

## CAMPANULA ROTUNDIFOLIA L SPECIE ENDANGERED WITH EXTINCTION, CONSERVED THROUGH IN VITRO TECHNIQUES

Agud Eliza Maria \*

\*University of Oradea, Faculty of Environmental Protection, 26 Gen. Magheru, St., 410048 Oradea, Romania: [eliza\\_agud@yahoo.com](mailto:eliza_agud@yahoo.com)

### Abstract:

*It was followed the conservation of Campanula rotundifolia L. specie through in vitro multiplication, which from a zoologically is a part of the EN=endangered group of conservation (according to the last red list of the vascular plants), paleoendemic and Dacian geo-element with poor populations meet in the Carpathians. The explant of the initiation of the in vitro culture was a very young floral bud, detached from the recalled specie which after prior sterilization was cultivated on the basal medium according to SH + MS vitamins in simple variants without hormones (Mt and V<sub>1</sub>) and variants V<sub>2</sub> up to V<sub>6</sub>, with different doses of auxin (AIB – 0,5-1,0mg/l) and cytokinins 2iP and BA (0,5, 1,5 și 2mg/l). After about 20-15 days from the in vitro incubation of the juvenile bud of Campanula, it regenerated only 2-12 plantlets/explant depending on the composition of the variant, but they were unrooted. The final observations were made only after 50 days of in vitro culture, following the percentage of regeneration, multiplication and rooting, the average number of plants and roots, and also the acclimatization capacity of the neoplantlets (Table 2). The best results were obtained on the mediums with BA: percentage of regeneration of 80-88% (depending on the concentration of BA), with multiplication of 83% and 92% rooting at the maximal concentration of BA (on V<sub>6</sub>). The other parameters gave the best results in the presence of BA too (V<sub>4</sub>, V<sub>4</sub> and V<sub>6</sub>) but also on the mediums with 2iP, but they were inferior to the benzyl amino purine (BA). The plants are completely organized and conformed; with a high capacity of acclimatization, depending on the value of the Radicular System, and the acclimatization of the new plantlets at the place of origin of the specie ensures an ecological reconstruction of the area and the enrichment of the poor area in which the specie was found. We recommend the extension of Campanula rotundifolia L specie, paleoendemic and Dacian geo-element from the flora of Romania and its conservation in the protected areas from where it disappeared, but we also recommend its introduction within the architectural landscape spaces of the country's mountain cities, National Parks and Botanical Gardens.*

**Key words:** Campanula rotundifolia L., Dacian geo-element, paleoendemic, EN= endangered specie, zoology, Murashige – Skoog (MS); Schenk – Hidebrandt (SH), floral bud, in situ, in vitro, ex situ, phytohormones: AIB, 2iP, BA .

### INTRODUCTION

The biodiversity at the level of the Earth suffered a great decline (between 1996 – 2004), being introduced within the red list over 8300 species of plants with different degrees of endangerment (Sarasan et al., 2006). IUCN Report since May 2006 established that climate changes too have a destructive effect on the species of plants, the rhythm of their disappearance being of 100 up to 1000 greater than the natural rhythm, being considered that more than 50% of the species have disappeared in the

last 20 years<sup>1</sup>. The Global Strategy for the Biodiversity of Plants (CBD, Haga, 2002) from the Convention for Biodiversity supports research within the biology of plant conservation, the importance of all forms of plant conservation, including *ex situ*<sup>2</sup>, a conservation form that follows up the realization of some “reserves” for ensuring, through this manner too, the protection of the endangered plants (Cristea and Denaeyer, 2004). The idea of conserving the genetic resources through any form cannot be but beneficial and is supported by numerous researchers (Bajaj, Y., 1986; Halmágyi and Butiuc-Keul, 2007; Cristea, 2010), the nature of these forms of conservation being watched and analyzed with a great deal of interest (Fay, 1992; Bavaru et al., 2007; Laslo et al., 2011a). The defense and protection of nature from Romania is manifested differently: by the presentation of some preclude field research on the status of the nature (Cristea et al., 1996), by the creation of new special protected areas (Sârbu, coord., 2007), by the highlighting the importance of biogenic reservations for the protection of the genofund (Toniuc et al., 1994), manifestations that are based on the red lists of the plants from the country (*Dihoru Gh.*, 1992; Toniuc et al., 1992; Olteanu et al. 1994; Sîrbu and Chifu, 2003; *Boşcaiu, N.*, and col., 1994; Dihoru and Dihoru, 2005), or the red book concerning the state of the vascular plants from Romania (Dihorul and Negreanu, 2009) to the elaboration of which there was considered the zoological framing of the specie (according to the categories established by IUCN) establishing the statute of the specie concerning the level of its endangerment at the level of the country.

