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Full Length Research Paper

The influence of gibberellic acid and paclobutrazol on induction of somatic embryogenesis in wild type and hairy root cultures of *Centaurium erythraea* Gillib.

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The effects of exogenous gibberellic acid (GA_3) and paclobutrazol on induction of somatic embryos in wild type and hairy root culture of *Centaurium erythraea* Gillib. were investigated. Both compounds were incorperated into 1/2 MS medium at 6 concentrations (0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 µM). Wild type root and hairy root explants cultured in the presence of GA_3 at all tested concentrations under 16-h photoperiod or in the darkness decreased the number of somatic embryos that were produced. Paclobutrazol (0.3 µM) induced the largest number (19.7, 16.5) of somatic embryos in wild type and hairy root cultures, respectively. Rooting of plants derived from somatic embryos as achieved on ½MS medium. These results indicate that paclobutrazol is beneficial for somatic embryo induction and formation in wild type and hairy root culture.

Key words: Hairy root, medicinal plants, root explants, somatic embryos.

INTRODUCTION

Centaurium erythraea Gillib. (Gentinaceae), commonly know as Centaury, is an important medicinal plant found in dry pastures and on chalky cliffs throughout the Europe, mostly in the Mediterranean. In herbal medicine, the aerial parts of *C. erythraea* are used as a tincture, infusion tonic, lotion, and tea for the treatment of several health problems. These medicinal properties are due to the presence of a secoiridoides and xantones (van der Sluis, 1985). Several factors, such as restricted distribution, small population in accessible areas and anthropogenic pressures on these populations, have contributed to the decline of *C. erythraea* in nature. Plant tissue culture is a well-known biotechnological tool that has proven to be effective for *in vitro* plant propagation of medicinal plants and commercial exploitation of valuable plant derived secondary metabolites (Ramachandra and Ravishankar, 2002). Somatic embryogenesis has been the common pathway for the large-scale production of important medicinal plants. *In vitro* regeneration in *Centaurium* species has been accomplished mainly through organogenesis (Laureová et al., 1986; Janković et al., 1997) and rarely via somatic embryogenesis (Barešova and Kaminek, 1984). In general, both morphogenetic pathways have been achieved using different explants (Barešova and Herben, 1985). Root cultures are generally suitable systems from the study and production of secondary metabolites (Kim et al., 2002; Sudha et al., 2003). However, they can also be used as model systems in the research focused on the effect of various substances on morphogenesis in root culture, e.g. plant growth regulators (Bálványos et al., 2001). Plants regenerated from the root explants are suggested to be genetically uniform (Sharma et al., 1993), emphasized that root culture could be used for germplasm preservation of many medicinal plants, including *C. erythraea*. The effects of some plant growth regulators, nutrient medium components and different light treatments on direct somatic embryogeneis and organogenesis have been investigated in detail in *C. erythraea* wild type and hairy root

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cultures (Subotić et al., 2006). Also, histological evidence of somatic embryogenesis formation from wild type root explants has been reported (Subotić et al., 2007). However, complete understanding of other plant growth regulators, especially gibberellins, in the regulation of different morphogenic pathways *in vitro* is lacking. The effect of exogenously applied gibberellins on *in vitro* morphogenesis is highly variable among species or tissues. Exogenously applied gibberellins exert a highly positive influence on somatic embryogenesis in cultures *in vitro* of *Iris germanica* L (Shimizu et al., 1997) and *Medicago sativa* L. (Ruduś et al., 2000). However, they exhibit an inhibittory effect on somatic embryogenesis in cultures of *Daucus carota* L. (Tokuji and Kuriyama, 2003), *Pelargonium x hortorum* Bailey (Hutchinson et al., 1997) and *Oncidium* (Chen and Chang 2003).

Exogenously applied plant growth regulators can interact with endogenous hormones in cultured explants involved in the determination of tissue specific embryogenic or organogenic potential (Jiménez et al., 2005). The involvement of endogenous GA_3 in the process of somatic embryogenesis varies among species. For example, embryogenic maize lines contained an elevated level of GA_3 (Jiménez and Bangerth, 2001), while in conifer the inhibition of GA_3 synthesis promoted somatic embryogenesis (Pullman et al., 2005).

