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HIGH BIOMASS PRODUCING ROOT CULTURES OF *GENTIANA PUNCTATA* L. TRANSFORMED WITH *AGROBACTERIUM TUMEFACIENS* C58C1 (pArA4b)

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Abstract - *Gentiana punctata* L. shoot cultures were transformed with *Agrobacterium tumefaciens* clone C58C1 harbouring *A. rhizogenes* A4 Ri plasmid (pRiA4b) to produce hairy roots capable of autonomous growth on growth regulator-free medium. Optimal medium comprised WPM macro-nutrient salts, MS micro-salts and iron, LS vitamins and 2% sucrose. Successful transformation was confirmed by PCR amplification of *aux 1* and *rol* C sequences. Excised, transgenic root cultures characterized by intensive lateral root branching enabled high biomass production. Fast growth required use of explants with small initial weight and frequent subculturing. Cultures were maintained for 10 years (more than 100 subcultures) without changes in morphology or physiological responses.

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INTRODUCTION

Roots and rhizome of *Gentiana punctata* are rich in bitter compounds commercially used for production of appetizers and in pharmaceutical industry. Uncontrolled rhizome harvesting made this species nearly extinct in Serbia. We therefore investigated alternative pathways for root biomass production among which culture of transgenic roots seems to be the most promising.

Experiments aimed at obtaining transgenic cultures were initiated in 1989 using several *Agrobacterium tumefaciens* and *A. rhizogenes* clones. These early investigations (Vinterhalter *et al.* 1993) indicated *A. tumefaciens* C58C1 (pArA4b) as the clone to which *G. punctata* was the most responsive. It provided constan-tly high biomass production and high phenotypic stability. Another clone of *G. punctata* root cultures transformed with A4M70GUS was thoroughly investigated in relation to its ability for spontaneous shoot regeneration followed by flowering *in vitro* (Vinterhalter *et al.* 1999). A great disadvantage of C58C1 (pArA4b) clone was the absence of suitable reporter genes. However, recent development of PCR techniques suitable for detection of transgene plants enabled us to confirm the transgene status of *G. punctata* root clones trasnformed with C58C1 (pArA4b).

MATERIAL AND METHODS

Shoot cultures and culture conditions

Shoot cultures of *G. punctata* established in 1988 were maintained on medium comprising WPM (Lloyd and McCown 1981) macro-nutrients salts, MS (Murashige and Skoog 1962) micro salts and iron and LS (Linsmaier and Skoog 1965) vitamins, 2% sucrose and 0.62% agar. Medium was supplemented with 0.25 *mg* L^{-1} BA and 0.1 *mg* L^{-1} IAA as previously reported (Vinterhalter and Vinterhalter 1998).

Conditions of the growth room were: temperature 25 ± 2 °C, photoperiod: 16 *h* 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).

Biomass production

Biomass presented as the index of biomass production was calculated as final fresh tissue weight divided by initial weight for a pre-defined vessel and medium volume. Each treatment containing 6-10 wide

neck 100 mL Erlenmayer flasks with 40 mL liquid medium and ≈ 400 *mg* root tissue was replicated at least twice.

Transgenic root cultures

Transgenic root cultures were obtained by wounding of shoots and inoculation with *Agrobacterium tumefaciens* strain C58C1(pArA4b) suspension. Roots obtained after wounding were excised and further cultured on agar solidified medium of the same composition as for shoot cultures except that growth regulators were omitted. Media also contained cefotaxime-Na (Jugoremedia, Zrenjanin) at 200 *mg* L^{-1}. Concentration of antibiotic was gradually decreased through several subcultures. Tests for detection of bacteria were performed and axenic cultures were further cultured on the antibiotic-free medium.

Non-transgenic root cultures

Non-transgenic excised root cultures were established by excision of adventitious roots induced on shoot cultures on medium with 2.0 *mg* L^{-1} IBA for 8 days and then transfered to hormone-free medium. Cultures of non-transformed roots were maintained by regular subculturing in liquid medium supplemented with 0.2 *mg* L^{-1} IBA and 0.1 *mg* L^{-1} GA_3 (Vinterhalter *et al.* 1993).

