IN VITRO REGENERATION OF *CENTAURIUM ERYTHRAEA* RAFN FROM SHOOT TIPS AND OTHER SEEDLING EXPLANTS

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ABSTRACT

Various explants from 30-day-old seedlings of *Centaurium erythraea* Rafn were evaluated for their morphogenetic capacity under in vitro culture conditions. Shoot formation from shoot tip explants was achieved mainly through adventitious bud differentiation. The highest number of shoots (up to 43.3 ± 2.2 from a single shoot tip) was obtained on Murashige and Skoog medium (MS) supplemented with indole-3-acetic acid (IAA) (0.57 µM) and 6-benzylaminopurine (BAP) (4.4 µM). Adventitious shoot regeneration was also achieved through organogenesis from calluses obtained from hypocotyls, cotyledons, roots and leaves on MS medium containing IAA (2.85 µM) and BAP (0.88 µM). Significant differences were noted between explant types in their effects on shoot regeneration. In the primary culture, the best response was obtained either from calluses derived from roots or leaves (44.4 ± 4.5 and 40.2 ± 6.0 shoots per callus, respectively). The number of subcultures of inoculated calluses affected both the multiplication rate (the number of shoots/explant) and shoot morphology (the frequency of shoot hyperhydricity). Shoots rooted with the frequency of 94-100% after culture on MS medium without growth regulators. Plantlets were successfully acclimatized (97%) under high relative humidity and then moved to the greenhouse.

KEY WORDS: *Centaurium erythraea* Rafn, Gentianaceae, micropropagation, organogenesis, adventitious shoots.

INTRODUCTION

Centaurium erythraea Rafn (Gentianaceae) is a biennial species occurring naturally all over Europe to Southern Scandinavia as well as in Asia. The plants are found also in North America. Aerial parts of *C. erythraea* represent official drug (*Centaurii herba*) included in the pharmacopoeias of almost all European countries and in the pharmacopoeia of the United States of America (Imbesi 1964; Van der Sluis 1985a). The drug is used in gastrointestinal tract diseases as tonicum, stomachicum and antihelminthic agent (Skrzypczak et al. 1993). The main group of medicinally important constituents of *C. erythraea* are bitter secoiridoid glucosides (gentiopicrin, erytaurin, swertiamarine, sweroside) (Van der Sluis 1985b), which are also used in preparation of some commercial beverages (Vágnerova 1992). This plant species has also attracted attention as a source of xanthones, compounds possessing various pharmacological properties (Menković et al. 2000).

C. erythraea is propagated by seeds. However, the cultivation of the plant is not easy, because of the high mortality rate of seedlings due to unfavorable conditions (Barešová 1988). Therefore, in spite of a great demand for *Centaurii herba* its supplies still depend on wild sources, which are rapidly disappearing due to unrestricted exploitation of the plants and the progressive clearance of its habitats. Now *C. erythraea* is listed as an endangered plant species (Holub et al. 1979). For these reasons an efficient micropropagation procedure would be useful for conservation of this threatened species and to ensure the supply of crude drugs from the herb. In vitro regeneration of shoots of *C. erythraea* directly from leaf segments and via callus phase has been described previously (Vágnerova 1992). Plantlets were also obtained through somatic embryogenesis (Barešová 1988). In this case, however, there were problems associated with differences in developmental stage of embryos induced from suspension culture of *C. erythraea*. In this paper we report for the first time our success in developing plants from shoot tip explants. *C. erythraea* plant regeneration via organogenesis from calluses derived from hypocotyl, cotyledon, root and leaf seedling explants is also considered in the present study. The obtained results can lead to an improvement in efficiency of the previously published culture systems for micropropagation of *C. erythraea*.

