IN VITRO CULTURE INTRODUCTION FOR *EX SITU* CONSERVATION OF SOME RARE PLANT SPECIES

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Many valuables plants species are nowadays threatened. To stop the continuous loss of plant biodiversity, the Global Strategy for Plant Conservation promotes the development of both *in situ* and *ex situ* conservation methods of rare and vulnerable species. While it is always preferable to conserve and to protect species and to preserve their native habitats, in many situations the *ex situ* conservation is also imposed. In our study, we have the main goal to introduce *in vitro* for *ex situ* conservation purpose of nine rare plants species from different families: *Artemisia tscherneviana* Bosser, *Artemisia alba* Turra, *Doronicum orientale* Hoffm. *Doronicum carpaticum* (Griseb.et Schenk) Nyman, *Dianthus glacialis* ssp. *gelidus* Schott, Nyman et Kotschy, *Gentiana lutea* L., *Iris halophila* Pallas, *Primula halleri* J.F. Gmelin, *Veronica mulifida* L. ssp. *capsellicarpa* (Dubovik) A. Jelen. In almost all species tested, there were induced *in vitro* tissue cultures and plants were regenerated successfully.

Key words: in vitro culture, rare plant, *ex situ* conservation.

INTRODUCTION

There are thousands of plant species which occur in Europe only (Ozinga, 2005). These endemic species can be considered as Europe's specific contribution to the Global Biodiversity. The endemites are the plant species from the ancient flora, with a differential evolution and with specific local conditions (Pop, Sălăgeanu, 1965).

At present, a lot of these European species are threatened (IUCN Red Data Books). These species need special nature conservation efforts in Europe. However, only several hundreds of species are protected under European regulations.

The total number of species for Romania is 3 976, 131 are target species for conservation, 61 under law protection, 79 – global threat (Ozinga, 2005).

In Romania, 3 976 plant species are rare, threatened or endangered (Dihoru & Dihoru, 1994). In the Romanian Red List of Vascular Plants, 1438 taxons and infrataxons are mentioned, 3% are endangered and 12% vulnerable (Oltean *et al.*, 1994).

The Global Strategy for Plant Conservation (2002) has as long-term objective the stopping of the current and continuous loss of the plant diversity. The Strategy provides a framework to facilitate harmony between the existing initiatives aimed at plant conservation, and promote the development of *ex situ* conservation methods related with *in situ* conservation of rare and vulnerable species.

While it is always preferable to conserve and protect species by preserving their native habitat (known as *in situ*), the *ex situ* conservation is also needed.

The genetic fund of endangered plant species can be *ex situ* conserved through the use of *in vitro* tissue cultures (Bromwell, 1990; Fay, 1992; Filippini, 1994; Zăpârțan, 1996; Engelman 1991, 1997; Cachiță *et al.*, 1999).

Ex situ conservation using vegetal biotechnologies required:

- the establishment and improvement of the methods for collection, disinfection, regeneration and conservation of the plant species,
- the genetic stability/variability evaluation of the plant material preserved in gene banks or collections.

To assure the genetic stability of micropropagated and *in vitro* conserved material is preferable the non-adventive system, but for the species with problems, any multiplication way is good.

The aim of our work was the *in vitro* culture introduction for the initiation of a tissue cultures collection for *ex situ* conservation in nine rare plant species.

MATERIAL AND METHODS

Several rare plant species belonging to different families were tested concerning their *in vitro* reactivity (Table 1).

The plant material used as explants for some species (*Veronica multifida* L. subsp. *capselicarpa* Dubovik. A. Jelen, *Artemisia tscherneviana* Bosser, *Artemisia alba* Turra, *Doronicum orientale* are collected from "D. Brandza" Botanical Gardens, Bucharest by kindness of Negrean Gavril PhD. The geographical distribution is presented in figure 1.

Different types of explants and different duration of sterilisation using mercuric chloride according to the species were used to assess the aseptic *in vitro* cultures (Table 2).

