# *Gentiana lutea* L. – CONSIDERATIONS FOR A SUCCESSFUL PROTOCOL ON MICROPROPAGATION

**SAVA SAND Camelia**

**Abstract.** Lately, has been increasingly important to ensure the conservation of biodiversity and look for alternatives to support the survival of the species. Endangered species list grows from year to year and climate change emphasizes this phenomenon. Adoption of conservation strategies to appeal to the tools offered by biotechnology has been more frequent after 2000. The aim of this paper is to provide information on successful micropropagation of the species *Gentiana lutea* L., as an endangered species for our country.

**Keywords:** *Gentiana lutea*, inoculation, micropropagation, *in vitro* cultivation.

**Rezumat. *Genţiana lutea* L. - Consideraţii privind un protocol de succes pentru micropropagare.** În ultima perioadă este tot mai important pentru asigurarea conservării biodiversităţii, găsirea de alternative care să asigure perpetuarea speciilor. Lista speciilor pe cale de dispariţie creşte de la un an la altul, iar schimbările climatice accentuează acest fenomen. Adoptarea de strategii de conservare care să apeleze la instrumentele oferite de biotehnologii este tot mai frecventă după 2000. Scopul acestei lucrări este acela de a oferi informaţii cu privire la micropropagarea cu succes a speciei *Gentiana lutea* L., periclitată pentru ţara noastră.

**Cuvinte cheie:** *Gentiana lutea*, inoculare, micropropagare, cultivare *in vitro*.

## INTRODUCTION

Most of the known plant species have been accessed for millennia by humankind for useful products: food, textile, medicine, wood, perfumes, dyes, food; some have been known for thousands of years, others are still waiting to be discovered (SHAHID & MOHAMMAD, 2013). Inventory of valuable genotypes of endangered plant species and cloning them through *in vitro* culture support today the obtaining of superior active principles that are highly valuable (PATHAK & ABIDO, 2014). *In vitro* inoculation and micropropagation are safety tools for the environment in protecting medicinal plants at an advanced stage of endangerment, according to commitments taken under the Bern Convention (LEE & ACKERMAN, 1993).

Romania also needs to comply with these political commitments and therefore should finance projects to safeguard the heritage of flora, as these national priceless treasures are not irretrievably lost yet (COSTE et al., 2012). This technique allows the creation of models (lots) of experimental producers of medicinal plants, given the high requirements of medicinal plants both for internal and external market (JUMA et al., 2014). The idea of associating arable land on large areas to be profitable it is still rejected, forcing us to find solutions for farmers who do not have large areas of land, especially in hilly and foothill areas where they can cultivate their land with herbs (BARROWS et al., 2013). Currently there are two major trends of particular practical interest, but unfortunately antagonistic: replacing medicinal chemical synthesis with the natural world, particularly derived from plants, on the one hand, and preserving biodiversity and protecting vegetable species, on the other hand (MARIENHAGEN & BOTT, 2013).

The World Health Organization announced that 75-80% of the world population is treated with herbal remedies (PANDEY et al., 2015). This is another reason that the culture of medicinal plants should be done as effectively, both in terms of raw material quality and economic production at a price that would make it competitive as many markets. As wild medicinal plants present a high genetic variability it makes difficult the industrialization processes of extraction for the same quality in active principles leading to increased costs.

This technology, originating from the beginning of the last century allows the revival of culture of medicinal plants, especially those belonging to endangered species, addressing new technologies and farming practices both beneficial to environment. However, it supports the idea of sustainable agriculture practiced in Europe not only for the noble purpose of ensuring food for a growing population but also to sustainable exploitation of the Earth: respect for nature that is still so generous and desire you keep your balance. It is noteworthy that in countries of Western Europe there is no interest in the cultivation of medicinal plants on a large scale due to the high cost of labour for harvesting and therefore they prefer to import plants in less developed areas in eastern Europe, obviously criticizing them especially for destroying biodiversity and protected species.

Cultivation of medicinal plants widely obtained by *in vitro* culture is such an international priority (BERMAN et al., 2013). In Romania, the wild flora is represented today by 3,700 native species of which there have been identified 800 species of medicinal interest and 370 species of these have already been tested in terms of pharmaceutical effects (DELIU et al., 2013). Herbs processed are used from simple teas to aromatic mixed fruit, tinctures, oils, extracts, syrups, concentrates for soft drinks, condiments, cosmetics and medicines, homeopathic products, functional foods, products for aromatherapy, bioproducts for plant protection organization of the organic crops, etc. (PÂRVU, 2002).

