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# MORPHOLOGY, SECOIRIDOID CONTENT AND RAPD ANALYSIS OF PLANTS REGENERATED FROM CALLUS OF *CENTAURIUM ERYTHRAEA* RAFN.

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*Centaurium erythraea* plants obtained by indirect organogenesis are described in the paper. The plants were initiated from a single adventitious shoot regenerated from callus derived from the cotyledon of a 30-day-old seedling. The shoot was multiplied on MS medium supplemented with IAA (0.1 mg·L$^{-1}$) and BAP (1.0 mg·L$^{-1}$). The multiplication rate (28 shoots per culture within 4 weeks) was highest at the first subculture and decreased in further subcultures. The shoots were rooted on MS medium. The effect of IBA (0.1 mg·L$^{-1}$) on the number of shoots forming roots differed depending on the composition of the basal medium (MS). The rooted shoots were transplanted to soil and grown in a greenhouse with 90% effectiveness. RAPD analysis was done with adventitious shoots of *C. erythraea* from in vitro culture. In shoots and whole plants regenerated from the callus tissue, secoiridoid content was determined by the HPLC method. We showed significant differences in morphology (leaf size, fresh and dry weight and height of plants) and changes in the DNA profiles as compared to earlier reports for shoot tip-derived shoots and plants of *C. erythraea*, but the two groups of plants biosynthesized the same qualitative pattern and similar levels of secoiridoids, up to 150 mg·g$^{-1}$ dry weight; the increased biomass of plants regenerated from callus tissue makes them a better source of secondary metabolites.

**Key words:** *Centaurium erythraea* Rafn., somaclonal variation, shoot culture, secoiridoid glycosides, indirect organogenesis.

## INTRODUCTION

*Centaurium erythraea* Rafn. is an important medicinal plant belonging to the family Gentianaceae. The plant is known to produce secoiridoid glycosides in aerial parts, mainly gentiopicroside, swertiamarine and sweroside. The compounds exhibit fungistatic, antibacterial, choleretic, pancreatic and hepatoprotective activities (Skrzypczak et al., 1993; Kumarasamy et al., 2003). These glycosides are also used in the food industry as ingredients of some beverages (Vágnerova, 1992).

Excessive exploitation of *C. erythraea* has led to a significant decrease in its natural population. It is a protected species in Poland. Plant tissue culture techniques can be used as alternative methods for mass production of pharmacologically important plants (Liu et al., 2004). Our previous studies on in vitro cultures of *C. erythraea* showed bioreactor shoot cultures and a liquid culture system to be suitable for the production of secoiridoids (Piatczak et al., 2005a,b). We reported regeneration of *C. erythraea* from seedling-derived explants and showed that the plants were morphologically identical with those initiated from seeds (Piątczak and Wysokińska, 2003). We also found that one of the calli derived from the cotyledon produced a single shoot whose phenotype was different from other *C. erythraea* shoots developed in vitro and from the original plants. It is well known that the phenotype of plants regenerated in vitro, especially callus-derived plants, may differ from the original mother plants. The changes induced by in vitro conditions are termed "somaclonal variation" (Larkin and Scowcroft, 1981). They include alterations at the DNA level and/or karyotypic changes in chromosome structure (duplication, translocation) or in the number of chromosomes (polyploidization) (de Klerk, 1990). Fronk (1996) and Tariq and Paszkowski (2004) suggested that the mechanism of

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somaclonal variation may be of epigenetic nature and that it leads to variation of the DNA methylation level. Brown (1989) described and explained the significant changes in the methylation status of DNA in somaclonal variants. The occurrence of somaclonal variation depends on several factors such as the explant type, culture medium composition, particularly its growth regulator content, and culture duration (Skucińska, 2001). The phenomenon of somaclonal variation is undesirable in culture in vitro if the goal is micropropagation and/or transformation of plants. However, somaclonal variation is one way to generate desired variation, such as somaclonal variants with improved secondary metabolite yield (Čellárová et al., 1994, Thomas et al., 2006).

In this study we characterized shoot culture and whole plants of *C. erythraea* originated from a single shoot formed on cotyledon-derived callus, evaluating their morphology, shoot multiplication rate, root-forming ability of shoots, survival of regenerated plantlets in soil, and production of secoiridoid glycosides. We used RAPD analysis to detect genetic changes in shoots developed from the cotyledon callus, and compared the DNA profiles of these shoots with those of shoots obtained from shoot tip explants.

