REACTION OF SOME TAXONS OF DIANTHUS GENRE AT THE *EX SITU* CONSERVATION THROUGH *IN VITRO* MULTIPLICATION TECHNIQUES

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Abstract

Some species of Dianthus genre met in our country's spontaneous flora: (Dianthus spiculifolius Schur. Dianthus serotinus Waldst & Kit., Dianthus diutinus Kit.), were analysed botanically and sozologically and then, there was initiated a comparative study related to their in vitro reaction whit the purpose of their in vitro multiplication and conservation. The vegetal material was composed of young floral buds harvested from the areas of origin of the species, which were inoculated on the MS (1962) medium with a balanced content of phytohormones (an auxin and a cytokinin 0.5-1.0mg/l) or with natural extracts (corn germ extract), with the role of substituting the phytohormones. After 55 days of in vitro culture there were followed: the capacity of regeneration (%), multiplication, the radicular system and the ex vitro acclimatization capacity of the plants, and there were established the following: D. spiculifolius specie reaches the best values in the presence of Zeatin(D_1): 100% regeneration, 68% multiplication with an average of 48 neo-plantlets/explant, and acclimatization of over 50%. D. serotinus evolves good on the medium with the higher concentration of BAP-1.0mg/l (D_2): with 70% regeneration, 78% multiplication and about 27 neo-plantlets/explant, and acclimatization is of over 30%. The evolution of diutinus specie is similar but with values somewhat smaller than the ones obtained on serotinus. We recommend: Murashige-Skoog medium as a basal medium for all dianthus species; the presence of the cytokinins, especially Zeatin, in a moderate or small dose (0.5-1.0mg/l) is beneficial for Dianthus spiculifolius (but also BAP) alongside a small dose of auxin (0.5mg/l); serotinus has the best reaction also in the presence of a cytokinin in a moderate dose as well as diutinus (1.0mg/l BAP). We recommend a smaller dose of auxin for intensifying the vigour of the roots. From the present study it can be seen the possibility of maintaining Dianthus species in collections in vitro through an adequate technology but also by following the stages that ensure a high percentage of ex vitro acclimatization of the neo-plantlets, with the purpose of repopulating some natural habitats or protected areas.

Keywords: *Dianthus spiculifolius* Schur; *Dianthus serotinus* Waldst & Kit.; *Dianthus diutinus* Kit., apical tissue (meristem), conservation, sozological status, critically endangered (CR), *in situ*, *in vitro* multiplication, *in vitro* collections, *ex vitro* acclimatization, ecological reconstruction.

INTRODUCTION

Many species of *Dianthus* variety met in Romania's spontaneous flora, with scientific and ornamental value have aroused the interest of the researchers for their protection, being studied for the purpose of their *in vitro* multiplication and conservation (Zăpârțan, 1995, 2001; Cristea 2010; Holobiuc et all., 2010;

Laslo, 2011, Agud, 2016). There was even followed the initiation of photoautotrophic culture at some taxons of this genre, with a certain sozological status (Cristea 2004, 2010). Meanwhile there was developed the idea of initiating some *in vitro* collections made up of species endangered with extinction (Mavchan, et all., 2004), which are maintained in collections through subcultures repeated on simple mediums, in the absence of phytohormones, thus making the technique more advantageous (Sarasan et all. 2006). The species from the *in vitro* collections represent a very valuable biological material that if acclimatized could ensure the reconstruction of the area where they come from. This practice of replanting them in outside collections or even at their place of origin is practiced for decades at the Royal Botanical Garden from Key – England (Fay, 1994). The purpose of the *ex situ* conservation is the protection of the vegetal genetic resources (Bajaj, 1986), but the need for the conservation of the botanical element was also extended on the basis of the advantages of some unconventional multiplication methods (Dodds, 1991: Agud, 2014a).

