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TRATAMENT WITH LOW TEMPERATURES APPLIED TO THE BULBS OF *NARCISSUS POETICUS L* FOR THE STIMULATION OF THE *IN VITRO* REGENERATION AND BULBIFICATION

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Abstract

The object of the study was the ex situ conservation of Narcissus poëticus L specie deriving from the protected area of "the Forest with Daffodils from Alparea (Oşorhei village)" from Bihor County, a vulnerable specie, conserved in vitro, with the purpose of multiplying and reconstructing the area where it originates. The advantage of the method consists in obtaining a large number of explants, identical with the mother plant, being a unique method of multiplying asexual plants. Ex situ multiplication in free collections has some deficiencies (the destruction of the specie by the attack of some diseases, by natural disasters, etc.), which raised interest for the in vitro conservation, the method becoming a certitude. At the tuber-bulbous plants, obtaining the vegetal material for the multiplication (the bulbs) requires a period of low temperatures (vernalization stage). Narcissus poëticus L was cultivated in vitro from scale detached from the bulb, with the disk area, after the bulb donor of the explant was treated with cold up to 4 months. The explant was cultivated on four basal mediums: MSM, MS, Heller and Gamborg, with different hormonal variants. After about 4 months from the in vitro inoculation, it was followed the evolution of the explant in relation to the period of cold, to the dose of phytohormones and lass to the nature of the medium. We point out the necessity of the treatment with cold for Narcissus poëticus L specie, involved in eliminating the profound repose of the bulbous plants. The rhythm of regeneration after a month of treatment with cold at 6 ^oC proved weak; after 2 months of treatment at 4 ^oC it is in slight increase with the formation of real leaflets; and after 3 - 4 months of cold at 2 – 3 ^oC applied to the bulb donor of explants the rhythm of regeneration reaches up to 30 – 50 %, with caulogenesis and the formation of 3 - 4 bulbils/explant. We recommend in vitro multiplication of Narcissus poëticus L specie, after normal periods of vernalization, or the replacement of this period with a treatment with cold for about 4 months at 2 – 4 ^oC, and even with high doses of cytokinins, for the natural extracts within the medium for obtaining in vitro bulbils at a low cost.

Key words: *Narcissus poëticus L., in situ, in vitro,* vernalization, treatment with cold, rhythm of *in vitro* regeneration

INTRODUCTION

Narcissus genus encompasses plants with bulbs, over 40 spontaneous species outspreaded in the South of Europe, The Caucasus and Asia up to China and Japan. They are plants with colored flowers, elegant port, some of them with a decorative value, cultivated in parks and gardens (Săvulescu, 1966). In Romania the specie is outspreaded in Cluj, Bihor, Alba and other Counties, in a spontaneous form (Fig. 1). *Narcisuss poëticus* L, Poet's Daffodil can be found in a spontaneous form in a few regions, in protected areas or "meadows with daffodils" (e.g. Alparea, Bihor, experimented by us). It is a specie with a large ecological amplitude at ground level,

amphitolerant to its pH and with moderate demands at heat (4,5 ^{o}C - 7,5 ^{o}C). It multiplies through bulbs, its flowers are white hemstitched with red, solitary, ordered (Fig. 2), it flowers in the months of April – May, and its fruit is a capsule (Pârvu, 2004).

