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Callus culture and micropropagation of *Eustoma grandiflorum* Shinn.

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Abstract

Regenerated plants and callus tissue were obtained by embryo culture and micropropagation of explants from shoots and leaves of *Eustoma grandiflorum* Shinn. (*Gentianaceae*), a medicinal plant. This species, which does not naturally occur in this country, but is found in the southwestern regions of North America, can be propagated in vitro in a relatively short time. Regenerated plants were obtained in amounts enabling extensive phytochemical analyses to be conducted.

Key words: in vitro, callus culture, micropropagation, Eustoma, Gentiancaceae

INTRODUCTION

It seemed purposeful to initiate studies on the in vitro culture and micropropagation of species from the family *Gentianaceae* since in tissue culture they synthesize pharmaceutically active substances (Wesołowska et. al. 1985). In addition to continuation of our work on the genus *Gentiana* L., we began attempts to culture in vitro a species from *Gentianaceae*, alien to our domestic flora, *Eustoma grandiflorum* Shinn. = *E. russellianum* Griseb. = *Lisianthus russellianus* Hook. (Shinners 1957, Zenkteler and Zenkteler 1987). This species occurs in the southwestern regions of North America. As a decorative plant it was introduced into production in the United States of America, then in Japan. Due to its large and colorfull flowers, this plant evoked the wide interest of producers as cut or potted flowers (Halevy and Kofranek 1984, Roh and Lawson 1984, Krause 1986, Hetman

Abbreviations: GA_3 – gibberellic acid; kin. – kinetin; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – β-indolyl-3-acetic acid; BAP – benzylaminopurine

1986). The chemical composition of this plant was also studied. Secoiridoid glucosides (Uesato et. al. 1979) were found in its above-ground parts while flavonoids were isolated from its flower petals (Asen et. al. 1986).

The aim of the first stage of our studies, begun in the spring of 1986, was to obtain regenerated *E. grandiflorum* plants by in vitro culture in such amounts as to enable chemical analyses to be conducted.

MATERIAL AND METHODS

Callus cultures. *Eustoma grandiflorum* Shinn. seeds were kept for 14 days at 4°C. Next, they were transferred to Petri dishes, covered with distilled water and left to imbibe for 48 hrs at a temperature of about 25°C. The seedlings and seeds were sterilized first with 70% ethanol, then with 0.1% sublimate and washed several times with sterile distilled water. The material prepared in this way was transferred to growth medium.

For the initiation of callus cultures, three variants of the Murashige and Skoog (1962, MS) and B_5 (Gamborg et al. 1968) mediums were used. They were chosen on the basis of our own comparative studies done previously. The mediums contained GA_3 (0.2; 0.5 and 1.0 mg dm^{-3}) and kin (0.1; 0.25 and 0.5 mg dm^{-3}).

The MS medium supplemented with IAA (0.5 mg dm^{-3}) was used for rooting. For transferring undifferentiating callus tissue, the MS medium containing kin (1.0 mg dm^{-3}) and 2,4-D (0.25 or 0.5 mg dm^{-3}) or just 2,4-D was used.

Micropropagation. The following explants from the sterile plants obtained earlier from differentiating callus were used to initiate the propagation of *E. grandiflorum* in vitro: shoot apices (40-60 mm long), shoot fragments with a nodule (0.5-1 cm long) and fragments of or whole leaves (about 1 cm^2 in area). The explants were placed on a modified MS medium containing (in mg dm^{-3}): NaH_2PO_4 H_2O – 170, adenine sulphate – 80, inositol – 100, thiamine – 0.4, agar – 8000, sucrose – 30 000 (Zenkteler 1984). The medium was enriched with the following growth substances (in mg dm^{-3}): zeatin – 0.5 (medium A), IAA – 2.0, kin. – 2.0 (medium B) and BAP – 1.0 (medium C).

The cultures were placed in a culture room at a temperature of 25°C, relative humidity 60-70% and constant fluorescent illumination at 2000 lx.

At the same time *E. grandiflorum* was cultivated from seeds.

RESULTS AND DISCUSSION

In vitro cultures of *Eustoma grandiflorum* Shinn. were initiated from embryos and seedlings obtained from seeds. Satisfactory results were obtained using modifications of the MS medium. After three weeks on the GA_3 and

PLATE I

Callus cultures of *Eustoma grandiflorum* Shinn.