The lacks of the classical conservation methods of the species of plants due to some causes (natural catastrophes, the destruction of the collection of plants as a consequence of the attack of diseases, etc.) represented the basis for finding some (unconventional) methods of multiplication, of stocking and conservation of some species threatened with extinction (Witheres, 1990). Initially, vegetal biotechnologies had some implications in the fast cloning multiplication of the species with an economical importance (Cachiță and Ardeleanu, 2007), but much later the technique is considered a means of conservation of the vegetal resources (Bajaj, 1986; Fay, 1992; Zăpârțan, 1995-1996; Laslo et al. 2011b). It is known the traumatic action of the technique of *in vitro* culture (Jain, S., 2001) and also the implication of some factors (the culture method, the nature of the hormonal balance, the type of explant, the subcultures, the nature of the specie and its physiological state, etc.) within the somaclonal variability (*Larkin and Scowcroft*, 1981). All these do not represent a barrier

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<sup>1</sup> ([http://www.natureserve.org/aboutUs/PressReleases/IUCN\\_Red\\_List\\_release.pdf](http://www.natureserve.org/aboutUs/PressReleases/IUCN_Red_List_release.pdf)).

<sup>2</sup> ([http://www.bgci.org.uk/files/7/0/global\\_strategy.pdf](http://www.bgci.org.uk/files/7/0/global_strategy.pdf)).

for the conservation and multiplication of the endangered species and for the stocking of the vegetal material obtained *in vitro* (Cachiță et al., 2004), for the initial technique of *in vitro* culture and then for finding the ideal formula (of medium and of the culture conditions) for the *in vitro* multiplication of the specie (Butiuc-Keul et. all., 1996; Zăpârțan, 2001), establishing the stages of a laboring protocol. After the genomic analysis of the plants obtained *in vitro* and after the *ex vitro* acclimatization there can be initiated actions of repopulating the endangered habitats, after a prior consultation with the specialists from the natural reservations and from the protected areas (Primack, 2002; Zăpârțan, 1994b).

Within this study there was followed the conservation of the *Campanula carpatica* L. specie through *in vitro* multiplication, a Dacian geo-element met in the Carpathians and in the surroundings (Fig. 1), a specie threatened with extinction (EN). Through the older conservation methods the specie was placed in the National Park of Măcinului Mountains, in the Carpathians, in the channels from Hârșova Harbor, in Cheia Jurassic reef, etc., and also *ex situ*, within the collections of the Botanical Gardens, a space where a periodic control of the state of the population must be ensured. The species conserved at us came from the Botanical Garden of the University from Cluj-Napoca (seeds and young plants).

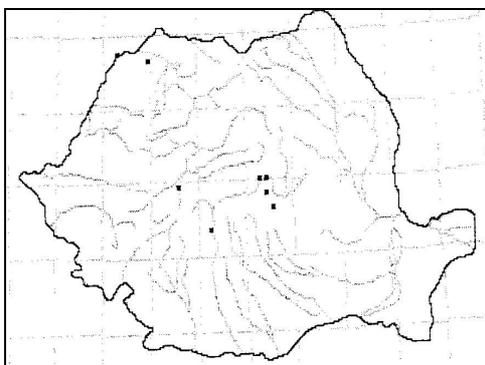


Fig. 1. Mapping area of *Campanula rotundifolia* L. (syn. Carpatica) specie, on the Romanian territory (Dihoru and Negreanu 2009 p. 116)

*Campanula rotundifolia* (syn. carpatica) is presented for the first time by Borza, 1923 and then described by Morariu 1964, and it can be met at the limit of three counties from Transylvania: Cluj, Bihor and Maramureș. Taxonomically it is perennial (with rhizomes), Campanulaceae genre, with a few subspecies (families of taxons): *rotundifolia*, *carpatica*, *romanica* (flora Europaea. Hayeck, 1933, Flora RSR, 1952, Beldie, 1961, Rațiu and Ghergely, 1961, quoted by Dihorul and Negreanu, 2009). Being considered a geo-element with poor populations, but over time also extended to other areas from our country and from the neighboring countries, it multiplies

from seeds but also through vegetatively through the detachment of the mature bush after flowering (in early autumn) or before the flowering (in early spring). The perennial material can be found in the collections of the botanical gardens (Encyclopédie de botanique et d'horticulture, 1997). Morphologically the specie is a paleoendemic Dacian element (*Toniuc, A*, 1987, 2000), and *rotundifolia* subspecie studied by us along with the botanical interest also has an ornamental value (according to the red List of the vascular plants, 2009).