The tested medium variants used for *in vitro* culture induction and micropropagation were based on MS formula (Murashige & Skoog, 1962) or N6 formula (Chu, 1978) supplemented with Gamborg vitamins (Gamborg, 1968) and different growth factors (cytokinines, gibberelins and auxins) concentrations.

In the case of the less reactive or recalcitrant species, in the first step there was induced the growth of *in vitro* tissues mass and then different medium variants tested for differentiation. Different supplements (MES, PVP and active charcoal) were used to counteract the oxidative stress effects and the phenolic compounds release.

In other species, (*Campanula carpatica* Jacq. *Doronicum carpaticum* (Griseb. et Schenk) Nyman, *Dianthus glacialis* subsp *gelidus (Schott, Nyman et*

Kotschy), Gentiana lutea L, *Primula halleri* J.F. Gmelin) the mother plants or explants were collected from Sinaia, Vârfu cu Dor, Stâna Regală, Buşteni, Valea Caraimanului and Jepi.

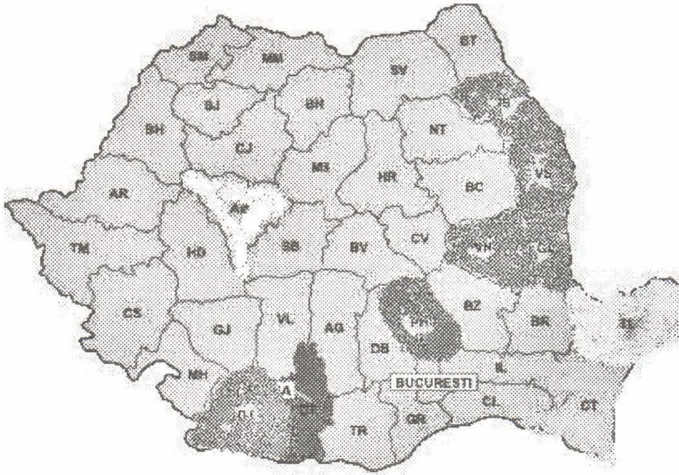

Fig. 1 – The geographical distribution of the plant species studied (white – *Artemisia alba*, grey – *A. tschernieviana*, green – *Iris halophila*, yellow – *Doronicum orientale*, the other species are spread in the Carpathian Mountains).

Table 1

Rare plant species introduced *in vitro* culture

Species	**Family**	**Current name**	**IUCN category**	**Other characteristics**
1	2	3	4	5
Artemisia alba Turra	*Compositae*	Pelin	Rare	Perennial, 30–100 cm, 2n = 36, rocks, calcareous, submedit-erranen, Alba, Harghita, Sibiu, Caras-Severin districts
Artemisia tschernieviana Besser (*A. arenaria* D.C)	*Compositae*	Pelin	Rare	Perennial, seaside sands Constanța and Tulcea districts
Campanula carpatica Jacq.	*Campanulaceae*	Cadelnita	Rare Carpathian endemit	Perennial, 2n = 34, calcareous rocks
Doronicum orientale Hoffm.	*Compositae*	–	Very rare	Perennial, 20–50 cm, 2n = 60, wet, woody lands, Tulcea district, Comana forest

Table 1

(continued)

1	2	3	4	5
Doronicum carpaticum (Griseb. et Schenk) Nyman	*Compositae*	Cujda	Subendemic Rare	Perennial 10–50 cm, shadow wet rocks
Dianthus glacialis subsp. *gelidus* (Schott, Nyman et Kotschy)	*Caryophyllaceae*	–	Endemic Rare	Perennial 5 cm, sunny rocks S-E Carpathians
Gentiana lutea L.	*Gentianaceae*	Ghintura galbena	Vulnerable/ rare	Perennial, 40–120 cm, 2n = 40, Alpine grasslands, abrupt slopes
Iris halophila Pallas	*Iridaceae*	–	rare	Perennial, 2n = 20, 40–80 cm, Dolj, Prahova, Ialomita, Vrancea, Galați, Vaslui, Iaşi districts meadows
Primula halleri J.F. Gmelin	*Primulaceae*	Anghelina	rare	Perennial 10–30 cm, sunny alpine grasslands
Veronica multifida L. ssp *capsellicarpa* (Dubovik) A. Jelen	*Scrophulariaceae*	–	rare	Perennial, 15–30 cm, sunny steppe, Central Pontic Dobrogea

