IN VITRO CONSERVATION AND MULTIPLICATION OF *DIANTHUS SPICULIFOLIUS* SPECIA DERIVING FROM THE PROTECTED AREAS OF VALEA GALBENEI AND PIATRA BULZULUI, SPECIE CRITICALLY ENDANGERED (CR)

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Abstract

In vitro culture of the rare and endangered species ensures their multiplication and the repopulation of the areas and habitats from where these species come. The specie that was the object of research in this experimental study - Dianthus spiculifolius Schur. (Fam. Caryophyllaceae) - comes from the protected areas of Valea Galbenei and Piatra Bulzului, community sites from Bihor, Romania, sozological, specie critically endangered (CR). The purpose of the experiment was the conservation and the protection of the dianthus (by its popular name) through in vitro multiplication of the specie from the spontaneous flora of Romania, with the following coordinates: floral rarity of scientific importance, Dacian-Pontic endemit, with a restricted area and poor populations, but also a decorative plant located in a few sites from PNMA (from Bihor, Cluj and Alba Counties). The multiple copies derive from the recalled sites having the sozological status of critically endangered plant (according to IUCN, 2006).

The research have proven that the rare spontaneous species, endangered and vulnerable lend themselves at the ex situ multiplication (in this case in vitro), with the purpose of their conservation and extension in their origin areas and in the landscape spaces, based on the ornamental value of the specie. The success of the culture depends on the nature of the specie, on its capacity to adapt to the in vitro conditions and on the capacity of the tissue to rerun the metabolism.

The regenerative capacity of the tissue is dependent on the nature, the provenience and on the age of the explanted tissue (explant) and also on the composition of the culture mediums conceived for the in vitro culture. In vitro multiplication of Dianthus spiculifolius specie was done starting from the ***apical tissue (apex)*** *detached from the mature plants and inoculated on mediums in March – April, being determined by the presence of a cytokinin in the medium (Z of BA) in a moderate dose (1mg/l) and of a small dose of auxin (AIB-0,5mg/l). The regeneration capacity on the mediums with the recalled composition (V_2 and V_3) can reach up to 68-98%, and the number of plantlets per explant to an average of 21-28 neoplantlet/explant. The Radicular System formed reaches to an average of 23/25 roots/neoplantlet (on the same variants) with a very good multiplication percentage of 69-100%. Ex vitro acclimatization is ensured by the vigorousness of the Radicular System and by the complete organization of the neoplantlets and it can reach a relatively good percentage, of 44-52%, depending on the capacity of the specie to differentiate vigorous and healthy roots, which leads to a good percentage of survival of the neoplantlets obtained in vitro in free conditions (ex vitro), hence ensuring the ecological reconstruction of the protected area from where the specie derives.*

Keywords: protected areas, conservation, biodiversity, species critically endangered (CR), ex situ, in situ, red list, red book, *Dianthus spiculifolius L., Schur. var. Transilvanicus*, in vitro multiplication, ex vitro acclimatization, ecological reconstruction.

INTRODUCTION

Environmental factors action over the plants and make so that they survive and mutually balance, but if such a factor becomes predominant, it can put advantage on some species and populations and it can disadvantage others, hence some species become rare and vulnerable, amplifying the interest of the researchers for their protection. The number of rare species from Romania is big (due to the continuous area), some of them being at the limit of the area (Cristea, et al. 1996). The action of conserving the species of plants which are in danger is of interest for the conservatorist specialists from other fields, as it is our case, in the field of vegetal biotechnologies (Cachiță, and Ardeleanu, 2009). By the Bern Convention (1979) there were taken measures of preventing some dangers which lead to the extinction of the species of plants and some concerning the development of some gene banks (Bavaru, et al., 2007). According to IUCN, 2006, the rhythm of disappearance of the plant species on the globe is of 100 up to 1000 times more intense, man and his activity being the main cause of their extinction, one out of 8 species of plants is threatened with extinction, and a percentage comprised between 20-40% from the world flora is falling into decline (Farusworth, E. and Sahotra S. 2007). The "red lists" and the "red books" (Olteanu et all., 1994; Boșcaiu,, et al., 1994; Dihoru and Negrean, 2009) comprise the endangered species from the entire country, and their conservation implies monitoring-protection plans of the habitats where these species can be found (Cristea, 2006: Domuța, (coord. 2013), recovery plans even of a single endangered specie. The ex situ conservation activity (Halmagyi, and Butiuc-Keul, 2007) of these species through unconventional methods, for example in vitro, presents a great interest and has a future (Engelman, 1997), being experimented at the horticultural species and also at the ones form the spontaneous flora. In vitro multiplication method has more advantages only if we recall the fact that for obtaining a few hundreds of samples we should start from one single plant (Bajaj Y., 1986), a seed, a single explant (the top of the sprout, floral bud, part of the leaf and stem, etc.), an element considered essential because rare or vulnerable plants are in a small number, so this way their origin place will not be endangered (Zăpârțan, 1996). The techniques of in vitro multiplication at the rare, endangered and endemic species from Romania, for their conservation were applied at a great number of species (Zăpârțan, 2001: Cristea at al., 2004; Laslo et al., 2011a; 2011b), and the implications of the vegetal biotechnologies proved their efficiency at the species which are multiplying through the classical method with difficulty (Laslo, 2013). The field of in vitro multiplication was also extended to the autotroph cultures, to the rare,