#### MATERIAL AND METHODS

At the initiation of a *in vitro* culture for the species from the spontaneous flora it will be considered the botanical framing of the specie, using almost all the tissues or parts of the plant (explants): apex (of the top or lateral), leafs, parts of the leaf (pod, limb, rib, etc.), stem (nod, internod), root, inflorescence, flower organs, etc. (Cachiță, 1987); to other species the initiation of the *in vitro* culture was possible only from germoplasma, from seeds germinated *in vitro*, from which there were developed plantlets from which there were then detached different explants (meristem, nod, leaf, root, etc.). Usually *in vitro* germination requires some preliminary operations: treating the seeds mechanically, chemically, physically for favoring germination, a treatment applied depending on the nature of the seed and on its age (Zăpârțan, 1994a). Each type of explant cultivated *in vitro* differentiates through its own capacity of regeneration and multiplication *in vitro*, a statement supported by the results obtained at different species of spontaneous plants by foreign researchers and by our co-nationals (Fay, M. F., 1992; Zăpârțan 2001; Laslo, 2013). The explant was a **very young floral bud**, detached from *Campanula carpatica* L specie, gathered from the opened (Fig. 2) which after prior sterilization was cultivated on the basal medium (MB) according to SH (Shenk-Hildebrandt, 1972) + vitamins MS (Murashige-Skoog, 1962), in the variants specified in Table 1.



Fig. 2 *Campanula rotundifolia* L mother plant, donor of explants

There are species which behave very well *in vitro* on simple mediums, as it is the case of the specie researched by us: MS1/2 (Mt.) medium with halved concentration of microelements and macro elements, or on MS with full concentrations (V<sub>1</sub>) and on which took place a satisfactory or even a good regeneration and multiplication depending on the specie and on the time of initiating the culture. The presence of some phytohormones in the culture medium led to organogenesis, caulogenesis, multiplication and even to the initiation of the *in vitro* multiplication organs (for example the potato tubers according to *Butiuc- Keul*, et all., 1996; *Agud et all.* 2013), the evolution depending on the nature, concentration of some phytohormones and on the sampling period.

Table 1

The composition of the culture mediums for the *in vitro* multiplication of *Campanula rotundifolia* L specie

Var	Basal medium (MB)	AIB (mg/l)	2iP (mg/l)	BA (mg/l)	Observations concerning the evolution of the bud
Mt	½ SH + vit. ½ MS	-	-	-	A control variant without hormones, with macro and microelements 1/2
V <sub>1</sub>	SH + vit. MS	0,5	0,5	-	A variant on which the <i>in vitro</i> regeneration and the rooting of the specie takes place in a satisfactory percentage
V <sub>2</sub>	SH + vit. MS	0,5	1,5	-	Values close to the ones of variant V <sub>1</sub>
V <sub>3</sub>	SH + vit. MS	1,0	2,0	-	Values close to V <sub>1</sub> and V <sub>2</sub>
V <sub>4</sub>	SH + vit. MS	0,5	-	0,5	A good variant, an evolution similar to the one on the variant with a high dose of 2iP (V <sub>3</sub> )
V <sub>5</sub>	SH + vit. MS	0,5	-	1,5	A very good variant with values somewhat lower than the ones on V <sub>6</sub>
V <sub>6</sub>	SH + vit. MS	1,0	-	2,0	The medium formula considered the best concerning the value of all parameters

The organogenesis and the *in vitro* multiplication rate of the specie is dependent on the nature of the specie, on the composition of the culture medium, on the type of explant, on the physiological phase in which the

tissue is (age of the donor mother plant), on the time of year in which takes place the sampling of the tissue, etc. (Cachiță, 1987).

## RESULTS AND DISCUSSION

The observations were made after 50 days of *in vitro* culture, and the average of the results is presented in Table 2 from which we can see the value of the neoplantlets (the number, the length and the differentiation of the Radicular System), and also the percentage of regenerated and multiplied plantlets. The average of the results is presented graphically in a suggestive manner in Figures 3, 4, 5 and 6.