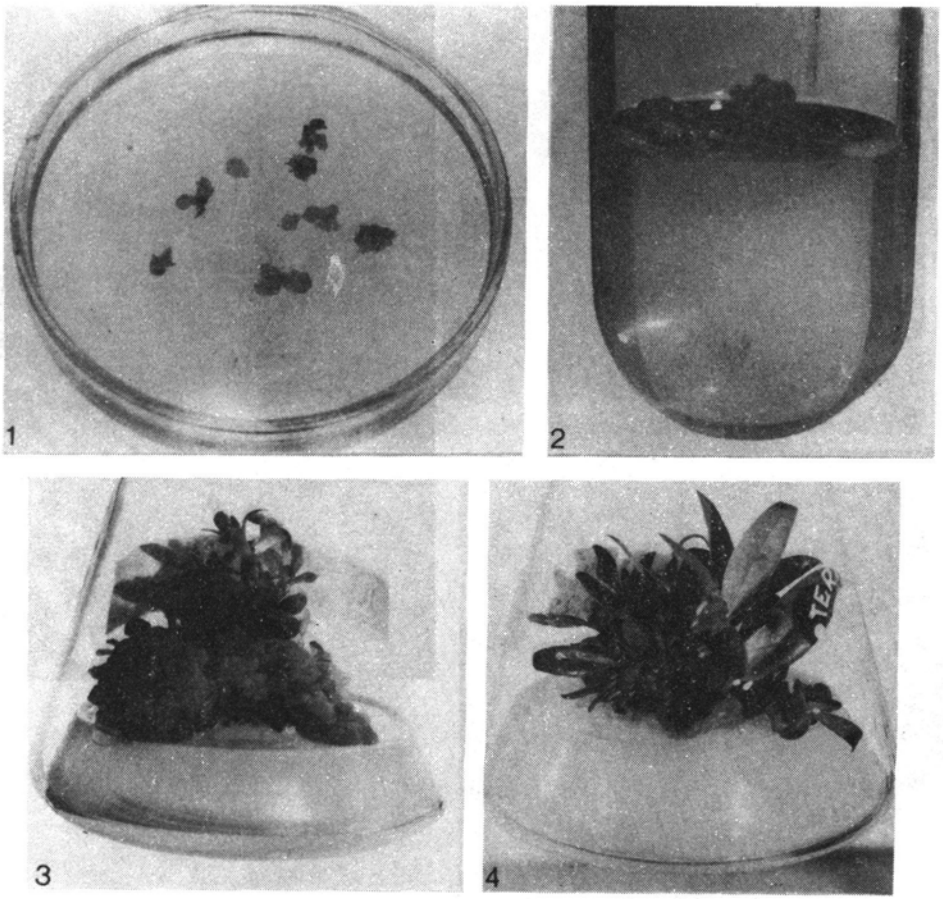
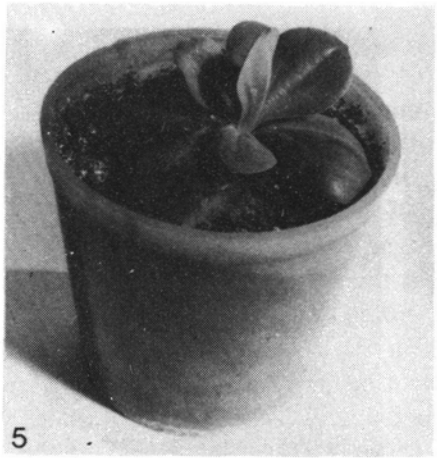
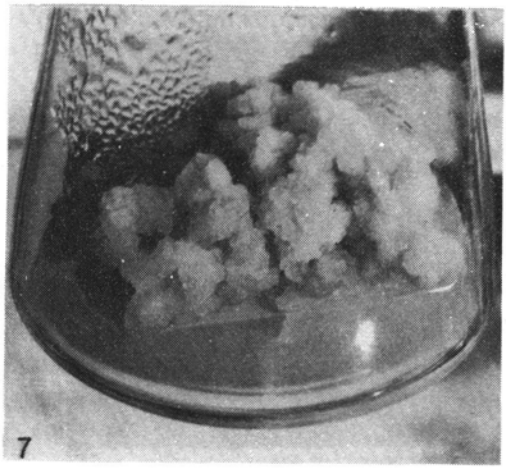


Fig. 1. Callus-forming embryos. Fig. 2. Transferred callus tissue, × 2.6. Fig. 3. Embryo-derived callus regenerating buds, × 1.1. Fig. 4. Developing shoots

PLATE II





Figs. 5 and 6. *E. grandiflorum* plants regenerated from callus tissue × 1.2 and 7, respectively. Fig. 7. An undifferentiated line after several transfers of embryo-derived callus. × 1.2

PLATE III

Micropropagation of *Eustoma grandiflorum* Shinn.

