

IN VITRO CULTURE OF THE PROTECTED RARE SPECIES *GENTIANA LUTEA* L. FOR CONSERVATIVE PURPOSE

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Abstract: The medicinal plants have a therapeutic well definite role, taking into account the toxicity associated to the indiscriminate use of synthetic drugs and antibiotics, the interest for the utilization of medicinal plants and plant based drugs being revived throughout the world. Most of them became rare owing to the intensive exploitation of the natural resources, many people of developing countries depending on the traditional medicine and obtaining incomes from plant harvesting and trade. *Gentiana lutea* L. is a medicinal plant, considered as vulnerable species at national and European level, for its conservation being necessary besides the natural habitats protection, the use of alternative strategies rely on the modern biotechnologies. Our aim is to perform *in vitro* tissues cultures for *ex situ* conservation in *Gentiana lutea* species. For the initiation of *in vitro* cultures, capsules with seeds were used and different explants were collected from the aseptic germinated seedlings. By testing different media variants, we were able to induce in *Gentiana lutea* different developmental ways as callusogenesis, morphogenesis (shooting and rooting) and direct and indirect embryogenesis with high regeneration rate. Murashige–Skoog (MS) medium supplemented with BAP (1mg/l), NAA (0.1 mg/l) and glutamine (0.4 g/l) ensure a good multiplication through direct morphogenesis. The regenerated shoots rooted with difficulty. Embryogenic callus was induced on medium MS added with 2, 4, 5-T. The maturation and the development of embryos occurred on mannitol supplemented medium (30 g/l). The *in vitro* cultures have been maintained over two years and viable plants were successfully regenerated.

Key words: *in vitro*, *Gentiana lutea*, plant regeneration, conservation.

Introduction

The Biodiversity Conservation became in the XXIth century an essential preoccupation of the scientific community and of the authorities at local, regional and global level.

The medicinal plants have a therapeutic well definite role, taking into account the toxicity associated to the indiscriminate use of synthetic drugs and antibiotics, the interest for the utilization of medicinal plants and plant based drugs being revived throughout the world. The majority of pharmaceutical industry is dependent on the natural plant populations which supply the raw materials. Due to lack of proper cultivation practices and destruction of plants habitats, many medicinal plants are severely threatened. The main causes of alpine species vulnerability are the intensive harvesting, the alpine tourism, the alteration of alpine pastures, the global climatic changes. The climate changes have already affected the distribution of the species in many parts of the world, more than half of the species could become vulnerable until 2080 [43].

About 85% from the people from the developing countries rely on traditional medicine for their primary health care needs, involving plant extracts.

Gentiana lutea L. is a perennial plant growing in alpine pastures, at 1000-2500 m altitude, in Europe and Asia, being a medicinal, rare, vulnerable, plant species at national and European level. *Gentiana lutea* represents a medicinal plant known and appreciated from the ancient times (cited as a cure by Plinius the Older and Dioscoride), being used for anorexia, gastric atonia treatment, having a stimulatory gastric, sialagogue and colagogue effects. It has also anti-helminthic, anti-inflammatory, antiseptic, febrifuge and tonic effects, the tonic bitter active principles being used for obtaining one of the strongest tonic that stimulates the liver and stomach functions, and fights against the debility.

The gentian roots, harvested after age of 5-7 years, contain gentiopicroside and amarogenin, the oligosaccharides gentianose, gentiobiose, phenolic acids (gentisic and capheic and protocatechuic acids), polysaccharides (inulin, and pectin), tannin, triterpenes (lupeol and amyirin), xanthones (gentisin, isogentisin, gentioside). The plant flowers after 3 years of vegetation and is cultured in many European countries. The exploitation in the natural habitats is forbidden having the status of protected species and being submitted to commercial restrictions, including in Romania.

It is mentioned in The Red List of the Vascular Plants [33]. Dihoru and Dihoru [13] also cited as vulnerable. Boscaiu et al., [4] considered to be in danger of extinction. In "The Critical List of Vascular Plants from Romania" [34] the species is considered critically endangered.

Several authors sustained the importance of *in vitro* methods in the *ex situ* conservation of rare plants [2, 9, 14, 15, 47]. The tissues culture methods in the controlled environmental conditions on artificial medium and reduced space, offer a viable and efficient alternative for the propagation and conservation of valuable rare medicinal plants from the entire world [30, 32, 45].