A possible relationship between the ability of cultured tissues for somatic embryiogenesis induction and GA_3 has been supported by studies on some embryogenesis-related genes. Expression of AGL15 gene enhances the capacity for somatic embryogenesis (Harding et al., 2003). PICKLE (*pkl*), encodes a chromatin remodeling factor which represses embryogenic capacity (Ogas et al. 1997) and plays a role in gibberellin-dependet responses (Henderson et al., 2004). Plant growth retardants (especially inhibitors of gibberellins biosynthesis) are known to reduce stem elongation, resulting in reduced internode lengths of plants without changing their developmental patterns. These compounds are well known to inhibit growth in some tissue by reducing cell expansion and lowering the rate of cell division, caused by blocking gibberellins activity (Smith et al., 1990). Concerning GA_3, there are contrasting reports about its involvement in somatic embryogenesis, based on results from experiments employing inhibitors of their biosynthesis. The inclusion of plant growth retardant such as paclobutrazol increased SE development in cultures of geranium (Hutchinson et al., 1997) and *Oncidium* (Chen and Chang 2003). Rajaseka ran et al. (1987) observed a neutral effect, since neither paclobutrazol nor the reduced levels of GAs, which may have resulted from its application, altered the rate of embryogenesis of *P. purpureum*. A negative effect was reported by (Mitsuhashi et al., 2003), who found that uniconazole, another inhibitor of GA biosynthesis, induced shrunken embryos when applied during the development of somatic embryos in carrot. Similarly, the use of paclobutrazol in alfalfa significantly decreased the number of somatic embryos formed (Rudus´ et al., 2002). Another effect of uniconazole is the afore mentioned promotion of secondary somatic embryos in carrot (Tokuji and Kuriyama, 2003). Finally, (Pullman et al., 2005) recently found an improvement in the initiation of somatic embryogenesis in several conifers using paclobutrazol. In this report, we used *C. erythraea* wild type and hairy root cultures to examine the effect of GA_3 and plant growth retardants, paclobutrazol, on somatic embryogenesis.

MATERIALS AND METHODS

The seeds of *C. erythraea* used in this study were collected in their natural habitat. Seeds were washed with local liquid detergent and rinsed three times under running water. They were then surface sterilized with 30% (v/v) commercial bleach (Varikina Pompa, Biohemija Imneh, Serbia) for 10 min then rinsed in sterile distilled water three times. Disinfected seeds were then transferred on to a filter paper placed in Petri dishes (55 × 15 mm) with 2 ml of sterile distilled water for germination. Roots were excised from three-week-old seedlings and cut into 15 mm long pieces. Wild type root cultures were established from excised root tips of sterile seedlings *in vitro* on ½MS medium containing half-strength macronutrients, full-strength micronutrients and vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.7% (w/v) agar and 100 mg l^{-1} myo-inositol. Transformed hairy root cultures of *C. erythreaea* were initiated by inoculating two days old explants with *Agrobacterium rhizognes* (strain A4M70GUS) according to procedures described previously (Subotić et al., 2004). Only one clone, with the highest grow rate were selected for the next study. Both wild type and hairy root cultures were subcultured every 30 days by excising 15 mm apical tips and transferring them to fresh medium.

GA_3 and paclobutrazol were compared at 6 concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 µM). GA_3 solution were filter-sterilized (0.22 µm) and then added to ½ MS autoclaved culture medium after cooling to 55°C. Paclobutrazol solution was introduced into ½ MS medium before autoclaving. The pH was adjusted to 5.8 prior to autoclaving (114º C, 20 min). Ten root explants were plated per Petri dish containing 20 ml of a gelled medium. Cultures were grown in a growth chamber under 16-h photoperiod, with photosynthetic photon flux density 50 µmol m^{-2} s^{-1} (cool white fluorescent Tesla tubes, Pančevo, Serbia, 60 W) and in darkness, all at 25 ± 2°C. Three independent experiments were performed to evaluate the effects of on GA_3 and paclobutrazol on induction of *in vitro* morphogenesis. At the end of each subculturing period, the average numbers of adventitious shoots were recorded. Each treatment consisted of 5 replications (Petri dishes containing 15 ml medium and 10 explants). The results of all experiments are presented as mean values with standard deviation. Statistical analyses were performed using StatGrafics software version 4.2 (STSC *Inc*., Rockville, Maryland, USA). Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P \leq 0.05$.

RESULTS

Wild type root cultures were successfully established on ½MS medium. This root culture had very low growth rate. Hairy root cultures established after two successive subcultures grown using procedures described previously

(Subotić et al., 2004) had high growth rate increased high lateral branching and lack geotropism.

