THE *IN VITRO* REACTION OF THE *DROSERA INTERMEDIA* HAYNE SPECIES, A CRITICALLY ENDANGERED SPECIES OF ROMANIAN FLORA

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Abstract

The Drosera intermedia Hayne species presents scientific interest due to its rarity and biological specificity. It is a carnivorous species, initially enjoying a vast habitat which has been restricted, after becoming endangered, to south-eastern Europe. In Romania, it is all but extinct, with only rare elements being present in the mountain ranges, with a compact population in the Gilău Mountains (in the Western Carpathian Range). Due to it becoming Critically Endangered (CE), we have tried to conserve it via in vitro multiplication and acclimation, ecologically reconstructing its presence in its original habitat. The vegetal material we used consists of juvenile bud of Drosera intermedia Hayne, harvested in the Gilău Mountains, which was cultivated in vitro on a base medium after Murashige-Skoog, 1962, with the variants and their effects as presented in the table below (see table 2):

V_o = MS1/2, weak regeneration, no multiplication (xx);
V_1= MS+0,1mg/lAIB+2mg/lBA, very good regeneration and multiplication (xxxxxx);
V_2 = MS+0,1mg/lAIB+5mg/lBA satisfactory multiplication (xxxx) + 1mm callus Ø;
***V_3** = MS+0,1mg/lAIB+2mg/lZ, exceptional regeneration and multiplication (xxxxxxx);*
V_4 = MS+0,1mg/lAIB+5mg/lZ, satisfactory multiplication + 2cm embryogenic callus Ø;
***V_5** = MS +0,1mg/lAIB+5g/lGP, very good multiplication (xxxxxx);*
V_6 = MS +0,1mg/lAIB+2mg/lBA+5g/lGP, 2 cm embryogenic callus Ø);
V_7 = MS+0,1mg/lAIB+2mg/lZ+5g/lGP, 4-5cmØ regenerative callus, 1-2 plants.

After 2-3 months of in vitro culture, the bud regenerated (forming completely organized plants), multiplied or differentiated callus according to the composition of the culturing medium. The presence of cytokinins in a lower dosage 2mg/l Zeatine (V_3) and benzylaminopurine-BA (V_1) stimulated the regeneration and multiplication of plants: 57 neoplantules/bud on V_5, completely conformed (leaf rosette of 40-22mmØ and 2-8 inflorescences) and rooted (circa 7-4 roots of circa 3cm). A high dose of cytokinin (5mg/l) led to the satisfactory stimulation of multiplication, but also callus production (V_2 and V_4). The corn germ extract +0,1mg/l AIB stimulated very good multiplication (V_5), approx. 46 neoplantules/bud, but in combination with 2mg/BA or Z (V_6 and V_7), it determined the formation of embryogenic callus, and in the case of V_7, even regenerative callus of a few centimeters in diameter, resulting in 2-3 neoplantules from the embryoids on the callus mass. We can conclude that the 2mg/l dose of cytokinins and 0,1 mg/l auxine stimulates regeneration, multiplication and obtainment of completely conformed and adaptable plants, the same as variant V_5 with 5g/l GP, and the 5 mg/l Z or BA + auxine dose stimulates the differentiation of a mass of embryogenic callus of approx. 2-5cmØ, most often regenerative. We recommend the in vitro multiplication of the species, from floral buds cultivated on media with moderate doses of cytokinins, small doses of auxines and an additive of natural corn germ extract, which we consider to be a favorable medium.

Key words: *Drosera intermedia* Hayne, sozological category CE (critically endangered), red list of plants, conservation, *in vitro*, regeneration, multiplication, floral bud, Murashige-Skoog medium, regenerative embryogenic callus, embryoids.

INTRODUCTION

Drosera intermedia Hayne, synonymous with *Drosera longifolia* auct., is a critically endangered (CE) species from a sozological perspective; taxonomically, it is a perennial plant, with rosulate leaves, with a lateral scalp longer than the 2-3 cm long leaves, with 3-7 ornamental flowers, with white petals (Flora RSR, 1955), obovate leaf lamina, glabrous petiole of approx. 3-4 cm (Fig. 1), the fruit is a capsule with 3-4 longitudinal trenches (Dihorul, Negreanu, 2009).