Botanical and sozological considerations of the studied species: *Dianthus spiculifolius Schur. Dianthus serotinus* Waldst & Kit., *Dianthus diutinus* Kit determined us to analyse them in a comparative manner under the aspect of their *in vitro* behaviour. *Dianthus spiculifolius Schur*. specie was seen on Romania's territory at the middle on the last century (Flora RPR), now being sporadically found only in Transylvania along some rivers and in a few points from the reservation Apuseni Mountains Natural Park (Western Carpathians). It is also seen in some sites from Bihor County (Galbenei and Sighiştelului Valley sites, and the site Piatra Bulzului); and also in Alba and Cluj Counties (Fig. 1). It is a geoelement „*Dacian-Pontic endemit with a restricted area and with very poor populations*, with the sozological status of specie critically endangered (Coldea, Ghe. et all., 2008), of national interest, the Germplasm being conserved in botanical gardens and in gene banks (Olteanu, M. et all., 1994). The habitat of the specie is of a community interest, 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 (UICN http://www.iucnredlist.org). *Dianthus serotinus* Waldst & Kit. specie, *transilvanicus* Novák var., presents a scientific interest, also a Dacian-Pontic endemit (Dihoru and Dihoru, 1994) with a sozological status of critically endangered, with very small populations (Boşcaiu et. al., 1994). Located in a few points from Transylvania, in Olt River Gorge (Dihoru and Negreanu,

2009), Râpa Roșie and Lancrăm (Alba County), from where the vegetal material for the initiation of the *in vitro* culture was brought.

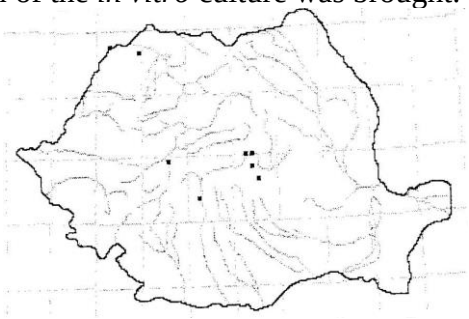


Fig. 1. Mapping areal of *Dianthus* species (spiculifolius and serotinus), on the Romanian territory (Dihoru and Negreanu 2009 p. 211)

The third specie, *Dianthus diutinus* Kit, is considered the specie the most endangered with extinction among all the species of *dianthus* variety. Rarely met in the area of Arad County and very rarely in Bihor, at the eastern limit of these areas, between the Criş Rivers (Ardelean, 1999). It is also known in Europe as an European endemit and Dacian-Pontic geoelement (Tutin at all.,1996), and some assert that it is "an endemic or rare specie in Serbia and Romania/*specie aliae*" "(R. Soó, 1980). *Dianthus diutinus* Kit. is charted on Romania's map in the spots marked in Fig. 2 (Dihoru and Negreanu, 2009), and alongside its botanical value the specie also has a distinguished ornamental value (Fig. 3).

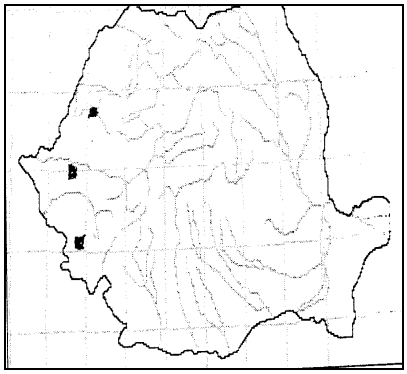


Fig.2 Prevalence areal in Romania for *Dianthus* Kit. (Dihorul, Negreanu, 2009)

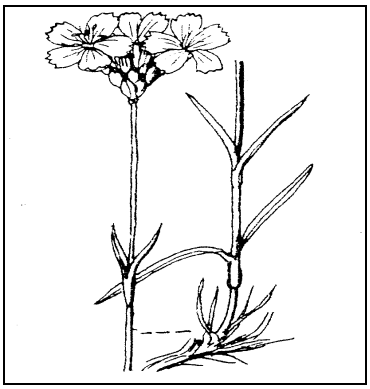


Fig.3. Dianthus diutinus Kit. diutinus general aspect of the plant (Bot.Ency.1997)

There are known *in situ* and *ex situ* conservation actions of the specie in some reservations on Crişul Alb river, acclimatized in the collections of some Botanical Gardens (Cristea, 2010), or the Germplasm in gene banks, similar to other species with severe sozological framing (Engelman, 1991), or with an economical value (Halmágyi and Butiuc-Keul, 2007). In our country, the concerns for the *in situ* conservation of the aboriginal flora dates from the middle of the last century, marked through the elaboration of some reference works (Flora, RPR,1952-1974), through the creation of some books or red lists (Dihoru, Negreanu, 2009), and then through studies on the field (Täuber, 1980), through their charting on the map of the country in the existing places.