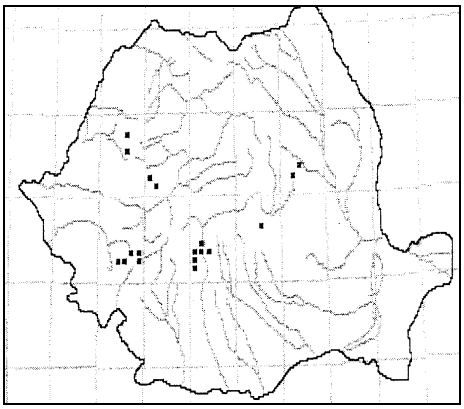

Fig. 1. The outspread of *Narcissus poëticus* L specie

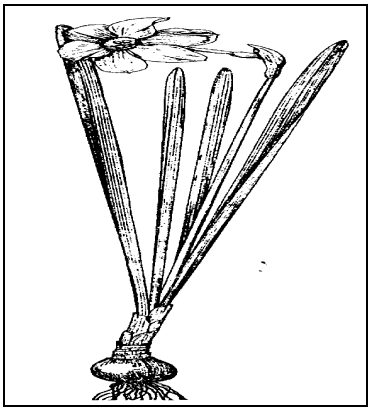

Fig. 2. Flower of Poet's Daffodil

A honey plant in medicine, also cultivated as an ornamental plant, with a technology which is typical to the rustic bulbous plants: it needs to be planted in autumn (for passing the vernalization period), at the end of September in a fertile soil, if flowers in April and after the drying of the flower and of the foliar system, the organ of multiplication (the bulb) is getting into the rest until autumn (Encyclopédie de botanique et d` Horticulture, 1999). *Narcissus poëticus* was taken under observation, monitored and conserved *in vitro* and *ex situ* due to its sozological status of vulnerable specie (Oltean et al., 1994; Boșcaiu et al., 1994; UICN, 2006). The conservation of the biodiversity of plants represents a major concern because the data support the fact that it has suffered a great decline proved by its insertion on the red list of thousands of plants with different sozological degrees between 1996 - 2000 (Sarasan et al., 2006). The biological diversity is permanently affected (about 50 % among the species have disappeared over the last 20 years) (http://www.natureserve.org/ aboutUs/PressReleases/IUCN_Red_List_release.pdf), at the Convention for Biodiversity (The Hague, 2002) CBD (CBD = The Global Strategy for Plant Biodiversity) was established, a strategy that supports the conservation of the vegetal patrimony, research of ecology, systematic, etc. and also the importance of *ex situ* conservation of Earth's biodiversity (http://www.bgci.org.uk/files/7/0/global_strategy.pdf).

At the bulbous plants, obtaining the vegetal material of quality for multiplication (the bulbs), requires covering a period of low temperatures, the vernalization stage (Tampsett, 1980), imperative for the growth process

and for the flower induction, a period that can be replaced with a treatment with cold or with chemical substances with implications in the differentiation of the floral plums (e.g. gibbrellin, GA_3). The treatment with gibbrellin (GA_3) replaced the low temperatures and interfered in the break of the repose (Yamagishi, 1993) inducing the flowering. Injected in the bulbs of Narcissus and Hyacinthus (horticultural forms), GA_3 replaced the cold and stimulated the floral induction in another period than the normal one (Zăpârțan, 1990). Classical multiplication has some deficiencies which led to the need of finding some unconventional methods which to ensure a more secure storage of the endangered species and which to relate the classical and the modern, complementing each other (Engelman, 1997). Initially, the *in vitro* method was considered only a way of rapid multiplication of the species, while it became a certain way of conserving the vegetal resources (Withers, 1990). The capacity of *in vitro* bulbification is superior through its use as explan of the juvenile bud from the young inflorescence (Zăpârțan, 2004). The obtaining of the planting material *in vitro* starting from the sprouts grown from the potato tubers is a technique of great economical interest, the results depending on the period when the sampling is done, on the variety and on the hormonal balance, and more accurately on the presence of a cytokinin (Agud et al., 2010), all of them ensuring the obtaining of a planting material at a low cost, the technique proving to be an economical one (Agud, 2011). A great number of the threatened species are included in *ex situ* conservation programs, in recovery plans even of a single specie with a certain sozological status (Bajaj, 1986).

MATERIAL AND METHOD

The vegetal material for the *in vitro* multiplication of *Narcissus poëticus* L specie consisted of *a longitudinal section of a scale detached from the bulb, a portion of disk* (a portion with the highest capacity of proliferation), inoculated on the culture mediums with the cut section and with the portion of the disk tightened on the culture medium. In the previous experiments the explants from the bulbs induced the differentiation of bulbils *in vitro* at the bulbous species, as for example the regeneration of Lilium (Zăpârţan et al., 2000) and Fritillaria, obtaining a larger quantity of bulbils towards the classical method for the propagation of the specie (Zăpârțan, 1996).

