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Protocol for efficient micropropagation of spring gentian and sand jurinea

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ABSTRACT: Protocols for the *in vitro* propagation of two endangered plant species in the Czech Republic, *Gentiana verna* L. and *Jurinea cyanoides* (L.) Rchb., were established. In *G. verna*, the induction of organogenesis on vegetative shoots was successful on the basal 6% agar WPM medium with 200 mg·l^{-1} of glutamine, 200 mg·l^{-1} of casein hydrolysate, 30 g·l^{-1} of sucrose, 0.2 mg·l^{-1} of BAP, and 0.1 mg·l^{-1} of IBA. The multiplication of primary explants was achieved on 6% agar basal MS medium with the same concentrations of substances mentioned above. In *J. cyanoides*, the induction of organogenesis and multiplication of its vegetative shoots were successful on MS medium with the same concentration of added substances used in *G. verna*. On the basal MS medium of 1/3 concentration with 3 mg·l^{-1} of IBA 70–75% rooting efficiency of *G. verna* microcuttings and 40% rooting efficiency of *J. cyanoides* microcuttings were reached. The mortality during acclimatization did not exceed 20% for *G. verna* and 25% for *J. cyanoides*.

Keywords: *Gentiana verna*; *Jurinea cyanoides; in vitro* reproduction; organogenesis

Gentiana verna L. (spring gentian) and *Jurinea cyanoides* (L.) Rchb. (sand jurinea) are listed as critically endangered species in the Czech Republic. Their current occurrence is fragmentary compared to the historically documented one.

G. verna is a perennial plant growing on sunny alpine meadows and moorlands throughout Eurasia. At present, in the Czech Republic it occurs only in a locality of the Rovná Natural Preserve Park in the South Bohemian region. Nowadays, the number of gentian plants is so low that their natural reproduction is not ensured.

J. cyanoides is a perennial plant found on sandy grasslands, mainly of Eastern Europe and Southern Siberia. In the Czech Republic, it grows only in open vegetation on the sandy dune and in the rare pine forest in an isolated locality of the Tišice Natural Preserve Sand in Central Bohemia. Only several individuals of this plant species occur there, and therefore their generative reproduction is very rare.

In this study, the induction of organogenesis, multiplication, rooting, and acclimatization procedures were developed for reproduction of these plant species. The *in vitro* culture propagation of *J. cyanoides* has not been investigated and reported until now.

At present, the cultures of both species are preserved *in vitro* in the Bank of Explants of the Forestry and Game Management Research Institute, Jíloviště, Czech Republic.

MATERIAL AND METHODS

G. verna vegetative shoots approximately 1 cm long from six donor plants and *J. cyanoides* vegetative shoots approximately 4 cm long from four donor plants were sampled in the spring (end of May) since 2008 to 2010. The shoots were sterilized in 10% Sekusept® forte (Farmak a.s., Olomouc, Czech Republic) for 5 min, in 1% NaClO (Savo, Bochemie a.s., Bohumín, Czech Republic) for 10 min, and washed three times in sterile distilled water.

Induction of organogenesis

G. verna sterilized shoots were cultured in 6% agar basal Woody Plant medium (WPM) (Lloyd, McCown 1981) with 0.2 mg·l^{-1} of BAP, 0.1 mg·l^{-1} of IBA, 200 mg·l^{-1} of glutamine, 200 mg·l^{-1} of casein