At international level, there are many works concerning the *in vitro* conservation of the rare, medicinal plants [12, 24, 25, 26, 27, 37, 38, 39, 40, 44].

The classical gene banks (or field collections) are expensive due to the labor costs, having disadvantages as the vulnerability to the natural hazards and the necessity of large spaces. An alternative strategy of plant *ex situ* conservation involves *in vitro* tissues cultures, the cyclic material multiplication using micropropagation schemes, the germplasm evaluation and distribution in active and base gene banks [2].

Materials and Methods

As plant material for the initiation of *in vitro* cultures were used immature capsules with seeds. The sterilization of the capsules was made washing in running tap water for 2 hours, the immersion in 70° alcohol for 1 minute and subsequently the exposure to mercuric chloride 0.1% for 20 minutes, followed by three washing in sterile distilled water.

The sterilised capsules were dissected and the seeds were inoculated in Petri dishes with solid Murashige-Skoog medium (MS) [30] with reduced at ½ of salts concentration, supplemented with benzyl aminopurine (BAP) 0.1 mg/l, alfa-naphtyl acetic acid (NAA) 0.01 mg/l, 20 g/l sucrose and maintained at 25 °C in dark.

From the seedlings obtained through the aseptic germination of the seeds, were collected different explants (fragments of hypocotils, roots, cotyledons) and cultured on different media variants based on MS formula [30] supplemented with Gamborg vitamins [16] mixture (Table 1).

For seed germination and calli induction, the *in vitro* cultures were maintained in a growth chamber at 25°C, in dark. For embryos germination and plant regeneration, the tissues cultures were transferred at 6h/day, at 2000 lux illumination. The roots developed on 2, 4-D supplemented medium (M3), were subsequently sectioned and used for calli induction.

Different *in vitro* developmental ways were analysed. The possibility to regenerate plants and to *in vitro* preserve regenerative cultures was also studied.

Results and Discussion

In Romania, researches concerning the use of *in vitro* methods for the conservation of the rare species *G. lutea* have not been performed.

The use of *in vitro* methods for the conservative purpose involves the induction and the maintenance of proliferative and regenerative cultures with the establishment of reproducible methods. The choice of the optimum explant type and the modulation of the specific culture conditions enable the induction of *in vitro* developmental processes (dedifferentiation, redifferentiation), callusogenesis, morphogenesis and somatic embryogenesis.

The establishment of an active gene bank is based on the collection of explants, on the initiation of tissues cultures, on the propagation, the maintenance and distribution of plant material.

At international level, there are some studies in few species of the *Gentiana* genus concerning *in vitro* multiplication, conservation and utilization for medicinal and aesthetic purposes [1, 28, 29, 36, 37, 39]. In *G. lutea*, *G. cruciata*, *G. purpurea* and *G. acaulis*, Momčilović et al., [29] studied the micropropagation process starting from nodal stem fragments excised from aseptically grown seedlings. Axillary shoots were initiated from lateral buds. In later subcultures, a basal callus tissue developed on the shoots, giving rise to de novo formed buds. Benzyladenine and indolyl-acetic acid combinations for shoot development were established.

In Romania, there are some approaches concerning *in vitro* conservation in rare plant species [3, 5, 6, 7, 8, 10, 11, 17, 18, 20, 21, 22, 23, 35, 41, 42, 46, 47, 48, 49, 50].

In a related species, *Gentiana cruciata*, a medium multiplication rate through indirect morphogenesis was recorded by Butiuc-Keul and Deliu, [6] on medium supplemented with 2-iP and IBA with a 7.6 regenerants/ callus induced from root and 8.3 regenerants/ callus induced from stem. Calli were induced from fragments of roots, stems aseptically germinated seedlings in presence of growth factors such as 2, 4-D and BAP or zeatin and IBA.

In the same related species, Mikula et al., [28] induced successfully somatic embryogenesis starting from explants from aseptically germinated seedlings. The effect of auxins (2, 4-D, NAA, dicamba) and cytokinins (BAP, zeatin, kinetin) in various concentration and combinations were studied. The indirect embryogenesis occurred sporadically on variants containing 2,4-D and NAA with low rate (0.38 % -1.64 % responding explants).

In the ornamental species *Gentiana pneumonanthe*, Bach & Pawlowska [2003] used leaves and apical meristems for the induction of indirect somatic embryogenesis on media supplemented with 2, 4-D or picloram and benzyl-aminopurine. The germination of the embryos occurred on MS ½ or MS medium with 50 % rate of conversion.

