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In vitro regeneration and transformation of *Blackstonia perfoliata*

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Abstract

In vitro root culture of yellow wort (*Blackstonia perfoliata* (L.) Huds.) was initiated on Murashige and Skoog (MS) medium. In the presence of benzylaminopurine (BAP) numerous adventitious buds formed, which developed into shoots. Presence of indole-3-butyric acid (IBA) in media significantly decreased number of buds, but increased development of lateral roots. On hormone-free medium shoots successfully rooted and developed flowers and viable seeds that formed another generation. Shoot cultures of *B. perfoliata* inoculated with suspension of *Agrobacterium rhizogenes* strain A4M70GUS developed hairy roots at 3 weeks and they were cultured on hormone-free MS medium. Spontaneous shoot regeneration occurred in 3 clones.

Additional key words: *Agrobacterium rhizogenes*, hairy roots, regeneration, root culture.

Introduction

Blackstonia perfoliata (yellow wort) (L.) Huds. (*Chlora perfoliata* L., *Gentiana perfoliata* L., *Seguiera perfoliata* O. Kuntze), *Gentianaceae*, is an annual plant, 10 - 60 cm high, with long internodes, triangular leaves, sometimes narrowing towards the base (Jovanović-Dunjić 1973). It is critically endangered plant in Yugoslavia that is a weak competitor in its habitats. *B. perfoliata* is widespread in European and African regions of the Mediterranean and in western Europe (Tutin 1972, Van der Sluis 1985).

There are several studies about *in vitro* culture of *B. perfoliata* (Skrzypczak *et al*. 1992, 1996, Bijelović *et al*. 2000, 2001) and on other species from the *Gentianaceae* family (Wesolowska *et al*. 1985, Viola and Franz 1989, Skrzypczak *et al.* 1993, Momčilović *et al.* 1997a, Menković *et al*. 1998, Vintehalter and Vinterhalter 1998, Mikula and Rybczynski 2001).

Since *B. perfoliata* could be used in medicine instead of *Radix Gentianae*, this plant can be produced in great biomass in culture *in vitro*. Generally, it has been shown that hairy root cultures have much faster growth in comparison to untransformed root cultures (Tepfer and Casse-Delbart 1987). There are a few reports about the transformation of some species from the family *Gentianaceae* with *Agrobacterium rhizogenes* (Momčilović *et al.* 1997b, Vinterhalter *et al.* 1999). However, there are no reports about transformation of *B. perfoliata* with *Agrobacterium rhizogenes* to date.

Materials and methods

In vitro cultures were established from the seeds that were collected near Igalo, Kotor bay (Montenegro), in August 1997. The seeds were sterilized with 20 % commercial solution of NaOCl (8 % active Cl) and rinsed three times in a sterile distilled water. Seeds were germinated in distilled water, under red radiation,

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog`s (1962) medium.
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obtained from fluorescent tubes *TL 20/15* (*Philips*, Hamburg, Germany) with plastic filter *Röhm and Haas* (Darmstadt, Germany) *No. 501*. Five mm long seedlings, were transferred to Murashige and Skoog (1962) (MS) medium with half-strength macronutrients, without hormones. Shoot culture was successfully established and rooted one month later. Basal medium (BM) contained Murashige and Skoog mineral salts, vitamins and iron stock solution, 3 % sucrose, 100 mg dm^{-3} myo-inositol and 0.70 % agar. The pH of the media was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min. All cultures were grown in a controlled environment room at temperature of 25 ± 2 °C, 16-h photoperiod and irradiance of 50 μmol m^{-2} s^{-1}.

Excised root tips were used for obtaining root culture on the same medium, supplemented with 0.1 μM indole-3-butyric acid (IBA). Vegetative propagation of *B. perfoliata* was carried out by transferring seedlings with two nodes to MS medium supplemented with constant concentration of IBA (0.5 μM) and different concentrations of 6-benzylaminopurine (BAP) (0 - 20 μM).

With the aim to investigate the effects of different growth regulators on plant regeneration, *in vitro* root culture of *B. perfoliata* was established from excised 10 mm long apical root segments, as well as from non-apical root segments. The root segments were cultured on MS medium supplemented with different concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM) of IBA or BAP.

