THE USE OF BIOTECHNOLOGY FOR SUPPLYING OF PLANT MATERIAL FOR TRADITIONAL CULTURE OF MEDICINAL, RARE SPECIES *Arnica montana* L.

Iuliana PANCIU[1], Irina HOLOBIUC[2], Rodica CĂTANĂ[2]

e-mail: lily.panciu@yahoo.com

Abstract

Taking into account the importance of *Arnica montana*, the attempts to improve the culture technologies are justified. Our study had the aim to optimize *in vitro* plant multiplication and growth as a source of plants for traditional culture in this species. Aseptic germinated seedlings were used as explants, apical meristem being the origin of the direct morphogenesis process. For induction of regeneration, to promote plant growth and rooting, we used some combination of growth factors and supplements as ascorbic acid, glutamine, PVP and active charcoal added in culture media based on MS formula. We improved the efficiency of micropropagation, the best values were recorded on variant supplemented with PVP –.7 regenerants/explant in the first 4 weeks and increasing at 17/ initial explant (mean 14.62) after 8 weeks. Concerning the germination capacity of the seeds scored after 2 weeks in sterile condition, the rate was 47.76 and in non-sterile conditions, the rate varied depending of the substrate used. Comparing to the plants obtained through traditional seeds germination, *in vitro* plants grew faster and were more vigourously. The micropropagation protocol in *Arnica montana* L. allowed us to regenerate healthy, developed and rooted plants in the second subculture cycle. This *in vitro* methodology can provide plant material for initiation of a conventional culture after acclimatization of the obtained vitroplants.

Key words: *Arnica montana*, vitroplants, improved regeneration rate.

A. montana is a vulnerable taxon, valuable as medicinal plant in traditional medecine. The habitats fragmentation, grazing, overexploitation through excesive harvesting conducted to the diminshig of the natural populations.

This taxon is introduced in Annex D of EU Council Regulation No.338/97 and Annex V (b) Habitats Directive (92/43 EC), being included in the red lists (Boscaiu N. et al., 1994, Oltean M. et al., 1994, Oprea A., 2005).

Arnica montana is a valuable medicinal plants, having an anti-inflammatory, antiseptic (antibacterial and antifungal) and reparatory effects, owing to its active compounds as sesquiterpene lactones. As plant organs used in phytotherapy and cosmetics are collected inflorescence and rhyzomes.

The species is cultivated in different countries to supply plant material for pharmaceutical purpose, meanwhile reducing the collecting pressure in the natural populations.

In Romania, there are some cultures of this species but on relatively reduced areas.

Taking into account the importance of *Arnica montana*, several studies concerning its culture, seeds germination and *in vitro* culture were reported (Chonchou O. et al., 1992; Malarz J. et al., 1993; Nichterlein K., 1995; Lê C., 1998; Weremczuk-Jezyna I. & Wysokinska H., 2000; Zăpârţan M. & Deliu C., 2001; Butiuc-Keul A. & Deliu C., 2001; Butiuc–Keul A. et al., 2002; Trejgell A. et al., 2009; Ştefanache C.P. et al., 2010; Duţă M. et al, 2010; Petrova M. al., 2011; Nikolova M. et al., 2013).

The rate of regeneration is not so high in this taxon in comparison to other related species.

Our study has the aim to improve the *in vitro* plant multiplication and growth and to compare with traditional seeds germination as source to provide plant material for the establishment of a field culture in this taxon.

We tested appropriate growth factors combination to improve the regeneration, growth of vitroplants and rooting.

[1] University of Agronomic Sciences and Veterinary Medicine, Bucharest
[2] Institute of Biology, Romanian Academy, Bucharest

MATERIAL AND METHOD

The plant material was represented by seeds purchassed from Germany, which were sterilized through washing in runnig tap water for 2 hours, short immersion 1 minute in 70º ethylic alcohol, sterilization in 0.1% mercuric chloride, three washing in sterile distilate water. The sterilized seeds were cultivated for one week in sterile distilled water supplenmented with 5 mg/l gibberelic acid and than transferred on MS(Murashige T. and Skoog F., 1962) medium free of growth factors for plant development.

Despite the first seeds geminated after 7 days of culture, the germination capacity (% of germinated seeds) were recorded after 2 weeks.

Different media variants based on MS formula of micro and macroelements, added with Gamborg vitamins (Gamborg O.L. et al., 1968), 30 g/l sucrose, 7 g/l Agar Duchefa Biochimie, adjusted at pH 5.8 and supplemented with different compounds were tested for direct morphogenesis (*table 1*). The explants were represented by aseptic germinated seedlings of ~ 1cm height.