In 1990, the largest area of a total surface of 9,350 ha planted with herbs was occupied by coriander (8,675 ha), followed by poppy (50 ha), cumin (12 ha) and fennel (5 ha), with a total production of approximately 5,390 tons (ISTUDOR, 2001). However, plants from spontaneous flora have at this time the largest share of the total processing and, worse, in some

cases imported herbs, so increasing the number of cultivated species, while increasing the cultivated area should be a national priority as it is estimated that the global trade may come with 2.5 billion $/year (BARATA et al., 2016). Given that many species of medicinal plants are under law protection, many research conducted in Romania are geared towards protecting the country flora heritage by providing feedstock crops. In Romania, there have already been published protocols for *G. lutea* collected from the wild (ZĂPÂRAȚAN, 1996; HOLOBIUC & BLINDU, 2006).

The scope of this article is to introduce the *in vitro* conditions characteristic to this species as a selected population from the wild named 'Săcel' (TANASE et al., 2007) in order to set up a micropropagation protocol and to test new culture media. This species is a monument species for the natural heritage and protected by the national regulatory framework and therefore it would be suitable to contribute for a balanced *in situ* and *ex situ* conservation strategy for our country (ANTOFIE, 2011).

## MATERIAL AND METHODS

**Plant material.** The plant material is represented by mature healthy plants of *Gentiana lutea* L, population 'Săcel' acclimated for field cultivation and originating from the experimental field in Brașov. Apical fragments of 10 cm each (i.e. including meristems, leaves and petioles) have been cut from the plants and prepared for sterilization.

**Sterilization.** The plant fragments have been maintained under cold running tap water for more than 15 min followed by a washing in jars of 800 ml with water and TWEEN (3 drops) for other 15 min followed by three repeated washing under the cold tap water 3 min for each. These jars are all prepared for the next steps in sterilization into the laminar flow hood. It is used Domestos (a commercial detergent containing 4.5% sodium hypochlorite and most environmental friendly compared to other sterilizing agents) for 1 ml at 100 ml sterilized water for 3 min and continuing stirring of the jar. The sterilization followed three rinsing of 5 min each using sterilized water. The sterilized plant material is maintained into the closed jars up to inoculation. Inoculation comprises shoot apexes, leaf fragments and petiole fragments and it was realized meristem inoculation under the stereomicroscope - using the same culture medium.

**Culture media.** Meristems, petiole and leaf fragments have been inoculated on MS62 culture medium in three variants according to table 1. Agar was added after pH correction at 5.8 and before sterilization. There have been used jars of 400 ml with 50 ml culture medium. The micropropagation medium consists of the same composition and for each variants there have been removed the cytokinins and added 0.4 mg/l of NAA (1-Naphthaleneacetic acid). All reagents are of Merck origin (Table 1).

Table 1. The variants of culture medium used for *Gentiana lutea* inoculation.

| Composition | V-I | V-II | V-III |
|---|---|---|---|
| Macro-elements | 50 ml | 100 ml | 100 ml |
| Micro-elements | 10 ml | 1 ml | 1 ml |
| Fe EDTA | 30 mg | 30 mg | 30 mg |
| Inositol | 100 mg | 100 mg | 100 mg |
| $NaH_2PO_4$ x $H_2O$ | 85 mg | - | - |
| Adenine sulphate | 80 mg | - | - |
| Tyrosine | 100 mg | - | - |
| Thiamine | 15 ml | 0,1 ml | 5 ml |
| Pyridoxine | 1 ml | 0,5 ml | 0,1 ml |
| Nicotinic acid | 10 ml | 0,5 ml | 1 ml |
| Kinetin | 9 ml | 0,2 ml | - |
| Indole-3-acetic acid | 0,4 mg | - | - |
| 1-Naphthaleneacetic acid | - | 0,1 ml | 0,1 ml |
| Sucrose | 30 g | 30 | 30 g |
| Agar | 6 g | 6 g | 6 g |

**Growth room conditions.** The temperature was 20°C during the day (16 h) and 18°C during the night (8 h). It was applied a light intensity of 2000 lux with cold light Philips.

## RESULTS AND DISCUSSIONS

In this study, there have been used 120 explants collected from 10 donor plants, in a healthy status during June 2014. From these explants, there have been inoculated 90 meristems (i.e. 30 per variant) and 120 petiole and leaf fragments (i.e. 40 of each per variant) a total of 330 explants. Following the inoculation all meristems have been transferred on a new culture medium, with the same composition, after 10 days, according to our experience that was also applied by different authors (ZĂPÂRȚAN, 1996).

During the first passage it was registered the first removal due to infections with different microorganisms or necrosis (Table 2). Also, it was observed that the plant fragments tissues already evolved presenting hypertrophy at the basal part and also following the cut-edges of petiole and leaf fragments. It was recorded a good evolution for all explants cultivated on V-I (i.e. all explants presented a visible hypertrophy with a survival rate of 80%).

