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MICROPROPAGATION STUDIES AND ANTIOXIDANT ANALYSIS OF THE ENDANGERED PLANTS OF BULGARIAN YELLOW GENTIAN (*Gentiana lutea* L.)

Maria I. Petrova[1], Ely G. Zayova[1], Lyudmila I. Dimitrova[1], Maria P. Geneva[2] ✉, Kamelia D. Miladinova-Georgieva[2]

[1] Department of Applied Genetics and Plant Biotechnology, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. George Bonchev, bl. 21, Sofia 1113, Bulgaria

[2] Department of Plant-Soil Interactions, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. George Bonchev, bl. 21, Sofia 1113, Bulgaria

ABSTRACT

In order to develop an efficient micropropagation system, it is essential to establish the appropriate concentration of growth regulators for seed germination, shoot formation and rooting. Nodal segments from *in vitro* obtained seedlings of *Gentiana lutea* L. were cultured *in vitro* in Murashige and Skoog's medium supplemented with BAP, Thidiazuron and Zeatin (0.5, 1.0 and 2.0 mg L^{-1}). A maximum number of shoots with the highest height was recorded at 2.0 mg L^{-1} BAP. For further optimization of the process, we used nutrient media containing BAP and Zeatin with a combination of low concentration of Indoleacetic acid. MS medium containing 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA resulted in maximum numbers of shoots 94.3) with shoot height 2.5 cm. The multiple plants were successfully *ex vitro* acclimatized with 65% survival. The presence of growth regulators (2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA) in the nutrient media resulted in an effective antioxidant activity in *G. Lutea* determined by the low molecular antioxidant metabolites such as phenols and flavonoids and activities of antioxidant enzymes – catalase, ascorbate peroxidase, guaiacol peroxidase, and superoxide dismutase. The described protocol allows the establishment of numerous micropropaged plants of rare and endangered *G. lutea*.

Key words: *Gentiana lutea* L., seed germination, micropropagation, plant growth regulators, antioxidants

INTRODUCTION

Gentiana lutea L. (yellow gentian, *Gentianaceae*) is a perennial herb native to the mountains of Central and Southeastern Europe. The plant is included in the Red Data Book of the Republic of Bulgaria in the category of endangered species [Peev et al. 2015]. Its medicinal efficiency is due to the bitter secoiridoid glucosides, xanthones, di- and trisaccharides, pyridine alkaloids, phenolic acids etc. located in the roots [Aberham et al. 2007] (Radix Gentianae). The species has a long history of use for treatment of digestive disorders and is an ingredient of many medicines. It is also applied as an immunostimulant and exerts antitumor, antivirus, antibacterial and antioxidant effects [Šavikin et al. 2009, Mirzaee et al. 2017]. The reproductive capacity of *G. lutea* in natural habitats is limited, because the germination rate is very low. Furthermore, the presence of competing species hinders the survival of seedlings and possibilities for

✉ boykova2@yahoo.com

vegetative propagation are restricted [Sidjimova et al. 2014]. Biotechnological approaches are suitable for preservation of plant material of medicinal plants threatened with extinction. The long generative cycle (six to eight years until flowering) of *G. lutea* and a low germination rate of seeds highlight the needs of its *in vitro* cultivation. Previous investigations aimed at establishing the optimal culture media and conditions of micropropagation, induction of calogenesis with subsequent *de novo* buds formation, organogenesis and regeneration [Momcilovic et al. 1997].

The initiation of plant organs – roots and shoots, is one dramatic morphogenetic transition imposed to *in vitro* plant systems. For this reason, plants need to activate antioxidant systems (enzymatic and low molecular metabolites) to protect themselves against adverse growth conditions [Niki 2011]. The addition of plant growth regulators to the nutrient media results in further antioxidant system stimulation. Therefore, the study of the effects on the antioxidant metabolism of the addition of plant regulators to the culture medium is of growing interest. During the initiation phase, superoxide dismutase (SOD) and catalase (CAT) activities have been higher in nodal stem sections, compared to less meristematic intermodal explants, which only had trace levels of catalase [Benson and Roubelakis-Angelakis 1992].