## MATERIALS AND METHODS

In our previous study we described shoot regeneration via organogenesis of cotyledon-derived callus of *C. erythraea* (Piątczak and Wysokińska, 2003). Briefly, callus culture was obtained on agar-solidified MS (Murashige and Skoog, 1962) medium supplemented with IAA (0.5 mg·L$^{-1}$) and BAP (0.2 mg·L$^{-1}$). After 5 weeks of culture, a single shoot was regenerated from the callus (Fig. 2a). The experiments described in this paper started with the single shoot of adventitious origin. The shoot was isolated from the callus tissue and transferred to fresh medium (MS agar medium containing 0.1 mg·L$^{-1}$ IAA and 1 mg·L$^{-1}$ BAP) to multiply more shoots. After 4 weeks, clusters of shoots were separated into individual shoots which were placed on MS medium supplemented with IAA (0.1 mg·L$^{-1}$) and different concentrations of BAP (0.5, 1, 2 mg·L$^{-1}$) in order to evaluate the effect of the cytokinin on shoot multiplication. We recorded the number of shoots initiated from each shoot base on each type of medium after 4 weeks. Observations were repeated in five successive subcultures and then in the tenth subculture (Fig. 1). For further multiplication, shoots were grown on MS agar medium supplemented with IAA (0.1 mg·L$^{-1}$) and BAP (1 mg·L$^{-1}$). The stock culture was routinely subcultured at 4 weekly intervals. For all multiplication treatments, each culture tube (2.5 cm diam) contained 25 ml medium. The pH of

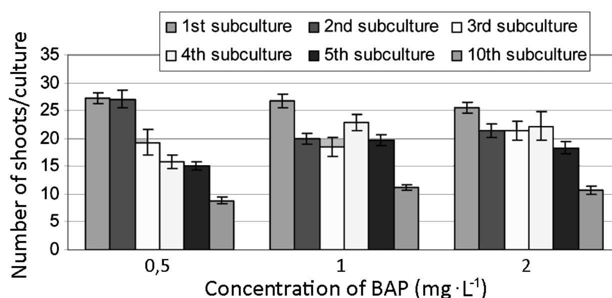


**Fig. 1.** Effect of BAP concentration and subculturing on *C. erythraea* shoot multiplication. After initial culture (4 weeks) the shoots were subcultured every 4 weeks on MS agar-solidified medium supplemented with IAA (0.1 mg·L$^{-1}$) and different concentrations (0.5, 1, 2 mg·L$^{-1}$) of BAP. Bars represent the mean±SE (9–13 replicate treatments).

all media was adjusted to 5.6–5.8 with 0.1 M NaOH before autoclaving at 121°C and steam pressure 1 kg·cm$^{-2}$ for 17 min. The culture tubes were kept in a growth chamber under cool-white fluorescent light with photosynthetic photon flux density (PPFD) of 40 μmol/m$^2$s (18 h photoperiod) at 26±2°C for 4 weeks.

### SHOOT ROOTING AND PLANTLET ACCLIMATIZATION

Individual shoots were excised from multiple clusters growing on multiplication medium (MS + IAA 0.1 mg·L$^{-1}$ and BAP 1 mg·L$^{-1}$) and transferred to rooting medium, that is, full-strength MS basal solidified medium without growth regulators. Shoots from different subcultures (8th and 30th) were rooted under these conditions. Additionally, for shoots taken from the 32$^{nd}$ subculture, half-strength MS medium (1/2 MS) alone or containing auxin (0.1 mg·L$^{-1}$ IBA) and full-strength MS medium with 0.1 mg·L$^{-1}$ IBA were used. The cultures were maintained under the same conditions as for shoot multiplication. The percentage of rooted shoots, root lengths and number of roots per shoot were recorded after 4 weeks; the experiments were done three times (Tab. 1). The rooted shoots (29) were transferred to pots (10 cm diam) containing a sterilized mixture of soil, sand and peat (4:3:3 v/v/v). Plantlets were kept for 2 weeks in a growth chamber and then transferred to a greenhouse. Survival rates were recorded 6 weeks later.

### DNA EXTRACTION AND RAPD ANALYSIS

Fresh shoot samples were collected from the plant material obtained from each treatment. The samples were derived from micropropagated shoots (4 weeks of culture on agar-solidified MS medium supplemented with IAA (0.1 mg·L$^{-1}$) and BAP (1 mg·L$^{-1}$).