endemic (Cristea, et. al. 2010) taxons of Dianthus, also threatened with extinction.

Dianthus spiculifolius Schur. Specie was signaled in the Romanian flora in 1953 by the botanist Iuliu Prodan, and at the present moment it can be found sporadically along the Someş and other rivers, and also in a few points from Transylvania (PNMA), the most sites where the specie was signaled being in Bihor (Valea Galbenei and Valea Sighiştelului sites, and the site called Piatra Bulzului, from Bihor County); and also in Alba and Cluj Counties (Coldea, Ghe. et all., 2008). The specie is a geoelement with a sozological status of critically endangered plant, with a scientific importance because it is a *Dacian-Pontic endemit with a restricted area and with very poor populations*, a limiting factor being the area with very poor populations, protected where it is found in reservations (e.g. Râpa Roşie) and conserved ex situ, in botanical gardens or the germoplasma in the gene banks. According to the Romanian law[1], *Dianthus spiculifolius* specie is found in saxicol associations, in the sites form Bihor County as rare specie but also of national interest (Olteanu, M., et all., 1994) and in different grassy cenosis where the specie is also rare and lives in associations with other species (Coldea, et all., 2008).

The habitat of the specie is of a community interest (Natura 2000), formed of alpine and subalpine calcified meadows, with small surfaces, conserved in its actual (in situ) form, through periodical monitoring of the perimeter of the site, applying a management related to the vulnerability of the specie at the anthropic pressure, through uncontrolled tourism and unreasonable economic exploitation of the area[2].

MATERIAL AND METHODS

The vegetal material used for the in vitro multiplication of *Dianthus spiculifolius* specie was ***meristematic tissue – apex of about 0,5 mm***, and the *culture mediums* were conceived in a balanced way, with the basal medium according to Murashige – Skoog, from which there were formed three variants with hormones (an auxin and a cytokinin) in a concentration of 0,5 – 1,0 mg/l (Table 1). It was followed the *evolution of the explants* after about 40 – 50 days of *in vitro* culture: concerning the percentage of regeneration, multiplication, rooting and acclimatization, and also the number of differentiated plantlets/explant. The time of the year when the experiment was initiated was March – April, and the duration of the

[1] Law no. 5/2000 concerning the approval of the arrangement plan of the national territory
[2] Lista Roşie (Red List) UICN (http://www.iucnredlist.org)

incubation of the in vitro tissues was of 30-40 days. The vegetal material was harvested from the protected area with great care in order not to affect the specie as it was in a small number, occasionally present and in poor populations and it was harvested only from the places where the species were in a larger quantity, a single sample only (a single plant) which was planted in flowerpot in a cold greenhouse (considered mother plant donor of explants). It could have also been applied the sampling of a single inflorescence or sprout (from the plant from that particular area) from which it was incised a young bud or apex, without affecting the plant, operation that must be done in the day of the experiment or a day before, for maintaining the properties of the tissue (Laslo, V., et all., 2011a). In order to sterilize the material, the apex of *Dianthus* was detached and was inoculated on a medium according to Murashige – Skoog, 1962, with the conceived variants: the witness variant and the variants with a hormonal in addition and supplemented with other substances specified in Table 1.