The aim of the present study was to establish the optimized culture conditions for micropropagation of *G. lutea* plants and to evaluate their antioxidant metabolism.

MATERIAL AND METHODS

Sterilization and germination of seeds, and shoot micropropagation. The seeds were collected from a natural population of the species in the region of Vetrovala, Nature Park "Vitosha", Bulgaria. Cold stratification (six months at 4°C) was carried out to stimulate the seeds germination. Seeds were surface washed with tap water and detergent. After that, the following sterilization trails were used – soaking in 70% C_2H_5OH for 1 min and thereafter 50% ACE (commercial bleach), which is a component of sodium hypochlorite (4.85%) for 5 min. The seeds were washed three times with sterile distilled water. The seeds germination was initiated on Murashige and Skoog [1962] (MS) basal medium containing ascorbic acid (ASC) and gibberellic acid (GA_3) at different concentrations and combinations (Tab. 1). Four culture media were used: MSG_0: MS control, MSG_1: MS + 10 mg L^{-1} ascorbic acid + 10 mg L^{-1} GA_3; MSG_2: MS + 10 mg L^{-1} ASC + 20 mg L^{-1} GA_3; MSG_3: MS + 10 mg L^{-1} ASC + 30 mg L^{-1} GA_3. The percentage of germinated seeds was measured after 30 days. The *in vitro* obtained seedlings were grown on modified MS nutrient media for further growth and multiplication stage. In order to determine the propagation frequency, the mean number of shoots per explant and the mean height of shoots, three types of cytokines (0.5 and 1.0 mg l^{-1} BAP, TDZ or Zeatin) were tested. For optimization of micropropagation, nutrient media containing BAP and Zeatin with a combination of low concentration of auxin IAA, were examined after 4 weeks of cultivation.

Rooting and adaptation of plants. Silver nitrate was added to the rooting medium to prevent bacterial contamination. After 4 weeks of cultivation on MS nutrient medium supplemented with 1.0 mg L^{-1} $AgNO_3$ and 1.0 mg L^{-1} IBA, percentage of rooted plants and number of roots per plant were measured. Three different mixtures of peat were evaluated for *ex vitro* plants adaptation. The plants were established in plastic pots (7 cm diameter). The pots were placed in a box with a transparent plastic cover that was removed after two weeks, thus allowing gradual adaptation to the lower air humidity in room conditions. The survival percentage of plants was assessed after six weeks.

Culture conditions. All media were solidified with 7 g L^{-1}. The media pH was adjusted to 5.8 with 1N NaOH or 1N HCl and sterilized. The *in vitro* cultures were maintained under growth room conditions at a temperature of 22 ±2°C, relative humidity of 70% and a 16 h photoperiod under 40 μmol $m^{-2}s^{-1}$ illumination.

Antioxidant capacity assays. Dry plant samples (0.3 g) from 4 weeks micropropagated plants on MS medium containing 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA were ground and exhaustively extracted with 96% (v/v) methanol. Free radical-scavenging activity was determined using 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant [Tepe et al. 2006]. The ferric reducing antioxidant power (FRAP) was monitored colorimeter-

ically by reduction of ferric ions [Benzie and Strain 1996]. Concentrations of total phenolic compounds were determined spectrophotometrically using the Folin–Ciocalteu reagent and calculated as caffeic acid equivalents [Pfeffer et al. 1998]. Flavonoids in plant tissues were measured spectrophotometrically according to Zhishen et al. [1999], using the standard curve of catechin.