TABLE 1. Influence of age of the multiplication culture on rooting of *C. erythraea* shoots. Shoots were cultured on MS or ½ MS media with or without auxin (IBA 0.1 mg·L$^{-1}$) for 4 weeks

| Medium | No. of shoot subcultures on multiplication medium | Shoot rooting (%) | Average number of roots/shoot | Average length of roots [mm] |
|---|---|---|---|---|
| MS | 8 | 100.0 | 2.8$^{a}$ ± 0.4 | 4.0$^{a}$ ± 0.5 |
| | 20 | 50.0 | 4.2$^{b}$± 0.7 | 8.0$^{b}$ ± 1.2 |
| | 30 | 11.8 | 2.0$^{a}$± 1.0 | 3.1$^{e}$ ± 0.1 |
| | 32 | 22.9 | 1.8$^{a}$ ± 0.5 | 12.9$^{f}$ ± 5.4 |
| ½ MS | 32 | 100.0 | 3.3$^{c}$ ± 0.5 | 15.5$^{f}$ ± 1.4 |
| MS + IBA | 32 | 47.4 | 1.9$^{a}$ ± 0.3 | 6.2$^{c}$ ± 0.4 |
| ½MS+ IBA | 32 | 80.0 | 3.2$^{c}$ ± 0.6 | 10.4$^{d}$ ± 0.6 |

Values are means ±SE of three independent experiments. 19–22 shoots were raised for each experiment. Means followed by the same letter within column do not differ significantly at P≤0.05 by the Mann Whitney U test.

Shoots from 10-week-old plants derived from seeds and grown in soil were the control material. All samples were frozen at -80°C. DNA was extracted from 50–70 mg plant tissue following the procedure described by Pirttilä et al. (2001) with slight modifications. The DNA concentration and purity were assessed with a NanoDrop 1000 spectrophotometer (Thermo Scientific). At least two PCR reactions per primer were run for each sample. Ten nanograms of purified genomic DNA (OD260/OD280 1.8; OD260/OD230 2.0) per reaction were used in a final volume of 25 µl under the following conditions: 5 mM buffer [with $(NH_4)_2 SO_4$], 0.2 mM each dNTP (Fermentas), 8.0 pmol primer and 1.0 unit Taq DNA Polymerase (Fermentas). The RAPD reaction was performed using a UNO II Biometra thermocycler and PCR profile consisting of a 94°C denaturation step (3 min) followed by 45 cycles consisting of 3 min at 94°C, 1 min at 40°C and 2 min at 72°C, with a final elongation step of 7 min at 72°C (Kawiak and Łojkowska, 2004). Amplification products were separated on 1.5% agarose gels (Prona) in 0.5 × TBE buffer at 5 V cm$^{-1}$. The gels were stained with 0.5 µg ml$^{-1}$ ethidium bromide and then photographed under UV.

### SECOIRIDOID ANALYSIS

Secoiridoid glycosides were extracted from air-dried and powdered plant material (0.5 g) with methanol (5 ml) as described earlier (Piątczak et al., 2005a). HPLC analysis employed a Hewlett-Packard 1100 Series HPLC instrument (Waldbronn, Germany) combining a quaternary pump, vacuum degasser and diode array detector. For instrument control, data acquisition and analysis, Hewlett-Packard ChemStation software and a Vectra computer were used. Analytical separation was done on a Hypersil ODS column (250 × 4 mm; 5 µm, Hewlett Packard) with a solvent system of 3% (v/v) acetic acid (A) and acetonitrile (B). The gradient was as follows: 0 min – 5% B; 28 min – 20% B; 32 min – 50% B; 35 min – 90% B; 40 min – 5% B. The methanolic extract injection volume was 20 µl, flow rate was 0.8 ml/min, and detection was performed at 260 nm. Secoiridoids (gentiopicroside, swetiamarine, sweroside) were identified by comparing retention times and the UV spectra of peaks detected in the methanolic extract with those of standard compounds. The secoiridoids were quantified using calibration curves prepared with pure compounds.

The results, expressed as mg·g$^{-1}$ dry weight, are means of three replicates (3 independent samples from plant material) (Fig. 4).

### STATISTICAL ANALYSIS

The results presented in Tables 1 and 2 were subjected to ANOVA and the significance of differences in values was checked with the Mann-Whitney U Test at P≤0.05 in the Statistica package (StatSoft'98).