In *Gentiana punctata* L., another rare related species from Romanian Flora, Butiuc-Keul et al.[8] reported the regeneration through organogenesis, plant multiplication was 2.6 plantlets/explant on media supplemented with different combinations of 2-iP (2-isopentenyladenine) or zeatin with indolyl-acetic and/or maize extract. The roots formation occurred with difficulty in this case on medium supplemented with NAA (1mg/l), 2-iP (1 mg/l), maize extract (1mg/l).

In our study, the capsules were efficiently sterilized. After 2 months of culture on Murashige-Skoog medium (MS)[30] with macro and microelements concentration reduced at ½, supplemented with BAP (0.1 mg/l), NAA (0.01 mg/l), 20 g/l sucrose, 90% of seeds matured, germinated and developed plantlets.

The seedlings represent the best source for *in vitro* culture initiation, the hypocotyls being appropriate for calli induction and the cotyledons for adventive shooting.

The *in vitro* response of *Gentiana lutea* to different composition of media used was different (Table 2). The presence of 2, 4-dichloro-phenoxy acetic acid favoured the non-embryogenic friable calli formation. M3 medium induced an active rhizogenesis process (7-10 roots/callus) - Fig.1a. The exposure to light determined the green colour of the roots.

Among of the three auxins tested (2, 4-D, 2, 4, 5-T and NAA), 2, 4, 5-T (at 2 mg/l concentration) was efficient for somatic embryogenesis induction (Fig. 1b).

The majority of the embryos did not converted into plants on auxin supplemented media. In some cases, directly on the roots fragments cultured on M5 variant an adventive somatic embryogenesis (just a few isolated embryos) can appear (Fig.1c).

Table 1: The composition of the culture media used in *Gentiana lutea* L.

Components		Media variants													
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
Macroelements		MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS 1/2
Microelements		MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
B 5 vitamins complex		B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	-
Growth factors (mg/l)	2,4,5,-T	-	-	1	1	2	2	-	-	-	-	-	-	-	-
	2,4- D	2	2	-	-	-	-	-	2	0.1	-	-	-	-	-
	NAA	-	-	-	-	-	-	2	-	0.1	0.1	-	-	-	-
	BAP	-	-	-	-	-	-	-	-	1	1	-	-	-	-
	Kin	-	-	-	0.1	-	-	0.2	0.25	1.5	-	-	-	-	-
	IBA	-	-	-	-	-	-	1	1	-	-	-	-	-	-
	GA ₃	-	1	-	-	-	1	-	-	1	-	1	-	-	-
Other compounds g/l	Sucrose	30	30	30	30	30	30	30	30	30	30	30	30	30	20
	Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	30	-
	Glutamine	-	-	-	-	-	-	-	-	-	0.4	-	-	-	-

Legend: MS- Murashige-Skoog medium [1962]; B5 -Gamborg vitamins mixture [1968], BAP- benzyl aminopurine; Kin- kinetin; NAA- alfa-naphtyl acetic acid; IBA- indolyl butyric acid; 2.4-D - 2,4 dichlorophenoxyacetic acid; 2,4,5 – T- 2,4,5 trichlorophenoxyacetic acid; GA₃- gibberellic acid.

Table 2: The *in vitro* response of *Gentiana lutea* L.

Media variants	Callus formation	Regeneration	Observations
M1	Light yellow friable callus	-	-
M2	Callus degeneration	-	-
M3	Friable, proembryogenic callus	absent	Short roots formation
M4	Friable proembryogenic callus	absent	Do not allow the long maintaining through periodical transfer
M5	Embryogenic callus	Indirect somatic embryogenesis; embryos in different stages	Subsequently subcultures can be performed; Globular and cotyledonar embryos formation; Occasionally, occurs the adventive somatic embryogenesis on roots, the embryos conversion is made with very low rate (2-3/ callus)
M6	Callus degeneration	Non-regenerative	-
M7	Proembryogenic callus	The embryos did not develop	The active growth of callus
M8	Non- regenerative callus	-	Reduced rate of callus growth
M9	-	Direct morphogenesis	2-5 shoots/explant, without roots
M10	-	Direct morphogenesis	15-20 shoots/explant, without roots
M11	-	-	Did not allow the embryos conversion into plants
M 12	-	-	Did not allow the maturation and germination of the embryos
M 13	-	-	Ensure the maturation and germination of the embryos and conversion into plants (90-100%).Efficient regeneration of plants and roots growth (three fold longer as plant length)
M14	-	-	Ensure the growth of neo-formed plants