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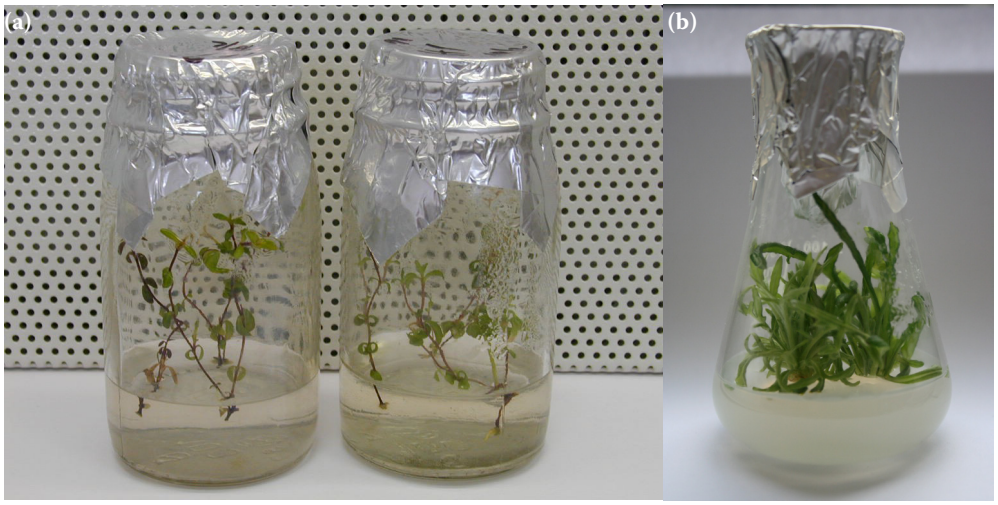

Fig. 1. Multiplication of *Gentiana verna* (a), *Jurinea cyanoides* (b)

hydrolyzate and 30 mg·l^{-1} of saccharose (all ingredients Sigma-Aldrich, CR), pH adjusted to 5.8. The shoots were cultivated in constant conditions at 24°C and 16 h photoperiod under white fluorescent light (30 µmol·m^{-2}·s^{-1}).

J. cyanoides sterilized shoots for inductions of organogenesis were cultured in 6% agar basal Murashige-Skoog medium (MS) (Murashige, Skoog 1962) supplemented with the same concentrations of substances mentioned above, pH 5.8. The shoots were grown in the same culture conditions as described for *G. verna*. After two or three transfers of growing shoots of both plant species to the fresh medium (one passage took approximately 4–5 weeks) the stabilized explant cultures were obtained.

Multiplication

The MS medium of the same composition as in the induction of organogenesis was used for multiplication of both species. Multiplied shoots were used for further multiplication or for rooting. The cultures of both plant species were cultivated in constant conditions at 24°C and 16 h photoperiod under white fluorescent light (30 µmol·m^{-2}·s^{-1}) (Fig. 1).

Rooting

The multiplied shoots of *G. verna* and *J. cyanoides* were transferred for rooting to the basal MS medium of 1/3 concentration without cytokinins but with 2 mg·l^{-1} of glutamine, 2 mg·l^{-1} of glycine, 10 g·l^{-1} of saccharose and 3 mg·l^{-1} of IBA. Both species were cultivated in darkness at 24°C for 1 week, then they were grown on the same medium without auxin in the same cultivation conditions as during the induction of organogenesis and multiplication (Fig. 2).

Acclimatization

The rooted cultures of both plant species were transferred to the pots containing perlite (Perlit, Ltd.,

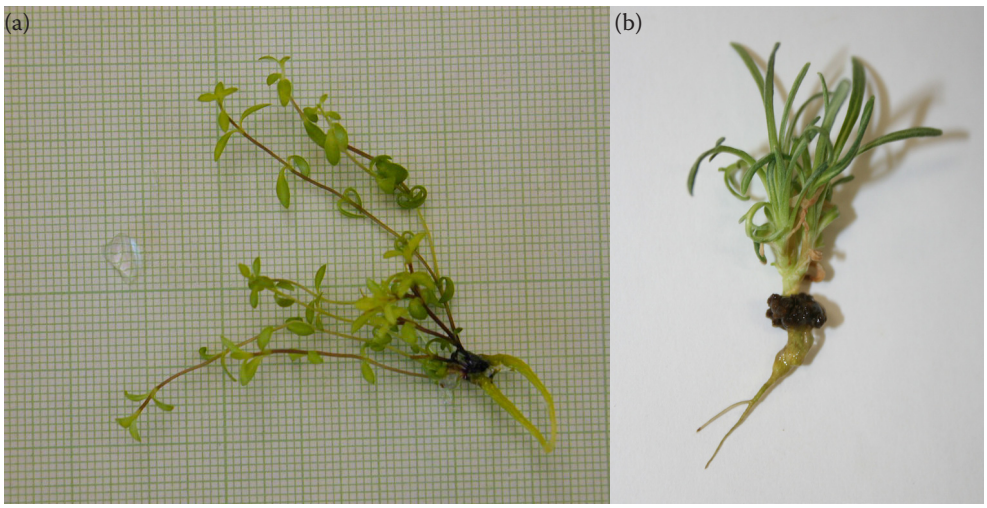

Fig. 2. Rooting of *Gentiana verna* (a), *Jurinea cyanoides* (b)

