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THE ANTIOXIDANT PROPERTIES OF *Gentiana lutea* ROOT CULTURES

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Abstract

Gentiana lutea is known as one of the most valuable medicinal plant, most of the chemical compounds being synthesized in the roots. The aim of this study was to evaluate the antioxidant properties based on total phenolic content, flavonoids concentration and reducing capacity of the extracts from in vitro differentiated roots of the protected plant species G. lutea. Explants represented by leaf and root fragments originated in a six years old culture of somatic embryos, were inoculated on liquid and solid nutritive Murashige & Skoog media variant supplemented with mannitol. 3% mannitol in liquid media was able to induce the best result concerning number of roots and root length, but a higher antioxidant capacity correlated with flavonoids oncntraation was founded in the root cultures induced on solid medium variant. A viable method for root culture should be use for improving the biomass of the gentian and exploitation of the secondary metabolites. In this way, the protection of the endangered species is ensured.

Key words: *flavonoids, mannitol, protected plant species root culture.*

INTRODUCTION

Gentiana species play an important role in ethnobotany, pharmacology and horticulture (Hayta et al., 2011), *G. lutea* being one of the most valuable medicinal plant (Sava, 2015).
Different *in vitro* studies showed that gentian extracts have antimicrobial, antispasmodic, anti-inflammatory, antioxidant, choleric and secretory activities (Nastasijević et al., 2012).
The main chemical compounds of this species are represented by bitter terpenes, xanthones, alkaloids, small amounts of tannins, sugar, essential oils mainly being synthesized in roots (Edwards et al., 2015). For a good exploitation of active ingredients, the gentian roots have to be collected after 4-5 years (Heltmann and Silva, 1970).
Taking into account the importance of *G. lutea* and other relatives, several studies were made during time concerning characterization, ecology, biotechnology and applications summarized in two volumes of The Gentianaceae book (Rybczyński et al., 2014, 2015).
Over the world, there was an increasing demand for raw material (1,500 tons of gentian roots); this reason caused a significant damage in their natural habitats (Ando et al., 2007). Nearly 100 species of *Gentiana* genus are protected by law in many European countries (Mikula et al., 2005).
In Romania, *G. lutea* is a protected plant species, listed as critically endangered (Oprea, 2005), collection and exploitation in the natural habitats being restricted under national and international regulations.
Despite the large amount of seeds, plant development and reproduction is influenced by its biological peculiarities: low seed germination, pest and diseases, soils and climatic conditions requirements, specific pollinators and mycorrhizal fungi (Drobyk et al., 2015).
In this way, an alternative technique for providing plant material for pharmaceutical industry is represented by biotechnological approaches, applied for sustainable use of endangered and/or protected medicinal and also for conservation of these species. The biotechnological approaches used for medicinal plants are based on *in vitro* techniques (to multiplicated the plant material) and biochemical or molecular techniques (to analyses the secondary metabolites with medicinal properties).

The aim of our study was to evaluate the antioxidant properties based on total phenolic content, flavonoids concentration and reducing capacity of extracts from *in vitro* differentiated roots induced in moderate osmotic stress of the protected plant species *G. lutea*.

MATERIALS AND METHODS

Root cultures were established starting from leave and root fragments detached from germinated somatic embryos (Figure 1).

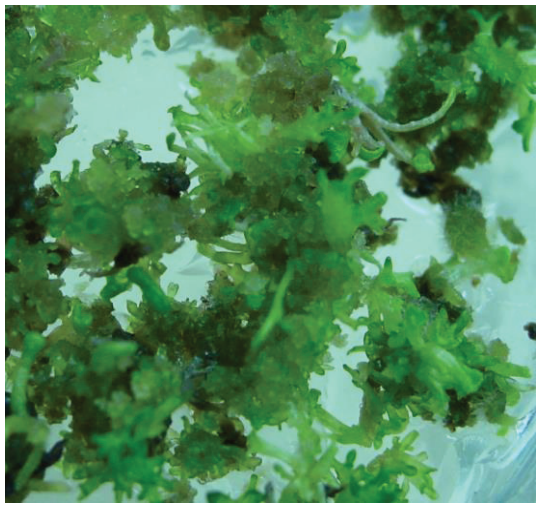

Figure 1. Six years - old highly embryogenesis culture used as explants source

A six year - old highly embryogenesis culture maintained in condition of moderate osmotic stress induced by 3% mannitol (Holobiuc and Catană, 2012) represent the source of somatic embryos.

The recurrent somatic embryos cultures were maintained on solid (M2) MS medium (Murashige and Skoog, 1962) supplemented with Gamborg vitamins (Gamborg et al., 1968), 3% sucrose and 3% mannitol. No plant growth factors were used.

