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Grădina Botanică "Alexandru Borza"
Cluj-Napoca

THE ROLE OF NATURAL EXTRACTS ON THE IN VITRO MULTIPLICATION OF *ARNICA MONTANA* L.

Anca BUTIUC-KEUL [1]***, Alexandra ŞUTEU*** [2]***, Martin KEUL*** [1]***, Constantin DELIU*** [1]
[1] Institutul de Cercetări Biologice, str. Republicii, nr. 48, **RO-3400 Cluj-Napoca**
[2] Universitatea "Babeş-Bolyai", Grădina Botanică "Alexandru Borza",
str. Republicii, nr. 42, **RO-3400 Cluj-Napoca**

Abstract: Micropropagation of *Arnica montana* L. using MS medium supplemented with growth regulators as benzyl adenine (BA), and β-indolilbutiric acid (IBA) in combination or not with natural extracts does not ensure the formation of high number of regenerated plants, maximum 2.66 neoplantlets/explant were obtained. Plants growth is good, after 4 weeks of culture obtained on medium supplemented malt extract or yeast extract, plants were 3.06-3.53 cm length, but root induction was obtained with good results only on media supplemented with pumpkin extract in combination with IBA. The clonal propagation of this species was improved by in vitro culture of from nodal segments using semisolid media supplemented with yeast extract in combination with growth regulators, phloroglucinol or adenine sulphate. Only three weeks after the inoculation plant multiplication as well as induction of roots were obtained The best results have been obtained on semisolid MS media supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 100-300 mg/l yeast extract.

Introduction

Arnica montana L. is a very important medicinal plant for its valuable anti-inflammatory and cicatrizing properties. These properties are due to the presence of sesquiterpene lactones of helenalin type in the inflorescence. The mentioned lactones also have a cardiotonic and cardiotoxic action [3]. For these reasons, *Arnica montana* has been excessively collected and became rare. According to Red List of Higher Plants from the Romanian Flora [5], this species is considered vulnerable and rare. Methods of preserving and restoring natural resources have been given much attention in the last years. Among these, in vitro micropropagation aroused great interest. The micropropagation technique has been pursued in this paper, using some natural extracts as maize extract, pumpkin extract, malt extract and yeast extract. The maize extract, due to its content of vitamins, mineral salts, glucose, lipids, cytokinins (zeatin), plays an important role in regeneration and multiplication of different plant species cultured in vitro [1,7]. There are some previous papers about in vitro multiplication of *Arnica montana* by using growth regulators [10] and maize extract on the semisolid media [2], but the multiplication of this species is generally low. The pumpkin, malt and yeast extracts are not usually used for the in vitro multiplication, but it is interested to

study their influence because of the content in vitamins, mineral salts, aminoacids that could replace some of the components of the culture media.

Material and methods

Culture media and conditions

The basal medium consist of Murashige and Skoog (MS) mineral salts and vitamins [4] plus 3% sucrose, solidified with 0.7% agar. The medium Ph was adjusted to 5.7 with NaOH before autoclaving (120°C for 20 min). The growth regulators, aqueous natural maize extract (EP), pumpkin extract (ED), malt extract (EM) and yeast extract (EY) were added to the media before autoclaving, in different combinations. The influence of growth regulators as benziladenine (BA), β-indolilbutiric acid (IBA) in combination or not with natural extracts was studied on the following media:

EP1 – MS + 1.0 mg/l BA + 1.0 ml/l maize extract;
ED1– MS + 1.0 mg/l BA + 1.0 ml/l pumpkin extract;
EM1– MS + 1.0 mg/l BA + 100 mg/l malt extract;
EY1 – MS + 1.0 mg/l BA + 100 mg/l yeast extract;
EP2 – MS + 1.0 mg/l AIB + 1.0 ml/l maize extract;
ED2 – MS + 1.0 mg/l AIB + 1.0 ml/l pumpkin extract;
EM2 – MS + 1.0 mg/l BA + 100 mg/l malt extract;
EY2 – MS + 1.0 mg/l BA + 100 mg/l yeast extract;
EP3 – MS + 5.0 ml/l maize extract;
ED3 – MS + 5.0 ml/l pumpkin extract;
EM3 – MS + 300 mg/l malt extract;
EY3 – MS + 300 mg/l yeast extract.

For maize and pumpkin extracts preparation germs were mortared in water (w/v) and after that centrifuged 15 min at 5000 rpm. The pellet was discarded and the extract was sterilized by thyndalization. The extract was stored at –17 °C. Malt extract and yeast extract were used as powder.

To improve the in vitro multiplication of *Arnica montana* an other experiment was carried out on the media supplemented with yeast extract in combination with growth regulators, phloroglucinol or adenine sulphate. The following semisolid media (0.4% agar) were studied:

I. MS + 1.0 mg/l BA + 1.0 mg/l AIB and 100 mg/l yeast extract;
II. MS + 1.0 mg/l BA + 1.0 mg/l AIB and 300 mg/l yeast extract;
III. MS + 100 mg/l yeast extract and 80 mg/l phloroglucinol;
IV. MS + 100 mg/l yeast extract and 40 mg/l adenine sulphate;

Cultures were maintained permanently in a growth chamber at 25-27°C with a total irradiance of 87 μmol/m^2/s provided by fluorescent tubes, under a 16 h daylight regime.