Bacterial strains

Bacterial strain C58C1(pArA4b) was constructed by Petit *et al.* (1983) by introduction of *A. rhizogenes* pArA4b plasmid into the disarmed C58C1 *A. tumefaciens* cell. It was maintained on agar (1.5%) supplemented YEB medium (Van Larebeke *et al.* 1977). Plants were inoculated with 24 *h* old bacterial suspension culture grown at 28 °C on a shaker adjusted to 220 r.p.m.. Suspension density was ≈ 10^8 cells mL^{-1}.

PCR analysis

Total genomic DNA was isolated from 100 mg of hairy roots according to Sul and Korban (1996). Reaction mixes were prepared according the Perkin Elmer protocol for Gene Amp7 Reagent Kit with Native Taq DNA Polymerase. In the PCR analysis of the *aux 1* sequence (656 bp) the following primers were used:

A_1, 5'-CTCAAGAGCGCTACTCCTTCAAGTG-3' and

A_2, 5'-CCTGTAGAAACCCCAACCCGT-3'.

In the analysis of *rol C* sequence (278 bp) the primers were:

C_1 5'- CCACGGGCTGCTGTACCTCTAC-3' and

C_2 5'- TTTCCCTTTGTCGAAGTTAGCTCC -3'.

In the first PCR cycle, the samples were heated to 95 °C for 4 min. This was followed by 30 cycles at 94 °C for 30 *s*, 55 °C (for *aux* 1) or 66 °C (for *rol C*) for 30 *s*, 72 °C for 45 *s*; and the last cycle of 72 °C for 5 min (Genius DNA Thermal Cycler - Techne). Amplified DNA was analyzed on 1.5 % agarose gels.

RESULTS

The efficiency of plant transformation with C58C1 (pArA4b) was 22.9% calculated on the basis of 268 inoculated shoots. Roots which appeared on the location of wounding were excised and subcultured on hormone-free medium (Fig. 1).

Root cultures developed from these explants were putative transformants. From 1990 to 2000. these transgenic root cultures have been continuously maintained by subculturing in hormone-free liquid and agar solidified media without visible morphological or physiological changes. Cultures were characterized by constant high branching ability and good elongation (Fig. 2.) High branching caused cultures to appear as clumps. Small clumps ≈ 400 *mg* of fresh weight could be used as excellent explants for subculturing when fast initial growth of cultures was desired.

PCR analysis was performed after nine years or approximately more than 90 subcultures. All samples from putative transformed root cultures which tested contained sequences of *aux 1* and *rol C* genes amplified by PCR (Fig. 3. a,b). In this way the presence of both TR- and TL- DNA was confirmed. The PCR analysis did not detect amplified fragments in non-transformed root cultures used as a control.

Table 1. Effect of liquid *vs.* agar solidified medium on the biomass increase of C58C1 transformed and non-transformed root cultures. Initial explants were single, 15 *mm* long excised roots, 10 roots *per* plate, treatment duration 35 days.

Culture type	Agar soldified or liquid	Lateral root number ± SE	Root elongation (*mm*) ± SE	Fresh weight of a single root (*mg*)*
Non-transformed	agar	3.24 ± 0.3	0.34 ± 0.2	4.58
	liquid	7.82 ± 0.4	15.72 ± 1.2	20.46
C58C1 transformed	agar	29.16 ± 1.8	7.26 ± 0.5	55.08
	liquid	31.64 ± 1.8	18.81 ± 2.1	96.77