## RESULTS AND DISCUSSION

A single adventitious shoot of *C. erythraea* regenerated from cotyledon-derived callus was cultured on MS agar-solidified medium supplemented with IAA (0.5 mg·L$^{-1}$) and BAP (0.2 mg·L$^{-1}$) for 5 weeks. The leaves of the shoot were dark green, larger and more oval (Fig. 2a) than the leaves formed on shoots obtained in our earlier studies of other explants of *C. erythraea* seedlings grown under similar experimental conditions (Piątczak and Wysokińska, 2003). These morphological changes were also observed in multiple shoot culture and plantlets originated from the shoot of cotyledon-derived callus (Fig. 2b,c). In this study we term this type of shoot/plantlet "altered," in contrast to the "unaltered" shoots/plantlets of *C. erythraea* developed from shoot tip explants. The latter plants were morpho-

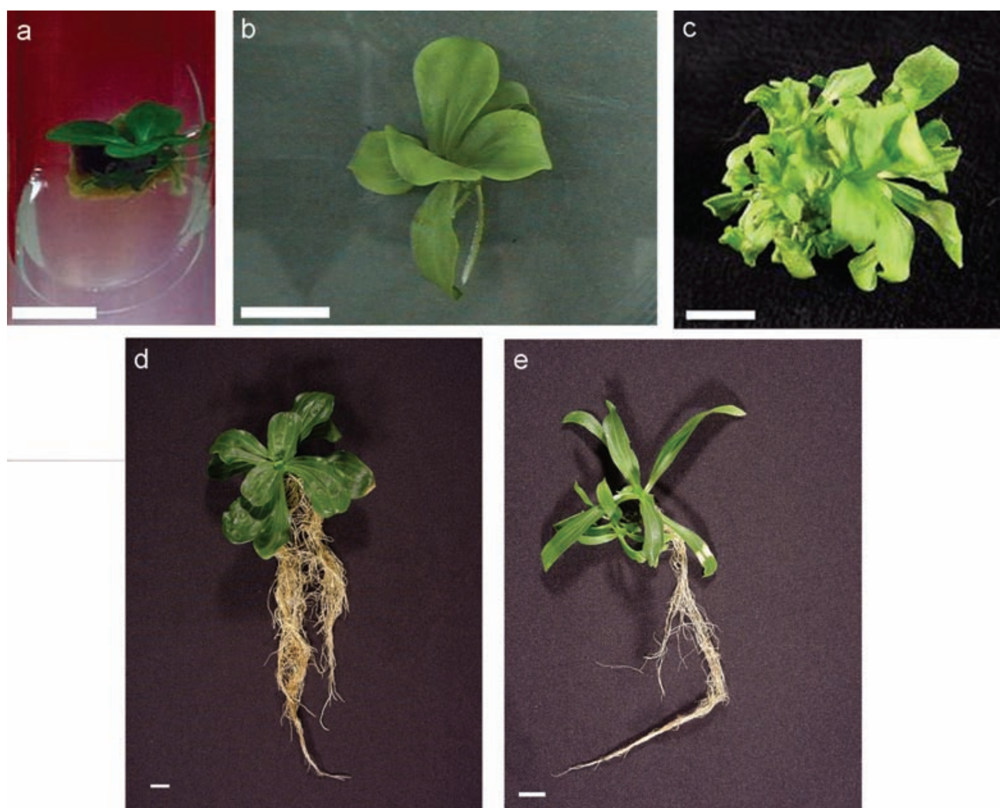


**Fig. 2.** Shoot differentiation from cotyledon-derived callus and plant regeneration of *C. erythraea*. (**a**) Callus formation and shoot regeneration from cotyledon explant (solid MS medium with 0.2 mg·L$^{-1}$ BAP and 0.5 mg·L$^{-1}$ IAA), (**b**) Shoot multiplication on MS medium supplemented with IAA (0.1 mg·L$^{-1}$) and BAP (1 mg·L$^{-1}$), (**c**) Rooting of shoot derived in vitro after 4 weeks of culture on MS medium without growth regulators, (**d**) 10-week-old acclimatized plant grown in soil under greenhouse conditions, (**e**) *C. erythraea* plant developed from shoot tip explant. Bar = 1 cm.

logically identical with mother plants of *C. erythraea*, that is, field-grown plants derived from seeds. It is known that plant tissues contain different types and concentrations of endogenous growth regulators. As a result they may respond differently to the culture media (Skucińska, 2001).

