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BRIEF COMMUNICATION

Direct regeneration of shoots from hairy root cultures of *Centaurium erythraea* inoculated with *Agrobacterium rhizogenes*

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Abstract

Stable transformation and expression of transgenes was achieved in *Centaurium erythraea* Gillib. using *Agrobacterium rhizogenes*-mediated system. Five hairy root clones exhibited the transformed phenotype. Shoot regeneration, with green organized structures, was apparent in 4 clones, after the first subculture on Murashige and Skoog (MS) half strength medium. The integration of Ri-plasmid T-DNA was confirmed by polymerase chain reaction (PCR) analyses.

Additional key words: adventitious shoot, centaury, Ri-plasmid T-DNA.

Like some other species of *Gentianaceae* family, *Centaurium erythraea* Gillib. (centaury) is well-known medicinal plant which aerial parts are used as a drug in popular medicine as well as for preparing commercial beverages. The populations of this plant are greatly endangered by collecting for medicinal purposes. This fact, explains the increase interest for *in vitro* propagation and genetic transformation of *Centaurium* species. Secoiridoid glucosides was found in the aerial and roots of centaury obtained from *in vitro* cultures as well as from plants collected from the natural population (Janković *et al.* 1997). Recent developments in research of transgenic plants have opened up the possibility of the metabolic engineering of biosynthetic pathways to produce high-value secondary metabolites (Ramachandra *et al.* 2002). In the *Gentianaceae* family regeneration of transgenic plants in hairy root culture of *Gentiana cruciata*, *G. purpurea* (Momcilovic *et al.* 1997) were described. Reports on successful regeneration of hairy roots into whole plants and alternations to their properties have stimulated interest in developing hairy root regeneration procedures. The aim of the present study, was to develop an efficient system for regenerating shoots from centaury hairy roots, for the first time.

Seeds of *Centaurium erythraea* Gillib. were surface sterilized with 1 g dm^{-3} NaOCl for 10 min, followed by rinsing with sterile distilled water and germinated in 6 cm Petri dishes with filter paper and 2 cm^3 sterile distilled water. After three weeks root pieces (15 mm in length) from seedlings were transferred on (Murashige and Skoog 1962) (MS) medium containing half strength macronutrients, full-strength micronutrients and vitamins, 3 g dm^{-3} sucrose, 7 g dm^{-3} agar and 100 mg dm^{-3} myo-inositol. A virulent *Agrobacterium rhizogenes* strain A4M70GUS, is harbouring with GUS construct integrated into TL region of pRiA4 plasmid (Tepfer and Casse-Delbart 1987). *In vitro* grown seedlings were inoculated with *A. rhizogenes* strains A4M70GUS by puncturing the internodes of stems with a sterile, hollow needle loaded with bacteria. Hairy roots appeared 14 d after inoculation. Apical segments of primary hairy roots were excised and subcultures on MS medium for three subculture, firstly with and subsequently without 250 mg dm^{-3} cefotaxime for selection. Root tips 15 mm in length from hairy root culture were transferred to MS medium and subcultured every month. Adventitious shoots were initiated under these conditions. When the shoots were 2 - 3 mm long, they were excited and

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Abbreviations: MS medium - Murashige and Skoog's medium; PCR - polymerase chain reaction.

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transferred to growth regulator-free MS medium for the shoot elongation and root initiation. Plants were maintained at temperature of 25 ± 1 °C and irradiance of 50 μmol m^{-2} s^{-1}, 16-h photoperiod. Significance of differences between means counts of adventitious shoots were evaluated by the Student's *t*-test.

The hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure (Sul and Korban 1996) was used for DNA extraction from transgenic roots and leaves. Polymerase chain reaction (PCR) analysis of genomic DNA was based on the standard protocol of *Perkin-Elmer* PCR (*Roche Molecular Systems*, Brancherburg, USA).

Hairy roots appeared at the inoculation site in almost 54 % 15 to 20 d after infection. The *Agrobacterium*-free hairy roots, which were established after two successive

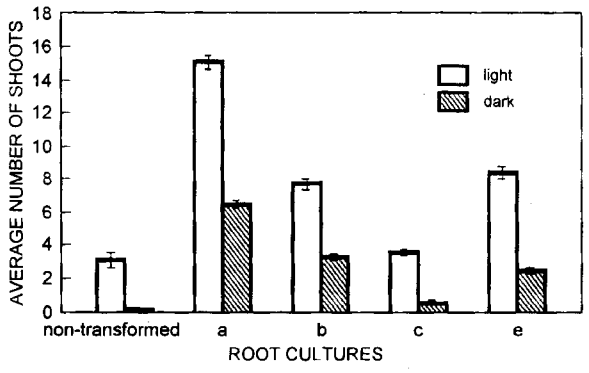

Fig. 1. Effect of light on adventitious shoots formation in root cultures of *Centaurium erythraea* clones a, b, c, and e. The data were recorded after one month of culture. Means ± SE, n = 50 roots. The experiment was repeated three times.