For the determination of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.1) and guaiacol peroxidase (GPO, EC 1.11.1.7) activities, fresh shoot material (0.5 g FW), collected from 4 weeks micropropagated plantlets was homogenized in 0.1 mM K-phosphate buffer (pH 7.8), containing 2.0 mM Na_2-EDTA, 1 mM PMSF (phenyl methyl sulfonyl fluoride), 2% polyvinylpyrrolidone K-40 (w/v) and 10% glycerol. The homogenate was centrifuged at 12 000 *g* for 30 min and the supernatant was used as a crude enzyme extract. Total SOD, CAT, APX and GPO activity was determined by Zayova et al. [2018]. Bradford [1976] method was used for soluble protein content measurement.

Statistical analysis. One explant was cultivated and 20 cultures were raised for each treatment. Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows).

RESULTS AND DISCUSSION

The cold stratification had positive effects on breaking the seed dormancy and 12.5% of seeds germinated on control medium (without GA_3 and ASC) (Tab. 1, Fig. 1). The seeds at room temperature did not germinate (data not shown). Seed germination was stimulated on MS nutrient medium containing ASC and GA_3. The best nutrient medium was MS medium supplemented with 10 mg L^{-1} ASC and 20 mg L^{-1} GA_3. The seeds cultured on this medium started to germinate after 10 days of culture and germination reached 70% on the 30th day of cultivation. At lower and higher concentrations of GA_3 in the medium, the germination rate was lower. For seeds sterilization the most authors applied sodium hypochlorite at concentrations 0.2–1.0% with a drop Tween 20 or 80 by 10–15 min exposure [Momčilović et al. 1997, Morgan et al. 1997] and commercial bleach Domestos (12–20%) for 15–20 min [Fiuk and Rybczynski 2008]. In our study due to the optimum exposure of sterilizing agents, no bacterial and fungi contamination of the *in vitro* cultured seeds of *G. lutea* were observed. The seeds of many species of the genus *Gentiana* germinate at low temperatures, which breaks the seed dormancy. Ohkawa [1983] implies that this is the reason the seeds germinate in the spring, because they are exposed to low temperatures during winter. Bicknell [1984] compared the effects of low temperatures and various concentrations of GA_3 on germination of *G. scabra*. He reported that 2–3 weeks at 2°C is sufficient to break the seed dormancy and indicated that GA_3 can increase the seed germination even in the absence of low temperatures. Bicknell [1984] and Morgan et al. [1997] used high concentrations of GA_3 (100–300 mg L^{-1}) for stimulation of seed germination in *G. scabra* and *G. corymbifera*. To increase the germination of seeds in *G. lutea*, *G. acaulis* and *G. purpurea* Momčilović et al. [1997], they used 0.2 mM GA_3, while for *G. cruciata* – 10 mM KNO_3.

After the first cultivation (one month in culture media for propagation), the plants grew only in height. Micropropagation was observed in the second

Table 1. Seed germination of *G. lutea* on different nutrient media

Nutrient medium	Cultivated seeds (number)	Germinated seeds (%)
MSG_0 control	40	12.5
MSG_1	40	37.5
MSG_2	40	70.0
MSG_3	40	57.5

Fig. 1. Seed germination of *G. lutea* (70%) on MS medium supplemented with 10 mg L–1 ascorbic acid and 20 mg L–1 gibberellic acid on the 30th day of cultivation

Table 2. Effect of different plant growth regulators on the micropropagation of *G. lutea* shoots after 4 weeks of cultivation