In the "altered" shoot culture and plants of *C. erythraea* we assessed the shoot multiplication rate, rooting frequency, survival rate and production of secoiridoid glycosides, and made RAPD analyses to detect alteration of the DNA profiles of "altered" shoots.

## SHOOT MULTIPLICATION

For multiple shoot induction, an "altered" shoot was excised from callus tissue and incubated on MS agar medium containing IAA (0.1 mg·L$^{-1}$) and BAP (1 mg·L$^{-1}$). After 4 weeks, media with other concentrations of BAP (0.5 and 2 mg·L$^{-1}$) were also used to evaluate the effect of the cytokinin on generation of new shoots. As seen in Figure 1, in the first subculture the shoot multiplication response in terms of mean number of shoots per culture was similar (25–28 within 4 weeks) on all used media containing 0.5, 1 or 2 mg·L$^{-1}$ BAP. The response decreased progressively in further subcultures. A decline in multiplication rates following continuous subcultures has often been observed in cultures of many plant species, for example in *Rosa damascena* Mill. (Jabbarzadech and Khosh-Khui, 2005) and *Ananas comosus* L. Merr (Hamad and Taha, 2008) or *Carlina acaulis* L. (Trejgell et al., 2009).

We also found that the shoot multiplication rate was higher after the 4$^{th}$–5$^{th}$ subcultures when media with a higher BAP concentration (1 or 2 mg·L$^{-1}$) were used. For example, almost 25 shoots per culture were produced within 4 weeks on MS medium supplemented with 0.1 mg·L$^{-1}$ IAA and 1 mg·L$^{-1}$ BAP, as compared to 15 shoots/culture on MS medium containing half the concentration of the cytokinin (0.5 mg·L$^{-1}$). Therefore we chose MS medium supplemented with IAA 0.1 mg·L$^{-1}$ and BAP 1 mg·L$^{-1}$ for multiplication of "altered" shoots of *C. erythraea* after the tenth subculture. Medium of the same composition was also recommended for multiplication of "unaltered" shoots of *C. erythraea*; after the 5$^{th}$ subculture, 20 shoots were produced from one

TABLE 2. Morphological differences between micropropagated *C. erythraea* plants with "altered" and "unaltered" phenotype. Plants grown in soil for 10 weeks; ± SE

| Morphological difference | "altered" plants | "unaltered" plants |
|---|---|---|
| Plant height (cm) * | 20.0ᵃ ± 0.2 | 11.0ᵇ ±0.6 |
| Average plant fresh weight (g) | 2.9ᵃ ± 0.3 | 1.6ᵇ ± 0.2 |
| Average plant dry weight (g) | 0.3ᵃ ± 0.1 | 0.1ᵇ ± 0.02 |
| Number of leaves/plant | 11.0ᵃ ± 0.1 | 9.0ᵃ ± 1.2 |
| Leaf area (cm²) | 12.0ᵃ ± 0.5 | 6.2ᵇ ± 0.5 |
| Length of leaf (cm) | 5.2ᵃ ± 1.6 | 5.8ᵃ ± 1.1 |
| Width of leaf (cm) | 2.3ᵃ ± 0.3 | 1.1ᵇ ± 0.2 |
| Maximum root length (cm) | 18.7ᵃ ± 1.2 | 9.0ᵇ ± 0.8 |

*plant height without the length of roots
Values followed by the same letter within row do not differ significantly at P≤0.05 by the Mann Whitney U test.