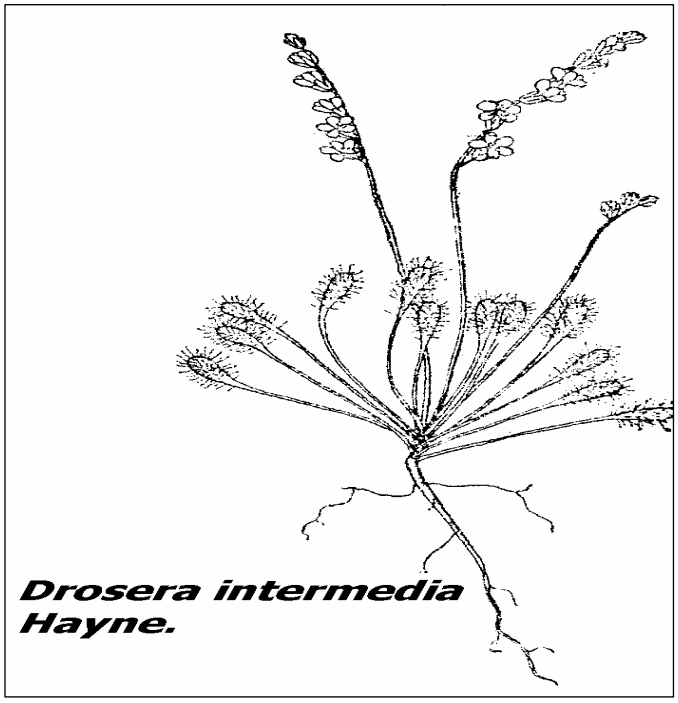

Fig. 1. *Drosera intermedia* Hayne (syn. Drosera longifolia)

It presents scientific interest due to its rarity and biological specificity, being a carnivorous species. Chronologically, its areal expands across the Cluj and Alba counties. It is certainly encountered, during the last 10-15 years, in the Gilău Mountains, Mare Mountain and at the Someșului Valley Springs (Boşcaiu et al., 1994). It is considered a glacial relict, with a habitat consisting of marshlands of oligotrophic peat, in association with species of Vaccinium, Carex etc. (Dihoru, Dihoru, 1994). As a limitative factor, its areal is restricted to North America and Europe (at the south-eastern limit of the European areal). It is now extinct in many mountain ranges in our country, such as the forests surrounding the city of Mediaș (Ciocârlan, 1988), yet it is still encountered in concentrated populations in the Gilău Mountains (Fig. 2), in the Cluj and Alba counties (Täuber, 1976),

an area in which rare and endemic flora elements are studies are being studied based on the relation between their classical protection (Cristea et al., 1996) versus unconventional conservation methods, such as *ex situ* micro-propagation (Cristea et al., 2004). At the debut of endangered species conservation efforts, Natural Reservations played an important role (Olteanu et al., 1994), as these species are protected either as germplasm (Delectus seminum quae… 1927-1991), or as fully grown plants, in botanical gardens (Maunder, Higgens, 1998). Conservation consisted of germplasm sampling and storing in gene banks (Blându, Holobiuc, 2008), or cryoconservation experiments in liquid nitrogen, starting from tissue, neo-plantules and callus obtained *in vitro* (Halámgyi, Butiuc-Keul, 2007).

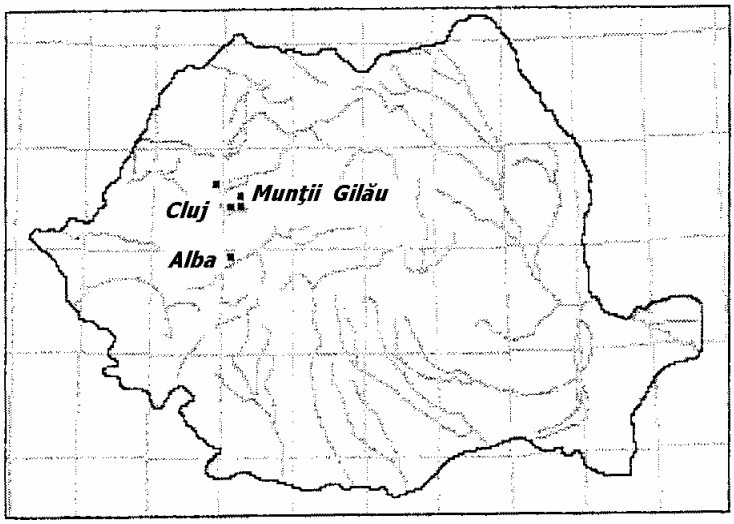

Fig. 2. Prevalence areal in Romania for Drosera intermedia Hayne species (Dihorul, Negreanu, 2009)

The studies connected to the conservation of endangered wild species are reported starting from the last two decades of the previous century (De Langhe, 1984), highlighting the role and advantages of the *in vitro* conservation technique. Subsequently, the studies become more extensive, tacking even issues connected to the management of the *in vitro* micro-propagation technique of rare and endangered plants (Frausworth, Sahotra, 2007). Our collective's preoccupations refer to *in vitro* conservation methods of germplasm (Laslo et al., 2011a), as well as unconventional methods conservation of rare and endangered elements of the Romanian spontaneous flora (Zăpârțan, 1994, 1996), and also finding the most advantageous methods of *in vitro* multiplication and acclimation of new plants (Laslo et al., 2011b).