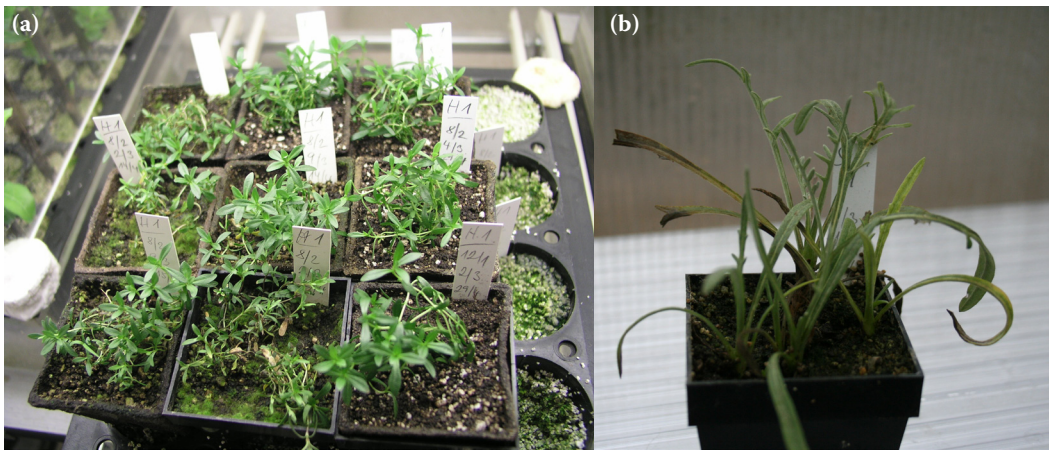

Fig. 3. Plantlets of *Gentiana verna* (a), *Jurinea cyanoides* (b)

Prague, Czech Republic) and watered twice a week with the basal MS medium (without phytohormones, glutamine, and saccharose) diluted at 1:10 with distilled water. The cultures were cultivated in constant conditions at 20°C and 24 h photoperiod under white fluorescent light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The plants of both species were transferred into the non-sterile peat substrate after 4 weeks and acclimatized for outplanting (Fig. 3).

Statistical evaluation

The efficiency of *Gentiana verna* micropropagation was evaluated during 8 months by the number of cultures growing *in vitro*. The efficiency of *Jurinea cyanoides* micropropagation was evaluated during 16 months by the number of cultures growing *in vitro*. The results were evaluated by ANOVA and Duncan's Multiple Comparison Test ($P = 0.05$).