MATERIAL AND METHODS

Plant material and culture conditions

Centaurium erythraea seeds obtained from the Botanical Garden of the Polish Academy of Sciences in Warsaw were used in this study. They were surface-sterilized with 2% so-

dium hypochlorite (NaClO) solution for 10 min., rinsed three times with sterile distilled water and aseptically germinated in glass tubes of 20 mm Ř, containing 25 ml MS (Murashige and Skoog 1962) medium supplemented with sucrose (3%). The pH of the medium was adjusted to 5.8 and the medium was solidified with 0.7% agar (Difco Bacto Agar). The cultures were kept in a growth chamber at 26 ± 2°C under continuous cool-white fluorescent light with Photosynthetic Photon Flux Density (PPFD) of 40 µmol · m^{-2} · s^{-1}. These incubation conditions were maintained throughout the whole course of this study unless otherwise stated. Shoot tips, hypocotyl segments, cotyledons, leaves and root tips were excised from 30--day-old seedlings and used as initial explants.

Shoot proliferation from shoot tip explants

Portions of shoot tips (3-5 mm in length), having one apical and two axillary buds, were transferred into MS medium supplemented with IAA (0.57 µM) and different concentrations of BAP (2.2; 4.4; 8.8 and 13.2 µM) and cultured for 4 weeks. MS medium containing IAA (0.57 µM) and BAP (4.4 µM) was applied at the beginning of five successive subcultures (28 days, each) using shoot tips from the previous subculture and maintaining the explant characteristics described for the primary culture. The number of shoots per explant as well as the presence or absence of hyperhydricity were evaluated in primary culture and after each subculture.

Shoot regeneration from other seedling explants

To test the effects of various explants on shoot formation, root tips, hypocotyl segments and whole cotyledons (about 3-5 mm in length), as well as young leaves (ca. 10 mm long) obtained from 30-day-old seedlings were placed horizontally on MS agar medium supplemented with IAA (2.85 µM) and BAP (0.88 µM). After five weeks the percentage of calluses forming adventitious shoots and the average number of shoots produced per callus were recorded. Caulogenic calluses initiated from various explants were cut into pieces (with one shoot bud, each) and subcultured under the same conditions, as those previously mentioned for primary culture. The number of shoots per callus after each subculture was calculated.

Shoot rooting and plant acclimatization

For rooting experiments, 4-5-week-old shoots from the primary cultures as well as from the eighth subculture were transferred individually into growth regulator-free MS basal medium. The percentage of rooted shoots, length of roots and the number of roots per shoot were evaluated after 2 and 4 weeks. Rooted shoots (4-week-old) were washed in water to remove agar from the roots and transferred into pots (Ø 10 cm) containing a sterilized mixture of soil, sand and peat (4:3:3 v/v/v). The number of plantlets transferred into pots was thirty one. Plantlets were covered with glasses to ensure high humidity. Glass covers were gradually opened during the acclimatization period and removed completely after 14 days. The potted plantlets were maintained inside a growth chamber at 90% relative humidity in conditions as described above, for 4 weeks. After this time pots with acclimatized plants were replaced into greenhouse and the survival of the plantlets was recorded 4 weeks later.

Statistical analysis

The presented results are the means ± SE of three replicates. The number of cultures per replicate varies and is listed for different experiments in the tables (1-3). Data on the effect of BAP concentration and explant type on shoot formation were analyzed with the Statistica program. Significant differences between means were assessed by Duncan's Multiple Range Test at a 5% probability.