The culture mediums used for stimulation of the differentiation of the *in vitro* minibulbils at *Narcissus poëticus* L specie, started from four different basal mediums (MB), to which there were conceived variants with phytohormones. The four basal mediums (MB) and the duration of the treatment applied to the material donor of explant are:

I. **MS Modified** = according to Murashige - Skoog, 1972, + 180 mg/l (**V_{om}**), from which there were conceived:

V_{1m} = MSM + 0,5 mg/l AIB + 1,0 mg/l BA,

V_{2m} = MSM + 0,5 mg/l AIB + 2,0 mg/l BA. To which it was applied **a month of treatment with cold at 6 ^{o}C**;

II. **MS** = MB according to Murashige - Skoog (**V_o**)**:**

V_1 = MS + 0,5 mg/l AIB + 1,0 mg/l BA,

V_2 = MS + 0,5 mg/l AIB + 2,0 mg/l BA. To which it was applied **2 months of treatment with cold at 4 ^{o}C;**

III. **He** = MB, according to Heller + vit.MS (**H_o**): **H_1** = Ho + 0,5 mg/l AIB + 1,0 mg/l BA, **H_2** = Ho + 0,5 mg/l AIB + 2,0 mg/l BA, **H_3** + Ho + 1,0 mg/l BA + 8 mg/l 2,4D. Which **were treated for three months with cold at 2-3 ^{o}C**;

IV. **B_5** = MB, according to Gamborg (B_5) + vit.MS (**Bo)**:

B_1 = Bo + 0,5 mg/l AIB + 1,0 mg/l BA,

B_2 = Bo + 0,5 mg/l AIB + 2,0 mg/l BA,

B_3 = B_o + Bo + 1,0 mg/l BA + 8 mg/l 2,4D. Which **were treated for four months with cold at 2 - 3 ^{o}C**.

The cytokinins in the culture medium in dose of 1,0 – 2,0 mg/l proved efficient for the *in vitro* tuberization of some potato varieties, for obtaining a superior planting material from a qualitative and a quantitative point of view (Agud et al., 2008). A small concentration of auxin (0,5 mg/l AIB) with a medium dose of cytokinin favors the Radicular System and the forcefulness of the spontaneous plants (Agud, 2014), and at the species with bulbs, it induces the differentiation of the bulbils *in vitro*.

The mature bulbs donors of explants were ***treated with cold for replacing the period of vernalization*** necessary for the species with bulbs (*liliaceae, amarilidaceae* etc.), the duration of the treatment with cold and the temperature values applied are presented in table 1: four periods of treatment, of 1, 2, 3, and 4 months with temperatures from 6 ^{o}C to 2 – 3 ^{o}C, applied to the bulbils in the refrigerated containers programmed for these temperatures. After the inoculation on the medium of the explants treated with cold according to Table 1, the phials were kept in certain *conditions of incubation in vitro*. The explants detached from the bulb are maintained after the inoculation at dark (about 4 - 5 days), with an effect over the activity of the auxins from the medium and with a beneficial effect on the *in vitro* regeneration and differentiation of bulbils (Laslo et al., 2011). We mention the effect of the conditions of the in vitro incubation applied to the young bud detached from the inflorescence of *Clivia miniata* and the treatment with dark for the floral induction (Zăpârțan, 1992).

Table 1

Duration of the treatment with cold (vernalization) and the time of the year when the explant of *Narcissus poëticus* L bulb was sampled

No. of experiments	Date of the experiment	Duration of the treatment with cold and the conditions of vernalization (months)	General evolution
I	4.0.07 2014	1 month, at 6 ^{o}C	Weak
II	05.08.2014	3 months, at 4 ^{o}C	Slow
III	07.09.2014	4 months, at 2 – 3 ^{o}C	Improved
IV	06.10.2014	6 months, 2 – 3 ^{o}C	Good

RESULTS AND DISCUSSION

The observations were made after 3 - 4 months from the *in vitro* inoculation of the *Narcissus poëticus* L explants, during the performance of the four experiments.