The final purpose of the technology of *in vitro* culture and multiplication of the plants either in the spontaneous flora, the species with an economical importance (agricultural, horticultural, medicinal, flavouring, etc.) or with an ornamental value is *ex vitro* acclimatization of the material obtained through the retracement of some binding steps, with different difficulty degrees, through which there can be avoided the state of shock (Laslo, 2013). The quality of the obtained plants is given by a good development and by their complete organization (Zăpârțan 2001), vigorous aerial organs, with a well developed radicular system and which is characteristic to the specie represents the essential condition for acclimatization, completed by the shading of the foliar system about a week from the direct protection of the solar rays (Agud, 2014b). Removing unfavourable conditions that can generate infections in the soil or in the atmosphere can be ensured through the initial disinfection at the level of the soil or even of the plant (Boxus, 1995), or by eliminating some infections through the technique of the molecular markers (Ng, 1999). The exchange of the vegetal material obtained *in vitro*, imposes adequate phytosanitary conditions, for ensuring an advantageous exchange of biologic material in the country and abroad (Badea, Săndulescu, 2001).

MATERIAL AND METHOD

The species of *Dianthus* variety experimented by us: *Dianthus spiculifolius* Schur., *Dianthus serotinus* Waldst & Kit. *transilvanicus and Dianthus diutinus* Kit. *var.* multiplied *in vitro* from *young floral bud* harvested from the exemplars form some areas of spreading specified in Table 1 (*spiculifolius* from Bihor - Piatra Bulz Site; *serotinus* - Râpa Roșie and *diutinus* - Criş Valley).

Table 1

Sozological situation of Dianthus species conserved *in vitro*

Dianthus specie	**Sozological status**	**Spreading of the specie**	**Situation of the specie /importance**
Dianthus spiculifolius Schur.	CR	Sporadic in Transylvania in the following regions: Cluj, Alba, Bihor. In Buhor is sites as: Galbenei Valley, Sighistelului Valley, and in Piatra Bulzului	Dacian-Pontic geoelement with few and poor populations in the area of origin. Botanical, scientific value.
Dianthus serotinus Waldst&Kit.	CR	Sporadic in Transylvania and in Olt Gorge, isolated in Alba County (Râpa Roșie and Lancrăm)	Dacian-Pontic endemit, small but pure populations
Dianthus diutinus Kit.	CR	At the eastern limit of Alba and Bihor Counties (on the Valleys of Criș River)	European endemit, Dacian-Pontic geoelement

Vegetal material consisting of young floral bud was inoculated at the beginning of summer (during the first stage of flowering) on the MS (Murashige- Skoog, 1962) basal medium, from which there were composed variants with a balanced hormonal composition and with natural extracts (corn germs extract), with the role of substituting the phytohormones. The conceived variants of medium were based on our previous experiences on some of these species and are presented in Table 2.

Table 2

Culture mediums for the *in vitro* multiplication of DIANTHUS species

Dianthus species	Var.	MB	ANA mg/l	BAP mg/l	Z mg/l	Additional additives (mg/l: g/l)
DIANTHUS spiculifolius Schur.	D_o	MS	-	-	-	3 g/l CV
	D_1	MS	0.5	-	1.0	-
	D_2	MS	0.5	1.0	-	-
DIANTHUS serotinus Waldst & Kit.	D_o	MS	-	-	-	-
	D_1	MS	0.5	0.1	-	-
	D_2	MS	0.5	1.0	-	-
DIANTHUS diutinus Kit.	D_o	MS	-	-	-	-
	D_1	MS	0.5	1.0	-	-
	D_2	MS	0.5	-	-	5g/l EP

(MB= basal medium; MS = Murashige-Skoog; ANA = α naftil acetic acid; BAP = benzylaminopurine; Z = Zeatin; EP = corn germ natural extract; CV = vegetal coal)

Following the Table we can see the witness sample (D_o) which comprised the MS basal medium, and in the case of *spiculifolius* specie there was added 3g/l of vegetal coal and with the hormonal variants that comprised concentrations of 0.5mg/lANA and 1.0mg/l BAP and Z: for *serotinus* there was experimented the same concentration of ANA and only BAP (0.1-1.0mg/l): in choosing these concentrations we started from the best results obtained at these

species Laslo, 2011; Agud, 2016). The variants in the case of *diutinus* specie comprised auxin ANA, then 1.0mg/lBAP and a variant with corn germ extract with the purpose of replacing BAP (Table 2).