During the first week on the all tested media the explants from wild type root culture enlarged. The earliest sign of somatic embryo formation was visible in the second week of culture. This developmental change was not observed in hairy root culture on medium without plant growth regulators. The absence of somatic embryos induction in medium without plant growth regulators was not surprising, as it has been previously reported in many hairy root cultures. In same species, hairy roots cultured under light and/or in the darkness formed somatic embryos on phytohormone-free medium. Somatic embryos appeared spontaneously on explants surface from wild type root culture without callus production. Small, somewhat bipolar embryo-like structures appeared directly on the wild type root explants grown in the treatments incubated under 16-h photoperiod after 10 days (Figure 1a). The embryos that developed on root explants cultured on ½MS medium supplemented with paclobutrazol (0.3 µM) became distinct bipolar structures producing the first leaf after 20 days (Figure 1b). The pattern of development was independent of the tissue in which the embryogenic structures had formed, and the process was asynchronous: somatic embryos at different stages of development coexisted on the same explant. Under the influence of the applied culture conditions, the embryos could not continue their normal development but progressively changed into shoot-like structures at an cotiledonary developmental stage (Figure 1c). From each developing somatic embryos a single shoot elongated within 20-25 days. These shoots attained a height of 2-3 cm in 30 days bearing between 4 to 9 leaves. Paclobutrazol was found more effective than GA_3 as seen by means number of somatic embryos formation after 30 days in culture (Figure 1d). The plantlets were of morphologically similar to plants regenerated from control root culture. Somatic embryos formed on media with any tested concentrations of GA_3 were etiolated, and most of them remained 5 cm in size during the end of subculture (Figure 1e). Isolated somatic embryos failed to root on ½MS medium without the supplementation of any growth regulators. In this culture condition about 99% of isolated somatic embryos developed roots within 12-15 days of culture (Figure 1f). The regenerated plants did not show any detectable difference in morphological or growth characteristics when compared with plants from natural populations. Light and dark conditions as well as concentrations of GA_3 and paclobutrazol were strong determining factors for the induction of somatic embryogenesis from the wild type and hairy root explants of *C. erythraea.* In wild type root cultures, GA_3 was found to have an inhibitory effect on the development of somatic embryos. Maximum inhibition, to about of the control was observed at 3.0 µM concentration in wild type root culture under 16-h photoperiod. Similarly in dark condition on all tested concentrations of GA_3 the average number of somatic embryos were drastically reduced (Table 1). In hairy root cultures, of the various levels of GA_3 tested, 0.3 µM proved to be effective, as on this medium maximum number 1.31 somatic embryos were developed per explants (Table 1). The strong stimulatory effect of paclobutrazol added to the ½MS medium and somatic embryos formation was visible over all concentrations tested (0.01-3.0 µM) in normal root culture. The applications of paclobutrazol at 0.3 µM during differentiation appeared to be the most effective treatment in enhancing somatic embryos production to about seven times more than in control (Table 2). However, regeneration of somatic embryos in hairy root cultures was significantly lower than in normal root cultures (Table 2).

DISCUSSION

The data presented above demonstrated clearly that *in vitro* morphogenesis from *C. erythraea* normal and hairy root culture was promoted by paclobutrazol, but retarded by GA_3. These results are in congruence with those obtained in studies of the induction of somatic embryogenesis in leaf segment culture of *sp. Oncidium* Chen and Chang (2003) where GA_3 in 0.1 - 1.0 mg l^{-1} concentration inhibited significantly the process of direct somatic embryogenesis. Some observations support the premise that GA_3 added exogenously exert part of its effect by modifying the concentrations of endogenous plant growth regulators. Hutchinson et al. (1997) reported an increase in the endogenous level of IAA during the induction of somatic embryogenesis. The level of endogenous auxins in that stage is a critical factor for further and normal development of somatic embryos. In *Arabidopsis*, somatic embryos are observed in the root of pkl mutants and exogenous application of GA_3 reduces the formation of the somatic embryos it is enhanced by the addition of uniconasole (Ogas et al., 1997). These results indicated that GA_3 keeps the epidermal cell in somatic state, and inhibits the expression capacity in root explants of *Arabidopsis*. Paclobutrazol is a thiazol that inhibits the conversion of ent-kaurene into ent-kaurenic acid, thus reducing the level of gibberelins in plant tissue (Radermacher et al., 1987). The enhancement of somatic embryogenesis by inhibitors of GA_3 synthesis has also been observed in *Echinochloa* (Sankhala et al., 1992), asparagus (Li and Wolyn 1995), *Pelargonium x hortorum* Bailey (Hutchinson et al., 1997) and *Oncidium* (Chen and Chang 2003). In this study the application of paclobutrazol, an inhibitor of gibberelic acid synthesis, had a stimulating effect on the processes of *in vitro* morphogenesis in *C. erythreae*. These results show that the absence or endogenous reduction of GA_3 enables the redifferentiation of root cells into embryogenic cells, like as in root of pkl mutant. To our knowledge, this is the first report of any effect of exogenously applied GA_3 and paclobutrazol on the induction of somatic embryos in wild type and hairy root culture of *C. erythreae*.