Table 2

Disinfection procedures used for the explants of different plants species

Species	**Explants type**	**Disinfection protocols**
1	2	3
Artemisia alba Turra	leaves, petioles, stem fragments	Washing in running tap water for 2 hours; pre-sterilisation in ethyl alcohol 70° for few seconds; sterilisation with $HgCl_2$ 0.1 % added with Tween 80 for 5–6 min; rinsed 3 times with sterilised distilled water
Artemisia tschernieviana Besser (*A. arenaria* D.C)	leaves, petiole, uninodal fragment	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % 5–6 min; rinsed 3 times with sterilised distilled water

Table 2

(continued)

1	2	3
Campanula carpatica Jacq.	Uninodal fragment, leaves	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.05 %, 2 min and then sterilisation with $HgCl_2$ 0.1 %, 2 min; rinsed 3 times with sterilised distilled water
Doronicum orientale Hoffm.	Leaves, petiole	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilization with $HgCl_2$ 0.1 % for 4–5 min; rinsed 3 times with sterilised distilled water
Doronicum carpaticum (Griseb. et Schenk) Nyman	Leaves, petiole	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % 4–5 min; rinsed 3 times with sterilised distilled water
Dianthus glacialis subsp *gelidus (Schott,Nyman et Kotschy) Nyman*	Uninodal fragments	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % for 5–6 min, rinsed 3 times with sterilised distilled water
Iris halophila Pallas	Immature seeds capsules	Washing in running tap water for 1 hour; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % for 10 min, rinsed 3 times with sterilised distilled water
Gentiana lutea L.	Capsule with seeds	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % for 20 min, rinsed 3 times with sterilised distilled water
Primula halleri J.F. Gmelin	Immature seed from ovaries	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % for 15 min, rinsed 3 times with sterilised distilled water
Veronica multifida L. ssp *capsellicarpa* (Dubovik) A. Jelen	Leaves, young inflorescences, uninodal fragments	Washing in running tap water for 2 hours; pre-sterilisation in 70° ethyl alcohol for few seconds; sterilisation with $HgCl_2$ 0.1 % 6–7 min, rinsed 3 times with sterilised distilled water

Table 3

The tested variants culture media used for the initiation of *in vitro* cultures and for micropropagation

Components		Culture media variants													
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
Macroelements		N6	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
B complex		B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	–	–	–
Hormones (mg/l)	BAP	0.1	1	1	1	1	1	1	1	1	2	–	–	–	0.1
	Kin	–	–	–	–	1	1	1	1	1.5	–	0.25	0.2	–	–
	NAA	0.01	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.25	0.5	–	1	–	1
	IBA	–	–	–	–	–	–	–	–	–	–	1	–	–	–
	2,4-D	–	–	–	–	–	–	–	–	–	–	1	1	2	–
	GA_3	–	–	–	–	–	–	–	–	0.5	–	–	–	–	–
Carbon sources (g/l)	Sucrose	20	30	30	30	30	30	30	30	30	30	30	30	30	30
Other compounds (g/l)	Ad	–	0.05	–	–	0.08	–	–	–	–	–	–	–	–	–
	MES		–	–	0.1	–	–	–	0.2	–	–	–	–		
	PVP		–	10	–	–	–	–	–	–	–	–	–		
	Glut	0.2	–	–	–	–	0.25	–	–	–	–	–	–	–	–
	CA		–	–	0.5	–	–	2	–	–	0.5	–	–	–	0.5
Agar (g/l)		10	10	10	10	10	10	10	10	10	10	10	10	10	10
pH		5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.7