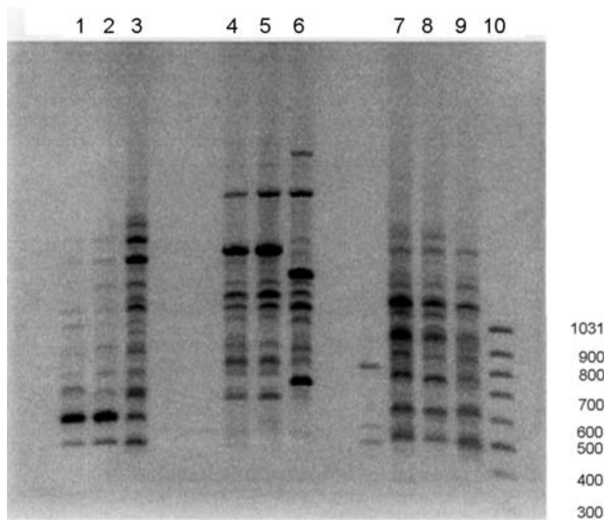


**Fig. 3.** Gel electrophoresis of RAPD fragments in *C. erythraea* shoots with "altered" (lanes 3, 6, 9) and "unaltered" (lanes 1, 2, 4, 5, 7, 8) phenotypes, with primers OPA-18 (lanes 1–3), OPA-17 (lanes 4–6) and OPA-03 (lanes 7–9). Lane 10 – Gene Ruler™ DNA ladder. Lanes 1, 4, 7 – "unaltered" shoots from 10-week-old plants grown in soil derived from seeds (control). Lanes 2, 5, 8 – "unaltered" shoots (4 weeks of culture on agar-solidified MS medium + 0.1 mg/l IAA + 1 mg/l BAP). Lanes 3, 6, 9 – "altered" shoots (4 weeks of culture on agar-solidified MS medium + 0.1 mg/l IAA + 1 mg/l BAP).

shoot tip within 4 weeks (Piątczak and Wysokińska, 2003). It is evident from the data that the shoot multiplication ability of "altered" and "unaltered" shoots of *C. erythraea* is similar.

MOLECULAR CHANGES IN "ALTERED" SHOOTS

In RAPD-PCR using DNA isolated from *C. erythraea* shoots grown in vivo, "unaltered" shoots grown in vitro (control) and shoots grown in vitro characterized by somaclonal variation and having an altered phenotype shown in Figure 2, there were differences between the control and "altered" shoots in the products obtained with three primers: OPA 03 [5' – AGTCAGCCAC – 3'], OPA 17 [5' – GACCGCTTGT – 3'] and OPA 18 [5' – AGGTGACCGT – 3']. The control shoots and the shoots grown in vivo did not differ in their genetic profile. The other primers tested either gave no products of the amplification reaction or else yielded 1 or 2 products, not enough for analysis (Fig. 3). Somaclonal variation involves genetic changes in DNA (in the nucleus, mitochondria and chloroplasts). The changes can be hereditary and comparable to spontaneous mutations, or epigenetic, which are usually nonhereditary and caused by changes in gene expression (Karp and Bright, 1985). Changes in genetic information may occur in cells of the maternal plant or during culture. Chemical and physical conditions that induce rapid cell division

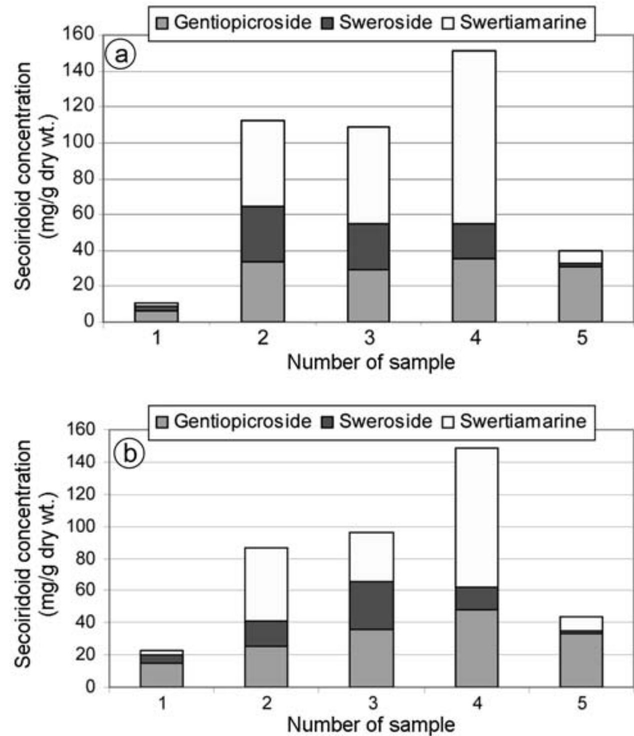


**Fig. 4.** Concentration of secoiridoid glycosides (gentiopicroside, sweroside, swertiamarine) in *C. erythraea* shoots and plants regenerated in vitro with "altered" (**a**) and "unaltered" (**b**) phenotypes. 1 – shoots cultured on MS solid medium supplemented with BAP 1 mg·L$^{-1}$ and IAA 0.1 mg·L$^{-1}$; 2, 3 – shoots of plantlets regenerated in vitro after 4 weeks of culture on MS medium without growth regulators. Before transfer to rooting medium the shoots were cultured on multiplication medium (MS solid medium supplemented with BAP 1 mg·L$^{-1}$ and IAA 0.1 mg·L$^{-1}$) for 16–20 subcultures (2) or 26–30 subcultures (3); 4, 5 – shoots (4) and roots (5) of plants regenerated in vitro, grown for 10 weeks in soil in the greenhouse.

caused by plant growth regulators in the medium can influence changes in DNA organization (Sarasan et al., 2006). The conditions of culture in vitro promote alterations of metabolic pathways, which can occur due to changes in the expression of particular genes (Sabała and Orlikowska, 1993).