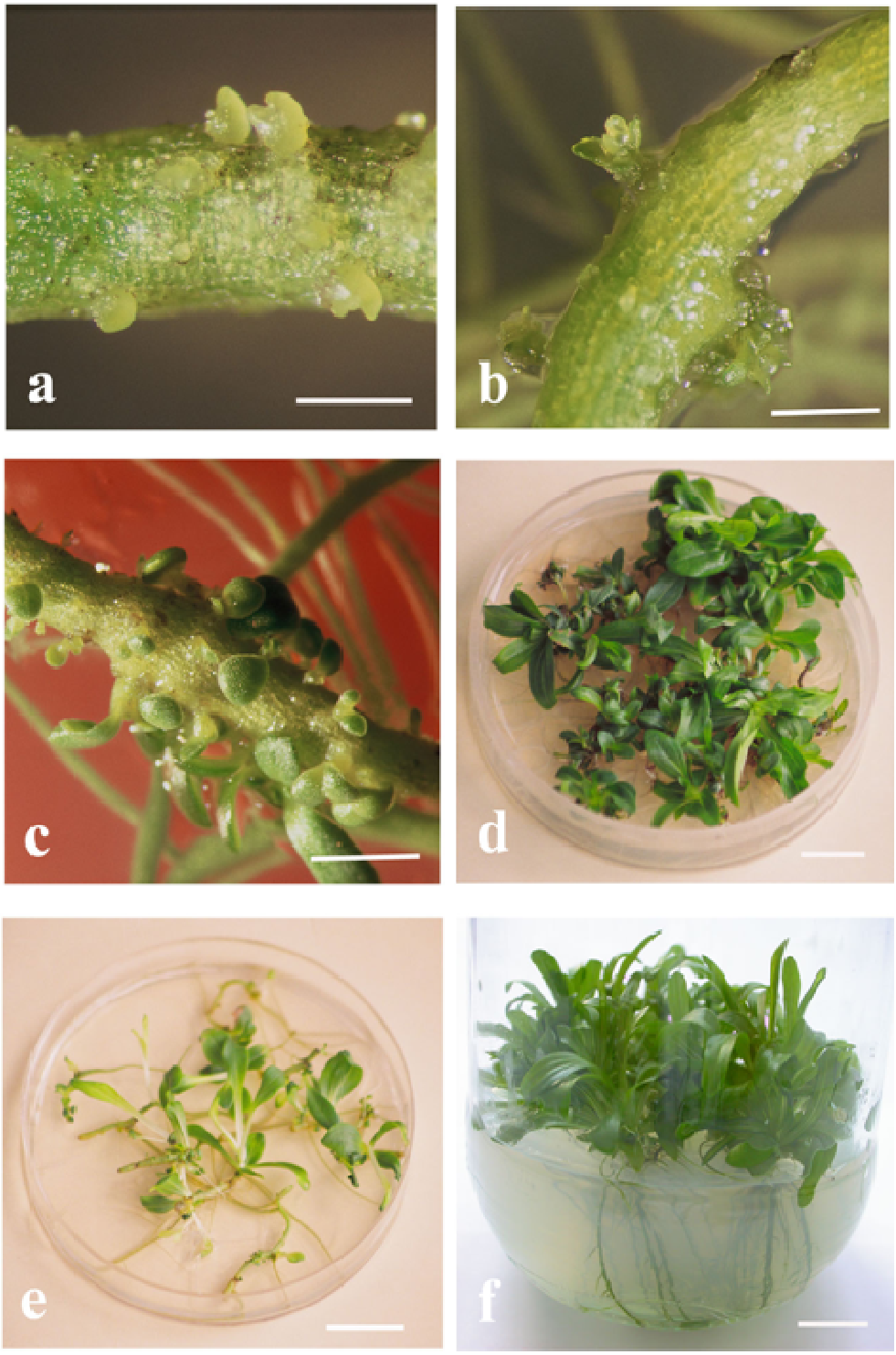

Figure 1. Plant regeneration throught somatic embryogenesis in wild type root culture of *C. erythraea*. *(a)* Somatic embryos directly regenerated from the wild type root explant on the ½MS medium supplemented with paclobutrazol (0.3 µM), after 10 days in culture. *Bar* = 2 mm. *(b)* Somatic embryos directly regenerated from the wild type root explant on the ½MS medium supplemented with paclobutrazol (0.3 µM), after 15 days in culture. *Bar* = 2 mm. *(c)* Detail of wild type root explant with well developed somatic embryos. *(d)* Root culture with well developed somatic embryos grown on ½MS medium with paclobutrazol (0.3 µM) after 30 days in culture. *Bar* = 2 mm. *(e)* Root culture three weeks after culturing on ½MS medium supplemented with (0.1 µM) GA_3. *Bar* = 4 mm. *(f) In vitro* rooting of regenerated plantlets on the ½MS medium. *Bar* = 4 mm.

Table 1. Influence of GA_3 on somatic embryos formation from wild type and hairy root cultures of *C. erythraea* after 3 weeks of culture in 16-h photoperiod or in the dark.

GA_3 (µM)	Number of somatic embryos (mean ± S.E.)			
	In wild type root cultures		In hairy root cultures	
	Light	Darkness	Light	Darkness
-	3.08 ± 0.45 [a]	0.12 ± 0.03 [d]	-	-
0.01	0.13 ± 0.03 [c]	0.30 ± 0.01 [bc]	0.23 ± 0.09 [d]	0.12 ± 0.04 [c]
0.03	0.47 ± 0.05 [ab]	0.50 ± 0.02 [c]	1.76 ± 0.11 [ab]	0.14 ± 0.06 [bc]