The agropin-type strain *Agrobacterium rhizogenes* A4M70GUS is harbouring cointegrative plasmid with GUS construct integrated into T_L region of pRiA4 (Tepfer and Casse-Delbart 1987). GUS construct contains *uidA* sequence under the enhancer-doubled 35S CaMV promoter. Bacterial strain was maintained on agar (1.5 %) solidified YEB nutrient medium (Van Larebake *et al.* 1977), supplemented with 100 mg dm^{-3} neomycin sulfate (*ICN Biomedicals Inc*., New York, USA). Bacterial suspensions were incubated with shaking at 220 rpm for 24 h prior to inoculation, at 28 °C. Suspension at a density of 10^8 cells cm^{-3} was used for inoculation.

Bacterial suspension of *A. rhizogenes* strain A4M70GUS was used for *in vitro* inoculation of *B. perfoliata* by injecting bacterial suspension into area of the second node. After 48 h of incubation, the wounded shoots were transferred to solidified basal medium supplemented with 300 mg dm^{-3} *Cefotaxime-Na* (*Jugoremedia*, Zrenjanin, Yugoslavia). When adventitious roots developed, their tips were excised and transferred to the *Cefotaxime* supplemented MS media with half-strength of macronutrients.

For multiplication and elongation root explants were placed on BM. We have obtained nine clones of *in vitro* root transformed cultures.

Genomic DNA for PCR analysis was isolated from transformed and control shoots (Dellaporta *et al.* 1983). PCR analysis was performed using the primers GUS 392 (5'-CCCGGCAATAACATACGGCGTG-3') and GUS 22 (5'-CCTGTAGAAACCCCAACCCGTG-3'), which amplified a 366 bp fragment of the *uid*A coding region. PCR reactions were carried out with 100 ng of plant DNA in 0.05 cm^3 volume containing 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % (m/v) gelatin, 200 μM dNTPs, 200 pM of each primer and 2.5 U native Taq DNA polymerase (*Perkin Elmer*, *Roche Molecular Systems, Inc*., New Jersey, U.S.A.). PCR amplification was performed on *Genius* (*Techne*, Cambridge, UK).

Prior to amplification, samples were denatured at 95 °C for 4 min, followed by 30 cycles which one consisted of denaturation step at 94 °C lasting for 30 s, annealing at 60 °C for 30 s and a polymerisation reaction at 72 °C for 30 s. Amplified DNA was separated by electrophoresis on 1.5 % agarose gel, and photographed under UV.

Results and discussion

In vitro culture of yellow wort was successfully established. Plants from shoot culture rooted on MS medium without phytohormones and gave viable seeds that germinated to yield on next generation (Fig. 1*A*). Those plants grown in culture *in vitro* were morphologically similar to those observed in wild populations.

The percentage of rooted plants was high (77.8 %) as well as the percentage of flowered plants (64.4 %). The average number of roots per plant was 6.9 ± 1.1, while the average length of roots was 22.9 ± 0.5 mm.

In vitro shoot culture of *B. perfoliata* was successfully established on MS medium supplemented with IBA (0.5 μM) and different concentrations of BAP (0 - 20 μM) (Fig. 1*B*). The effect of BAP and IBA on number of adventitious and axillary shoots was tested. The number of developed adventitious shoots was much lower when plants grew on medium without or with high BAP concentrations (10 and 20 μM), when compared with those on a medium with 1 and 5 μM BAP (Fig. 2). The highest rate of multiplication, number of adventitious and axillary shoots was on medium supplemented with 5 μM BAP.