Table 2

*In vitro* regenerative capacity of the bud of *Campanula rotundifolia* L (after 50 days)

Var.	Average no. pl./l. (cm)	Average no. of roots/L (cm)	% Reg.	% Multiplic.	% Rooting	Bonification
Mt.	1,0 / 1,5cm	0	12	15	0	x
V <sub>1</sub>	2,5 / 1,2cm	2,0 / ,5cm	45	15	8	xxx
V <sub>2</sub>	5,0 / 1,2cm	2,0 / ,8cm	60	57	20	xxxx
V <sub>3</sub>	15,0 / 1,0cm	6,0 / 0,5cm	70	70	70	xxxxx
V <sub>4</sub>	7,0 / 0,8cm	7,5 / 0,7cm	80	47	34	xxxx
V <sub>5</sub>	18,5 / 1,0cm	8,0 / 0,5cm	80	72	48	xxxxxx
V <sub>6</sub>	31,0 / 1,0cm	16,8 / 1,0cm	88	83	92	xxxxxx

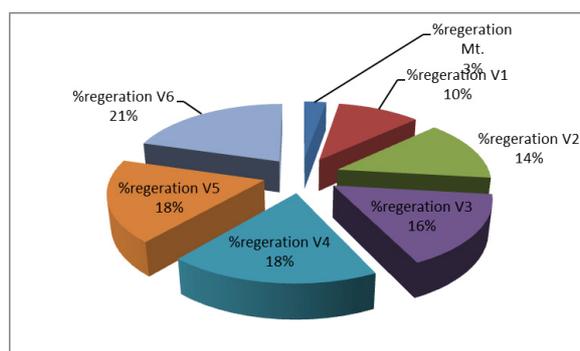


Fig. 3. Regenerative capacity (%) of the bud of *Campanula rotundifolia* L (after 50 days)

The regeneration percentage is between 12% and 88% depending on the composition of the variants of medium (the absence, the presence and the concentration of phytohormones). In Fig. 3 we can see only 12% regeneration on Mt. and 45% on V<sub>1</sub> (both variants without phytohormones) and between 60% and 88% to the other variants (with phytohormones). In the presence of auxin (AIB) and of benzyl amino purine (BA) in different concentrations, regeneration is of over 80%, on the highest concentration reaching 90%. On the mediums with izopentiladenine (2iP) regeneration is lower but it reaches 70% on V<sub>3</sub> (SH+ vitamins MS+2 mg/l 2iP+ 1,0mg/lAIB). The association of a basal medium according to SH with vitamins according to MS leads to an *in vitro* regeneration of the explants of

about 45% even in the absence of phytohormones. On the withes sample Mt. regeneration can be seen in a percentage of 12%, probably due to the halved concentration of macro and microelements from the basal medium.

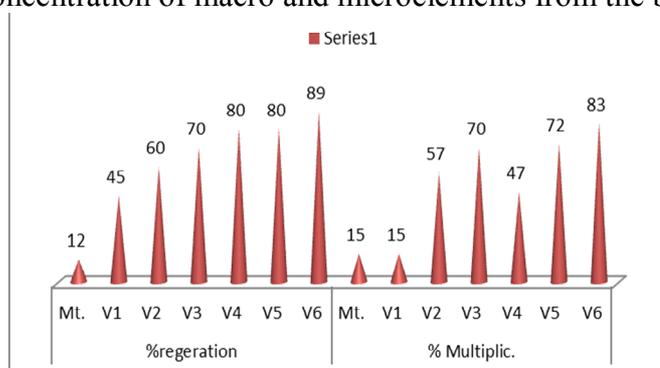


Fig. 4 Comparative presentation of the percentage of regeneration and multiplication of the bud of *Campanula rotundifolia* L. (after 50 days)

The percentage of regeneration and multiplication are parameters which have a similar evolution (depending on the formula of medium used), with insignificant value differences. Figure 4 presents the average percentage of the regenerative capacity, in comparison to the capacity of multiplication of the juvenile bud of *Campanula rotundifolia* L., after 50 days of *in vitro* culture. The best multiplication is signaled at the highest concentration of cytokinins: of 92% in the presence of 2,0mg/l BA (on V<sub>6</sub>); 72% on the medium with a medium concentration of 1,5mg/lBA (V<sub>5</sub>) and 70% on the medium with 2mg/l 2iP (V<sub>3</sub>). On the mediums with small doses of cytokinins, the multiplication is of 47-57%; on the variants without additional phytohormones of only 15% (Fig. 4). We consider that the presence of the cytokinins in a dose of 2mg/l is beneficial for the regeneration and multiplication of the floral bud of *Campanula* cultivated *in vitro*, obtaining well conformed vigorous plantlets.