RESULTS AND DISCUSSION

Shoot proliferation from shoot tip explants

All shoot tips with a single apical and two axillary meristems produced multiple shoots during a 4 week culture on MS medium containing IAA (0.57 µM) and different concentrations of BAP (2.2; 4.4; 8.8 and 13.2 µM) (Table 1). IAA in combination with BAP (4.4 µM) are recommended as growth regulators for shoot tip cultures of other species of the family of Gentianaceae (Skrzypczak et al. 1993). It was observed, that for *C. erythraea* shoot proliferation the optimum concentration of BAP was also 4.4 µM. Under those conditions as many as 43.3 ± 2.2 shoots were formed from one shoot tip within 4 weeks (Table 1). The number of shoots was not significantly different in the presence of 2.2 or 8.8 µM of BAP. However, when the concentration of BAP was increased to 13.2 µM, the number of shoots per explant (33.8 ± 3.3) was significantly lower. Microscopic observation of the longitudional sections through the formed shoot buds showed that *C. erythraea* shoots were of adventitious rather than axillary origin. Most of shoots (93-96%, dependent on BAP concentration) were derived from the caulogenic tissue, formed at the basal end of explants. No difference in the axillary and adventitious shoot morphology was observed. All developed shoots had small elongated green leaves arranged in a basal rosette. Shoot tips excised from these shoots were repeatedly subcultured on MS medium supplemented with IAA (0.57 µM) and BAP (4.4 µM) at 4-week intervals. It was observed that the number of shoots in repeated subcultures ranged from 20 to 39 shoots per explant (Fig. 1). The comparison of the results presented in Table 1 and Fig. 1 indicates, that subculturing of shoot tips obtained from in vitro cultured shoots on multiplication medium (MS supplemented with 0.57 µM IAA and 4.4 µM BAP) enabled continuous production of shoots, but the number of shoots in subcultures was always lower than that in the primary culture. Similar results were obtained with shoot tip culture of *Catharanthus roseus* (L.) G. Don (Bajaj et al. 1988) and *Arnica montana* L. (Conchou et al. 1992). In contrast, the multiplication rate

TABLE 1. Effect of BAP on multiple shoot formation through shoot tip explants of *Centaurium erythraea* cultured for 4 weeks on MS medium supplemented with IAA 0.57 µM (primary culture).

BAP concentration (µM)	Number of explants	Mean number of shoots/explant* ± SE
2.2	29	37.6 ± 2.6[a]
4.4	36	43.3 ± 2.2[a]
8.8	33	42.4 ± 1.7[a]
13.2	21	33.8 ± 3.3[b]

*Values followed by the same letter are not significantly different from each other at a 5% level by Duncan's multiple range test.

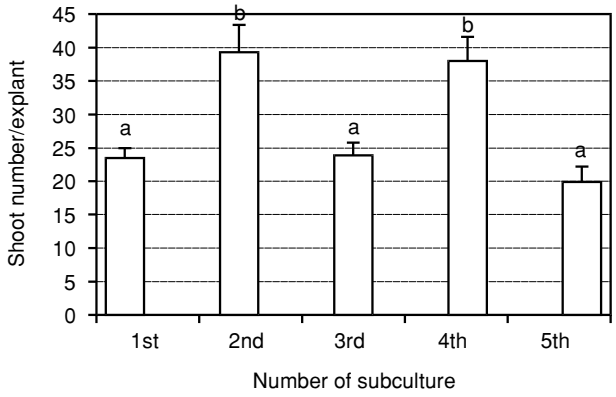

Fig. 1. Effect of subsequent subcultures on shoot multiplication of shoot tip culture of *Centaurium eythraea*. Subcultures were performed every four weeks on MS medium containing IAA (0.57 µM) and BAP (4.4 µM). The presented results are the means of 9-13 explants. Standard errors are shown by bars. Different letters show statistically significant differences at a 5% level by Duncan's multiple range test.

increased in the subcultures of shoots of *Aconitum carmichaeli* Debx. (Hatano et al. 1988) and *Melissa officinalis* L. (Tavares et al. 1996).

We also noticed certain important qualitative differences between the shoots of *C. erythraea* excised from the primary culture and those harvested from older culture. The latter showed often symptoms of hyperhydricity and had translucent, easily breakable leaves. By the end of the fourth passage, about 50% of adventitious shoots were less or more hyperhydrous. This is not surprising as the age of culture is a known factor increasing the hyperhydricity in in vitro raised cultures (Debergh et al. 1992).