Table 1

Medium formulas used for the in vitro multiplication of Dianthus specie from Şes Mountain area (**AIB** = indolyl butyric acid***; BA*** = benzyl adenine; **Z** = Zeatin)

SPECIE	**Var.**	**MB**	**AIB mg/l**	**BA mg/l**	**Z mg/l**	**Additional additives gr./l or mg/l**
Dianthus	V_0	MS	-	-	-	-
	V_1	MS	-	-	-	3g/l vegetal coal (CV)
	V_2	MS	0.5	1.0	-	825mg/l NH_4NO_3
	V_3	MS	0.5	-	1.0	-

Some endangered species from the area of Bihor and from the surrounding areas were successfully conserved in vitro; hence we recall rare species from Piatra Craiului Mountain, (Blându, R., andI. Holobiuc., 2007), rare, endangered, vulnerable and endemic Dianthus species from Gilău, Muntele Mare Mountain from PNMA (Cristea, V., et all., 2004a and 2004b). After the inoculation on the aseptic mediums, the explants were kept within the conditions of the growth chamber, at a luminous intensity of 16 hours light out of 24 hours, at a temperature of 26^0C and a humidity of 80%. The light and intensity varies depending on the followed purpose and on the nature of the specie (fluorescent diffuse light with an intensity of 2-10 klux, depending on the stage of development of the neoplantlet), a certain type of light and a certain intensity are necessary for inducing the organogenesis of the innoculous. For the species that need a period of incubation, another regime of light, temperature and humidity, there are used climatized closets capable of ensuring the conditions of temperature and of photoperiod desired or that are necessary for the specie.

RESULTS AND DISCUSSION

The phials with the inoculated tissue were kept in the conditions of the growth chamber, and after 40 and respectively 50 days there were made measurements and observations concerning: the percentage of regeneration, rooting, multiplication and acclimatization of the neoplantlets which came from the apex of Dianthus, and also the average number of regenerated neoplantlets from each explant. Table 2 encompasses the values of the parameters observed (the average number of differentiated plantlets, and also the percentage of regeneration, multiplication and acclimatization).

Table 2

The values of the parameters analyzed at the three species cultivated in vitro (after 40-50 days)

Experimented specie/ explant	Var.	Reg. %	No. pl./ expl.	Root. %	Multi.%	Acclim. %
Dianthus spiculifolius - apex	V_0	11	2	10	20	20
	V_1	54	16	20	38	25
	V_2	68	21	23	60	44
	V_3	**98**	**28**	**25**	**100**	**52**

The process of in vitro regeneration of the tissue detached from *Dianthus spiculifolius* specie follows the natural biological cycle of the specie. The capacity of in vitro regeneration is influenced by the conditions of culture, and also by the time of the year when the experiment takes place and by the nature of the specie. The favorable time for the classical multiplication of the plants is in early spring for the majority of the perennial or annual species and also in late autumn (at the end of November) for the ones that multiply from the bulbs (Encyclopédie universelle, 1999). The same recalled periods of time also proved to be favorable for the in vitro culture (Zăpârțan, M., 2001). The evolution of the apical tissue (apex) of *Dianthus spiculisolius* is based on the capacity of this tissue to regenerate in vitro in a percentage of 98%, in a medium with a content of cytokinin, in the case of this experiment with a content of Zeatin (Z), under these conditions multiplication is also in a very good percentage, of 98% even up to 100%.

Figure 1 presents in a comparative manner the capacity of regeneration and the one of multiplication, after about 45-50 days of in vitro culture. We can see the high values on the mediums with cytokinins (V_2 and V_3) and even within those variants, we signalize some differences depending on the nature of the cytokinin (the values are different in the presence of benzyl adenine towards Zeatin, on the medium with Zeatin the values are superior). Hence, in the presence of Zeatin (V_3) the percentage of

regeneration is of 98% and of acclimatization of even 100%, results that justify our recommendation for using a cytokinin in the medium in moderate doses (of 1,0mg/l). Following Table 2 and also Figures 1 and 2, we can see that on all the variants with phytohormones takes place a good regeneration and multiplication, the differences in the evolution being determined by the dose and the nature of the phytohormone.