The average number of plants formed from an explant reaches the highest values on V<sub>6</sub>=SH+vit.MS+1,0mg/lAIB+2,0mg/lBA, on about 31 plantlets/explant and of 18 neoplantlets/explant on V<sub>5</sub>=SH+vit.MS+0,5mg/lAIB+1,5mg/lBA; on the variants with 2iP on V<sub>3</sub> (2,0mg/l2iP+ +1,0mg/lAIB+2mg) there is forming the highest number of about 15 neoplantlets/explant; on the variants without phytohormones the number is of 1-2 neoplantlets/explant (Mt, V<sub>1</sub>).

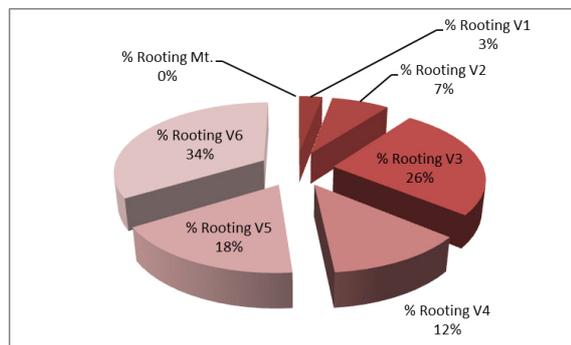


Fig. 5 Percentage of rooting of the *Campanula* neoplantlets

The rooting of the *Campanula* neoplantlets is conditioned by the presence of auxin  $\beta$  indolyl butyric acid (AIB) in both concentrations. On the variants with a maximal concentration of AIB ( $V_3$  and  $V_6$ ), the percentage of rooting reaches 70% and respectively 92% (Fig. 5), the association between the high dose of cytokinins (BA and 2iP/2mg/l) and the high dose of auxin (AIB-1mg/l) proves to be beneficial. On the other variants the percentage of rooting is of 20-48%; on the withes sample (Mt.) neoplantlets do not root and on  $V_1$  the percentage reaches only to 8%.

For the rooting of the neoplantlets of *Campanula rotundifolia* L. differentiated *in vitro* it is absolutely necessary the presence of an auxin in a average concentration of 1,0mg/l which will lead to obtaining a good number of vigorous roots, disposed in a wisp with the role of enlarging the capacity of acclimatization to the *ex vitro* conditions of the neoplantlets obtained *in vitro*.

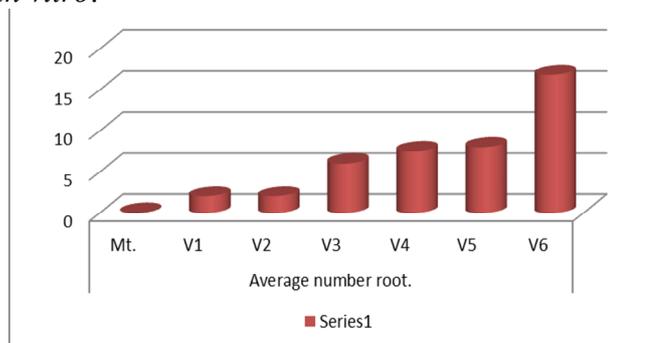


Fig. 6. The average number of roots obtained at *Campanula* after 50 days of *in vitro* culture

It is obtained the *highest average number of roots/explant* on the variants with auxin and cytokinins in the highest concentration: of 2mg/l cytokinin and 1mg/l auxin ( $V_6$  and  $V_3$ ). Figure 6 presents the average number of roots differentiated on the seventh variants from which we can see the following: on  $V_3$  (MB+1,0mg/lAIB+2,0mg/l2iP) with 2iP -2mg/l, about 6 roots/plantlet, and from the variants with BA  $V_6$

(MB+1,0mg/lAIB+2,0mg/lBA) on which the average rises three times (about 17 roots/plantlet) and V<sub>5</sub> (MB+0,5mg/lAIB+1,5mg/lBA) on which it reaches to 8 roots/plantlet; on the other the values are smaller. We can see the favorable effect of the combination between the high doses of BA and AIB on the average number of roots/plantlet.