RESULTS AND DISCUSSION

Micropropagation by means of organogenesis is referred to as a reliable and convenient method for the propagation of a number of plant species including trees (Malá et al. 2010) and those plant species that are listed as endangered. It allows the exclusion of factors limiting natural regeneration and environmental causes eliciting serious damage *in situ*. This method guarantees genetic identity of the propagated plant material (D'Amato 1978). For micropropagation, we used only a small amount of the plant material (nodal segments), the donor plants remained undamaged. The main aim of this work was to establish the protocol for effective micropropagation of the critically endangered plant species *G. verna* and *J. cyanoides* growing in the Czech Republic. The optimized procedure of micropropagation is mentioned above. We compared the efficiency of MS and WPM media for the induction of organogenesis and tested several combinations of phytohormones. Induction of organogenesis was also successfully used for the propagation of many *Gentiana* species such as *G. pneumonanthe* (Lamproye et al. 1987), *G. kurroo* (Sharma et al. 1993), *G. acaulis*, *G. cruciata*, *G. lutea*, *G. purpurea*, (Momčilović et al. 1997; Hayta et al. 2011), *G. cerina*, *G. corymbifera* (Morgan et al. 1997), *G. punctata* (Butiuc-Keul et al. 2005), *G. austriaca* (Vinterhalter et al. 2008) and *G. dinarica* (Vinterhalter et al. 2012; Krstič-Miloševič et al. 2013). Optimizing micropropagation is not straightforward since it is a complex process involving a sequence of developmental stages that are influenced by numerous endogenous and exogenous stimuli, which include especially phytohormones, such as auxins and cytokinins. The focus here is on the cytokinin/auxin ratio, which has been shown by many authors to play an important regulative role during organogenesis (e. g. D'Angeli et al. 2001; Caboni et al. 2002). These authors recommend for the induction of organogenesis of *Gentiana* species different BAP concentrations ranging from 0.1 $mg \cdot l^{-1}$ up to 2 $mg \cdot l^{-1}$. Successful induction of organogenesis was reached by using BAP (2 $mg \cdot l^{-1}$) in combination with IAA(0.2 $mg \cdot l^{-1}$) but also with lower concentrations of phytohormones or growth regulators, e. g. with 0.1–2 $mg \cdot l^{-1}$ of BAP or with kinetin in combination with 0.1 $mg \cdot l^{-1}$ of NAA (Butiuc-Keul, Deliu 1999). Generally, it was demonstrated that high concentrations of BAP inhibited subsequent rhizogenesis (Bollmark et al. 1988). These findings are in agreement with our experiments with *G. verna*, in which low concentrations of BAP (0.2 $mg \cdot l^{-1}$) in combination with IBA (0.1 $mg \cdot l^{-1}$) were sufficient for successful induction of organogenesis. While using low concentrations of phytohormones, no inhibition effects of BAP were observed regarding rhizogenesis. In *G. punctata*, the

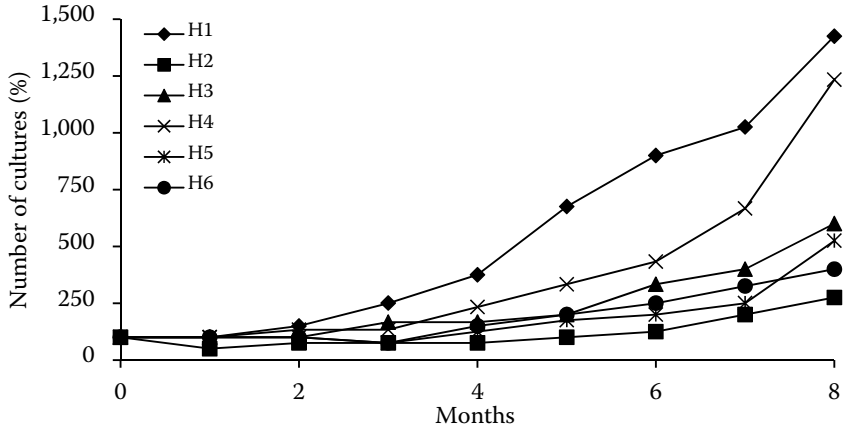

Fig. 4. Efficiency of *Gentiana verna* micropropagation, number of cultures growing *in vitro* during 8 months, the multiplication efficiency was significantly higher in clone H1 in comparison with clones H2, H3, H5 and H6; the results were evaluated by ANOVA and Duncan's Multiple Comparison Test ($P = 0.05$)

inhibition of rhizogenesis was described by using high concentrations of 2iP and zeatin (Butiuc-Keul et al. 2005). In our experiments WPM medium was more efficient for the induction of *Gentiana* explants than MS medium. On the contrary, Hosokawa et al. (1996) recommended MS medium for the induction of various *Gentiana* explants. On average, 2–3 adventitious shoots of *Gentiana verna* were developed during 4–6 weeks in our experiment. *In vitro* cultures of six clones show a different regeneration potential during subcultivations (Fig. 4). The multiplication efficiency was significantly higher in clone H1 in comparison with clones H2, H3, H5, and H6.