*average for 10 roots present on each plate

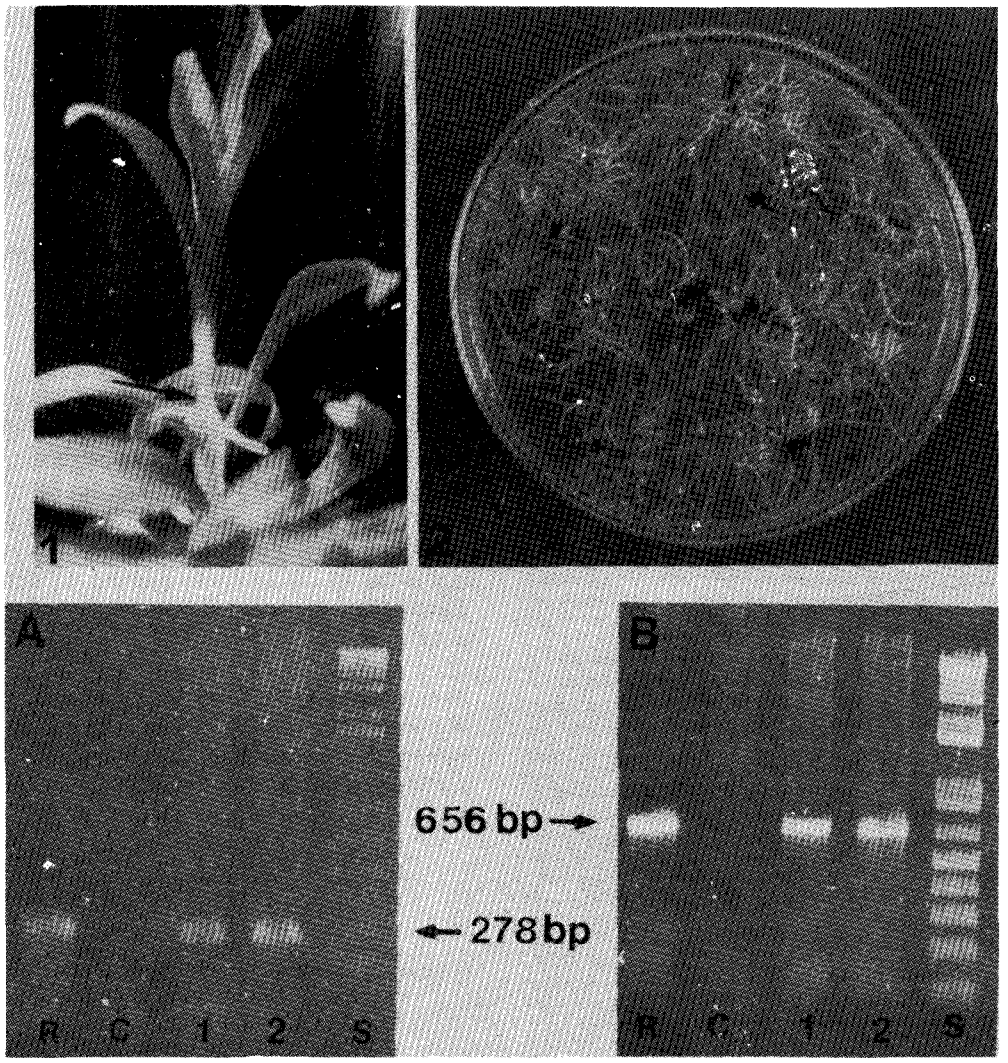

Fig. 1. Initiation of hairy roots on *in vitro* cultured *G. punctata* shoots wounded and inoculated with C58C1 (pArA4b).
Fig. 2. Petri dish with C58C1 (pArA4b) induced hairy root cultures on agar solidified medium.
Fig 3. PCR analysis of *G. punctata* C58C1 (pArA4b) hairy root cultures showing the presence of 278 bp DNA fragments from *rol C* gene (A) and 656 bp DNA fragment from *aux 1* gene (B). R- C58C1 (pArA4b) positive control, C - nontransformed roots, 1 and 2 transformed root clones, S - size marker.

Effect of the explant size, sucrose concentration and agar solidified *vs.* liquid medium were investigated in both transformed and non-transformed cultures. Non-transformed root cultures were maintained in WPM medium supplemented with IBA 0.2 *mg* L^{-1} and GA_3 0.1 *mg* L^{-1}. The biomass yield of transformed root cultures was several times higher than in non-transformed, also liquid medium was far superior to agar solidified medium in both types of cultures (Table 1). In liquid medium elongation of transformed roots was only 20% higher than in non-transformed ones but the increase of biomass yield was 4.7 times higher. Thus the high biomass yield of single root apices in the liquid medium comes from increased root branching rather than from plain root elongation.

To evaluate the effect of sucrose on the biomass increase, explants consisting of ≈400 *mg* transformed roots were cultured in 40 mL liquid media at 8 different concentrations ranging from 0.1 to 8% sucrose (Table 2).

Maximum for the biomass increase was achieved at 2% sucrose. At concentrations higher and lower than the maximum the cultures perished from necrosis which decreased the biomass yield.

Table 3. contains the data on the effect of initial explant size (weight) on biomass increase. It is evident that biomas yield decreases with increasing weight of the initial root explant. In the case of explants consisting of single roots with initial weight of ≈10 *mg* /10 roots (data in Table 1) the index of biomass increases reached the values over 60.