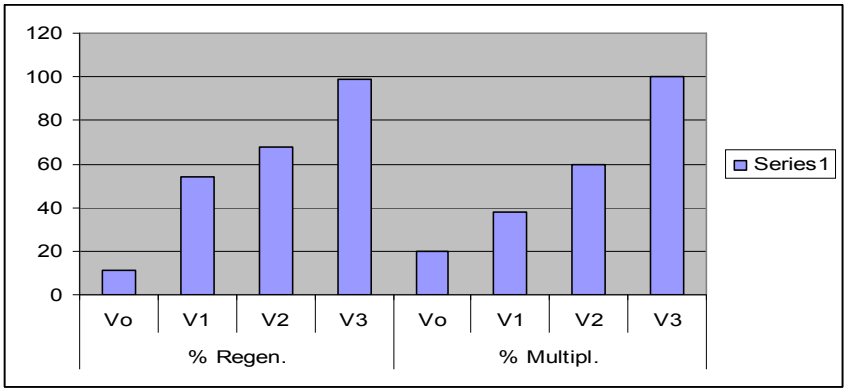

Fig. 1 The capacity of regeneration, multiplication and acclimatization (%) of the apex of *Dianthus spiculifolius* (after about 50 days)

Acclimatization to the ex vitro conditions is analyzed in relation to the value of the Radicular System differentiated in vitro, being known the fact that, the more vigorous the Radicular System, the greater the percentage of acclimatization. *The best rooting percentage* of 23% and respectively of 25% takes place on the variants with cytokinins (V_2 and V_3) and in the presence of auxin, which also bring with them the best acclimatization percentage of over 50%, suggestively presented in Figure 2. Following the figure we can see a directly proportional relationship between the presence of the auxin AIB - 0,5mg/l (the auxin being involved in the formation of the roots) and the value of the Radicular System. The presence and the concentration of cytokinins, associated with the conditions of formation of a vigorous Radicular System, an auxin, leads to a superior acclimatization percentage, an aspect also attested by other experiments of in vitro cultivation of *Dianthuis spiculifolius* specie (Zăpârțan, M., 1995: Agud E., 2014), and even to other species of economic (Agud, E., 2011) or ornamental (Fay, M. F., 1992; Laslo, V., 2013) importance.

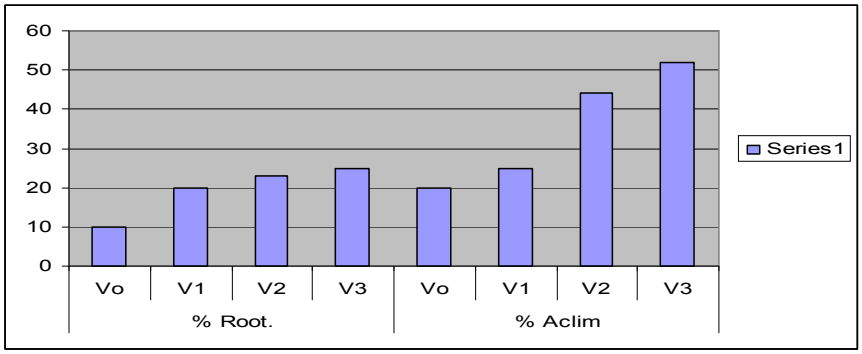

Fig. 2 The evolution of the Radicular System with its acclimatization capacity (%) of the neoplantlets of *Dianthus spiculifolius*

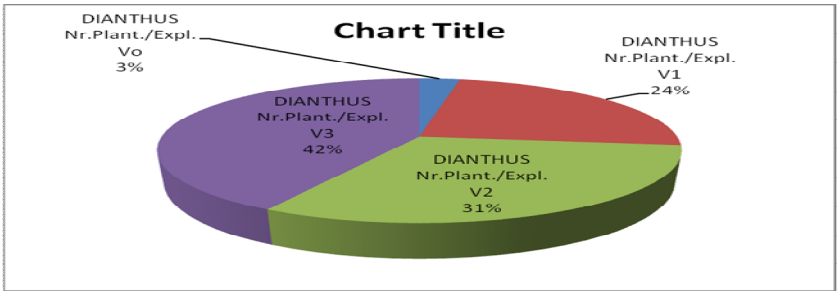

Fig. 3 The average number of Dianthus spiculifolius plants differentiated from the apex (after about 50 days)

The average number of plantlets differentiated from an explant is comprised between 16 – 28 plantlets/apex (Fig. 3), and to this specie, the number of neoplantlets depends on the presence or on the absence of cytokinins (on the used medium formula).