## CONCLUSIONS

1. *Ex situ* conservation through *in vitro* multiplication of the species from the spontaneous flora ensures obtaining a large number of specimens, in a relatively short period of time, phenotypically and genotypically identical with the mother plant from which the tissue was sampled;

2. For the initiation of the *in vitro* culture it can be used a single mother plant, a seed, a leaf, an apex, a meristem, a cell, etc., (an explant), without compromising the plants from the nature which are as it is few, a major advantage for the protection of the plants in their habitats;

3. *In vitro* multiplication of *Campanula rotundifolia* L. specie is dependent on the nature of the specie, on the composition of the culture medium (the nature and the concentration of phytohormones), on the age of the mother plant, on the type of explant, on the physiological phase of the tissue and on the period when the explant was sampled;

4. Completely conformed plants, with a good Radicular System were obtained after about 50 days of *in vitro* culture on V<sub>3</sub>, V<sub>5</sub> and V<sub>6</sub>, mediums with a content of cytokinins (BA, 2iP) in a dose of 1,5 - 2 mg/l and an auxin (AIB) in a concentration of 0,5 - 1,0mg/l.;

5. *Campanula bud* proved a very good *in vitro* regenerative capacity of 90% and a multiplication capacity of over 80% on the medium with the highest concentrations of phytohormones (V<sub>6</sub>);

6. After covering the preliminary stages (protection under a hand-glass, adjustment of temperature, humidity and light depending on the needs of the specie), the plants acclimatized in a percentage of over 80% ensuring a vigorous and quantitatively and qualitatively valuable material;

7. We recommend *ex situ* conservation of the plants of *Campanula rotundifolia* L multiplied *in vitro*, through the reconstruction and the repopulation of the areas where the specie was extincted or is endangered and through its maintaining in a architectural landscape space (in the Botanical Garden).

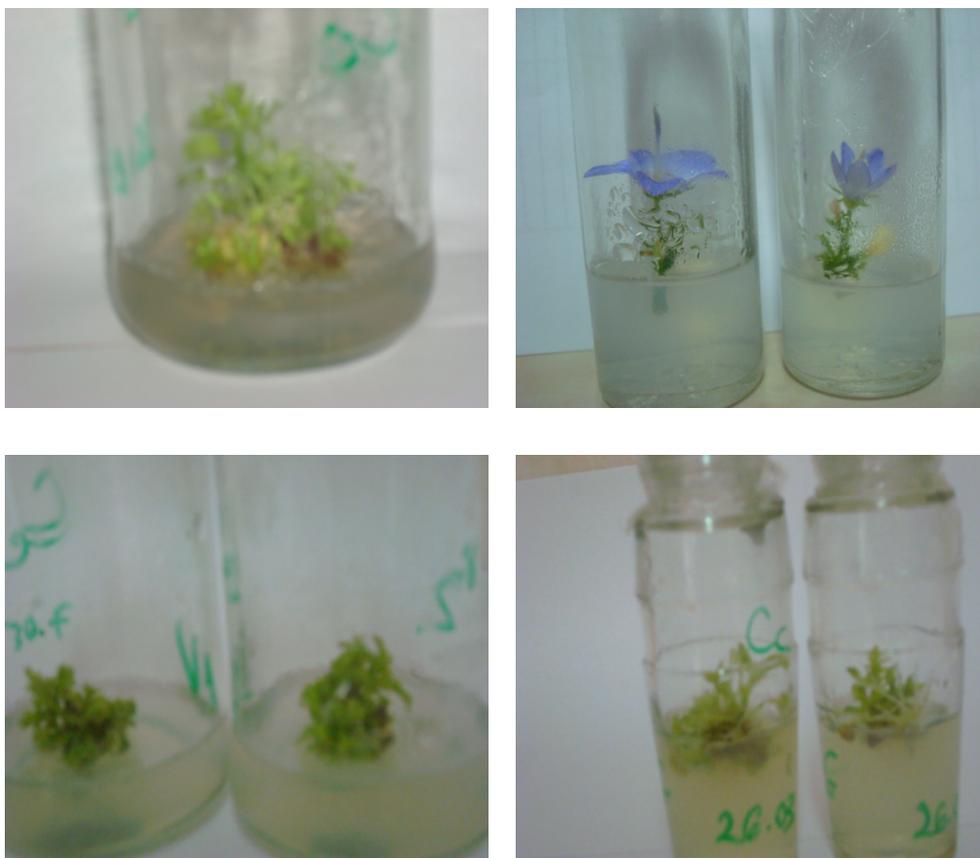


Photo.1, 2, 3, 4 *Campanula* bud regenerated and multiplied *in vitro* after 50 days

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