Plant growth regulators	Cytokinins (mg L^{-1})	Micropropagation frequency (%)	Number of shoots (explant^{-1})	Shoot height (cm)	Rooting (%)
BAP	0.5	60	2.1 ±0.11^{b}	1.8 ±0.17^{c}	–
	1.0	80	3.0 ±0.24^{d}	2.2 ±0.19^{d}	–
	2.0	65	3.6 ±0.27^{e}	2.4 ±0.18^{de}	–
Zeatin	0.5	55	1.9 ±0.23^{b}	2.7 ±0.23^{fg}	–
	1.0	75	2.1 ±0.16^{b}	2.5 ±0.22^{ef}	45
	2.0	50	2.5 ±0.17^{c}	2.9 ±0.24^{g}	–
TDZ	0.5	45	1.0 ±0.06^{a}	0.9 ±0.08^{b}	–
	1.0	50	1.9 ±0.18^{b}	0.8 ±0.07^{b}	55
	2.0	40	1.3 ±0.12^{a}	0.5 ±0.04^{a}	–
	LSD		0.31	0.29	
BAP + IAA	1.0 + 0.1	70	2.1 ±0.21^{a}	2.0 ±0.18^{a}	–
	2.0 + 0.2	85	3.8 ±0.26^{c}	2.2 ±0.21^{ab}	–
Zeatin + IAA	1.0 + 0.1	77.5	2.7 ±0.23^{b}	2.8 ±0.26^{c}	–
	2.0 + 0.2	95	4.3 ±0.28^{d}	2.5 ±0.23^{bc}	–
	LSD		0.46	0.42	

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis

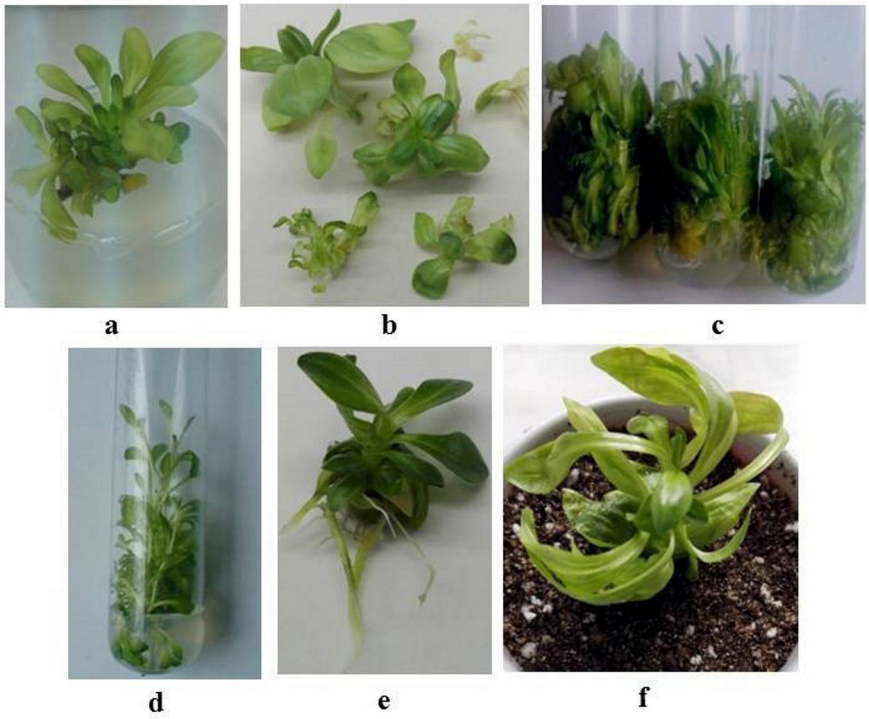

Fig. 2. Micropropagated plants of *G. lutea* on: a) and b) MS nutrient medium, supplemented with 1.0 mg L–1 BAP; c) MS nutrient medium, supplemented with 2.0 mg L^{-1} BAP; d) multiplied shoots cultured on MS medium supplemented with 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA; e) *in vitro* rooting on ½ MS medium with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} AgNO3; f) *ex vitro* acclimatization of *G. lutea* plants with Mix 3 – peat, perlite and sand (2 : 1 : 1, v/v/v)