Shoot regeneration from hypocotyls, cotyledons, leaves and roots

Several other seedling explants of *C. erythraea*, such as hypocotyl, cotyledon, root and leaf, were also tested to evaluate their morphogenetic potential. The explants were cultured on MS agar medium supplemented with IAA (2.85 µM) and BAP (0.88 µM). The induction of adventitious shoots occurred in all types of explants and was always proceeded by callus stage, although induced calluses differed in their organogenic capacity (Table 2). The best result in terms of percentage of calluses forming shoots (94%) was achieved for the root explants. Also, the highest mean number of shoots (44.4 ± 4.5 per callus) was observed in calluses initiated from root explants (Fig. 2), followed in leaf explants (40.2 ± 6.0), without significant difference between them. On the other hand, significant differences ($p \leq 0.05$) in the number of formed shoots were observed between calluses derived from roots, hypocotyls and cotyledons (Table 2). This is a new result in *C. erythraea*. Previous reports on shoot regeneration of this species

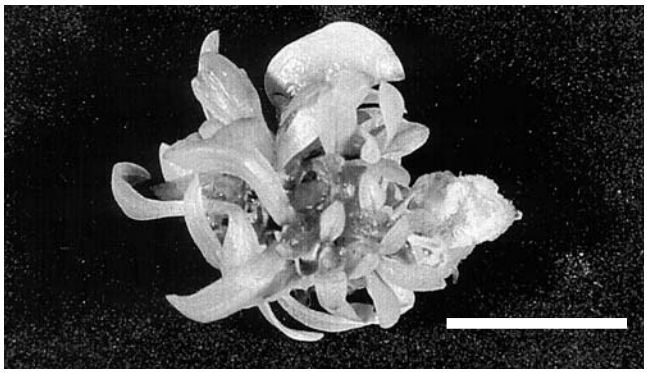

Fig. 2. Adventitious shoots of *Centaurium erythraea* regenerated from root-derived callus after 5 weeks on MS medium supplemented with IAA (2.85 µM) and BAP (0.88 µM). Bar = 1 cm.

showed, that leaf explants gave the best response. Using these explants Vágnerova (1992) reported 15-20 shoots per explant on Linsmayer and Skoog medium containing IAA (1.14-3.42 µM) and kinetin (0.93 µM). The improvement in organogenic response (two- to three-fold higher shoots per explant than those achieved by Vágnerova) observed in our studies was possible by changing the basal medium (MS instead of LS) and/or by BAP presence in the medium. The variations in the efficiency of shoot regeneration in calluses derived from different explants suggest, that selection of explant type may also be important in improving culture system for micropropagation of *C. erythraea*. Similarly, differences in efficiency of shoot regeneration among different explants were reported for many other species, such as *Eucalyptus grandis x E. urophylla* (Cid et al. 1999) and *Helianthus smithii* Heiser. (Laparra et al. 1997). This may be primarly due to the differences of endogenous growth regulator level in the explants.

The callus cultures of *C. erythraea* derived from different explants continued to regenerate new shoots through at least 7 months of incubation on MS medium containing IAA (2.85 µM) and BAP (0.88 µM) with a periodical transfer into a fresh medium every 5 weeks. The experiment indicates that the shoot regenerating ability of the calluses fluctuated in the successive subcultures (Fig. 3). Consequently, after five subcultures, the rate of shoot regeneration of *C. erythraea* remained high only in root-derived and hypocotyl-derived calluses (32 and 25 shoots per callus, respectively) (Fig. 3). It is well known, that the regeneration ability in callus cultures generally decreases and finally disappears during a long term subculture. The reason of lower morphogenetic capacity is unclear. It could be caused by disappearance of a specific factor in the primary explant (Rao et al. 1988) or due to polyploidisation and/or aneuploidisation of cells in cultures (Nehra et al. 1990; Moyne et al. 1993). Furthermore, in vitro culture generally induces a certain degree of rejuvenation (Franclet et al. 1987),

TABLE 2. Shoot formation from calluses derived from different explants of *Centaurium erythraea*. Observations were made after 5 weeks of culture on MS medium in the presence of BAP (0.88 µM) and IAA (2.85 µM).