CONCLUSIONS AND RECOMENDATIONS:

Conservation through in vitro multiplication of the species from the spontaneous flora ensures the obtaining of a great number of plants in a relatively short period of time, completely conformed and phenotypically and genotypically identical with the mother plant from which the tissue was sampled. The advantage of the method consists in the fact that through the initiation of the culture there can be used a single explant (a single mother plant, a seed, a leaf, a meristem, a cell, etc.), without being affected the plants from the nature.

1. There must be taken into consideration the totipotency of the vegetal cell, which depends on the age of the explant (the younger the donor tissue, the greater the totipotency), the fact that each organ (seed or part of the plant: root, leaf, etc.) has its own regeneration and multiplication capacity.

2. It is very important to establish the area of origin of the specie that we desire to multiply in vitro and it must be kept the genetic variability of the population. The sozological category in which the specie can be placed, the chronology and the area of spreading of the specie ensure the success of the reconstruction of the habitat of origin or of the architectural landscape.

3. The success of the in vitro culture depends on the specie, on its capacity to adapt to the in vitro conditions and on its capacity to rerun the metabolism: the nature, the provenance and the age of the explant, the regenerative capacity of the tissue and the time of the year in which the culture is initiated.

4. The climatic factors from the growth chamber and their intensity: light, temperature, humidity, must be adjusted depending on the demands of the specie.

5. The final stage of the acclimatization process ex vitro is ensured by the value of organization of the neoplantlets, on their capacity to differentiate a vigorous Radicular System, on running over the intermediary stages which determine a better percentage of survival.

6. In vitro multiplication of *Dianthus spiculifolius* specie is determined by the presence in the culture medium of the Z and BA in a moderate dose (1mg/l) or maybe even greater and of a small dose of auxin (AIB-0,5mg/l).

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REFERENCES

1. Agud, E., 2014, "Vulnerable and protected endemic species from the protected areas of Bihor County. Their conservation through in vitro multiplication", Internati. Symp. "Risk factors for environment and food safety" & "Natural resources and sustainable development", în: Analele Univ. Oradea, Fascicula: Protecția Mediului,vol.XXIII, Ed. Univ. Oradea; pp.553-565

2. Bajaj, Y., 1986, *In vitro* preservation of genetic resources, IAEA-SM-282/66 Vienna;

3. Bavaru A., Godeanu S., Butnaru G şi Bogdan A., 2007, Diversitatea şi ocrotirea naturii, Editura Academiei Române Bucureşti;

4. Blându, R., I. Holobiuc, 2007, Contributions ti *ex situ* conservation of rare plants from Piatra Craiului massif using biotechnology, Conference Proceedings The 1st International Conferance Environment – Natural Sciences – Food Industrx in European Context Ensfi 2007, 1st edition, p. 483-788

5. Boşcaiu, N., Coldea G., Horeanu C, 1994, Lista roşie a plantelor vasculare dispărute, periclitate, vulnerabile şi rare din Flora României. Ocrot. Nat. Med. Înconj., 38 (1), Buc.;

6. Cachiță., D.. Ardeleanu, A., 2009, Tratat de biotehnologie vegetală, Vol. II., Editura Dacia, Cluj – Napoca;

7. Coldea, Ghe., Fărcaş, S., Ciobanu, M., Hurdu, B., Ursu, T., 2008, Diversitatea floristică şi fitocenotică a principalelor situri protejate din Parcul Natural Apuseni;

8. Cristea M., 2006, Biodiversitatea, Ed. CERES, Bucureşti ;

9. Cristea, Victoria., M. Miclăuş, M. Puşcaş., C. Deliu, 2004a, Conservative micropropagation of some endemic or rare species from the Dianthus L. genus. In: In vitro Cult. and Hortic. Breeding, Fifth IV CHB Symposium Biotehnology, as therorz and Practice in Horticulture, 2004, p. 3-13;

10. Cristea, Victoria., M. Miclăuş, M. Puşcaş., C. Deliu, A. Halmagyi., 2004b, The micropropagation of some endemic or rare taxa from Gilău, M-tele Mare massif. Contrib. Bot., XXXIX, Cluj – Napoca, p. 201-209;

11. Cristea, V., S. Denaeyer., J.P. Herremans., I, Goia., 1996, Ocrotirea naturii şi Protecția Mediului în România, Ed. University Press., Cluj – Napoca, p. 36