To increase the number of roots, a liquid variant of the same medium was used. The pH was adjusted to 5.8. The solid media variants were solidified with 8 g/l Plant agar. A GFL 3017 Orbital shaker (110 rpm) was used to perform liquid root cultures. A solid MS without mannitol was used as control (M1) variant.

The conditions for maintaining of *in vitro* cultures were represented by a 16 h light/8 h dark photoperiod, with light intensity of 27 $\mu mol*m^{-2}*sec^{-1}$ and temperature 24±2°C.

Survival percentage of the explants (%), percentage of rooted explants (%), number of roots/explant and mean roots length (cm) represents the evaluated parameters of the *in vitro* root cultures. The results were recorded after 4 weeks.

The survival percentage was calculated as the number of viable explants after 4 weeks of culture on media variants/ total of initial explants * 100.

To check the statistical significance, one-way analyse of variance was used. Tukey test was used for multiple comparations (http://xltoolbox.sourceforge.net).

The extraction of *G. lutea* antioxidant compounds. Differentiated roots were dried on filter paper, grounded with quartz sand and extracted in a ratio of 1: 1 (v/v) with 100% methanol for 24 hours. The homogenates were centrifuged at 10000 G, for 20 minutes and the supernatant were used for the subsequent analysis.

Determination of the total phenolic content (TPC). TPC was determined according to the method described by Mihailovic et al., 2013 with some changes. The reaction mixture (0.5 ml of suitably diluted extract, 2.5 ml Folin-Ciocalteu reagent diluted 11-fold, 2 ml of 7.5% Na_2CO_3) was kept 30 minutes at room temperature. After that the absorbance was measured at 765 nm. Three repetitions of the same variant were made and the average represents total phenol content expressed as gallic acid/fresh weight (EAG mg/g sample).

Determination of the flavonoids. Estimated flavonoid content of methanol extracts was performed using a protocol described by Zhishen et al., 1999, with minor adjustments. Thus, suitably diluted 0.5 ml methanol extract of each sample was added with 2 ml of distilled water and 5% $NaNO_2$ solution and the mixture was equilibrated for 5 min. Then 150 µl of 10% $AlCl_3$ solution was added, and after 6 minutes of reaction was added 1 ml of 4% NaOH and brought to a total volume of 5 mL with distilled water.

Optical density at 510 nm was recorded against blank and flavonoid concentration estimation was done according to a calibration curve using the standard routine. Three repetitions of the

same variable were made and the average of flavonoid content was expressed as rutin equivalent/ f fresh weight (ERU mg/g sample).

Determination of antioxidant capacity by DPPH method. According to the method proposed by Marxen et al., 2007, corresponding diluted 100µl extract was mixed with 2.25 ml of methanol and 150 µl methanol solution of 1.27 mM DPPH. At the same time, control was replaced with the extraction solvent. After 30 minutes of incubation at room temperature and darkness absorbance was read at 515nm. Antioxidant capacity represented differences between the samples and control against a standard curve that used Trolox (synthetic antioxidant α-tocopherol analogue) as standard antioxidant. Three repetitions of the same variable were done and the average readings representing antioxidant capacity, expressed as Trolox/ fresh weight (mM Trolox/g sample).

Measurement of reducing power or determination of Fe^{3+} ions reduction. The reduction of Fe^{3+} to Fe^{2+} ions by the methanol extract was carried out according to the method described by Gursoy et al., 2009. The reaction mixture consisted in 1 ml of the diluted extract, 1 ml of 1% $K_3Fe(CN)_6$ and 1 ml of 0.2 M Na phosphate buffer, pH 6.6, incubated for 20 min at 50°C. 10% TCA was added and centrifuged at 10000 RCF, 10 min at room temperature. Subsequently, 1 ml supernatant was mixed with 1 ml of distilled water and 0.5 ml 0.1% $FeCl_3$ and absorbance was measured at 700 nm.
The measurement of reducing power was determined by reference to a standard curve with Trolox as a reducing agent. Three repetitions were made and the average of reducing power has been expressed in Trolox equivalents/fresh weight (mg Trolox/g sample).

RESULTS AND DISCUSSIONS

The root system of *G. lutea* from nature is forbidden to harvest being a protected plant species.
In this way, an *in vitro* root culture was applied for roots induction and for the analyses of the antioxidant properties.
In our case, it was observed that rooting response varied in terms of survival percentage of the explants, rooted explants (Table 1).

Table 1. Survival and rooting percentages of explants on media variants tested

	M1 (control)	**M2** (solid variant)	**M3** (liquid variant)
Survival percentage	16.66	100	100
Rooting percentage	0	60	50

Legend: M2 and M3 – media variants added with 3% mannitol.