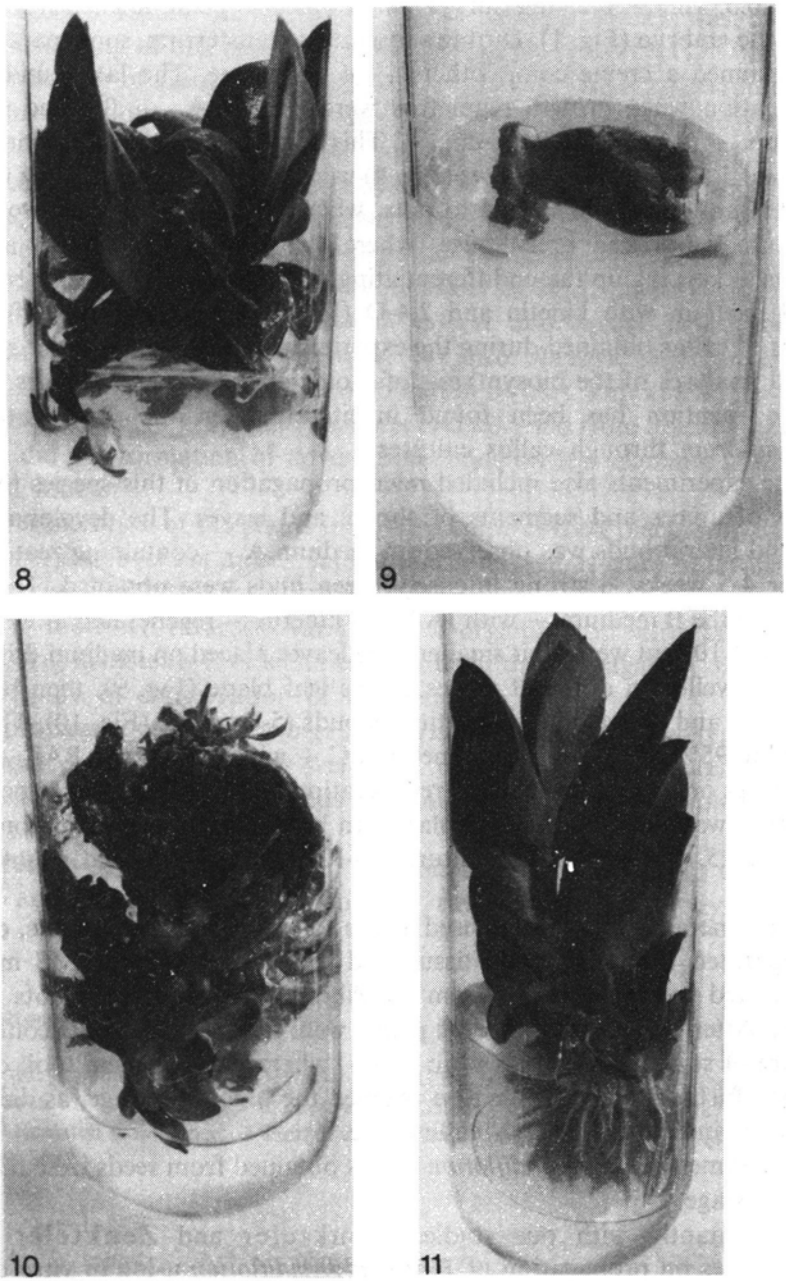


Fig. 8. Apical and lateral buds developed from a shoot apex, × 2.3. Fig. 9. A leaf blade with visible swelling after the third week of culture, × 2.7. Fig. 10. Numerous adventitious buds regenerating on a leaf after the sixth week of culture, × 2.3. Fig. 11. A rooted, regenerated shoot, × 2

kinetin-containing MS medium (GA_3 – 1.0, kin. – 0.5 mg dm^{-3}) the embryos grew and formed callus from the hypocotyl. During this same time, (GA_3 – 0.5, kin. – 0.25 mg dm^{-3}) callus was also being formed from other parts of the embryo (Fig. 1). During subsequent transferring, some parts of the callus assumed a creme color, others – a green hue. The latter underwent differentiation when growth regulators were used (GA_3 – 0.2 and 0.5 mg dm^{-3}; kin. – 0.1 and 0.25 mg dm^{-3}). This stage of the culture is shown on Figs. 2 and 3. The obtained shoots (Fig. 4) were rooted (IAA – 0.5 mg dm^{-3}), then the plants were transferred to pots, where their growth was observed in the culture room and greenhouse, where they bloomed (Figs. 5 and 6). Attempts at keeping up the undifferentiating callus of *E. grandiflorum* by using the MS medium with kinetin and 2,4-D (Fig. 7) were also successful. The amounts of callus obtained during the experiment were large enough to permit chemical analyses of the biosynthesis of biologically active substances. Up to now, no mention has been found in literature on the regeneration of *E. grandiflorum* through callus cultures.