It was followed the evolution of the explant of scale with a portion of disk, depending on the duration of the treatment with cold, the time of the year when the treatment is applied, the basal mediums and the hormonal variants conceived. *In vitro* evolution of the explant detached from *Narcissus poëticus* L specie, was reported at the period of cold applied and at the dose of phytohormones and less at the nature of the medium, Table 2 presenting the rhythm of regeneration (%) and the evolution of the tissue, depending on the time of sampling and of inoculation.

The regenerative capacity after a month of cold at about 5 – 6 ^{o}C is weak (2 %), signaled only on the variants with hormones (V_{1m} and V_{2m}) where there were differentiated 1 - 2 leaflets (the presence of wisteria can be favorable). For the stimulation of the evolution it is necessary a higher duration of cold and a lower temperature. After 2 months at 4 ^{o}C the regenerative capacity is growing at 8 – 12 % (Fig. 3), greater on the medium with 2,0 mg/l BA (V_2) where caulogenesis appears too.

In vitro evolution of the explant is significantly increasing only after 3 months of treating the bulbils with cold at 2 – 3 ^{o}C, the regenerative capacity reaching up to 30 – 40 % on the mediums with phytohormones (H_1 and H_2), on which differentiation of about 2 - 3 bulbils/explant takes place (Fig. 4-6).

On the medium for the formation of the callus tissue (H_3) is differentiating a callus muff around the explant of about 3 mm. After 4 months of treatment with the same temperature (Fig. 7-9), the regenerative capacity of the explant reaches at 40 – 50 % (Fig. 10) and the bulbification is intensifying, forming 3 - 4 bulbils/explant. After this treatment the callus muff formed on B_3 grows too, reaching up to 3 – 4 mmØ (Table 2).

Table 2

Rhythm of regeneration (%) of *Narcissus poëticus* L specie and the evolution of *Narcissus poëticus* L explant, depending on the time of sampling and of inoculation

Experiments	**Var.**	**Regen. (%)**	**Organogenesis**	**Observations/ Evolution**	**Bonif.**
I. June	V_{om}(MSM)	0	-	It stagnates	-
(1 month at 6°C)	V_{1m}	2	1 leaf (lf.)	Slow evolution	x
	V_{2m}	4	2 leaves	Easily improved evolution	xx
II. August	V_o (MS)	0	-	necrosis	-
(2 months at 4° C)	V_1	10	1 lf./explant	Slow evolution, a real leaf appears	xx
	V_2	15	4 lf./explant Caulogenesis appears	Better evolution	xxx
III. October	H_o(Heller)	1		It stagnates	-
(4 months at 2-3° C)	H_1	30	Bulbification	1-2 bulbs/expl.	xxxx
	H_2	40	Bulbification	2-3 bulbs/expl.	
	H_3	callus	Caulogenesis	0,5-1,0 mmØ	callus
IV. December	B_o(B_5)	1		It stagnates	
(6 months at 2-3° C)	B_1	40	Bulbification	3 bulbs/expl.	
	B_2	50	Bulbification	4 bulbs/expl.	
	B_3	callus	Caulogenesis	2,0-4,0 mmØ	callus