Explant type	Number of explants	Frequency of callusing (%)	Calluses forming shoots (%)	Mean number of shoots/callus ± SE *
Cotyledon	22	75.0	45.0	13.3 ± 2.6[a]
Hypocotyl	24	81.6	58.0	24.9 ± 3.9[b]
Root	31	88.9	93.5	44.4 ± 4.5[c]
Leaf	28	92.5	71.4	40.2 ± 6.0[bc]

*Values within a column followed by the same letter are not significantly different from each other at a 5% level by Duncan's multiple range test.

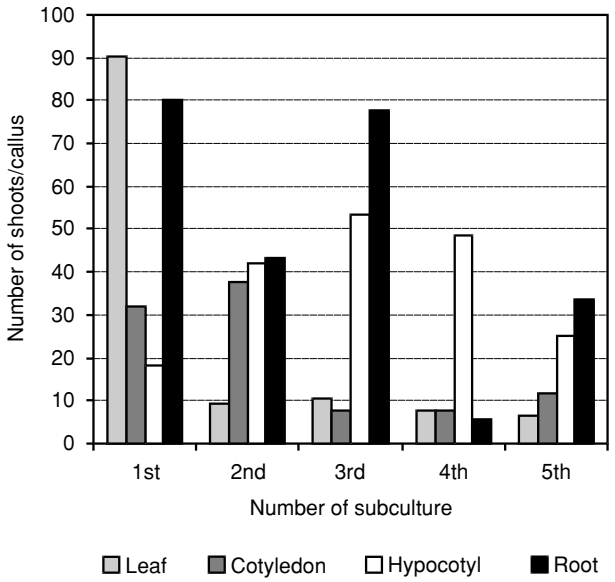

Fig. 3. Effect of subculture on shoot regeneration efficiency of *Centaurium erythraea* callus tissue derived from different explants. Subcultures were performed every 5 weeks on MS medium containing IAA (2.85 µM) and BAP (0.88 µM). The presented results are the means of 8-16 cultures.

which may in turn increase the sensitivity of the formed tissues to exogenous growth regulators. This would explain the differences found in the regenerative capacity of calluses derived from various seedling explants during subculturing. Duration of culture had also its effect on *C. erythraea* shoot appearance. Shoot hyperhydricity started after the first subculture and increased progressively in successive subcultures. After five passages, shoot hyperhydricity was observed in almost all cultures irrespective of their origin. It was also observed that one of the shoots obtained from cotyledon-derived callus possessed an altered size, shape and color of leaves. They were longer (about 6 mm in diameter, whereas normal value is 3 mm), more oval in shape and darker green in color. The shoot was excised from callus, transferred into fresh multiplication medium (MS with IAA 0.57 µM and BAP 4.4 µM) and about 30 new shoots with the above-described leaf morphology were produced within 4 weeks. The shoot stock is now maintained in in vitro culture. When the shoots were cultured on MS medium without growth regulators, 65% of them produced complete rooted plants. The plants growing in soil further differed in morphological characteristic from the rest of plants originating from callus tissues (Fig. 4A and B). Whether these variations have a genetic basis or represent epigenetic phenotypes induced by tissue culture remains to be analyzed. Somaclonal variations can be beneficial if it is heritable for useful traits, for example, for secondary metabolite production (Menendrez-Yuffa and Garcia de 1996). Further experiments are in progress to characterize the phytochemical profile of morphological altered plants of *C. erythraea* and evaluate their potential as a source of herbal raw material.