Different concentrations of IBA in root culture of *B. perfoliata* influenced the number and length of induced buds and lateral roots (Fig. 3*A*,*B*). Increasing auxin concentration raised the number of lateral roots, decreasing the number of induced buds. The best was 3 μM IBA. Higher concentrations of IBA had negative effects on bud development and bud and lateral root

elongation (Fig. 1*C*). The optimum IBA content in medium for plant regeneration and root development was between 0.03 - 0.1 μM. There was no significant difference between the effect of IBA on apical and non-apical root segments. However, Vuylsteker *et al*. (1998) indicated that lateral roots were formed within 4 mm of the apical root meristem in *Cichorium intybus*. The requirement of growth factors was shown to vary according to the stage of lateral root formation: auxins stimulated initiation of primordia but prolonged treatments inhibited their conversion into lateral roots (Pelosi *et al.* 1995). Indeed, in *B. perfoliata in vitro* culture, after six weeks IBA was enough efficient and lateral roots were developed in great number at certain IBA concentrations.

1 and 3 μM BAP stimulated number and length of induced buds (Fig. 1*D*), while 10 μM inhibited them (Fig. 3*C*,*D*). Higher concentrations of BAP in the

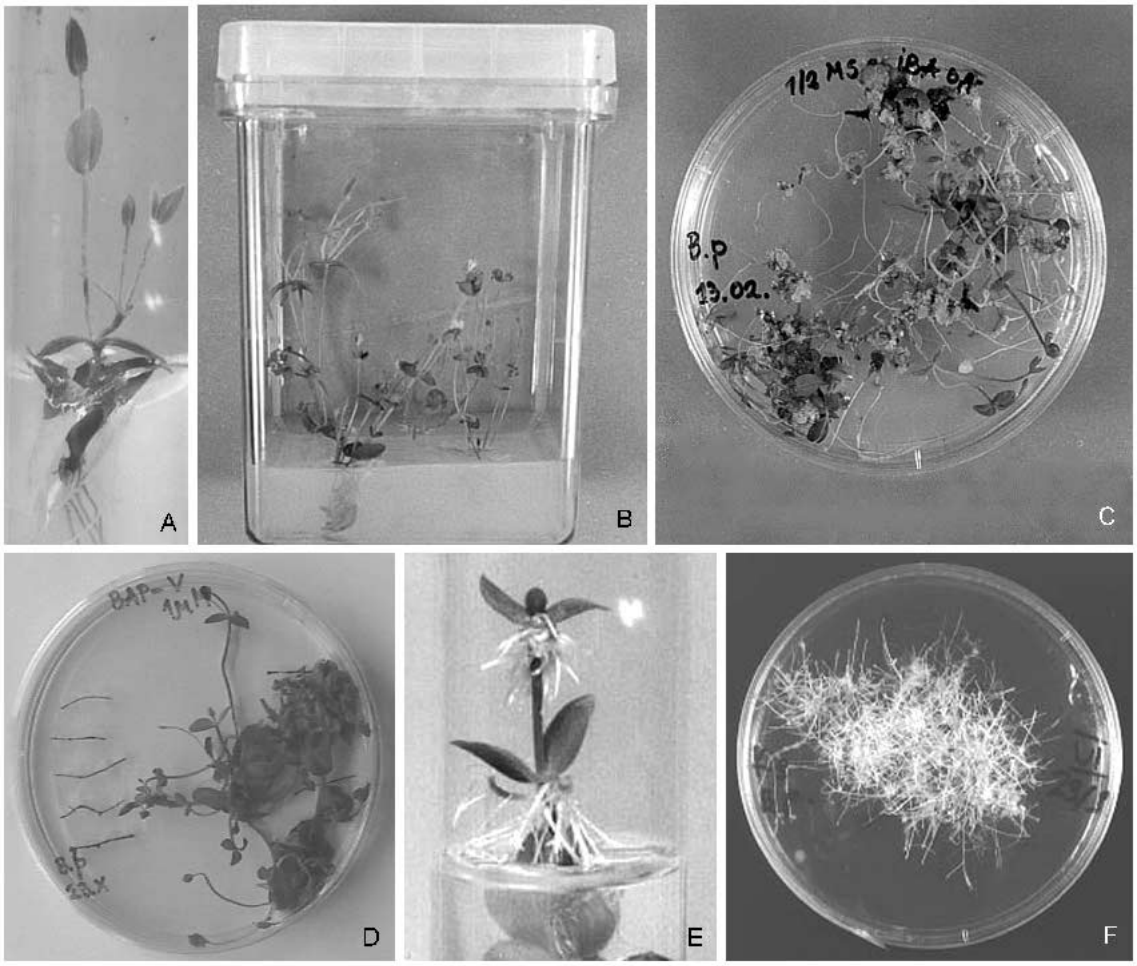

Fig. 1. *In vitro* culture of *Blackstonia perfoliata*: *A* - plantlet developed from viable seed produced by *in vitro* cultivated plants; *B* - flowering plant in *in vitro* culture; *C* - *in vitro* root culture on MS medium supplemented with IBA; *D* - *in vitro* root culture and regenerated buds on 1 μM BAP, *E* - roots developed on shoot of transformed plant; *F* - transgenic root culture.