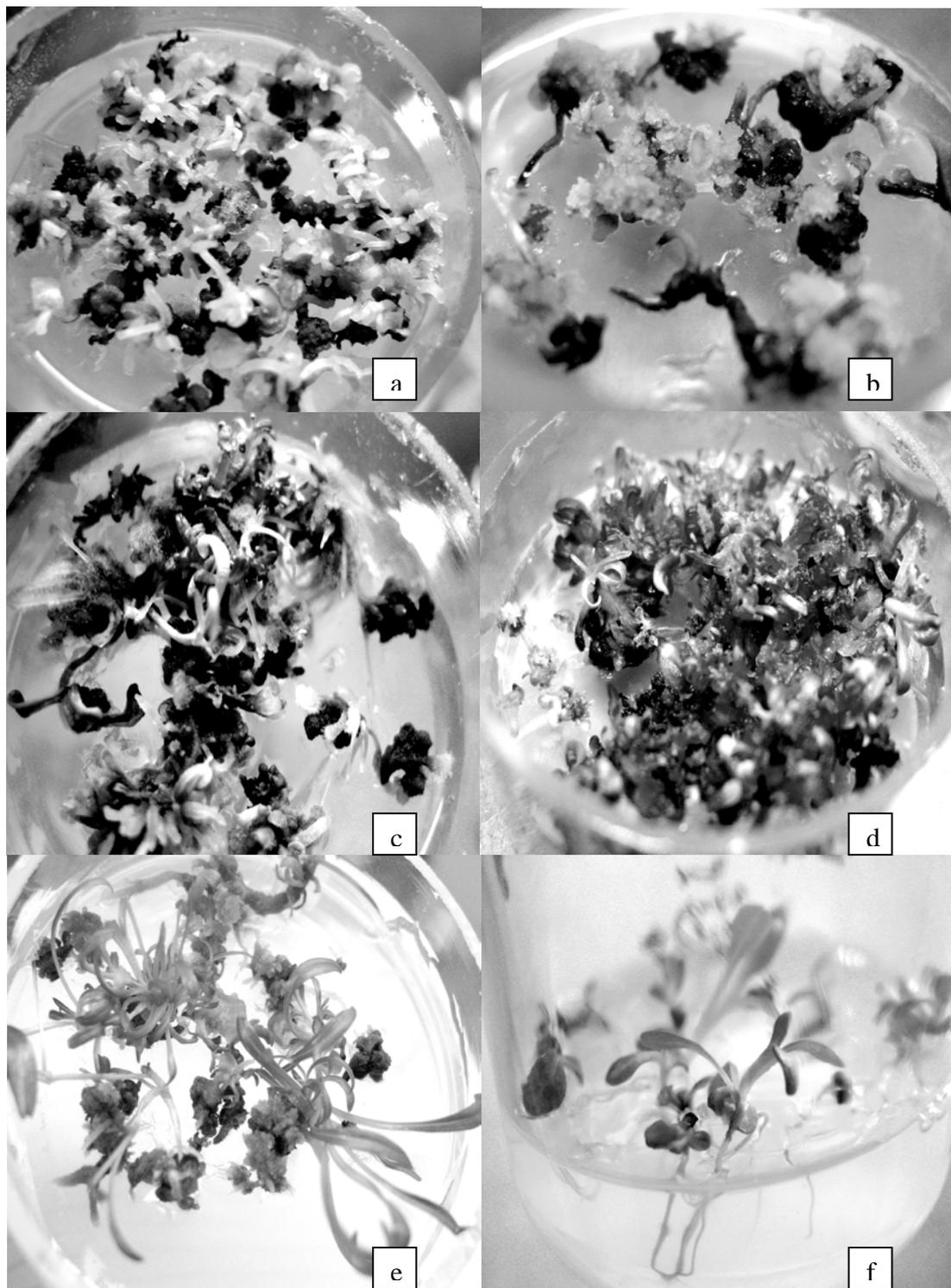


Fig. 1: a-Induction of roots in *Gentiana lutea* on M 3 medium supplemented with 2,4-D, after 40 days of culture; b-embryogenic calli induced on 2,4,5-T supplemented variant after 30 days; c-adventive somatic embryos formed on the roots after 60 days of culture; d-development of the somatic embryos on M13 variant with high rate after 60 days; e-shoots induction on M9 medium after 45 days of culture; f-*In vitro* rooted plants on M14 medium variant.