Shoot rooting and acclimatization of plantlets

The shoots excised from parent culture were transferred into root development medium (hormone-free MS medium). Roots were formed after two weeks, but four weeks were necessary to increase the percentage of rooting and the number of roots (Table 3). In some species, increased rooting competence has been found to be positively correlated with the number of shoot subculture (Patnaik and Debata 1996). In the case of *C. erythraea*, however, there was

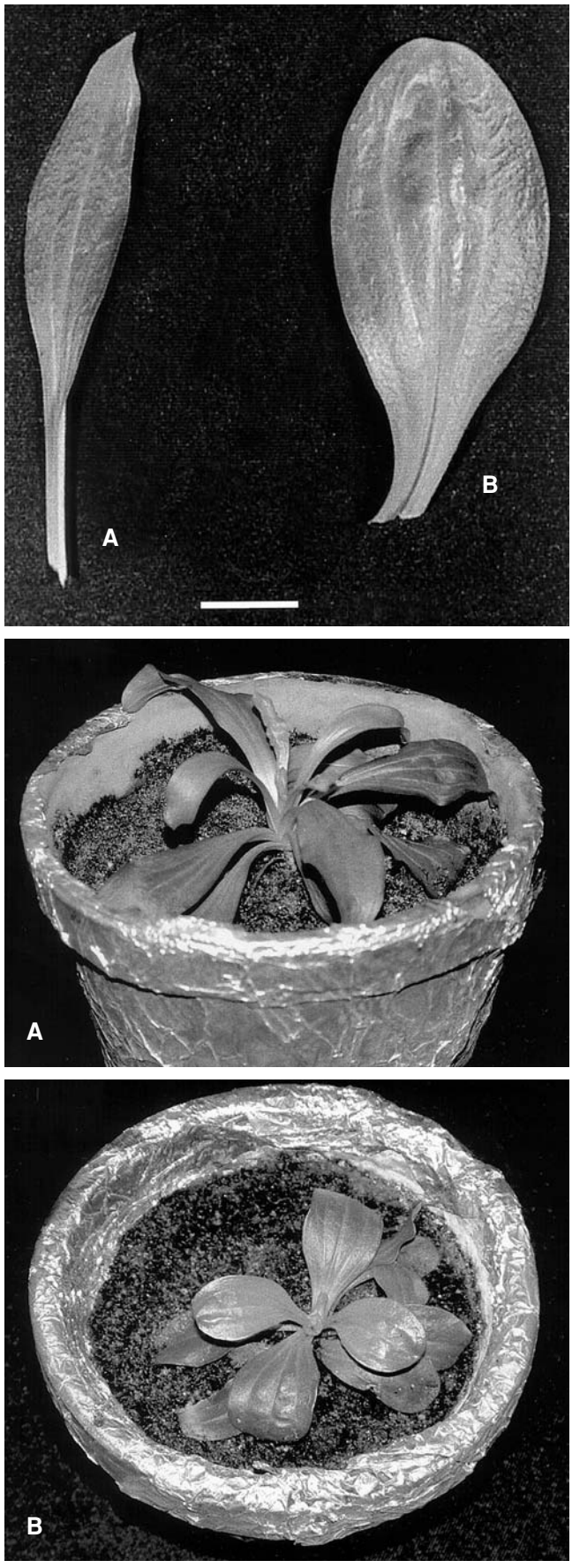

Fig. 4. *Centaurium erythraea* from in vitro after 5 months of growth in a greenhouse: A – regenerant with unaltered morphology; B – regenerant with altered morphology; (above) – leaves of the plants (A and B).

TABLE 3. Rooting response of in vitro regenerated shoots of *Centaurium erythraea* derived from primary culture (A) and the eighth subculture (B) on MS hormone-free medium.