medium decreased the number and length of lateral roots. Optimal development, number and length of lateral roots were obtained on medium supplemented with 0.01 - 0.3 μM BAP. The induction of buds and lateral roots at non-apical root segments were the most abundant on higher BAP concentrations (3 μM for buds and 0.3 μM for lateral roots), while in apical parts of roots it was the highest on lower concentrations (1 μM for buds and 0.03 - 0.1 μM for lateral roots) (data not shown). Based on our study, cytokinin (BAP) played major role on inducing bud development, and in the same time, auxin (IBA) was the key factor on inducing lateral root development. Cytokinins are considered to be inhibitors of lateral root formation, and they promote bud formation. Also, the auxin/cytokinin ratio has been shown to play a role in co-ordinating lateral root

production. However, different developmental phases have different requirements for cytokinins and auxins. It is hypothesized that cytokinins and low concentrations of auxins are responsible for lateral root initiation (Stirk and van Staden 2001).

The appearance of adventitious roots was the first sign of infection at the site of inoculation (Fig. 1*E*). Hairy roots appeared 3 weeks after inoculation. Tips of adventitious roots of *B. perfoliata* were excised and transferred to Petri dishes, on hormone free MS medium and observed as separate clones. The hairy root cultures of *B. perfoliata* were successfully obtained (Fig. 1*F*).

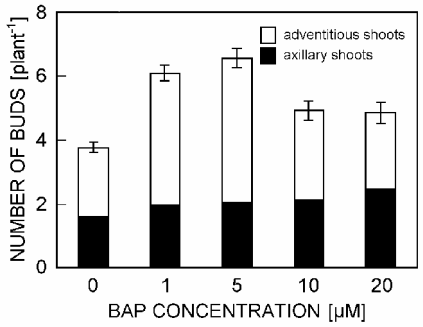

Fig. 2. Effect of BAP on shoot multiplication of *B. perfoliata*.