Table 2. Effect of sucrose concentration on the biomass increase of C58C1 transformed root cultures in 40 mL liquid medium after 35 days .

Culture type	Sucrose (%)	Initial fresh weight (*mg*) ± SE	Final fresh weight (*mg*) ± SE	Biomass increase
non-transformed	2	283.5 ± 16.5	682.3 ± 33	x 2.4
C58C1 transformed	0.1	419.9 ± 22.3	561.8 ± 27	x 1.3
	0.5	413.7 ± 30.9	1710 ± 80	x 4.1
	1	382.6 ± 21.3	3412 ± 80	x 8.9
	2	406.3 ± 4.4	5018 ± 399	x 12.4
	4	403.4 ± 6.4	2323 ± 338	x 5.8
	6	404.9 ± 7.2	1058 ± 79	x 2.6
	8	406.9 ± 1.9	861.0 ± 71	x 1.1

Table 3. Effect of the initial explant weight on the biomass increase of C58C1 transformed root cultures. Liquid hormone-free medium supplemented with 2% sucrose, measured after 35 days.

Initial weight group (*mg*)	Mean initial weight (*mg*) ± SE	Final weight (*mg*) ± SE	Biomass increase index
< 350	300.2 ± 13.9	5312 ± 914	x 17.7
350 - 450	406.3 ± 4.4	5018 ± 399	x 12.4
450 - 550	506.4 ± 87.3	5643 ± 423	x 11.1
550 - 650	576.0 ± 8.4	5589 ± 524	x 9.7
> 650	717.0 ± 33.5	6774 ± 156	x 9.5

DISCUSSION

Clone C58C1 (pArA4b) of transformed root cultures of *G. punctata* was highly stable, never manifesting signs of morphological or physiological changes, in contrast to A4M70GUS transformed roots in which spontaneous shoot regeneration followed by *in vitro* flowering was observed (V i n t e r h a l t e r and V i n t e r h a l t e r 1999). These two clones of *Agrobacterium* transformed root cultures showed marked and constant differences in the growth habit. Root cultures transformed with C58C1 (pArA4b) had a pronounced lateral root branching and required frequent subculturing since the burst of the initial growth was followed by early appearance of necrosis, usually after 5 weeks in culture. On the contrary, A4M70GUS transformed root clones had far better elongation than the branching. These steady growing clones could be maintained undisturbed for much longer periods requiring subculturing after 10-12 weeks. Comparison of fresh weight biomass production in optimal conditions (liquid medium + 2% sucrose - Table 2.) showed that C58C1-(pArA4b) transformed clones had three times higher yields than A4M70GUS (V i n t e r h a l t e r and V i n t e r h a l t e r 1999, Table 2). However, final judgment of the value of these two root clones should be made only after a study on the efficiency of their secondary metabolite productivity.

Transformed root cultures of *G. acaulis* and *G. cruciata* were obtained with A4M70GUS and in *G. lutea* and *G. purpurea* with ATCC 15834 (M o m č i l o v i ć *et al.* 1997). Unfortunately in none of these Gentiana species transformation with C68C1 (pArA4b) was attempted, therefore comparisons with the results which we obtained with *G. punctata* are not possible. It should however be noted that C58C1(pArA4b) transformed root clones of *G. punctata* although highly branched did not manifest fasciation as did the ATCC 15834 transformed root clones of closely related *G. lutea* (B u d i m i r *et al.* 1998).

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КУЛТУРА КОРЕНОВА *GENTIANA PUNCTATA* L. ТРАНСФОРМИСАНИХ ПОМОЋУ *AGROBACTERIUM TUMEFACIENS* C58C1(pArA4b)

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Културе изданака *Gentiana punctata* L. трансформисане су транскоњугованим сојем *Agrobacterium tumefaciens* клон C58C1 са убаченим *A. rhizogenes* A4 Ri плазмидом (pArA4b) који продукује коренове способне за аутономан раст на подлози без регулатора растења. Успешна трансформација показана је PCR амплификацијом *aux 1* и *rol C* секвенци у узорцима трансформисаних коренова. Културе трансгених коренова које су се карактерисале интензивним бочним гранањем, одржаване су током 10 година (90 и више субкултура) без промена у морфологији и физиолошким својствима. Оптимални услови за раст ових култура били су течна подлога и 2% сахароза. Брз пораст култура захтевао је мале почетне експлантате и честа пасажирања на свежу подлогу.