The subcultures were made at every 4 weeks.

For each variant were cultured 2 explants/ petri dish in 4 repetitions in the first stage and 4-5 explants/ Duchefa polypropylene autoclavable box of 9x10X10 cm. All the cultures were maintained in the growth chamber at 3000 lux illumination and 16/8 photoperiod and 25^0 temperature regime.

In the second stage of multiplication, we used media variants added with active charcoal AC (0.5g/l) to improve plant vigourosity and to help rooting. AC also prevents phenolic compounds accumulation in the culture medium.

The cultures were evaluated using 2 parameters: the mean number of regenerants/ initial explant scored after two time intervals (4 weeks, 8 weeks, respectively), and the maximum length of the developed plants (in cm).

Also the maximum height of plants were compared *in vivo* and *in vitro*.

Non-sterilized seeds originated from the same source were treated for 2 days with distilled water and 5 mg/l GA_3 and susequently were sowed on different substrates as V1(mixture of soil substate 50%+ perlite 50 %), V2 (mixture of soil substate 50%+peat 25% +perlite 25 %),V3(mixture of soil substate 50%+ sand 50 %.

The seeds were sowed ~ 100 for each treatment in 3-5 repetitions and were maintained at 25^0C temperature and normal day/night regime.

The germination of seeds after 2 weeks and the growth of *in vivo* seedlings after 8 weeks were recorded.

Graphic values are expressed as mean values ±SD. One-way analysis of variance (ANOVA) was applied to calculate the statistical significance at $p<0.05$.

RESULTS AND DISCUSSIONS

The *in vitro* developmental way in *A. montana*, similarly to other *Asteraceae* taxa is direct and indirect morphogenesis, but both for conservative purpose and also for multiplication the direct way is preferred because the regeneration is better and plants are more genetically stable.

Our work involves the establisment of an efficient *in vitro* regeneration protocol through direct morphogenesis, which was compared with traditional seeds germination.

In our study, we tested usual growth factors as citokinine benzyl-amino purine BAP, Kinetin or more expensive Zeatin associated with alfa-naphthyl acetic acid (more stable as IAA). For induction of higher regeneration rate, to promote plant growth and rooting, we used some combination of growth factors and active charcoal (0.5 g/l). 2, 4-D used as auxin instead of NAA, did not significantly improve the regeneration.

Gibberelic acid presence in the culture media help shoots to elongate.

Zeatin adding in M2 variant also did not improves significantly the regeneration comparing to other variants tested.

The use of classical culture media based on MS formula and supplemented with citokinins and auxins in 10/1 ratio, gibberelic acid (0,25 mg/l) and some other compounds as ascorbic acid, glutamine or polyvinyl pirrolydone, conducted to impoved regeneration (*figure 1*), good growth and vigour of plants. The best values were recorded on variant M7 supplemented with PVP -7 regenerants/explant in the first 4 weeks (*figure 3*) and increasing at 17/ initial explant (mean 14.62) after 8 weeks (*figure 4*). Rooting also occured on the same media variants (*figure 2*) without to be necessary the transfer of shoots on rooting medium –in phase III.

The *ex vitro* acclimatization of the rooted plants was made starting with 9-10 weeks after initiation phase on V1 variant, with 75% survival rate in the recorded after 3 weeks.

Our results were satisfactory when are compared to previously reported works.

Conchou O. et al.,(1992) reported 7.7 regenerants/explants on medium MS and 9 regenerants on B5 medium suplimented with BA (1 mg /l) and NAA (0,1 mg/l), after 6 weeks of culture.

Weremczuk-Jezyna & Wysokinska (2000) obtained plants through direct shooting on MS medium (Murashige & Skoog, 1962) added with IAA (beta indole acetic acid) 0,5 M /l and zeatin (0,05 M/l) with a medium rate of 6 shoots/ explant.

In Romania, Butiuc-Keul A. &Deliu C. (2001) obtained direct shoot formation on MS medium added with N6-[2-izopenthenyil] adenine (2-iP), zeatin and alfa-naftilacetic acid (NAA) with a maximum regeneration of 3,2 neoplantuls per explant. Butiuc–Keul A. et al., 2002, reported

later an improved regeneration of 3,6 shoots/explant on semisolide medium added with yeast extract, BAP 1mg/l and IBA 1mg/l.