The explants cultivated on the variant V-2 presented a survival rate of 31.2% and mainly meristems presented a positive evolution for morphogenesis. Almost the same survival rate 31% was recorded for the variant V-3. In this

case all leaf or petiole fragments ceased to develop presenting clear necrosis installation into the plant tissues. It is difficult to clear state that the survival rate was mainly due to the culture conditions and not to the manipulation of the excised material. Still, considering that the explants have been randomly inoculated it might be considered that the composition of the culture medium may have a clear influence on the development of the explants (i.e. the lack of cytokinins that supports cell division).

Table 2. The viability of explants of *G. lutea* 10 days after the inoculation.

| **Explant** | **Culture medium Murashige-Skoog 1962 (MS62)** | | | | | |
|---|---|---|---|---|---|---|
| | **Variant I (V-I) K 9 ml/l + 0.4 ml/l AIA** | | **Variant II (V-II) K 0.2 ml/l** | | **Variant III (V-III) ANA 0.1 mg/l** | |
| | % inoculum | Significance | % inoculum | Significance | % inoculum | Significance |
| Foliar explants | 63 % | A | 21.2 % | A | 25 % | A |
| Axillary meristems | 69 % | A | 25.8 % | B | 31 % | B |
| Petiole fragments | 10 % | D | 10.7 % | D | 0 % | - |

Further after inoculation, for micropropagation it was used the same culture medium and 6 weeks (i.e. 8 weeks of cultivation) after the first transfer of plant material cytokinins have been removed and the culture media have been supplemented with NAA at 0.4 mg/l. After another 8 weeks from the second transfer of explants, morphometric analyses have been realized for the resulted plantlets and results are presented in table 3. It can be said that the same culture medium is suitable for the inoculation of this species for *in vitro* cultivation (Table 3).

Table 3. Morphometry of the plantlets of *G. lutea* 3 months after the first inoculation.

| **Explant** | **Roots length (cm)** | | | **Roots no.** | | | **Leaf no.** | | | **Plantlets height cm)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variants | V-I | V-II | V-III | V-I | V-II | V-III | V-I | V-II | V-III | V-I | V-II | V-III |
| Foliar fragments | 2.3 | 1.6 | 2.5 | 3 | 3 | 4 | 4 | 3 | 4 | 6.5 | 7.3 | 7.5 |
| Axillary meristems | 1.8 | 1.4 | 2.2 | 2 | 3 | 3 | 4 | 3 | 5 | 6.1 | 7.0 | 7.3 |
| Petiole fragments | 1.1 | 0.9 | 1.8 | 3 | 2 | 2 | 3 | 2 | 4 | 6.3 | 6.8 | 7.0 |

Based on the analysis of the results presented in table 3 it can be said that it is easy to transfer into *in vitro* culture *G. lutea* and to apply biotechnology as a tool for supporting the conservation of the species (Fig. 1).

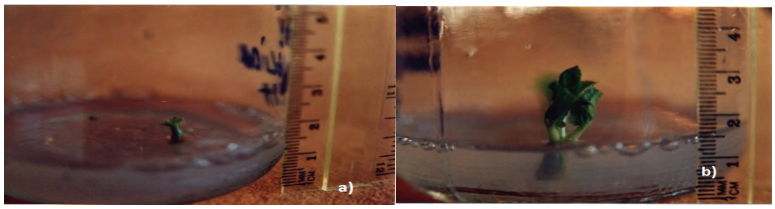


Figure 1. *Gentiana lutea* – in vitro inocula 4 weeks after inoculation of meristems (a) and micropropagated plantlets (b) (original).

Taking into account the results of HOLOBIUC & BLINDU (2006), we consider that the starting inoculum may have an important impact as they used as a starting material seeds collected from the wild. They also considered that MS62 was one the most appropriate culture medium for the inoculation of the species. Still, their experimental data stopped for that stage. In 1996, it was for the first time published a protocol (ZĂPÂRȚAN, 1996) for *G. lutea* using different culture media with comparable results.

## CONCLUSIONS

The analysis of the results of this study revealed that *G. lutea* is a species that is reactive for *in vitro* culture by taking into account the results on inoculation from 1996 to 2006. All tested explants generated micro-propagules and all organs of the species may be obtained: stems, leaves, roots. The first variant, similar to the original MS62 provided the best results supporting previously studies on this species. It can be considered that the species may be successfully introduced into *in vitro* conditions using these protocols depending on the laboratories facilities and resources.

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**Sava Sand Camelia**
Lucian Blaga University of Sibiu,
Faculty for Agricultural Sciences, Food Industry and Environmental Protection5-7 Dr. Ioan Ratiu Str, Sibiu, Romania.
E-mail: camelia.sand@yahoo.com

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