Both explants type (root and leaf fragments) cultivated on solid and liquid media variants added with 3% mannitol showed a 100% survival percentage. On the control, medium variant without mannitol, only 16.66% of explants survived (Table 1).
The rooting process started about 14 days after the initiation of root cultures.
Moderate stress induced by 3% mannitol was able to initiate and sustain the roots development with no significant differences ($p<0.05$) between solid (M2) and liquid (M3) media variants. No roots induction was observed on control variant. These data suggested that the combination of sucrose and mannitol sustain the root developmental. Our results are in accordance with results concerning shoot multiplication in a root tip culture of garlic, where MS media variant added with 0.8% mannitol and 3% sucrose had a better result than all the sucrose concentrations tested (Haque et al., 2003).
Number of roots/explant and roots length showed significant differences ($p<0.05$) between liquid and solid media variants added with 3% mannitol (Table 2).

Table 2. Number of roots and root length of *G. lutea* developed on the solid and liquid media variants

	M2 (solid variant)	**M3 (liquid variant)**
No. of roots/explant	1.16±0.93	3.91±0.9*
Root length	0.87±0.37	1.23 ± 0.21*

Data followed by * are significant different according to Tukey test ($p<0.05$).

During time, the importance of growth media variant and incubation conditions to produce specific medicinal compounds at a rate similar

to intact plants was recognized (Hussain et al., 2012).

Since the main chemical compounds of *G. lutea* are synthesized in roots (Edwards et al., 2015), some methods of root induction were developed by adding sucrose (Sharma et al., 1993), auxins (Momcilovici et al., 1997), silver nitrate (Petrova et al., 2011), *Agrobacterium*-mediated transformation (Hayta et al., 2011), fast-growing isolated root culture (Drobyk et al., 2015).

The organized cultures, especially root cultures, can make a significant contribution to phyto-chemicals production. According to Dobryk et al. (2015), isolated root cultures showed greater concentration of secondary metabolites than callus culture.

A root culture initiation represents a better way to obtain biomass than other tissue culture techniques due to short time and low-price necessary for initiation (Dobryk et al., 2015). Between numerous media variants, Murashige and Skoog medium was considered (since 1962) suitable for the production of secondary metabolites in suspension cultures. In our case, the liquid medium variant had a positive effect on the number of roots/explant than the solid variant (Table 2). These data are in accordance with Hassaneim (2010), who showed that liquid medium had a favourable effect on *Pelargonium* rooting compared with the solid medium variant. This may be explained by a better availability of the nutrients and water to the explants. Induction of the new roots may be ensured by the water supply (De Klerk et al., 1999).

Concerning the different capacity of the explant type to form roots, our results showed that roots induced from leaf fragments were longer (~4 cm) and the maximum number of new roots/ explant was 4, in one month. In the case of roots obtained from root explants, the length was shorter (~2 cm), but the number of new roots varied between 3 and 18/explant (Figure 2).

The type of explant seems to be of great importance in the process of morphogenetic response. There are numerous papers that underline this idea presented by Molina and Nuez (1995).

In a period of 30 days of *in vitro* root culture, 4-fold fresh weight of roots was obtained on the liquid MS variant. Through root cultures may be assure a high demand of biomass necessary for secondary metabolites extraction. In this way, the conservation of protected plants is assured.

Figure 2. Differences between new roots induced from leaf (a) and root (b) explants in liquid media variant added with mannitol

In the next step, we analyzed the antioxidant properties of roots induced on the same media variant with the original culture, respectively Murashige and Skoog medium added with 3% mannitol. In this aim, the new roots induced were detached from the explants and analyzed after 4 weeks of *in vitro* cultures.

On the solid medium variant were obtained higher level of analyzed parameters than on the liquid one (Table 3). The lower values of these parameters in the case of roots induced on liquid medium were determined maybe by the accumulation of water, our determination being reported to fresh weight.

Taking into account the total values (regardless the media variants), differences between total phenolic content and flavonoids are not observed in new roots induced on medium added with 3% mannitol (Table 3).

Flavonoids, near phenols compounds, represent support for bioactive properties of the plants, being responsible for the most part of antioxidant properties.

In the present study, a nonsignificant correlation was found between antioxidant activities and phenolic content in *Gentiana lutea in vitro* roots induced, R^2 value were 0.11.

Between antioxidant capacity and flavonoid content the R^2 value was 0.96. These results suggested that flavonoid compounds were responsible for antioxidant capacity in our case.