Trejgell A. et al. (2009) used aseptic germinated seedlings as explants source, just apical meristems showed morphogenic response, the best was recorded in presence of 3,0 mg /l BAP (2,5 shoots/ explant). The shoots were rooted on auxin free media.

Ştefanache C.P. et al., (2010)also used aseptic germinated seedlings collected from natural populations or inflorescences but the regeneration was quite reduced after 4 weeks, just 3-4 regenerants/ explants were obtained on MS medium + BAP 1mg/l or 2 mg/l associated with NAA 0,3 mg.l. Ştefanache C.P. et al., (2011) also made a comparative analysis between *in vivo* and *in vitro* obtained plant material concerning active compounds content

Duţă M. et al, 2010, used a ratio of 0.02 auxins:4 citokinins, 40g/l dextrose and active charcoal (0,3 g/l) to regenerate 5 plants/initial explant in the II stage of multiplication.

Petrova M. et al., 2011, reported indirect morphogenes at very low rate(0.86) regenerants/explant and direct morphogenesis (16,3 generants/explant) on MS + 1 mg /l BA +,1 mg /l IAA(beta indole acetic acid).The shoots were rooted after 4weeks on MS1/2 medium added with 0.5 g/l Indole buthylic acid (IBA).

Concerning the germination capacity (%) of the seeds scored after 2 weeks in sterile condition, the medium rate was 47.76 and in non-sterile conditions, the rate varied depending of the substrate used, better values were recorded on V1 and V3 (*table 2*).

Vârban D.I. et al., 2012 have also made a study concerning germinative energy and capacity of seeds from 3 different origins and used 4 different substrates, the best response being obtained on V1 variant consisted in 50% peat, 25% terra rosa, 25% sand.

Table 1

Media composition used for the induction of direct morphogenesis in *Arnica montana* L.

Components		**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**
Macroelements		MS	MS	MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS
Vitamins		B5	B5	B5	B5	B5	B5	B5
Growth factors (mg/l)	**BAP**	**1**	**-**	**1**	**1**	**1**	**1**	**1**
	Kin	**-**	**-**	**1**	**1**	**1**	**1**	**1**
	Zea	**-**	**1**	**-**	**-**	**-**	**-**	**-**
	NAA	**0.1**	**0.1**	**-**	**0.2**	**0.2**	**0.2**	**0.2**
	2,4-D	**-**	**-**	**0.2**	**-**	**-**		
	GA_3	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**
Other compounds (mg/l)	**A a**	**-**	**-**		**-**	**20**	**-**	**-**
	Glut	**-**	**-**	**-**		**-**	**200**	**-**
	PVP	**-**	**-**	**-**	**-**		**-**	**10.000**

Legend: MS- Murashige & Skoog medium ; B5 Gamborg- vitamins; BAP - benzyl aminopuryne; Kin- kinetin; Zea- zeatin, NAA - alfa-naphtyl acetic acid, 2.4-D-dichlor phenoxy acetic acid, GA3- gibberelic acid,A a Ascorbic acid, Glut- glutamine, PVP-polyvinyl pyrrolidone.

Figure 1 **Regenerated rooted plant in the phase II of culture on M6 variant - transplantation.**

Figure 2 **Regenerated rooted plant obtained in the phase II of culture on M7 variant before ex vitro**