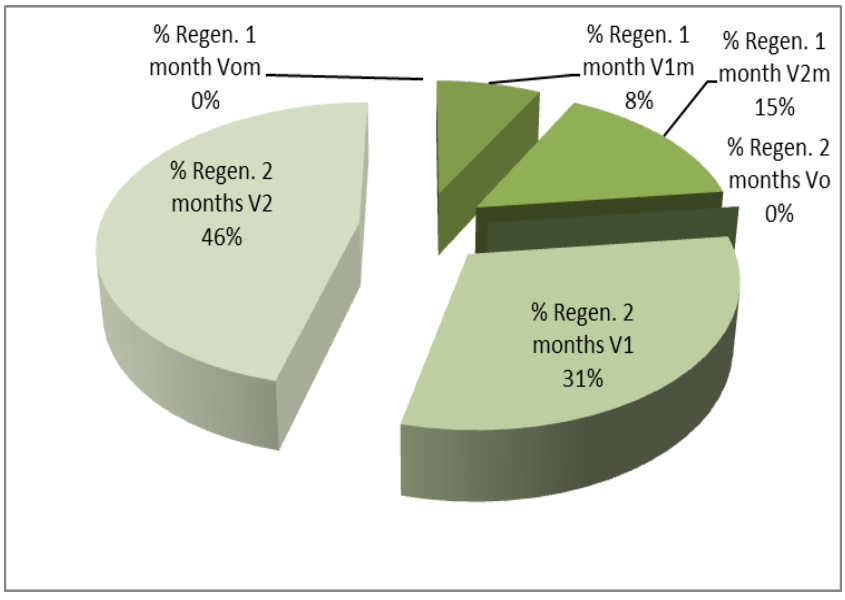

Fig. 3. Regenerative capacity after 1-2 months of treatment with cold at 6°C and respectively 4°C

Fig. 4-6. Photos after about 2 months of treatment with cold of about 4 ^{o}C

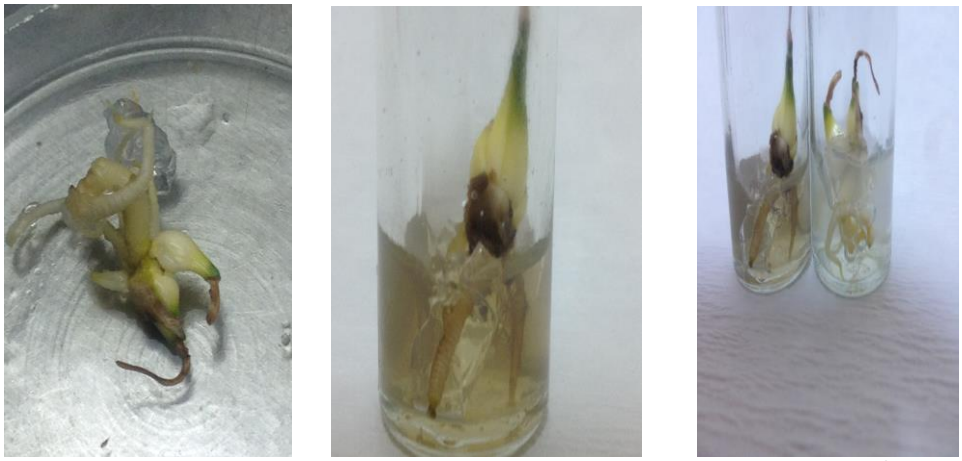

Fig. 7-9. Photos after 3 - 4 months of treatment with temperatures of 2 – 3 ^{o}C

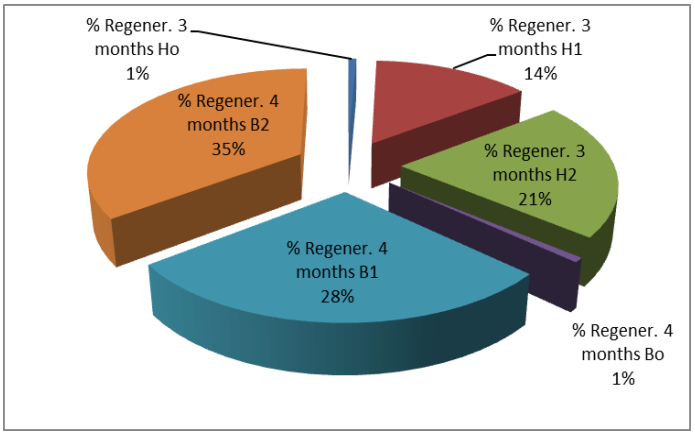

Fig. 10. Regenerative capacity after 3 - 4 months of treatment with cold at 2-3^{o}C

Figure 11 presents the overall of the rhythm of evolution of the explant from the scale detached from the bulb of *Narcissus poëticus* L, after the four periods with cold, where we can see the superior regenerative capacity on the variants maintained at cold of 2 – 3 ^{o}C for 3 - 4 months, in comparison with the ones maintained only a half a month and especially in the presence of benzyl adenine in a concentration of 1,0 - 2,0 mg/l both on the Heller-He basal medium (H_1, H_2), and on the Gamborg-B_5 medium (B_1 and B_2).