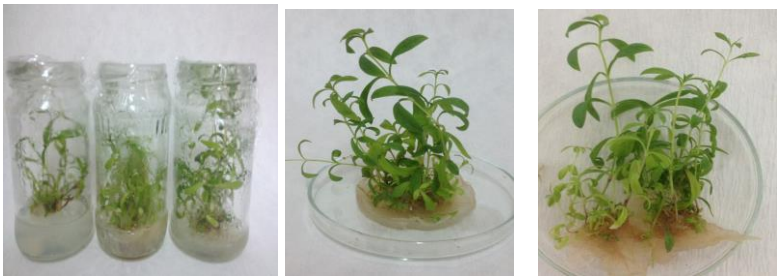
RESULTS AND DISCUSSION

After 55 days of *in vitro* culture there were followed the percentage of *in vitro* regeneration, of rooting (the value of the differentiated radicular system), the percentage of multiplication and the average of the number of regenerated neo-plantlets/explant. The percentage of acclimatized plants *ex vitro* is considered the most important parameter in the case of this type of experiments, because it has as a final purpose the conservation of the specie and the repopulation of the habitats where the species are found in extinction. Table 3 comprises the medium values and the percentages of the followed parameters for each specie, and in Figures 4 (a,b,c), 5 (a,b,c), 6 (a,b,c) we present in a comparative manner the values obtained at the three *dianthus* species.

Table 3

In vitro evolution of the bud detached from DIANTHUS species (after 55 days)

Dianthus species	**Var.**	**%Rege.**	**% Root.**	**%Multiplication/ Average no. pl/expl.**	**% Acclim.**	**Bonification**
Dianthus spiculifolius Schur.	**D_0**	12	5	11 / 2	20	xx
	D_1	**100**	**15**	**98 / 48**	**50**	**xxxxxxxxxx**
	D_2	60	13	68 / 35	**50**	xxxxxxxx
Dianthus serotinus Waldst & Kit.	**D_0**	9	3	8 / 2	15	xxx
	D_1	28	12	18 / 10	25	xxxxxxx
	D_2	**70**	**14**	**78 / 27**	30	
Dianthus diutinus Kit.	**D_0**	10	2	5 / 2	8	xx
	D_1	70	12	67 / 25	30	xxxxxxx
	D_2	48	10	45 / 14	25	xxxxxx



A. *Dianthus spiculifolius*

B Dianthus serotinus

C. Dianthus diutinus

I. The capacity of regeneration and multiplication of the explants of dianthus floral bud.

There were analysed comparatively the regeneration capacity of the explants and their multiplication capacity (expressed percentually) at each of the three species. **Fig. 4a** presents the two parameters of *spiculifolius*, from where we can see very good values on D_1 (with 1.0mg/LZ), that reach up to 98% regeneration and 100% multiplication: good values on D_2 (with 1.0mg/lBAP), exceeding 60% on both parameters: on the witness sample (D_o) due to the presence of the vegetal coal, there are obtained smaller percentages of regeneration and multiplication (of 9 and 6 times), but we mention that they are better than on the other witness samples (where the coal is missing). The percentage values of regeneration and multiplication at *Dianthus serotinus* specie are presented graphically in **Fig. 4b**, from which we can see values under the ones of *spiculifolius* specie, but in this case also on the medium with a high dose of cytokinin D_2 (1.0mg/lBAP), there are obtained good values (78% regeneration, 70% multiplication), in exchange, on the small dose of cytokinin of 0.1mg/lBAP, D_1 medium (with 0.1mg/lBAP), the values are of about three times smaller (18% and respectively 28%). Regeneration and multiplication on the control sample at this specie are reaching only 8-9%. We can assert the need for the presence in the medium of a higher dose of cytokinin for the success of multiplication.

Following **Fig. 4c** we can see values of the regeneration and multiplication of *Dianthus diutinus* specie that are similar with the ones of *serotinus* specie on the medium with a moderate dose of cytokinin: on the variant D_1 (with 1.0mg/lBAP), there took place 78% regeneration and 70% multiplication. In the case of this specie corn germ extract proved to be efficient, for replacing the cytokinins in the medium; on D_2 medium (with 5mg/lGP) regeneration reaches up to almost 45-50%, and multiplication is of 48-50%. The values on the control sample are also small (10%), which determines us to assert the need for adding a cytokinin in the medium or for replacing BAP and Z, GP for obtaining the desired results.