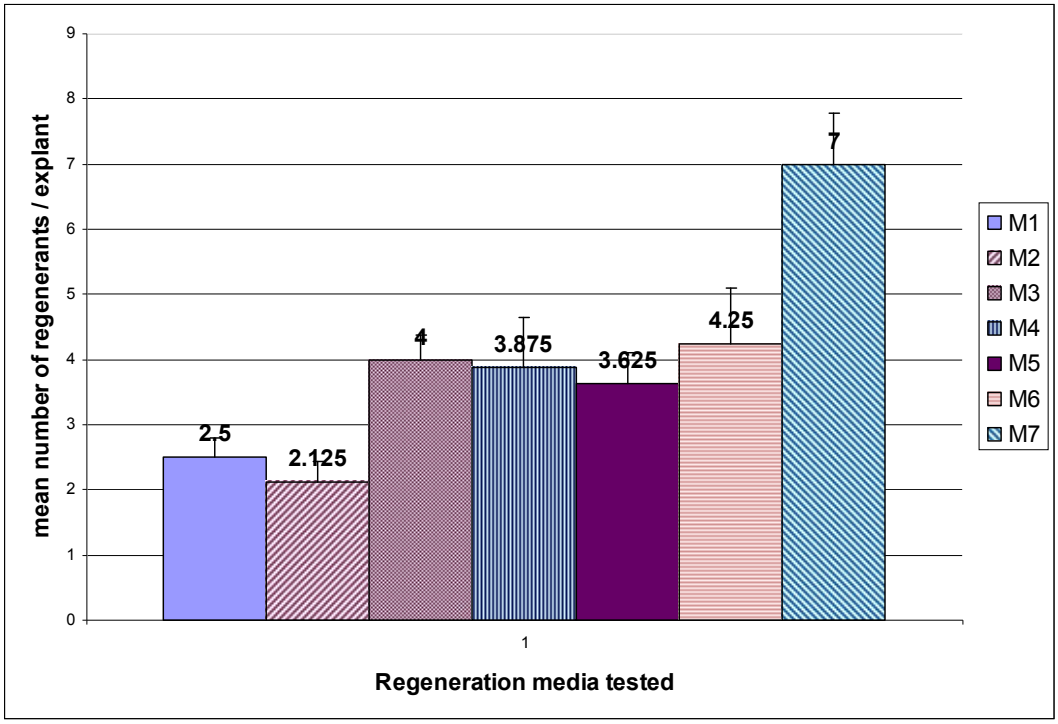

Figure 3 **The number of regenerants/explant after 4 weeks of culture (mean values+SD).**

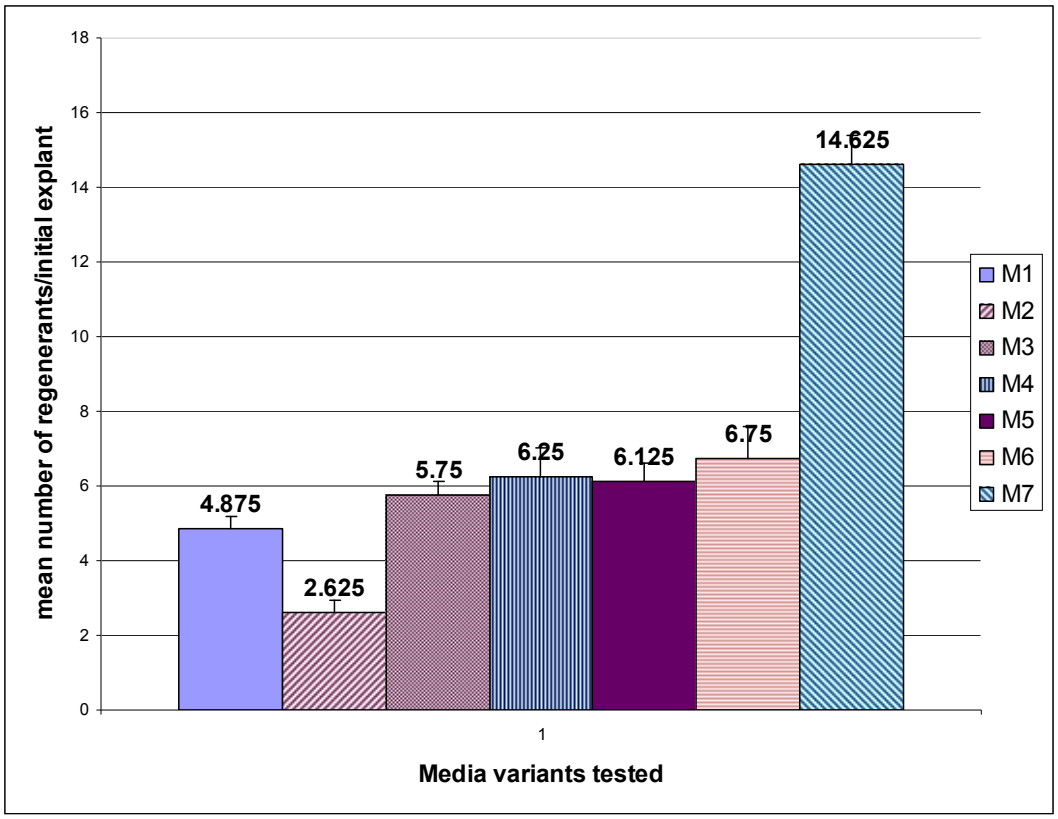

Figure 4 **The number of regenerants/initial explant after 8 weeks of culture (mean values +SD).**