Legend: MS – Murashige & Skoog medium (1962), N6 – Nitch media (Chu *et al.*, 1975), B5 – Gamborg vitamins (1968), BAP– benzyl aminopurine, NAA – alpha-naphthyl acetic acid, 2,4D – 2,4 dichlor phenoxyacetic acid, Kin – kinetin, IBA – indolyl acetic acid, AIA – beta indolyl acetic acid, 2,4,5-T – 2,4,5 trichlorophenoxy acetic acid, TDZ – thidiazuron, GA3 – gibberelic acid, PVP – polyvinyl pyrrrolidone, AC – active charcoal, Glut – glutamine; Ad – adenine; MES – morpholine ethanesulfonic acid.

Table 4

The *in vitro* reactivity of the rare species studied

	Experimental system	Variants media													
		M 1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M1 1**	**M12**	**M13**	**M14**
Species		*In vitro* reactivity													
1	*Artemisia alba* Turra	+–	–	–	–	++	–	–	–	–	–	–	+	–	–
2	*Artemisia tschernieviana* Besser	+	+	–	+–	+++	+–	–	–	++	+	+	++	+–	+
3	*Campanula carpatica* Jacq.	+–	–	–	+	+++	++	–	–	–	–	–	+–	–	–
4	*Doronicum orientale* Hoffm.	+–	–	–	–	+	–	–	–	++	–	–	+–	–	–
5	*Doronicum carpaticum* (Griseb. et Schenk) Nyman	+–	–	+–	+–	–	+–	–	–	+++	–	+	–	–	–
6	*Dianthus glacialis* subsp *gelidus (Schott,Nyman et Kotschy)*	+	+–	–	–	–	+	–	–	+++	–	–	–		
7	*Iris halophila* Pallas	–	–	–	–	–	–	–	–	–	–	++	+	–	–
8	*Gentiana lutea* L.	+	–	–	–	–	–	–	–	–	–	++	++	++	–
9	*Primula halleri* J.F. Gmelin	+	+	–	–	++	+	–	–	+++	++	–	–	–	–
10	*Veronica multifida* L. ssp *capsellicarpa* (Dubovik) A. Jelen	++	+–	+–	–	–	+++	–	–	+++	++	–	+	+	

RESULTS AND DISCUSSIONS

Except for *Dianthus glacialis* subsp *gelidus*, the other species had never been tested concerning their *in vitro* behavior in Romania.

The selection of the optimum explants and the positive response of the cultured tissues can assure the success of *in vitro* cultures. The plant species studied belong to different families, having some particularities concerning the most suitable explants, their *in vitro* reactivity and the possibility to micropropagate and to preserve them efficiently (Table 4).

In *Artemisia tscherneviana* and *Artemisia alba* species ***(Asteraceae family)***, the *in vitro* cultures were induced starting from leaves and petiole explants. A positive response was recorded on the M5 media variant added with BAP, Kinetin, NAA and adenine, the regeneration way was the direct morphogenesis (Fig. 2a). On M1, M2, M3 media variants, the regeneration way was indirect morphogenesis. The number of regenerants /explants varied between 10–20, depending on the media variant used. The M9 variant added with gibberelic acid allowed the obtaining of several plantlets regenerated from vitrified green calli. On M12-M14 variants were obtained different types of calli (tough light green, friable yellow-green). *In vitro* reactivity of *A. tscherneviana* and *A. alba* species was identical; the micropropagation protocol can be extended to other rare plant species of the same genus.

In *Campanula carpatica (Campanulaceae* family), the single node stem explants were disinfected using two different concentrations of $HgCl_2$. The M4 variant allowed the axillary shooting induction starting from the lateral meristem of buds. This species was highly reactive *in vitro* culture. On M5 and M6 media, 10 regenerants/explant by indirect morphogenesis were obtained (Fig. 2b).

In the case of *Doronicum carpaticum* and *Doronicum orientale* species, the explants prove to be susceptible to the sterilisation with $HgCl_2$, many trials being required.