No references about the *in vitro* propagation of *Jurinea cyanoides* are available. Very scarce information about the *in vitro* cultivation of any species of the genus *Jurinea* was published (Panayotova et al. 2008; Verma et al. 2012). The main aim of micropropagation of *Jurinea* species is an urgent need of their *ex situ* conservation. The induction of organogenesis of *J. cyanoides* was successful on MS medium, similarly Panayotova et al. (2008) and Verma et al. (2012) reported that MS medium was suitable for the *in vitro* propagation of *Jurinea* species.

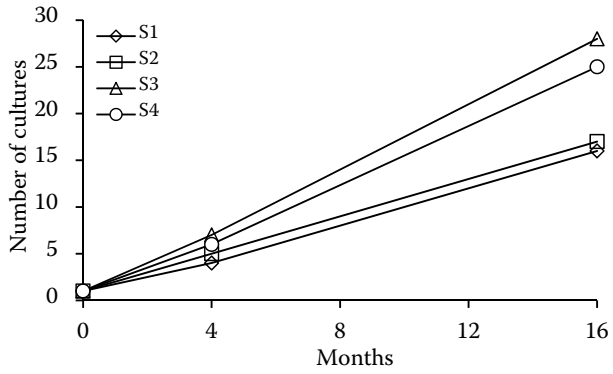

Fig. 5. Efficiency of *Jurinea cyanoides* micropropagation, number of cultures growing *in vitro* during 16 months, the multiplication efficiency was significantly higher in clone S3 and S4 in comparison with clones S1 and S2; the results were evaluated by ANOVA and Duncan's Multiple Comparison Test ($P = 0.05$)

On average, 2–3 adventitious shoots of *J. cyanoides* were developed during 4–6 weeks in our experiment. During 16 months of the *in vitro* cultivation of four clones of *J. cyanoides* 102 multiapex cultures were regenerated (Fig. 5). The multiplication efficiency was significantly higher in clone S3 and S4 in comparison with clones S1 and S2. For rooting, the propagated shoots of *G. verna* and *J. cyanoides* were transferred to rooting media with 3 mg·l^{-1} of IBA. Other authors used for the rooting of different species of the genus *Genciana* various concentrations of NAA (0.3–2 mg·l^{-1}) for 14 days, followed by the transfer of rooted plantlets to a growth regulator – free MS or WPM medium for subsequent 30 days (Momčilovič et al. 1997). In our experiments, the growth of root systems of *G. verna* and *J. cyanoides* was steady, no significant differences either in length or in numbers of roots were observed. During 2–4 weeks, the roots were formed with 40–75% efficiency in both species but their root systems were weak and also the stomata were dysfunctional. For acclimatization of regenerants, the first phase of cultivation of plants in wet perlite was crucial. The losses did not exceed 20% in *G. verna* and 25% in *J. cyanoides* during acclimatization. At present, the multiapex cultures of both species are stored in the Bank of Explants of the Forestry and Game Management Research Institute, Czech Republic.

CONCLUSIONS

We have verified the possibility of using micropropagation methods for conservation of the endangered species *Gentiana verna* and *Jurinea cyanoides.* Micropropagated plant material is maintained in the Bank of Explants of the Forestry and Game Management Research Institute, for the preservation of these plant

species. *In vitro* cultures can be further investigated in terms of genetic stability, vitality during long-term cultivation and possibility for their reintroduction.

References

Bollmark M., Kubát B., Eliasson L. (1988): Variation in endogenous cytokinin content during adventitious root formation in pea cuttings. Journal of Plant Physiology, ***132***: 262–265.

Butiuc-Keul A.L., Deliu C. (1999): Plant regeneration from nodal explant and callus of *Gentiana cruiata* L. In: Cachita-Cosma D., Ardelean A., Craciun C. (eds): *In Vitro* Cultures of Higher Plants. Cluj-Napoca, Risoprint: 74–79.