Random amplified polymorphic DNA (RAPD) analysis is often favored over phenotypic or cytological measurements, and generally reveals even small variations of the genome (Ehsanpour et al., 2007). This technique has been applied successfully in several species to detect somaclonal variation following micropropagation (Gavidia et al., 1996; Kawiak and Łojkowska, 2004; Chaudhuri et al., 2007; Cai et al. 2009; Ćwiklińska et al., 2010). The use of RAPD markers is more advantageous than RFLP markers, because large amounts of samples can be analyzed economically and quickly, the specific DNA fingerprints are obtained independent of ontogenic expression, and most of the genome can be sampled with a potentially unlimited number of markers (Bennici et al., 2004). It is expensive and time-consuming to use RFLP markers (Gao et al., 2009). The amount of somaclonal variation can also be estimated with other markers such as isoenzymes (Feuser et al., 2003), microsatellites (Litt and Luty, 1989) or simple sequence repeats (SSRs) (Jacob et al., 1991). Flow cytometry (FCM) can be used to estimate nuclear content and to assess the genome stability of plants regenerated in vitro (Sliwinska and Thiem, 2007).

### ROOT FORMATION

"Altered" shoots were subcultured on multiplication medium (MS + IAA 0.1 mg·L$^{-1}$ and BAP 1 mg·L$^{-1}$) for 8, 20, and 30 subcultures, after which they were excised and transferred to rooting medium consisting of MS nutrient medium without growth regulators. The rooting capacity of *C. erythraea* shoots in the medium varied and depended on the time that the shoots spent on the multiplication medium before transfer to the rooting medium. All shoots from the 8$^{th}$ subculture formed roots within 4 weeks of culture on MS basal medium (Tab. 1). Sometimes an additional single shoot developed from an axillary bud of a shoot. The rooting frequency decreased 50% in shoots after the 20$^{th}$ subculture. Rooting induction decreased further to 12% within 4 weeks in shoots after the 30$^{th}$ subculture on multiplication medium. We tested a longer period for root induction (6 instead of 4 weeks) but it did not lead to an increased percentage of shoots forming roots (data not shown). For shoots that failed to develop roots almost completely in 2.5 years of culture we tried other rooting media (MS with 0.1 mg·L$^{-1}$ IBA and MS medium without growth regulators or containing 0.1 mg·L$^{-1}$ IBA). Adding IBA to full-strength MS medium improved the rooting percentage of shoots (47%) as compared to MS medium without the auxin (23%) (data for shoots after 32 subcultures on MS growth regulator-free medium) (Tab. 1). However, in the presence of auxin the roots were significantly shorter and callus was formed at the basal part of the shoot. Optimal for rooting was MS medium without auxin, on which all shoots excised from the 32$^{nd}$ subculture developed an average 3 roots per shoot within 4 weeks.

### PLANT GROWTH CHARACTERISTICS

Rooted shoots were transferred to soil and grown in pots under greenhouse conditions. About 90% of the plants survived after acclimatization (8 weeks in the greenhouse). As shown in Table 2 and Figure 2d,e, the plants differed in morphology from those arising from shoot tip explants of *C. erythraea* ("unaltered" plants) of the same age and grown in the same conditions. The former were higher, with leaves more oval-shaped and darker green. Leaf size also differed between the two groups of *C. erythraea* plants regenerated in vitro (Fig. 2d,e). The "altered" plants had a better-developed root system with more extensive lateral branching (Fig. 2 d). These characters resulted in significantly higher fresh and dry weight in "altered" plants, by 80% and 300%, respectively, compared to "unaltered" plants (Tab. 2). We did not observe flowering of the "altered" plants during two years of growth. These observations and RAPD results indicate that the *C. erythraea* micropropagated plants of different origin had different genotypes and phenotypes. Somaclonal variation has not been reported previously for *C. erythraea*, but similar alterations in plants derived from callus tissue of many other species have been reported frequently. Significant variability of morphological characters, biomass production and hypericin formation have been reported in regenerants of *Hypericum perforatum* originating from the same genotype (Čellárová et al., 1994). Hunault and du Manoir (1992) found differences in morphology, essential oil composition and fruit production in somatic embryo-derived clones of *Foeniculum vulgare*. Saxena et al. (2000) obtained two somaclones of *Pelargonium graveolens* from nodal explant-derived calluses. The clones differed in morphology (leaf size and shape) and in essential oil yield and composition.