The Drosera species in the natural space of our country are supervised and studied with interest. Thus, it was established that *Drosera rotundifolia* can be considered a "*bio-test plant*" for *in vitro* cultures (Cachiță, Zăpârțan, 1991), many of the species of the Drosera genus being researched from the perspective of the ratio between regeneration, multiplication and acclimation capacity of new plantules obtained *in vitro* in the original habitat of the species or of the individuals from which explants were harvested (Zăpârțan, 2001).

MATERIAL AND METHODS

The *purpose* of this paper was the conservation of the *Drosera intermedia* Hayne species, using *juvenile bud* as *explant*, harvested from plants in the Gilău Mountains (The Western Carpathian Range), which after sterilization were cultivated on medium variants specified in table 1, and kept under growing room conditions.

Table 1

Medium variants for the *in vitro* cultivation of *Drosera intermedia Hayne*
(MS = Murashige-Skoog medium; AIB = β indolyl-butyric acid; Z = zeatine;
BA = benzilaminopurine; GP = corn germ extract)

Var.	Mediu de bază	AIB (mg/l)	Citochinine BA (mg/l)	Z	GP g/l	Bonificare
V_o	MS1/2	-	-	-	-	xx
V_1	MS	0,1	2	-	-	xxxxxx
V_2	MS	0,1	5	-	-	xxxx + callus
V_3	**MS**	**0,1**	**-**	**2**	**-**	**xxxxxxx**
V_4	MS	0,1	-	5	-	xxx + callus
V_5	**MS**	**0,1**	**-**	**-**	**5 g/l**	**xxxxxx**
V_6	MS	0,1	2	-	5 g/l	Embryogenic callus
V_7	MS	0,1	-	2	5 g/l	Regenerative embryogenic callus

In the preparation of variants, the MS (Murashige, Skoog, 1962) base medium was used, with different hormonal formulas, from simple mediums MS1/2 (V_o), to mediums with large and very large doses of cytokinins in combination with a small concentration of auxine (V_1 –V_4), also experimenting with the natural corn germ extract, alone or in different combinations (V_5 – V_7). Throughout our experiments, the addition of the corn germ extract proved to be advantageous for the *in vitro* culturing of numerous plant species for different purposes; we will only recount the effect on the *in vitro* tuberification of some potato variants which were cultivated *in vitro* (Agud, 2011), when used either on its own (for the substitution of cytokinins), or in combination with other phytohormones.

RESULTS AND DISCUSSION

After 2-3 months of cultivating the Drosera bud *in vitro,* on the previously mentioned mediums, observations were made on the number of plantules differentiated from a single bud, the diameter of the leaf rosette, the value of the root system, the differentiation of flowers and callus tissue (see Table 2).

Table 2

Average values of analyzed parameters for *Drosera intermedia Hayne, in vitro* cultivation (after 2-3 months)

Var.	Nr.pl/ expl.	Ø rosett fr.(mm)	Nr. rot.	Lung. r.(cm)	Nr. inflores. /expl(3luni)	Evaluation/ notes
V_o	1	10	1	0,2	-	xx
V_1	30	22	4	3,0	2	xxxxxx
V_2	17	18	2	1,0-1,5	4	xxxx + callus: Ø 1cm, hard, with 2 embryoids
V_3	**57**	**38-40**	**7,5**	**3,4**	**8**	**xxxxxxx**
V_4	25	20	2,5	1,8	4	xxx callus Ø 2cm, olive green, friable, 3-4 embryoids
V_5	**46**	**35**	**4,0**	**3,4**	**4**	**xxxxxx**
V_6	-	-	-	-	-	Embryogenic callus Ø 2 cm
V_7	**-**	**-**	**-**	**-**	**-**	**regenerative callus Ø 4-5 cm, olive green, with 2-3 plantules**