Butiuc-Keul A., Suteu A., Deliu C. (2005): *In vitro* organogenesis of *Gentiana punctata.* Notulae Botanicae Horti Agrobotanici Cluj-Napoca, ***33***: 38–40.

Caboni E., D'Angeli S., Chiappetta A., Innocenti A.M., van Onckelen H., Damiano C. (2002): Adventitious shoot regeneration from vegetative shoot apices in pear and putative role of cytokinin accumulation in the morphogenetic process. Plant Cell, Tissue and Organ Culture, ***70***: 199–206.

D'Amato F. (1978): Chromosome number variation in cultured cells and regenerated plants. In: Thorpe T.A. (ed.): Frontiers of Plant Tissue Culture. Calgary, University of Calgary: 287–295.

D'Angeli S., Lauri P., Dewitte W., van Onckelen H., Caboni E. (2001): Factors affecting *in vitro* shoot formation from vegetative shoot apices of apple and relationship between organogenic response and cytokinin localisation. Plant Biosystems, ***135***: 95–100.

Hayta S., Akgun I.H., Ganzera M., Bedir E., Gurel A. (2011): Shoot proliferation and HPLC-determination of iridoid glycosides in clones of *Gentiana cruciata* L. Plant Cell, Tissue and Organ Culture, ***107***: 175–180.

Hosokawa K., Nakano M., Oikawa Y., Yamamura S. (1996): Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana.* Plant Cell Reports, ***15***: 578–581.

Krstić-Milošević D., Janković T., Vinterhalter B., Menković N., Aljančić I., Vinterhalter D. (2013): Influence of carbohydrate source on xanthone content in root cultures of *Gentiana dinarica* Beck. Plant Growth Regulation, ***71***: 147–155.

Lamproye A., Crevecoeur M., Keveres C., Gaspar T. (1987): Multiplication végétative *in vitro* de *Gentiana lutea* et de *Gentiana pneumonate*. Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent, ***52***: 1255–1257.

Lloyd G., McCown B.H. (1981): Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot tip culture. Combined Proceedings, International Plant Propagators' Society, ***30***: 421–427.

Malá J., Máchová P., Cvrčková H., Šíma P. (2010): Biotechnologie v lesním hospodářství a šlechtění. [Biotechnology in forestry and breeding.] Lesnická práce, ***8***: 17–19.

Momčilović L., Grubišić D., Nešković M. (1997): Micropropagation of four *Gentiana* species (*G. lutea, G. cruciata, G. purpurea* and *G. acaulis*). Plant Cell, Tissue and Organ Culture, ***49***: 141–144.

Morgan E.R., Butler R.M., Bicknell R.A. (1997): *In vitro* propagation of *Gentiana cerina* and *Gentiana corymbifera*. New Zealand Journal of Crop and Horticultural Science, ***25***: 1–8.

Murashige T., Skoog F. (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum, ***15***: 473–497.

Panayotova L.G., Ivanova T.A., Bogdanova Y.Y., Gussev Ch.V., Stanilova M.I., Bosseva Y.Zh., Stoeva T.D. (2008): *In vitro* cultivation of plant species from sandy dunes along the Bulgarian Black Sea Coast. Phytologia Balcanica ***14***: 119–123.

Sharma N., Chandel K.P.S., Anderson P. (1993): *In vitro* propagation of *Gentiana kurroo* – an indigenous threatened plant of medicinal importance. Plant Cell, Tissue and Organ Culture, ***34***: 307–309.

Verma P., Mathur A.K., Jain S.P., Mathur A. (2012): *In vitro* conservation of twenty-three overexploited medicinal plants belonging to the Indian sub continent. The Scientific World Journal, ***2012***: 1–10.

Vinterhalter B., Janković T., Šavikin K., Nikolić R., Vinterhalter D. (2008): Propagation and xanthone content of *Gentianella austriaca* shoot cultures. Plant Cell, Tissue and Organ Culture, ***97***: 329–335.

Vinterhalter B., Krstić-Milošević D., Janković T., Milojević T., Vinterhalter D. (2012): *In vitro* propagation of *Gentiana dinarica* Beck. Central European Journal of Biology, ***7***: 690–697.

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