Response	After 2 weeks of culture		After 4 weeks of culture	
	A	B	A	B
% of shoots with roots (number cultured)	66.7 (18)	73.7 (19)	94.4	100.0
Mean number of roots/shoot ± SE	2.6 ± 0.6	2.2 ±0.3	4.8 ± 0.6	2.9 ± 0.3
Mean root length (mm) ± SE	2.7 ± 0.6	4.5 ± 0.3	12.7 ± 0.8	9.6 ± 0.8

no evident difference between shoots excised from primary culture and those from the eighth subculture, and 94% and 100% of the shoots developed an average of 5 and 3 roots per shoot, respectively (Table 3). The values were considerably higher compared to those achieved for morphologically altered shoots. In the case, the rooting frequency, the number of roots per shoot and mean root length were 65%, 2.8 and 4.0 mm, respectively. Owing to the ease with which *C. erythraea* shoots rooted, it was not necessary to use auxins in the medium at this stage. Such highly efficient root formation on auxin-free medium may be due to the availability of higher quantity of endogenous auxin in in vitro raised shoots (Minocha 1987). Vágnerova (1992) has also reported that medium without auxin but with increased concentration of sucrose to 50 g/l (146 µM) was suitable for the induction of roots in *C. erythraea* shoots. In our study high rooting frequency (94-100%) was achieved in the presence of 30 g/l (87.6 µM) of sucrose. This concentration of sucrose was also the best for rooting of *Lavandula viridis* L'Herr shoots in MS-basal medium (Dias et al. 2002). It is worth noting that a reversion of *C. erythraea* shoot hyperhydricity can be achieved, when the shoots were transferred into the hormone-free medium. The shoots rooted normally and following their transfer to the soil, plant hyperhydricity was no longer observed. Also, Bouza (1997) achieved a complete elimination of hyperhydricity in *Prunus tenella* Batsch. shoots by the use of a medium without growth regulators. Koroch et al. (2002) reported similar results for *Echinacea purpurea* L. shoots. Some plantlets of *C. erythraea* (20% of the total plants) formed flower-buds flowered when they were cultured on the MS rooting medium for a prolonged period (12 weeks) (Fig. 5). Consequently, it is possible to obtain in vitro flowering plantlets within several (4-5) months without the necessarily of field cultivation. Rooted shoots (4-week old) of *C. erythraea,* were transferred from the aseptic culture into sterilized mixture of soil, sand and peat (4:3:3 v/v/v). Humidity around the plantlets was controlled at the initial stage (for about 2 weeks). The survival rate of plants assessed after 8 weeks of their transfer to the soil was 97%. It would be important to further test the performance of in vitro propagated plants in the field in comparison to the mother plants.