### SECOIRIDOID CONTENT

We used the HPLC method to determine the content of three secoiridoid compounds (gentiopicroside, sweroside, swertiamarine) in shoot culture and "altered" plants of *C. erythraea* grown in vitro and ex vitro (greenhouse) (Fig. 4a). For comparison we give the content of these compounds in "unaltered" shoot culture and micropropagated plants (Fig. 4b).

"Altered" *C. erythraea* shoots grown in multiplication medium (MS+ IAA 0.1 mg·L$^{-1}$ and BAP 1 mg·L$^{-1}$) produced 10.5 mg·g$^{-1}$ dry wt secoiridoids, calculated as the sum of gentiopicroside, sweroside and swertiamarine. We noted that establishment of the root system improves the secoiridoid level. Analysis of shoots of 4-week-old plantlets rooted on MS medium without growth regulators showed that secoiridoid content was 10 times higher than in shoot culture without roots (Fig. 4a,b). There were no differences in secoiridoid quantity with respect to the time shoots spent on multiplication medium, and shoots of plantlets from subcultures 16 to 20 and from subcultures 26–30 produced similar amounts of the compounds (Fig. 4 a). The trend was similar in "unaltered" *C. erythraea* plant material (Fig. 4b). "Unaltered" shoots in culture in vitro accumulated a lower level of secoiridoids than shoots of rooted plantlets, and the level was twice the amount detected in the culture of shoots with an "altered" phenotype. Also, for "unaltered" plantlets the number of subcultures had a slight effect on the total content of secoiridoids in shoots rooted on MS basal medium (Fig. 4b). Secoiridoid content was highest (150 mg·g$^{-1}$ dry wt) in 10-week-old micropropagated plants grown in the greenhouse. The compounds were accumulated mainly in aerial parts of the plants. In shoots, swertiamarin was the dominant secoiridoid; in roots it was gentiopicroside. From the results shown in Figure 4 we infer that in spite of the morphological and genetic differences, both types of *C. erythraea* micropropagated plants biosynthesized the same qualitative profile and similar cell levels of the analyzed compounds. However, the results in Table 2 make it evident that the "altered" plants produced significantly more biomass (fresh and dry weight) than the "unaltered" plants at corresponding times (10 weeks). The global effect is high production of the metabolites in the former; average total secoiridoid content of an "altered" plant was 62.2 mg, versus 21.2 mg from an "unaltered" plant. It is also worth noting that in "altered" *C. erythraea* micropropagated plants the secoiridoid levels were higher than those in 10-week-old field-grown plants of *C. erythraea* initiated from seeds, which amounted to 47.4 mg (data not published). Earlier, Menković et al. (1998, 2000) reported that shoot cultures of two *Gentiana* species (*G. lutea* and *G. punctata*) contained more gentiopicrine than aerial parts of the plants collected from natural localities. Rathore and Shekhawat (2009) also demonstrated that organs of *Pueraria tuberosa* plants regenerated in vitro accumulated more isoflavone (puerarin) than the mother plant. Similar results were obtained with *C. erythraea* shoots cultured in vitro, which produced higher amounts of xanthones than from naturally growing plants (Janković et al., 2000). One reason for increased production in vitro could be exposure of plants under in vitro and ex vitro conditions to different types of stress, which can affect the secondary metabolism of these plants. From investigations of the production of secoiridoids in cultures of different Gentianaceae species in vitro it is clear that the type and concentration of plant growth regulators play an important role in secondary metabolite production in vitro (Menković et al., 1998, 2000; Janković et al., 2000).

Micropropagated plants can be exploited independently of their phenotype and regeneration pathway to produce bioactive secoiridoids. The increased yield of these compounds we achieved in "altered" plants obtained from cotyledon-derived callus was due mainly to the increased shoot and root biomass of these plants. The present study provides the first information on somaclonal variation in micropropagated *C. erythraea* plants at the molecular level, confirmed by RAPD analysis.

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