subculture. The rate of propagation depended on the concentration and the type of applied cytokinin in the culture medium. Concentrations of 0.5 mg L^{-1} (BAP, Zeatin or TDZ) at all were less effective for micropropagation of *G. lutea* (Tab. 2). Best results were observed on MS nutrient medium containing BAP at a concentration of 1.0 mg L^{-1}, wherein the average number of adventitious shoots per explant was 3.0 with 2.2 cm shoot height (Figs. 2a, 2b). The shoots were cultured on MS medium with 2.0 mg L^{-1} BAP (Fig. 2c) or 2.0 mg L^{-1} Zeatin, where in average 3.6 and 2.5 were produced per explant, respectively. In this culture media, the plantlets showed better growth and development. Callus was formed at the base of most of the explants grown in nutrient media with the TDZ and a small part of them produced shoots. In response to application of 1.0 mg L^{-1} TDZ into the nutrient medium, 50% of explants formed vegetative buds (in average 1.9 shoots per explant) and 55% rooting. The addition of BAP and Zeatin in combination with IAA had a positive impact on the efficiency of propagation of *G. lutea* (Tab. 2). The most effective medium for shooting of *G. lutea* proved to be MS medium containing 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA, resulting in 4.3 shoots per explant with mean height shoots 2.5 cm in 4 weeks of cultivation (Fig. 2d). The majority of authors have used MS medium for multiplication of species of the genus *Gentiana*. Morgan et al. [1997] applied BM (basal medium) containing MS salts and vitamins B5 [Gamborg et al. 1968]. An effective protocol for micropropagation of two species of the genus *Gentiana* – *G. acaulis* and *G. purpura* was elaborated applying WPM medium [Momcilovic et al 1997]. Some authors added cytokinin BAP to the bas-

Table 3. Effect of mixture substrate on the survival of *G. lutea* plants during of *ex vitro* acclimatization after six weeks of cultivation

Peat mixture	Number plant	Plant survival (%)	Plant height (cm)
Mix 1 – peat : perlite (2 : 1, v/v)	30	20	1.7 ±0.16[b]
Mix 2 – peat : perlite : soil (2 : 1 : 1, v/v/v)	40	30	2.4 ±0.22[ab]
Mix 3 – peat : perlite : sand (2 : 1 : 1, v/v/v)	40	65	2.8 ±0.25[a]

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis

Table 4. Antioxidant potential of extracts of *G. lutea* grown for shooting on MS medium containing 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA

		Unit	Value
Antioxidant enzymes	Catalase (CAT)	units mg protein^{-1} min^{-1}	31.43
	Ascorbate peroxidase (APX)	µmol asc mg protein^{-1} min^{-1}	0.57
	Guaiacol peroxidase (GPO)	nmol mg protein^{-1} min^{-1}	32.49
	Superoxide dismutase (SOD)	enzyme units	148.12
Antioxidant metabolites	Flavonoids	mg g^{-1} DW	19.74
	Phenols	mg g^{-1} DW	3.79
	DPPH$^{\bullet}$	%	92.84
	FRAP	µmols Fe^{2+} g^{-1} DW	30.71

al medium at a concentration of 0.1 to 6.0 mg L^{-1} for micropropagation of *G. lutea* [Zeleznik et al. 2002]. In most cases, BAP was combined with low concentration of some of the auxins – IAA or NAA. Viola and Franz [1989] observed high proliferation of shoots of *G. lutea* at the presence of 3.0 mg L^{-1} BAP. An average micropropagation rate estimated to be three of shoots per explant within 21 days on MS nutrient medium supplemented with 1.0 mg L^{-1} IAA and 0.1 mg L^{-1} BA [Zeleznik et al. 2002]. Drobyk et al. [2015] established differences of micropropagation frequency of *G. lutea* regardless of the genotype, of the BAP, kinetin and $CaCl_2$ content added to the nutrient medium. One of the most critical stages of *in vitro* cultivation of *G. lutea* was the rooting of microcuttings. A functional root system is very important for subsequent adaptation of plants to *ex vitro* conditions. The rooting of *in vitro* obtained *G. lutea* microcuttings required adding of plant growth regulators to the culture medium, because it is quite difficult to perform on hormone-free MS medium [Momčilović et al. 1997]. Mayorova et al. [2015] reported that efficiency of rooting of *G. lutea* microclones depends on a combination of the optimal composition of nutrient medium and favorable for rooting maintaining substrate. In our study, it was found that shoots from multiplication phase have to pass two subcultures on nutrient medium contained auxins to stimulate the process of rhizogenesis. Because the entire *in vitro* process goes through several passages, sometimes a bacterium-like contaminant was found during the rooting of *G. lutea* plants. This is a serious problem and subsequently it hampers the root formation. Due