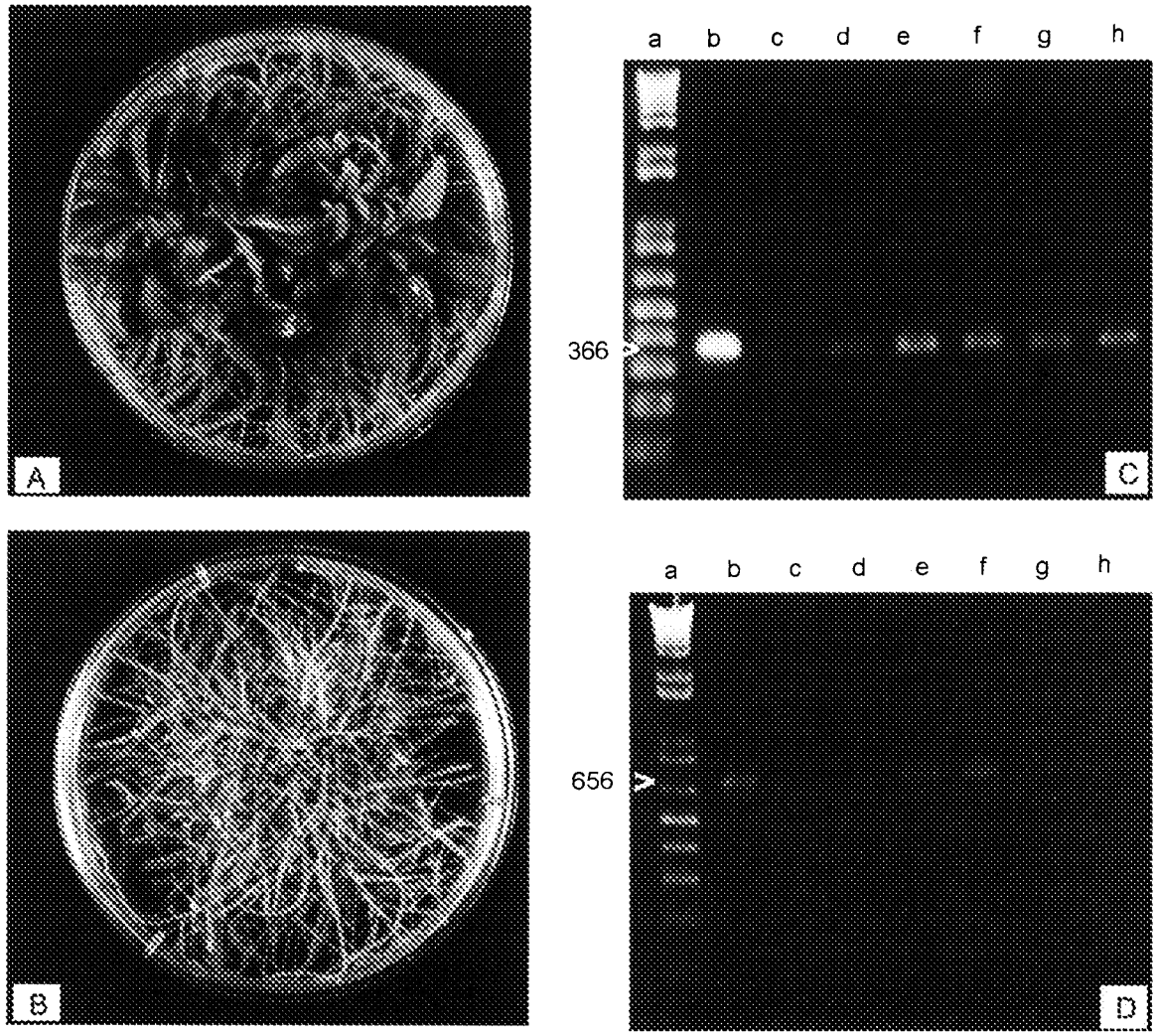

Fig. 2. *A,B* - Morphology of non-transformed root culture and clone d, respectively, three weeks after subculture. *C,D* - gel of PCR-amplified 366 bp *uidA* (*C*) and 656 bp *auxI* (*D*) gene fragments from genomic DNA: a - DNA standard, b - bacterial positive control, c - non-transformed culture; d - clone a; e - clone b; f - clone c; g - clone d; h - clone e.

subcultures and subsequently without cefotaxime, grew vigorously on growth regulators free medium and had typical characteristics of transformed roots such as fast growth, high lateral branching and lack of geotropism Five clones (a, b, c, d, e) were selected for the further study based on differences in their phenotype. The roots of four clones show normal growth on a MS medium without phytohormones. A faster growth rate, with a typically branched root appearance was observed only in root cultures of clone d. These hairy roots were thin, fibrous, non-pigmented with many root hairs (Fig. 2*B*). The phenotype of the root cultures has been perfectly stable over several years of maintenance *in vitro*. Non-transformed root cultures, used as a control, had very low growth rate. At the beginning of culture, adventitious shoot primordia developed only in the central portions of whole roots in the Petri dishes, which, being apart from the root apical meristem, are composed of older cells. In fact, numerous adventitious shoots thrust out of the root surface without visible callus formation. After 30 d of culture, adventitious shoots were formed at the both transformed and control root explants. Two alternative shoot morphologies were observed from hairy root cultured in light. There were shoots of normal size and morphology like shoot regenerated in control root cultures (Fig. 2*A*). Adventitious shoot formed in the dark were etiolated, most of them remained 5 mm in size during the subculture period in the dark. However, regeneration of shoots in non-transformed cultures was significantly lower than in transformed root cultures (Fig. 1). The obtained shoots were cut off and transferred to MS solid medium, where the whole plants were regenerated. The presence of *uid*A gene for β-glucuronidase was also detected by PCR amplification of 366 bp fragment in all examined clones which strongly suggested the incorporation of the TL-DNA (Fig. 2*C*). Presence of *auxl* gene and TR-DNA was comfirmed in four clones (Fig. 2*D*). Hairy root induction and shoot regeneration from hairy roots are important prerequisites for suecessful production of transgenic plants using *A. rhizogenes*. Only few reports on direct shoot formation after infection with *A. rhizogenes* have been published.

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