Table 3. Total phenolic content, flavonoids concentration, antioxidant activity, reduction power of *G. lutea* roots induced in the presence of 3% mannitol

	Solid media variant	**Liquid media variant**	**Total**
Total phenolic content (EAG mg/g FW)	1.035 ± 1.10	0.321 ± 0.118	1.356 ± 1.219
Flavonoid concentration (ERU mg/g FW)	1.471 ± 0.83	0.211 ± 0.088	1.682 ± 0.471
Antioxidant capacity (mM Trolox/g)	4.095 ± 0.72	1.055 ± 0.374	5.15 ± 1.102
Reduction power (mg Trolox/g FW)	4.165 ± 1.83	0.555 ± 0.148	4.72 ± 1.979

These data are not in accordance with previous reports, where the antioxidant activity of *G. lutea* correlates with the phenolic content (Bayliac et al., 2016; Nastasijević et al., 2012). There are studies which showed good correlation between phenolic content and antioxidant activity (Deighton et al., 2000) whereas another found no correlation (Gazzani et al., 1998).

Although our results showed that the antioxidant capacity is not correlated with total phenolic content, the reducing power showed that these compounds may act as primary and secondary antioxidants (Chanda and Dave, 2009). The reducing power reflects the electron donating capacity of bioactive compounds, being associated with antioxidant activity. Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action (Nabavi et al., 2009).

The primary antioxidants term refer to those which actively inhibit oxidation reactions, and secondary antioxidants refer to those which inhibit oxidation indirectly, by mechanisms such as oxygen-scavenging, binding pro-oxidants, etc.) (Shashidi and Wanasundara, 1992). Rice-Evans et al. (1997) considered phenolics to operate as secondary oxidants due to their ability to bind with potentially pro-oxidative metal ions.

Some papers were mentioned to evaluate the antioxidant activity of Gentian roots. Screening the antioxidant activity of *G. lutea* commercially dried root, Azman et al. (2014) obtained 12.3 g GAE/g DW total phenolic content and 15.89 μmol of TE/g DW antioxidant activity. In our case, the total value of phenolic content was lower (1.35 mg GAE/g FW and 5.15 mM Trolox/g FW DPPH activity). The quantitative differences might be referred to the environmental factors and current growth stage, our cultures were grown only for 4 weeks, whereas the commercially dried root was over three years old.

A comparative analysis of metabolites in *G. scarba* roots from different sources (2 months old normal roots grown in greenhouse and two different perennial *Gentiana* dried herbs available in the market) showed that secondary metabolite contents are greatly affected by age and source of origin of the plants (Huang et al., 2014).

Concerning type of explant, differences were observed not only at morphological but also at biochemical level. Differences of biosynthetic potential of organ cultures derived from various explants, plant genotypes, and structural and functional rearrangement of cell genomes in *in vitro* culture represent some factors which can caused changes in secondary metabolism (Kunakh, 2005).

Roots induced from leaf explants were characterized by higher flavonoid concentration (Figure 3).

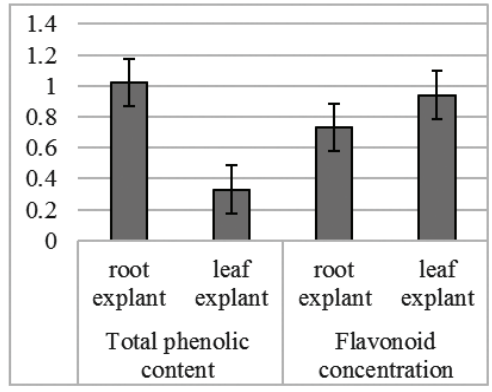

Figure 3. Total phenolic content and flavonoids concentration of roots induced from different explants

These results are due to the distribution of flavonoids virtually in all plant parts, particularly the photosynthesizing plant cells (Kumar and Pandey, 2013). Higher flavonoid

concentration in leaf than root where found in *Rhus pentaphylla* extracts (Itidel et al., 2013).
In or case, a higher total phenolic content was observed in the case of roots induced from root explant. These data are in accordance with those founded in *Rhus tripartita* and *R. pentaphylla,* where the total phenolic content was higher in stem cortex than in other plant parts (Itidel et al., 2013).

CONCLUSIONS

The root induction is depending on type of explant and the media variant used.
The MS liquid media variant added with 3% mannitol was able to induce a higher result concerning number of roots and roots length. 4-fold fresh weight of roots was obtained on liquid MS variant in 30 days.
Since root culture is less expensive, required less growth period and eco-friendly method, it might be a good alternative for production of important medicinal ingredients from protected *Gentiana*.
Roots obtained on solid medium variant showed a higher antioxidant activity correlated with flavonoids content.

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