor which affected their growth. Differences in the growth parameters between HR clones were not observed. The effect of sucrose on the growth of a typical clone, HR14 is presented in Table 2. The highest biomass production with a 9 - fold increase of fresh mass was registered at 2 % sucrose. Media with 1 and 4 % sucrose also provided high biomass production (6.5 fold increase). Shoot bud regeneration was highest on media with 1 and 2 % sucrose with 50.3 and 48.8 shoot buds per culture respectively (Fig. 1*D*). Sucrose at 4 % and higher concentrations had an adverse effect both on root growth and shoot differentiation. Roots and shoots became necrotic, developing a characteristic brown color. At still higher sucrose concentrations (6 and 8 %) brown colour was even more prominent, roots became thick and shoot buds which regenerated on roots failed to elongate.

The transgenic nature of hairy root and shoot cultures regenerated from them was obvious from their excellent

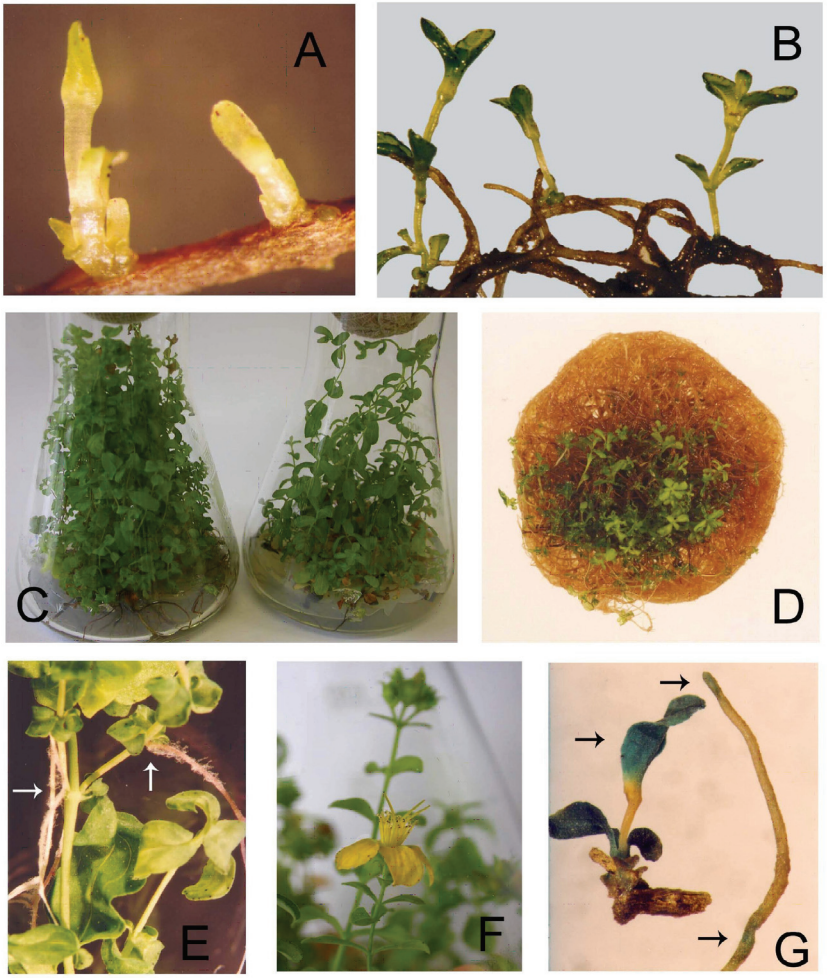

Fig. 1. *A* and *B* - Stages of shoot regeneration in hairy-root cultures of *H. perforatum* inoculated with *A. rhizogenes* A4M70GUS. *C* - HRRS 27 shoot culture on hormone-free medium (*left*) and NTS shoot culture on media with 0.5 mg dm^{-3} kinetin (*right*). *D* - Clump of hairy-roots removed from liquid hormone free medium with a cluster of regenerated shoots in the middle (from above). *E* - Induction of adventitious roots (*arrows*) on shoots of clone HRRS 14 at internodes high above the medium. *F* - *In vitro* flowering of clone HRRS 2. *G* - Histochemical staining with X-gluc. Blue colour indicating positive reaction in transformed shoots and roots (*arrows*).

growth on hormone-free medium. It was further confirmed histochemically with GUS assay and PCR analysis. Positive X-gluc staining reaction was obtained for all HRRS clones. In HR cultures the staining intensity was always less prominent than in shoots of corresponding HRRS cultures (Fig. 1*G*). Blue colour developed only in tips and some portions of the central cylinder of hairy roots. GUS reaction was negative in shoots and roots of NTS plants. PCR analysis confirmed the presence of GUS gene in the investigated HRRS clones. The 366 bp fragment of GUS gene was amplified in all transformed HRRS clones but not in non-transformed, control clone (Fig. 2).

It is well known that hairy roots can be cultured on hormone-free medium (Christey 2001). In *H. perforatum* this same feature is found not only in hairy-root cultures but also in hairy-root regenerated (HRRS) shoot cultures. HRRS shoot cultures can therefore be multiplied,

elongated and rooted all on a simple hormone-free medium. Such universal type medium can be a good choice for large-scale continuous biomass production systems.

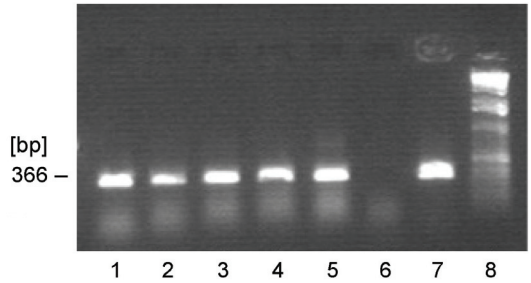

Fig. 2. PCR analysis of HRRS shoot cultures: *lanes 1 - 5* HRRS clones 2, 14, 15, 27, 29 respectively, *lane 6* - negative control (non-transformed shoots), *lane 7* - positive control (A4M70GUS), *lane 8* - 1 kb DNA ladder.

Shoot cultures of *H. perforatum* require exogenous cytokinins alone or in combination with auxins for good growth and multiplication (Čellárová *et al.* 1992, Zdunek and Alfermann 1992, Murch *et al.* 2000). Proper cytokinin/auxin balances is also required for the establishment and shoot regeneration of *H. perforatum* callus tissues (Čellárová *et al.* 1995, Preto and Santarém 2000). We showed that in some HRRS cultures shoot multiplication in absence of exogenous cytokinins occurs at the same rate as in NTS shoot cultures on media supplemented with 0.5 mg dm^{-3} kinetin.

Differences in growth parameters which we observed between clones although stable and characteristic for every clone were not acompanied with changes in plant morphology. Normal phenotype and frequent spontaneous shoot regeneration was observed in two other species transformed with the same *A. rhizogenes* strain (Nikolić *et al.* 2003/04, Subotić *et al.* 2003/04). However in A4M70 transformed hairy root cultures of *Gentiana punctata* shoot regeneration was a rare event and regenerated plantlets exhibited a complete set of phenotypic changes characteristic for hairy root cultures including: wrinkled leaves, reduced apical dominance, short internodes and plagiotropic roots (Vinterhalter *et al.* 1999). Thus the phenotypic stability of hairy root regenerants obtained with *A. rhizogenes* strain A4M70GUS seems to be species dependant.

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