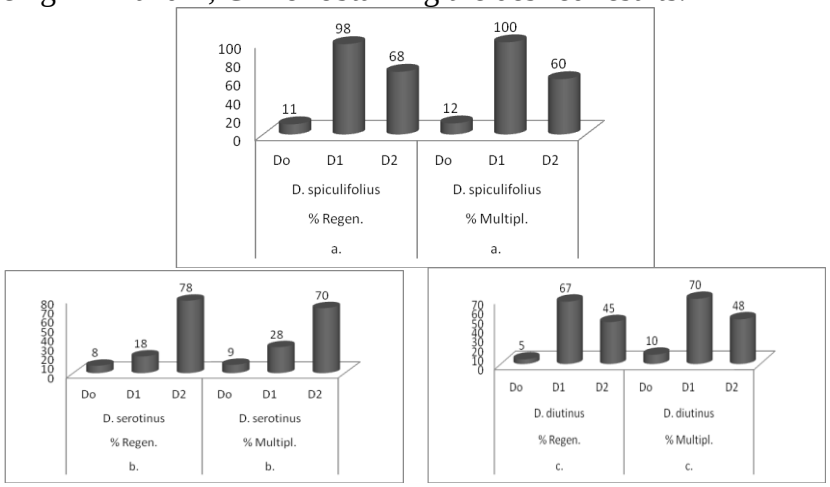


Fig. 4 Capacity of regeneration and multiplication at *Dianthus* conserved *in vitro*, depending on the specie and on the composition of the medium (after 55 days)
(a. = *Dianthus spiculifolius*; **b**. = *Dianthus serotinus*; **c.** = *Dianthus diutinus*)

II. Number of neo-plantlets/explant:

The average of the number of neo-plantlets obtained on each *dianthus* explant (bud) is presented in **Fig. 5** in a comparative manner between the three species. We can see, in the case of this parameter too that *Dianthus spiculifolius* specie, on the medium with Zeatin (D_1), has the highest number of neo-plantlets generated from an explant, about 48 neo-plantlets/bud, followed by the variant with the medium dose of BAP (D_2), with about 35 neo-plantlets/bud. On the witness sample D_o (MB+ 3g/lCV) the bud differentiates an average of only 2 neo-plantlets/explant: tall plants of about 10-12 cm, height stimulated by the presence of the vegetal coal.

The other two species *serotinus* and *diutinus* reach very close averages: 25-27 neo-plantlets/explant on the mediums with a high dose of BAP, and

barely 10 neo-plantlets on the small dose of BAP; and in the presence of 5mg/lEP over 14 neo-plantlets/bud (*Dianthus diutinus* on variant D_1). On the witness sample all species generate only about 2 neo-plantlets (without multiplication), which demonstrate the need for cytokinins, for obtaining a satisfactory number of neo-plantlets/explant.

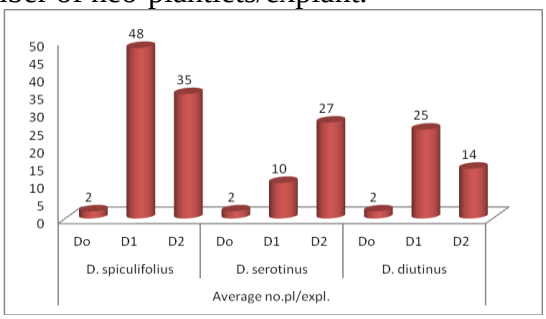
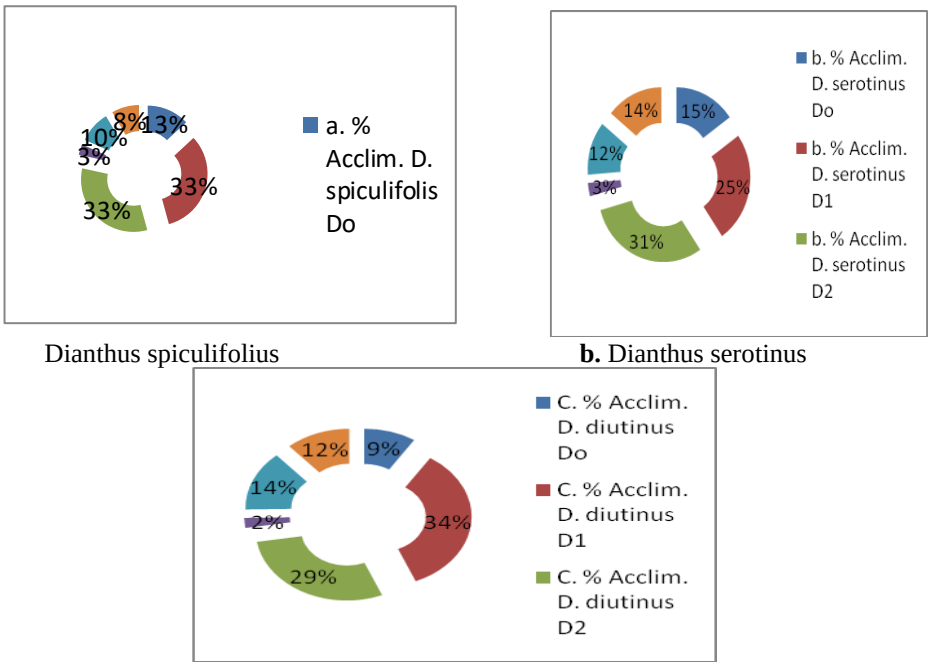