These experiments also included micropropagation of this species through explants of apices and segments of shoots and leaves. The development of apical and lateral buds was observed on medium A – containing zeatin (Fig. 8). After 4-5 weeks, 6 strong intensely green buds were obtained. The same explants on the B medium – with IAA and kinetin – regenerated more lateral buds (about 10) but were a bit smaller. The leaves placed on medium B initially (3 weeks) swelled in different places of the leaf blade (Fig. 9), then burst in those places and regenerated adventitious buds (5-6 weeks) (Fig. 10). This was observed in 95% of the leaves. On medium C – which contained BAP – from whole leaves or fragments of them, regeneration of adventitious buds occurred in a similar way directly on the explants. In both cases the propagation index was about 25, but for further culture, 10-12 large, well-formed shoots were chosen.

The regenerated shoots were used as the source of further explants, or they were separated from the initial tissue and transferred to basic MS medium supplemented with IAA (0.5 mg dm^{-3}). About 80-90% of the shoots rooted (Fig. 11). After 3-4 weeks the rooted plants were transferred to pots containing a mixture of soil and sand at a ratio of 1:1, where most of them took on and developed further. These plants also reached the blooming stage, as did those regenerated indirectly through callus (Fig. 6).

At the same time, *E. grandiflorum* plants obtained from seeds were reaching blooming stage.

Concomitantly with our studies, Zenkteler and Zenkteler (1987) started studies on propagation of *Eustoma grandiflorum* using in vitro culture methods. They achieved development of adventitious buds after seven weeks of culture on MS medium with BAP and IAA and an 18 hr photoperiod. Organogenesis in the explants was of a direct nature and occurred in the

subepidermal layer of leaf parenchyma. After three months, approximately 15-20 shoots were obtained from each explant.

The second report on micropropagation of this species was published during the editing of this paper. Semeniuk and Griesbach (1987) obtained the regeneration of *Eustoma grandiflorum* buds from shoot apices, internodal fragments of shoots and leaf segments using MS medium enriched with BAP or BAP and NAA. Depending on the type of explant and medium composition, an average of 4.5-14.5 new shoots was obtained. These are the only available data on the in vitro culture of *Eustoma grandiflorum*.

Our experiments on micropropagation of *Eustoma grandiflorum* from different explants confirm the favorable effect of BAP on the formation of adventitious buds and stimulation of lateral and apical buds. We used somewhat different culture conditions, but also obtained high propagation indexes. The use of zeatin and the combination of IAA with kinetin gave good results on attempts to propagate *Eustoma grandiflorum* from apical and lateral buds, as did the formation of adventitious buds on leaf explants induced by IAA and kinetin.

The regeneration of the plants in in vitro cultures took place from callus or existing meristems (shoot apices, lateral buds), as well as from other tissues in which the process of somatic organogenesis took place.

Due to the often observed variability of organisms in vitro, regeneration from existing meristems is of significant value in the propagation of medicinal plants because of conservation of the genotype.

The propagation of *Eustoma grandiflorum* Shinn. in vitro may find wider applications, in horticulture as well, due to the difficulties encountered in propagating this species from seeds and in vegetative propagation (Krause 1986).

The results of our study make it possible to quickly obtain in vitro callus cultures and *E. grandiflorum* plants as raw material for phytochemical analyses.

Acknowledgement

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Kultura kalusa i mikrorozmnażanie Eustoma grandiflorum Shinn.

Streszczenie

Z kultur zarodków i metodą mikrorozmnażania z eksplantatów pędów i liści otrzymano zregenerowane rośliny i tkanki kalusowe *Eustoma grandiflorum* Shinn. (*Gentianaceae*), rośliny leczniczej. Gatunek ten, obcy florze rodzimej, występujący na terenach południowo-zachodniej Ameryki Północnej, można rozmnażać w kulturze in vitro w stosunkowo krótkim czasie. Zregenerowane rośliny pozyskano w takiej masie, która pozwoli przeprowadzić wyczerpujące badania fitochemiczne.