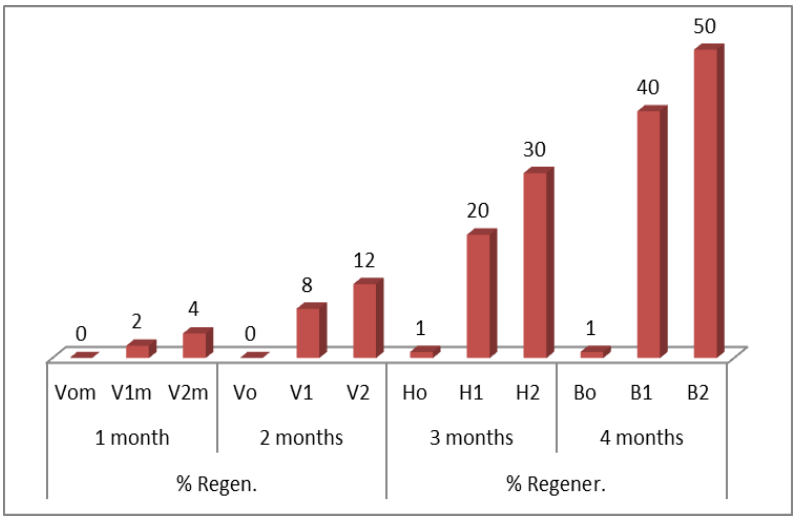

Fig. 11. Comparative presentation of the regenerative capacity of the explant detached from the bulb of *Narcissus poëticus* L, cultivated *in vitro*, after the four periods of treatment with cold

CONCLUSIONS

1. We mention the role and the necessity of the treatment with dark applied to the explants detached from the bulb and inoculated *in vitro*, with the purpose of stimulating the differentiation of the reproductive or multiplication organs (bulbs) in the case of the species from the tuber-bulbous families.

2. The treatment with cold at *Narcissus poëticus* L specie is involved in the reduction or even in the elimination of the deep repose (indispensable for the floral induction) of the specie.

3. A month of treatment with cold (30 days) proved insufficient, marked by a slow evolution and only in the presence of phytohormones.

4. After 2 months of treatment with cold at 4^{o}C the regenerative capacity is increasing, caulogenesis being easily signaled too (the formation of real leaflets).

5. The treatment with 2 – 3 ^{o}C applied for three – four months stimulates the regenerative capacity that reaches up to 30 – 50 %, with the differentiation of about 3 - 4 bulbils/explant.

RECOMMENDATIONS

We recommend the multiplication *in vitro* of *Narcissus poëticus* L specie, after running over the normal period of vernalization (naturally or forced through the treatment with cold). For the stimulation of the differentiation of a greater mass of callus it is necessary to modify the medium by adding a higher concentration of cytokinin (between 4 - 5 mg/l BA) in association with 8 - 10 mg/l 2,4D. The experiments must continue after running over the normal vernalization of the bulbils (in soil), or after the longer treatment with cold (about 4 months), and also with the testing of other medium and phytohormones formulas (Z, 2iP, ANA etc.), or even with natural extracts, for obtaining *in vitro* bulbils (the multiplication or the planting material) at low cost. We can certainly recommend a longer period of vernalization, of about 4 months of treatment with lower temperatures of about 2 – 3 ^{o}C, and we also recommend other types of phytohormones and higher doses of cytokinins, both for the *in vitro* differentiation of the micro bulbils and for the regeneration of the neoplantlets via-explant from the portion of the bulb, and also for the proliferation and multiplication of the specie of bulbous Narcissus plants via-callus.

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