12. Dihoru, Ghe., Negreabu, G, 2009, Cartea roşie a plantelor vasculare din România, Editura Academiei Române

13. Domuța, C. (coord.), Brejea, R., Şandor, Maria, Domuța, Cr., Borza, Ioana, Timar, A., Vuşcan., A., Ciobanu, Gh., Csep, N., Ciobanu, Cornelia., Bucureanu, Elena, Zăpârțan, Maria, Curilă, M., Romocea, Tamara, Sarca, Gh., Laslo, V., Pantea, Emilia, Samuel, Alina, Oneț, Aurelia, Oneț, C., Şerban, Eugenia, Costea, Monica, Gîtea, M., Agud, Eliza**,** Bodog, Marinela, Jude, E., Cozma, Alina (2013), "*Monitoringul mediului: lucrări practice",* Ed. Universității din Oradea;

14. Engelman F., 1997, In vitro conservation methods, in: Callow, J. A., Ford-Lloyd, B., Newbury, H. J., (eds.) Biotechnology and Plant genetic Resources, 119-161;

15. Farusworth, Elizabeth (Lead Author), Sahotra Sarkar (Topic Editor). 2007, Conservation and management of rare plant species. in: Encyclopedia of Earth. Eds. Culter J. Cleveland (Washington, D.C., Environmental Information Coalition, National Council for Science and the Environment).[Published in the Encyclopedia of Earth Ahgust 28, 2007; Retrieved January 16, 2008] :

16. Fay, M. F., 1992, Conservation of rare and endangeres plant using in vitro methods, In vitro Cell. Dev. Biol., 28P;

17. Halmagyi, A., Butiuc-Keul, A., 2007, Conservarea resurselor genetice vegetale, Ed. Tedesco, Cluj – Napoca;

18. Laslo V., Zăpârțan M., Agud Eliza**,** 2011a, "In *vitro conservation of certain endangered and rare species of Romanian spontaneons flora",* în: Analele Universității din Oradea, Fascicula: Protecția Mediului,vol.XVI A, Ed. Universității din Oradea,

19. Laslo V., Vicaş S., *Agud Eliza*, Zăpârțan M., 2011b" Methods of conservation of the plant germplasm. In vitro techniques", în: Analele Universității din Oradea, Fascicula : Protecția Mediului,vol.XVI B, Ed. Universității din Oradea,

20. Laslo Laslo, V., 2013, BIOTEHNOLOGII VEGETALE şi aplicațiile lor, Ed. Univ. din Oradea, 2013;

21. Murashige, T., F. Skoog, 1962, A revised medium for rapid growth and bioassays with tobacco tissue culture. In: Physiol. Plant., 15, pp. 374-497

22. Olteanu, M., G. Negreanu., A. Popescu, N. Roman., G. Dihoru., V. Sandală., S. Mihăilescu., 1994, Lista roşie a plantelor superioare din România, Studii Sinteze Documentații de Ecologie., Academia Română, Instit. de Biol. Buc.., pp. 16-31

23. Zăpârțan, M, 1995, Specii endemice rare şi ocrotite conservate prin tehnici de cultură *in vitro* (*Dianthus spiculifolius* Schur.), Anuarul Univ. Oradea, Biologie II, p. 42-49

24. Zăpârţan, M, 1996 Rolul culturilor de ţesuturi în conservarea unor specii rare pentru salvarea şi extinderea lor în cultură, Contrib. Bot. Cluj-Napoca, p. 217-221

25. Zăpârţan, M, 2001, Conservarea florei spontane prin înmulţire *in vitro*. Ed. ALC MEDIA GROUP, Cluj –Napoca.

*** Flora, RPR., T. Săvulescu, (ed.), de la Vol. I-XIII din 1952 – 1974;

*** Conferinţa ONU de la Rio de Janeiro, 1992;

***Convenţia asupra biodiverstsităţii; Reţeaua ecologică „Natura 2000"

*** IUCN: International Union for the Conservation of Nature

*** WWF: World Wildlife;

***Legea nr. 5/2000 privind aprobarea planului de amenajare a teritoriului naţional

*** Lista Roşie UICN (http://www.iucnredlist.org)

*** Encyclopédie universelle des 15.000 de plantes, 1999, Ed. Christopher Brickell, en association avec la Royal Hort. Soc., Editura LAROUSSE-BORDAS,

*** http://www.iucnredlist.org/info/2007RL_Stats_Table%202.pdf

***2007Planul de management al Parc Natural Apuseni.20PNAp%2030.11.2007.doc http://www.capdd-bihor.org/downloads/PNAP/1.%20Plan%20de%20