0.1	0.49 ± 0.07 [ab]	0.61 ± 0.03 [b]	2.11 ± 0.18 [a]	0.17 ± 0.03 [b]
0.3	1.31 ± 0.08 [b]	0.77 ± 0.08 [ab]	1.5 ± 0.12 [ab]	0.19 ± 0.07 [b]
1.0	0.51 ± 0.04 [ab]	0.40 ± 0.03 [bc]	1.49 ± 0.09 [ab]	0.35 ± 0.13 [a]
3.0	0.43 ± 0.07 [ab]	0.90 ± 0.09 [a]	0.51 ± 0.05 [c]	0.18 ± 0.08 [b]

Each treatment had 50 replications and was repeated thrice.
Means followed by same letters are not significantly different at $p \leq 0.05$ accroding to LSD test.

Table 2. Influence of paclobutrazol on somatic embryos formation from wild type and hairy root cultures of *C. erythraea*. After 3 weeks of culture in 16-h photoperiod or in the darkness.

Paclobutrazol (µM)	Number of somatic embryos (mean ± S.E.)			
	In wild type root cultures		In hairy root cultures	
	Light	Darkness	Light	Darkness
-	3.08 ± 0.45 [d]	0.12 ± 0.03 [e]	-	-
0.01	5.23 ± 0.23 [c]	1.98 ± 0.14 [d]	2.33 ± 0.12 [e]	1.98 ± 0.14 [d]
0.03	6.8 ± 0.14 [c]	5.78 ± 0.23 [c]	4.50 ± 0.25 [d]	5.78 ± 0.23 [c]
0.1	11.2 ± 0.56 [ab]	9.87 ± 0.34 [b]	9.87 ± 0.56 [c]	6.77 ± 0.34 [c]
0.3	19.7 ± 0.67 [ab]	16.78 ± 0.56 [a]	16.55 ± 0.67 [ab]	13.45 ± 0.56 [a]
1.0	10.89 ± 0.78 [ab]	8.98 ± 0.98 [b]	17.88 ± 0.78 [a]	11.78 ± 0.98 [ab]
3.0	8.99 ± 0.45 [b]	6.34 ± 0.89 [c]	11.22 ± 0.45 [b]	8.99 ± 0.89 [b]

Each treatment had 50 replications and was repeated thrice.
Means followed by same letters are not significantly different at $p \leq 0.05$ accroding to LSD test.

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