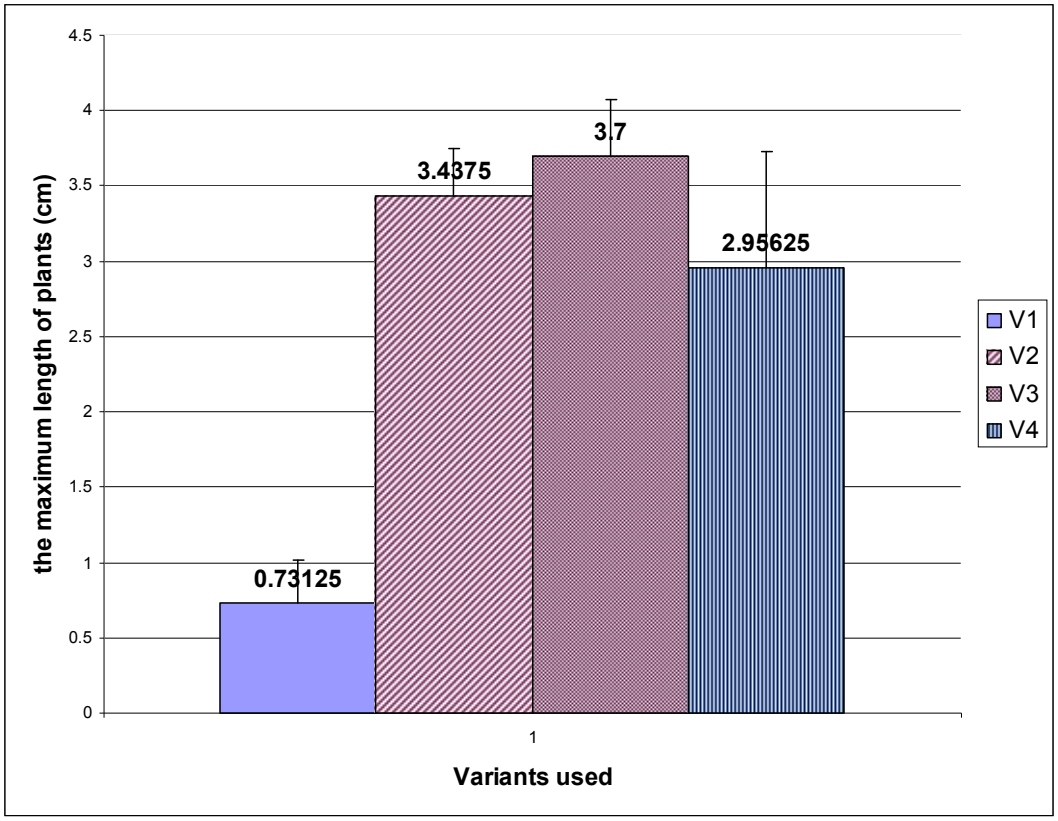

Figure 5 **The maximum growth of plants cultured in different conditions (V1- in non-sterile condition on soil/peat/sand mixture; V2- variant supplemented with Ascorbic acid and AC;V3- variant with glutamine and AC, V4- medium added with with PVP and AC (mean values +SD) after 2 months.**

Table 2

Seeds geermination in different experimental conditions (%)

Germination capacity of seeds	
V0 sterile condition	47.76 %
Non-sterile conditions on different substrates	
V1 soil 50%+perlite 50%	58.8%
V2 50 % soil+25% peat+25% perlite	20%
V3 50 % soil +50% sand	66.6 %

Our seeds showed a lower germination capacity, the best response was recorded on V3 variant, but provided us enough material to initiate *in vitro* cultures.

Concerning the growth of non-sterile seedlings and regenerated plants, we observed that *in vitro* plants grew faster and were more developed (V2-V4) compared to those obtained through classical germination-V1 (*fig.5*).

CONCLUSIONS

Our *in vitro* multiplication protocol for *Arnica montana* can provide healthy, developed and rooted plants in the second phase of culture, starting from aseptic germinated seeds as source of explants, through direct organogenesis originated from apical meristem of seedlings. The regenerants rate of growth is higher comparing to plants derived from the seeds traditionally germinated.

The protocol can ensure plant material for the initiation of traditional cultures after acclimatization of the regenerants.

Using affordable plant growth factors, but combined with some supplements and active charcoal help to improve regeneration, growth and rooting. It is not necessary the culture of neo-formed shoots separately on rooting media as in previous reports.

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