After 1 month of culture on M5 medium, the somatic embryogenesis was induced, but the embryos developed with very low rate.

The supplementation of the culture media with gibberelic acid (in M6 variant) or the transfer on hormone free media (M14) did not help satisfactory the conversion of the embryos into plantlets.

In our case, only M13 variant allowed the development of the embryos (Fig. 1d) with 90-100% frequency. The maturation of the embryos underwent at 25 °C and 16 hours photoperiod and 2000 lux. The growth of plants on the same medium was also stimulated the roots length was three fold greater as plants.

In our experiment, the media variants with cytokinin dominance (M9 and M10) determined the induction of shoots through direct morphogenesis with different rates. In the case of M9 medium, between 2-5 new shoots were regenerated (Fig. 1e) and on M10 variant supplemented with glutamine, the morphogenesis was more active, several shoots were regenerated (15-20).

Despite this acceptable regeneration rate, the rooting of the shoots is quite difficult to perform on hormone-free MS medium. On the contrary, in the case of somatic embryogenesis, the embryos germinated on M13 medium with high efficiency (Fig. 1d) and were converted into plants, forming easily roots in absence of any growth factor (Fig. 1f).

The somatic embryogenesis occurred continuously with successive cycles of adventive somatic embryos formation. The transfers of regenerative cultures were made at every 2-3 months and maintained for more than two years, the number of embryos being very high.

From these *in vitro* cultures can anytime be regenerated healthy plants.

Conclusions

- ◆ In *Gentiana lutea* L. were successfully induced *in vitro* regenerative cultures. The cultures have been *in vitro* maintained for more than 2 years.
- ◆ Using culture media variants supplemented with different growth factors, callusogenesis, rhizogenesis, morphogenesis and embryogenesis processes were induced.
- ◆ The optimum way to micropropagate and *ex situ* preserve *Gentiana lutea* is the somatic embryogenesis which occurs with high rate all round the year with good conversion of the embryos into plants. The embryogenic callus was induced on 2, 4, 5-T supplemented medium and somatic embryos maturation and germination was made on mannitol supplemented medium.
- ◆ The multiplication protocol can be used for the establishment of *in vitro* collection in this rare species.

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CULTURA IN VITRO LA SPECIA RARĂ, PROTEJATĂ GENTIANA LUTEA L. ÎN SCOPUL CONSERVĂRII

(Rezumat)

Plantele medicinale au un rol terapeutic bine definit, in contextul toxicității asociate cu folosirea fără discernământ a medicamentelor de sinteză și a antibioticelor, interesul pentru utilizarea plantelor medicinale fiind resuscitat în întreaga lume. Multe dintre plantele medicinale au devenit rare ca urmare a exploatării intensive a resurselor naturale, mulți oameni din țările în curs de dezvoltare depinzând de medicina tradițională, obținând venituri din recoltarea și comerțul cu aceste plante. *Gentiana lutea* L., este o specie medicinala, considerată

vulnerabilă la nivel național și european, pentru conservarea sa fiind necesare pe lângă protejarea habitatelor, utilizarea unor alternative bazate pe biotehnologiile moderne.

Scopul lucrării noastre este realizarea de culturi *in vitro* în scopul conservării *ex situ* la specia *Gentiana lutea* L.

Pentru inițierea culturilor *in vitro* au fost utilizate capsule cu semințe, diferite explante fiind prelevate de la plantulele germinate aseptice. Testând diferite medii de cultură, au fost induse la *Gentiana lutea*, diferite procese de dezvoltare ca morfogeneza (lăstărire și înrădăcinare), calusogeneza, embriogeneza indirectă și directă.

Mediul Murashige–Skoog (MS) suplimentat cu BAP (1mg/l), ANA (0.1 mg/l) și glutamină (0.4 g/l) a asigurat o rată bună de multiplicare prin morfogeneza directă. Lăstarii regenerați înrădăcinează însă cu dificultate. Calus embriogen a fost indus pe mediul MS suplimentat 2,4,5-T. Maturarea și germinarea embrionilor s-a realizat pe mediu suplimentat cu manitol (30 g/l) în absența factorilor de creștere. Culturile *in vitro* au fost menținute peste doi ani și au fost regenerare cu succes plante viabile.

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