For instance, both species had a low *in vitro* reactivity, possibly owing to the physiological status of the donor plants and the phase of late blossoming. Using M1 variant, there were obtained fragments of proliferative non-regenerative green calli. In the next step, these fragments were sub-cultured on different media variants. Indirect morphogenesis was obtained on M5 and M9 variants in the case of *D. carpaticum*. The regeneration rate varied between 2–5 shoots/explant (Fig. 2c). In *D. orientale*, it was induced only a green non-regenerative callus, further tests being necessary.

The *in vitro* response of *Dianthus glacialis ssp. gelidus* species was similar with the other *Caryophyllaceae* species already studied previously (Cristea, 2002, Cristea, 2004, Morgan, 1997). The best explant is single node stem fragments. The multiple axillary shoots were obtained on all the media variants with cytokinines predominance (Fig. 2d).

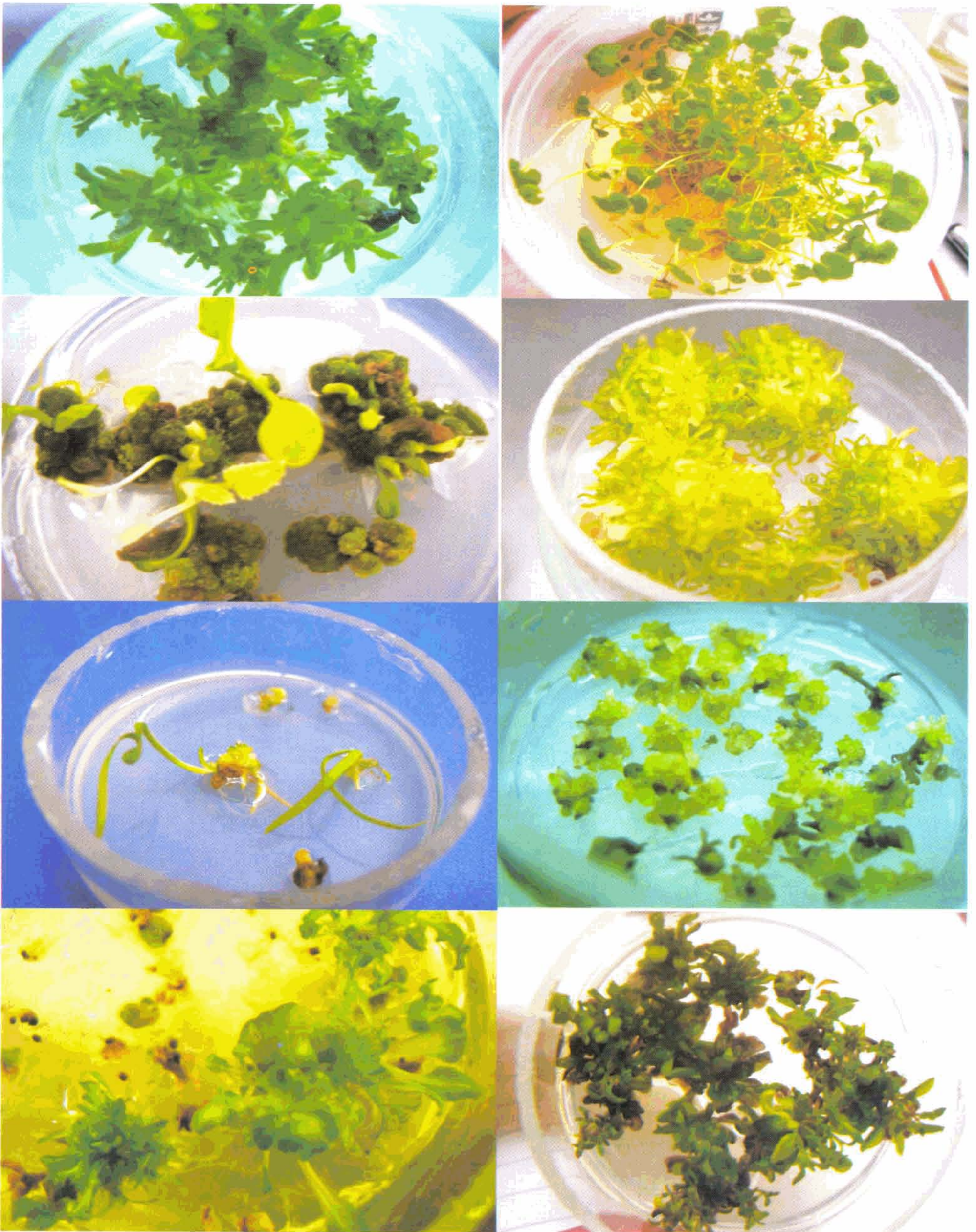

Fig. 2. a – Direct morphogenesis in *Artemisia tscherneviana*; b – Direct morphogenesis in *Campanula carpatica*; c – Indirect shoots formation in *Doronicum carpaticum*; d – Direct morphogenesis in *Dianthus glacialis* ssp. *gelidus*; e – *Iris halophila* shoots regeneration from the callus; f – Embryogenic calli in *Gentiana lutea*; g, h – Direct morphogenesis in *Primula halleri* and *Veronica multifida* ssp. *capsellicarpa*.

Gentiana lutea (Gentianaceae family) is considerred a natural monument and has valuable pharmacological properties, representing an important species of the Romanian Flora.

At international level, it has already been paid attention to micropropagation in valuable *Gentiana* species (Murashige, 1962, Zapartan, 1996). In our experiment, fragments of cotyledons, hypocotyls and roots were used as explants. These explants (hypocotyls and roots) were obtained from seeds germinated on M1 variant. M11 and M12 were the best media variants for calli formation (calli formation is very important for the synthesis of pharmaceutical compounds). M13 variant added only with an auxin (2 mg/l 2,4-D) promoted the formation of embryogenic calli, but did not sustain the embryo conversion to plants (Fig. 2e). The next step for this species is to establish an efficient plant regeneration and conservation protocol.