Analyzing the data in table 2, we can remark the evolution of the Drosera bud and the differences between variants. The best values from an *in vitro* multiplication standpoint were obtained on V_3 with 2mg/l Z and 0,1mg/AIB: approx. 57 neo-plantules/bud, with a large leaf rosette 38-40mmØ, approx. 8 inflorescences and a good root system (an average of 7 roots of approx. 3.5 cm in length). Next is V_5 which proves to be a good combination for multiplication (0,1 mg/lAIB+5g/l GP). The average number of neo-plantules/explant of Drosera regenerated *in vitro* is shown in fig. 3, in which the values of variants V_3 and V_5 are obviously distinguished from an *in vitro* species multiplication standpoint. The number of inflorescences/ neo-plantule is highest on the variants which encouraged the best multiplication as well (see Fig. 3) (8-4 inflorescences after 3 months).

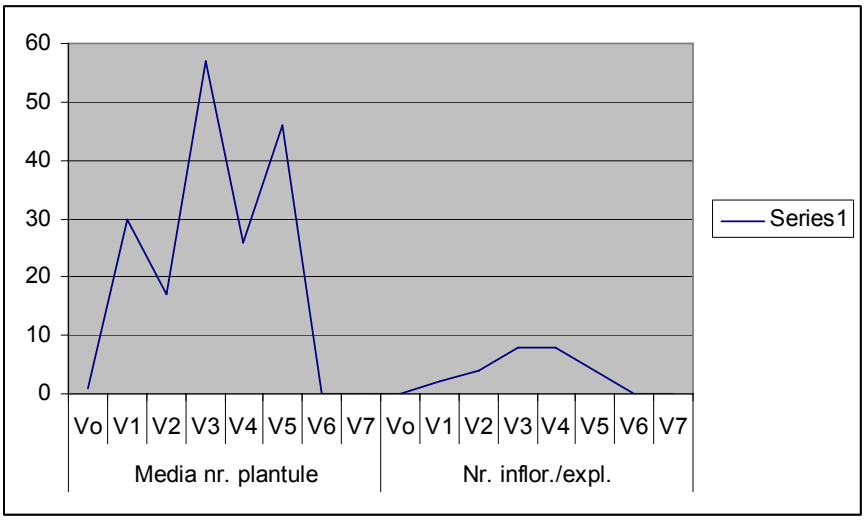

Fig. 3. Average plant number (after 2 months) and inflorescences/explant (after 3 months), regenerated *in vitro* by *Drosera intermedia* Hayne

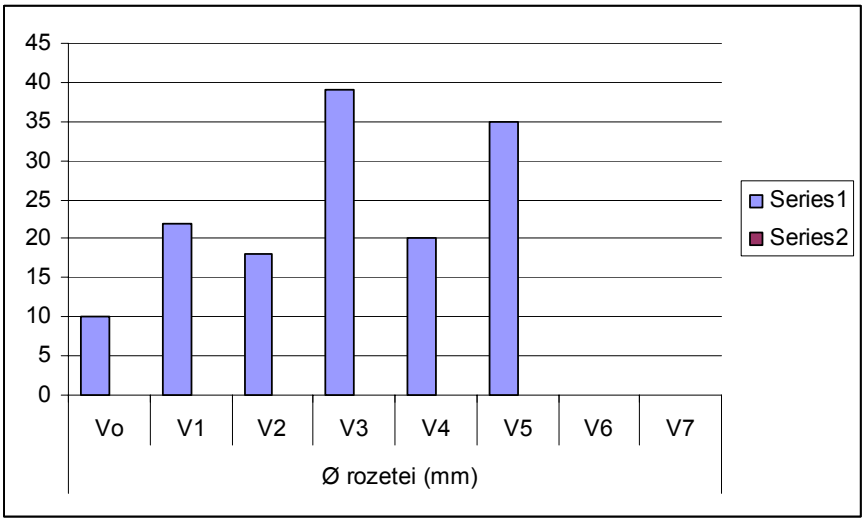

Fig. 4. Leaf rosette size (Ø cm), formed by *Drosera intermedia* Hayne plantules (after 3 months)

The diameter of the leaf rosette, a decisive elements for a complete conformation of the plant, was measured after three months of *in* vitro cultivation, when water pustules specific to carnivores appeared on the leaves, after which we moved on to *ex vitro* acclimation of the new plantules. Fig. 4 shows the diameter of the leaf rosette (mm), and yet again, in this case V_3 (MS+0,1mg/lAIB+2mg/lZ) and V_5 (MS +0,1mg/lAIB+5g/lGP) proved to be the best media.