Fig. 5. Average of the number of neo-plantlets obtained *in vitro* depending on the nature of the specie and on the composition of the medium (after 55 days)

III. Value of the radicular system (rooting %)

Knowing the direct relationship between the differentiation of a corresponding radicular system (as vigour and number of roots), the acclimatization capacity of the neo-plantlets in **Fig. 6 (a,b,c),** and the presence of an auxin in the culture medium (in a moderate dose), we decided to present the two parameters together in order to conclude in a correct manner the results. In **Fig. 6a** is presented the value of the radicular system in relation with the percentage of acclimatization at *Dianthus spiculifolius*: on the mediums with auxin (0.5mg/lANA) the average number of roots/plantlet is of 10-12 roots, with 15% percentage of rooting/variant. We consider that the value of the radicular system is good in order to ensure a percentage of acclimatization of over 50%. The percentage values of the radicular system and the *ex vitro* acclimatization capacity at *Dianthus serotinus* and *Dianthus diutunus* are very close and are presented in **Fig. 6b and 6c** (with the presentation of the percentages in columns). The values are inferior to *spiculifolius* specie concerning the number of roots/neo-plantlet, and the percentage of rooting is of 12-14% and *ex vitro* acclimatization at half of the plantlets from outside (about 25-30%). We can see the beneficial role of auxin (ANA) in dose of 0.5mg/l, which in certain conditions must be supplemented up to even 1.0mg/lANA, so that the thin and frail roots differentiated *in vitro* (Photos A and B) to develop more vigorously.



a. Dianthus spiculifolius **b.** Dianthus serotinus

c. *Dianthus diutinus*

Fig. 6 Percentage of acclimatization in relation to the percentage of rooting of Dianthus plants obtained *in vitro* (after 55 days)
(a=*Dianthus spiculifolius*; **b**. = *Dianthus serotinus*; **c.** = *Dianthus diutinus*)

CONCLUSIONS

1. The success of the acclimatization of the neo-plantlets obtained *in vitro* involves obtaining vigorous plants, completely conformed, with a well developed corresponding radicular system.
2. For a good *in vitro* regeneration and multiplication of the bud detached from *dianthus* species from the spontaneous flora is necessary the presence in the MS medium of a cytokinin (about 1.0mg/l -Z or BAP), and for a good radicular system an auxin (about 1mg/l ANA, AIA or AIB).
3. *Dianthus spiculifolius* specie reaches superior values (98% regeneration and 100% multiplication) towards the other two species: *diutinus* and *serotinus* (about 70-45%), values presented in Table 3.
4. The neo-plantlets obtained *in vitro* do not raise issues at their passing *ex vitro if there are considered the stages of acclimatization and the phases of development of the bodies of the plants*.

5. The study analysed the possibilities of maintaining the species of plants from the spontaneous flora in collections *in vitro*, establishing the binding stages that must be followed periodically: repeated passing, the rejuvenation of the vegetal material and the preparing of a fresh culture medium with adequate doses and phytohormones.
6. The good acclimatization involves obtaining vigorous plants *in vitro*, completely conformed, with a corresponding well developed radicular system (ensured by an auxin – about 1mg/l), the avoidance of the state of shock from the first days since their transfer outside due to some morpho sozological modifications.
7. Eliminating the risk of infections from the soil or atmosphere, through: the initial disinfection of the substratum of culture and even of the plantlets; then, a protection system (the shading of the foliar system) at the passing *ex vitro* with the reduction of hydration and of the effect of the direct solar rays.
8. The vegetal material obtained *in vitro* ensures an internal or international exchange of species, that imposes phytosanitary conditions for eliminating the danger of introducing the pathogens from one country to another.
9. The paper followed the establishment of a protocol that must be followed for ensuring a high percentage of acclimatization of the neo-plantlets obtained *in vitro*, having a particular purpose of repopulating some natural habitats or protected areas with the vegetal material obtained *in vitro*.

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