Iris halophila (Iridaceae family), a monocotyledonous species, had a particular *in vitro* behavior. The explants were represented by the immature zygotic embryos and endosperm excised from the immature seeds. On the media variants with auxin dominance calli and shoots were obtained. M11 was proved the best media variant used for this species. The presence of two auxins (IBA and 2,4-D) favoured the calli inductions. The plantlets obtained were successfully *ex vitro* acclimatized. The rate of regenerates/ callus did not exceed 2–3 (Fig. 2f).

In *Primula halleri* species (*Primulaceae* family), ovaries with immature seeds represented the explants cultured for 2 months on the M10 medium variant. The germinated plantlets were used as a source of explants for the multiplication protocols. Direct morphogenesis was induced on M5 variant and indirect morphogenesis on M9. The regeneration rate was very good exceeding 15–20 shoots/ cultured explant (Fig. 2g). The *ex vitro* acclimatisation of regenerants was successful.

Veronica multifida ssp. capsellicarpa (*Scrophulariaceae* family) had a very good reactivity on all tested media variants (with a different way of regeneration). The optimum explants were single node shoots and young inflorescences. The best multiplication way was multiple axillary shooting (on M6 variant). On M7 media the direct morphogenesis (Fig. 2h) and on M9 variant the indirect morphogenesis were induced. The new plantlets regenerated with a rate varied between 6–20 /explant had a simple rooting and can be easily *ex vitro* acclimatised.

CONCLUSIONS

– *In vitro* cultures were induced successfully in all species studied; it was only in *Doronicum caucasicum (orientale)* and *Gentiana lutea* that regenerants in this first step could not be obtained.

– The multiplication methods can be extended in other rare plants of the same genus.

– The micropropagated material was used to create a tissue cultures collection of rare plants in the Plant Cytobiology Department of the Institute of Biology, Romanian Academy.

– In *Gentiana lutea*, the establishment of an efficient micropropagation protocol is strongly imposed for *ex situ* conservation purpose.

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