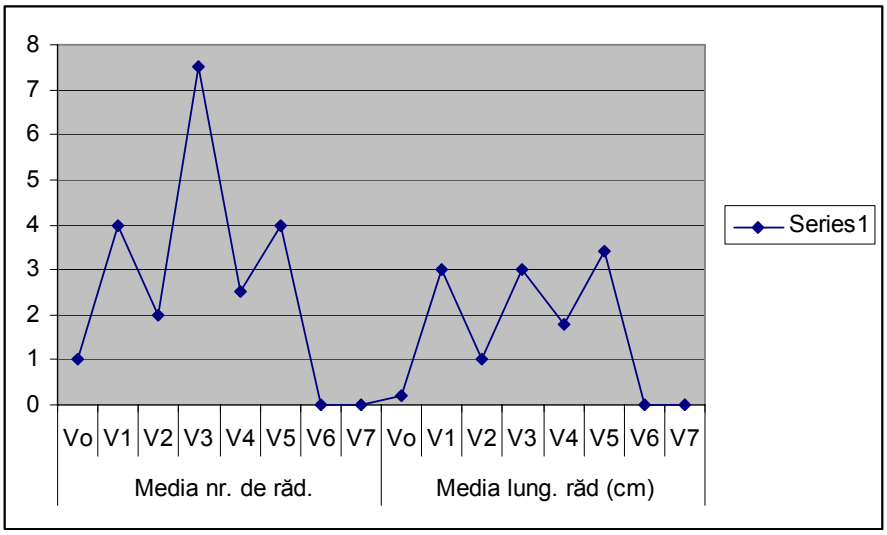

Fig. 5. The value of *in vitro* generated Drosera neo-plantule root systems

The acclimation of new *Drosera intermedia* Hayne plantules depends on the value of their *in vitro* differentiated root system, evolution which is shown in fig. 5, from which we highlight the superior value of the average number of roots, of their length and thickness, still on the V_3 (MS+0,1mg/lAIB+2mg/lZ) and V_5 (MS +0,1mg/lAIB+5g/lGP) media.

CONCLUSIONS

In regards to the general evolution of bud explants from *Drosera intermedia* Hayne, on the experimental media, we can conclude the following:

1. On V_o- witness (MS1/2), we register regeneration, but poor evolution (xx);

2. On (MS+0,1mg/lAIB+2mg/lBA), the explant manifests very good *in vitro* micro-multiplication reaction (xxxxxx), generating approx. 30 neo-plantules/bud, with a 22mmØ rosette and appropriate root system;

3. On V_2 (MS+0,1mg/lAIB+**5**mg/lBA), multiplication is weaker (xxxx) but we register the differentiation of a *mass of callus* (1cmØ with embryoids);

4. On V_3 (MS+0,1mg/lAIB+2mg/lZ), we register very good multiplication, approx. 57 neo-plantules, with a 40mmØ leaf rosette, very good root system and approx. 8 inflorescences: *this is the best and most advantageous culturing medium formula for the in vitro multiplication of Drosera intermedia species;*

5. On V_4 (MS+0,1mg/lAIB+**5**mg/lZ), multiplication is average (xxx), but embryogenic callus is formed (approx. 2cmØ, with 3-4

embryoids): *it seems that a high concentration of zeatine, next to an auxine, has a double effect – micro-propagation (weaker) and formation of regenerative callus;*

Fig. 6-7. *In vitro* regeneration and multiplication of drosera plants

6. On V_5 (MS+0,1mg/lAIB+**5**g/lGP), good multiplication is favored, approx. 46 neo-plantules/bud, completely conformed and perfectly adaptable to *ex vitro* conditions;

7. On V_6 (MS+0,1mg/lAIB+2mg/lBA+5mg/lGP), we do not register multiplication, only a mass of callus of 2 cm Ø, which does not prove to be regenerative;

8. On V_7 (MS+0,1mg/lAIB+**2**mg/lZ+5g/l GP), the bud does not multiply, only generating embryogenic callus of 4-5cmØ, out of which 2-3 plantules differentiate: *ideal formula for obtaining regenerative embryogenic callus*.

For the multiplication of the *Drosera intermendia* species, we recommend a medium with a dose of 2 mg/lZ + 0,1 AIB (V_3) or 5g/l GP, (cost-effective medium, due to the fact that is substitutes the cytokinins in the medium), and for the obtainment of embryogenic callus, we recommend Zeatine + 0,1mg/lAIB or in combination with Corn Extract (V_7).

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