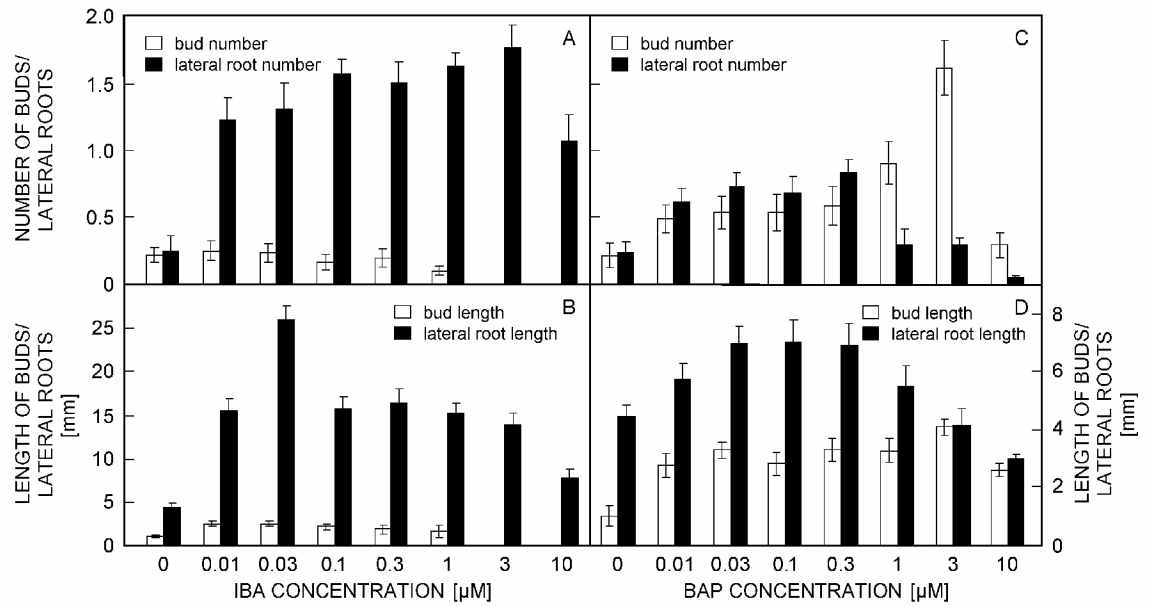

Fig. 3. Effect of IBA (*A*,*B*) and BAP (*C*,*D*) on number (*A*,*C*) and length (*B*,*D*) of regenerated buds and lateral roots.

These roots exhibited typical hairy root phenotype (rapid growth, very high branching and plagiotropism).

Spontaneous shoot regeneration occurred in clones Nos. 2, 3, and 5. Regenerated shoots were transferred to hormone-free MS medium and with a high shoot multiplication rate they were well preserved for three years in culture *in vitro*. All the regenerated plants displayed the phenotype typical for the presence of *rol* genes. However, only one plant spontaneously produced seeds and the percentage of germinated seeds was very low (data not shown). This could be explained by the fact that *B. perfoliata* is not a self-fertilized plant.

According to Handa (1992), Suginuma and Akihama (1995), Momčilović *et al*. (1997b), and Vinterhalter *et al*. (1999) spontaneous shoot regeneration in transformed root cultures was observed only in few species from the family *Gentianaceae* (*Gentiana cruciata, G. purpurea, G. punctata, G. scabra* and *Eustoma grandiflorum*). Also, established *in vitro* culture of transformed plants showed preserved morphogenetic capacity and potential for

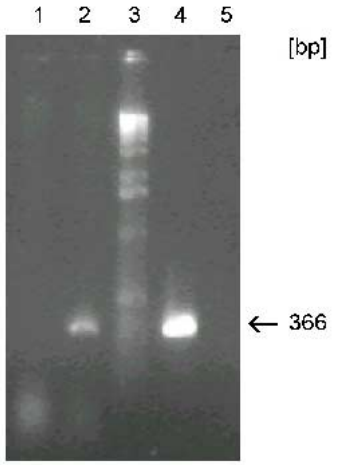

Fig. 4. PCR analysis of DNA from transformed shoots of *B. perfoliata*: *line 1* - non-transformed clone; *line 2* - transformed clone No. 2; *line 3* - standard DNA marker, 2 kb leader (*Gibco Brl*); *line 4* - *A. rhizogenes* A4M70GUS (positive control); *line 5* - negative control. 366 bp indicated amplified fragment.

application in biotechnology as a way of achieving mass production.

Successful transformation of *B. perfoliata* by *A. rhizogenes* A4M70GUS was confirmed by PCR analysis (Fig. 4). The presence of the *uid*A gene segment (366 bp) was shown in genomic DNA isolated from shoots spontaneously regenerated on the hairy root clone No. 2. The PCR product of the same size was obtained in the reaction where the DNA template was total DNA isolated from *A. rhizogenes*, serving as a positive control. The reaction was negative with non-transformed shoots (Fig. 4, *line 1*). To prove that PCR product was not an artefact we ran additional reaction with all components except DNA and it yielded no product (Fig. 4, *line 5*).

Although papers exist about transformation of some *Gentianaceae* species (Momčilović *et al*. 1997b, Vinterhalter *et al.* 1999), our findings present the first report on transformation of *B. perfoliata* and show a way for its efficient mass production *in vitro*. Also, it is concluded that *A. rhizogenes* could be a suitable vector for the transfer of desirable genes into *B. perfoliata* genome, as it was confirmed for some other species (Nikolić *et al*. 2003/4)

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