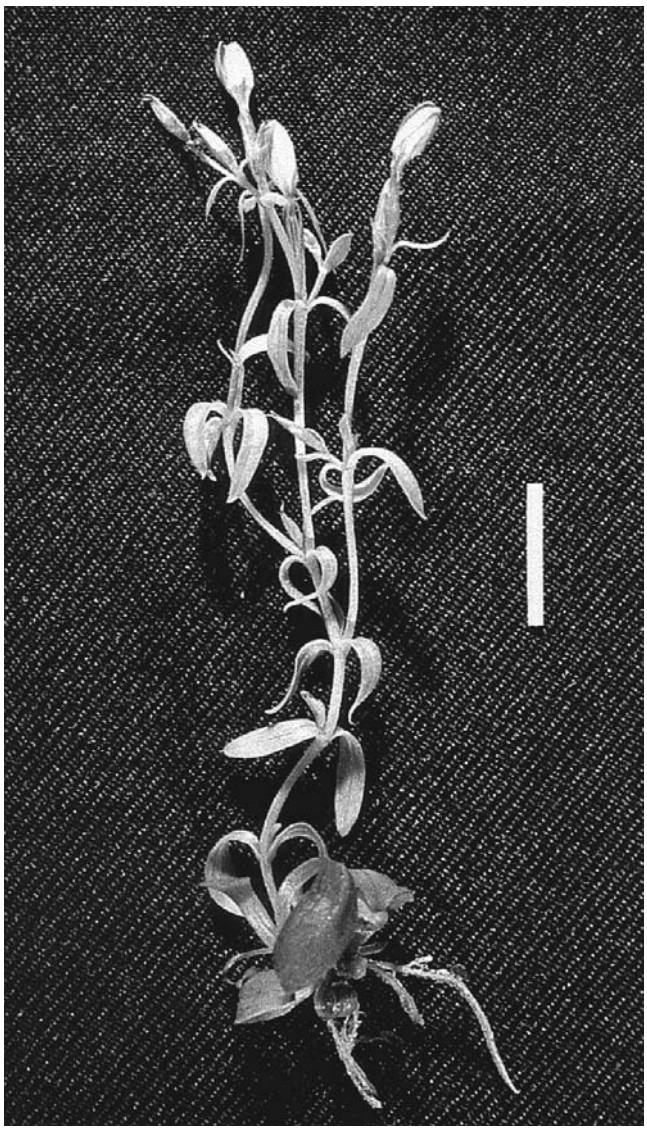

Fig. 5. Flowered plant of *Centaurium erythraea* regenerated in vitro after 12 weeks on MS medium without growth regulators. Bar = 1 cm.

In conclusion, both methods developed in the current work, i.e. multiplication of shoots by shoot tips and shoot production via organogenesis from callus cultures, are suitable for in vitro propagation of *C. erythraea*, providing high regeneration frequencies (even above 90%) associated with a high (up to 45) number of shoots per explant (data for primary culture). Moreover, some shoots (less than ten per explant) were formed in *C. erythraea* directly from leaves or roots placed on MS medium devoid of growth regulators (data not shown). On the basis of our experiments, roots seem to be superior to *C. erythraea* shoot organogenesis. Nevertheless, hypocotyls and leaves can also be effective for adventitious shoot induction. Rooting of regenerated shoots on a hormone-free medium, as well as successful transfer of plantlets into the soil, makes the procedure of *C. erythraea* in vitro propagation simple and highly reproductive. It may be used for the conservation of endangered plant species and to provide material for the production of biologically active compounds, such as secoiridoid glucosides and xanthones. The system via shoot organogenesis developed in this study can be potentially used for selecting mutants with desirable traits.

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REGENERACJA IN VITRO *CENTAURIUM ERYTHRAEA* RAFN Z WIERZCHOŁKÓW PĘDÓW ORAZ INNYCH EKSPLANTATÓW POCHODZĄCYCH Z SIEWEK

STRESZCZENIE

Badano zdolność do regeneracji pędów z wierzchołków pędów i innych eksplantatów pochodzących z 30--dniowych aseptycznie wyhodowanych siewek *Centaurium erythraea* Rafn. Tworzenie pędów z wierzchołków pędów zachodziło głównie z pąków przybyszowych. Największą liczbę pędów na eksplantat (43,3 ± 2,2) otrzymano na podłożu Murashige i Skoog'a (MS) z dodatkiem kwasu indolilo-3-octowego (IAA) (0,57 µM) i 6-benzyloamino-puryny (BAP) (4,4 µM). Regenerację pędów przybyszowych osiągnięto również drogą organogenezy z kalusów pochodzacych z hypokotyli, liścieni, korzeni i liści siewek hodowanych na podłozu MS uzupełnionym IAA (2,85 µM) i BAP (0,88 µM). Istotne różnice w regeneracji pędów zaobserwowano pomiędzy różnymi typami eksplantatów. W kulturze pierwotnej najlepszą odpowiedź uzyskano w przypadku kalusów pochodzących z korzeni i liści (odpowiednio 44,4 ± 4,5 i 40,2 ± 6,0 pędów na kalus). Pędy ukorzeniały się z częstotliwością 94--100% na pożywce MS bez regulatorów wzrostu. Ukorzenione pędy po przesadzeniu do doniczek aklimatyzowały się w 97%, a następnie wykazywały normalny wzrost w warunkach szklarniowych.

SŁOWA KLUCZOWE: *Centaurium erythraea* Rafn, Gentianaceae, mikrorozmnażanie, organogeneza, pędy przybyszowe.