to this contamination, the plantlets retarded its development. On the leaves and at the base of the explants, necrosis developed and the percentage of rooting was significantly reduced. Due to this contamination, a positive effect of the addition of silver nitrate ($AgNO_3$) at 1 mg L^{-1} was observed on ½ MS culture medium supplemented with 1.0 mg L^{-1} from IBA, which provided root formation and reduction of microbiological contamination. The results showed that addition of silver nitrate to the medium also stimulates the induction of roots. The percentage of rooting varies depending on the applied concentration of silver nitrate [Petrova et al. 2011]. At ½ MS nutrient medium, containing 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} $AgNO_3$ (Fig. 2e), where the shoots were derived from MS medium with 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA, the rooting was 90%.

Attempts to improve the conditions for the *ex vitro* acclimatization of *G. lutea* plantlets were made. Micropropagated plants with a well-developed root system were transferred into small plastic pots (7 cm diameter) containing three different mixtures (Tab. 3). When transferring the plants from *in vitro* to *ex vitro* conditions, many of them died. The most suitable mixture for the adaptation of plants was Mix 3: peat : pearlite : sand (2 : 1 : 1), where the survival rate is 65% after six weeks of adaptation (Fig. 2f). The *in vitro* conservation methods developed in this study allowed the micro-growth and preservation of *G. lutea*, given the limited distribution of this species in danger of extinction.

Antioxidant capacity of *G. lutea* plant extracts depended on the activity of antioxidant enzymes and the content of antioxidant metabolites (Tab. 4). The activities of SOD, CAT, GPO and APH were detected in the extracts of young plants grown on MS medium for multiplication containing 2.0 mg L^{-1} Zeatin and 0.2 mg L^{-1} IAA. No study to date has yet determined the potential of antioxidant enzyme activity in *in vitro* shooting of *G. lutea* plantlet extracts. CAT and peroxidases are known to play a role in growth and differentiation and their high activity could be correlated to the process of differentiation that occurred during the shoot or root induction [Molassiotis et al. 2004]. Tian et al. [2003] reported that antioxidant enzymes were involved in the process of shoot organogenesis in strawberry callus. Moreover, they act as the second messenger in several plant hormone responses [Kwak et al. 2006]. The content of flavonoids is higher than that of total phenols. Total polyphenol content usually considered responsible for the antioxidant capacity was found to correlate well with $DPPH^{\bullet}$ scavenging activity in *Gentiana lutea* extracts [Nastasijević et al. 2012]. Flavonoids are also strong scavengers of reactive oxygen species and were well connected with inhibition of FRAP equivalent capacity assay.

CONCLUSIONS

The present study provides an efficient method for micropropagation of Bulgarian Yellow Gentian. The results suggest that *in vitro* cultivation of *G. lutea* seeds allows to increase the seed germination up to 70%. A maximum number of shoots per explant on the MS medium supplemented with 2.0 mg L^{-1} Zeatin and 0.1 mg L^{-1} IAA was obtained. *In vitro* rooting and significant reduction in late bacterial contamination was achieved on ½ MS medium containing 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} $AgNO_3$. Extract from *in vitro* propagated *G. lutea* plantlets showed excellent antioxidant activity measured by DPPH scavenging assay. The results obtained contribute to the conservation of this valuable and endangered medicinal plant.

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