Plant material

The culture was initiated from nodal segments (10 mm long) of in vitro cultured plants obtained by seed germination. After 4 weeks of culture the explants

were cut out and inoculated into MS medium [4] supplemented with different growth regulators and natural extracts.

The culture was evaluated four weeks after the inoculation, the number and the length of regenerated plantlets/explant being followed as well as the number and length of roots/explant.

Subcultures were performed every six weeks, to ensure the plants material for other experiments. All experiments were performed three times and twenty explants were used on each treatment. Regeneration efficiency was calculated considering the mean number of shoots or roots per explant.

Results and discussions

In vitro multiplication of *Arnica montana* on media supplemented with different natural extracts in addition or not with growth regulators is shown in Fig. 1a. On ED3 and EM2 media plant regeneration was not obtained, explants died after two weeks of culture. On the EY2, EP1 And ED2 culture media, plant regeneration took place, but plant multiplication was very low. The best media for multiplication were EY1, where 2.66 plantlets/explant have been obtained, and EM1 media where 2.33 plantlets/explant have been obtained. On ED1, ED2, EM3, EP1, EP1, EP3, EY3 media there is no plant multiplication.

Plant growth on all of these media is good, plants were in average, 1.96-3.06 cm length. The best medium for plant growing is EY1, where plantlets were 3.53 cm length (Fig .1b).

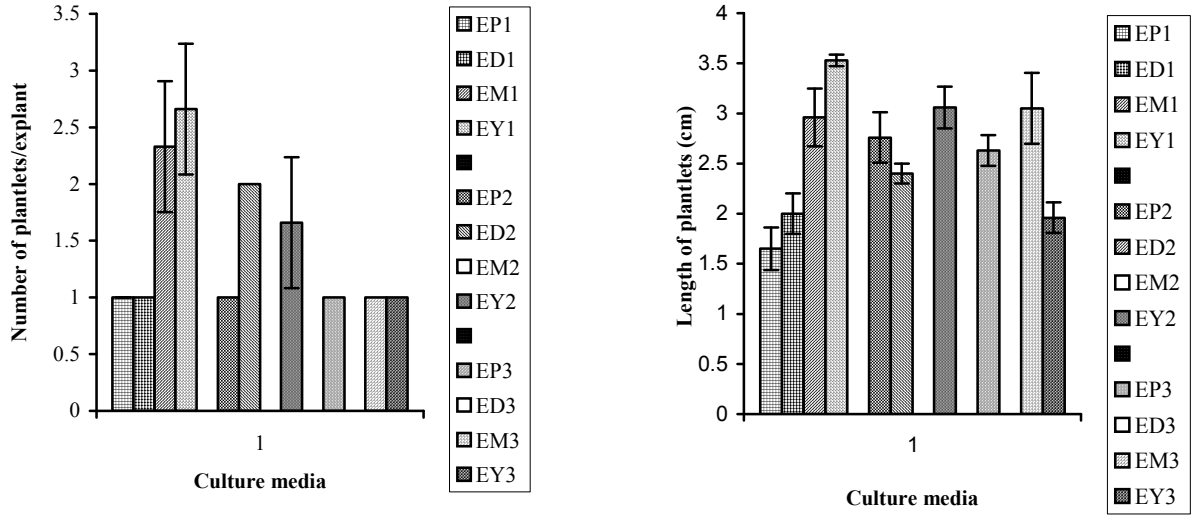

Fig. 1: Plant multiplication of Arnica montana on media supplemented with natural extracts and growth regulators (a - number of plantlets/explant, b - length of plantlets).

Root induction was obtained on media supplemented with maize extract 1.0 ml/l and IBA 1.0 mg/l (6 roots/explant have been obtained having 3.96 cm length). On the media supplemented with pumpkin and malt extracts root induction was not

obtained. Media supplemented with yeast extract in addition with 1.0 mg/l BA, 2.66 roots/explants having 1.16 cm length. On the media supplemented with yeast extract in addition with 1.0 mg/l IBA, 1.66 roots/explants having 1.06 cm length have been obtained.

The influence of semisolid media supplemented with yeast extract in combination of growth regulators, phloroglucinol or adenine sulphate on the in vitro multiplication of *Arnica montana* is shown in Fig. 2 a,b. Plant regeneration took place after three weeks of culture on all media tested. Plant multiplication was obtained on MS media supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 300 mg/l yeast extract (2,6 plantlets/explant having 4.44 cm length) but the best media was MS supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 100 mg/l yeast extract where 3.6 plantlets/explant have been obtained, having 4.18 cm length. On the media supple4mented with yeast extract and phloroglucinol or adenine sulphate, plant multiplication is low, but plant growing is good.

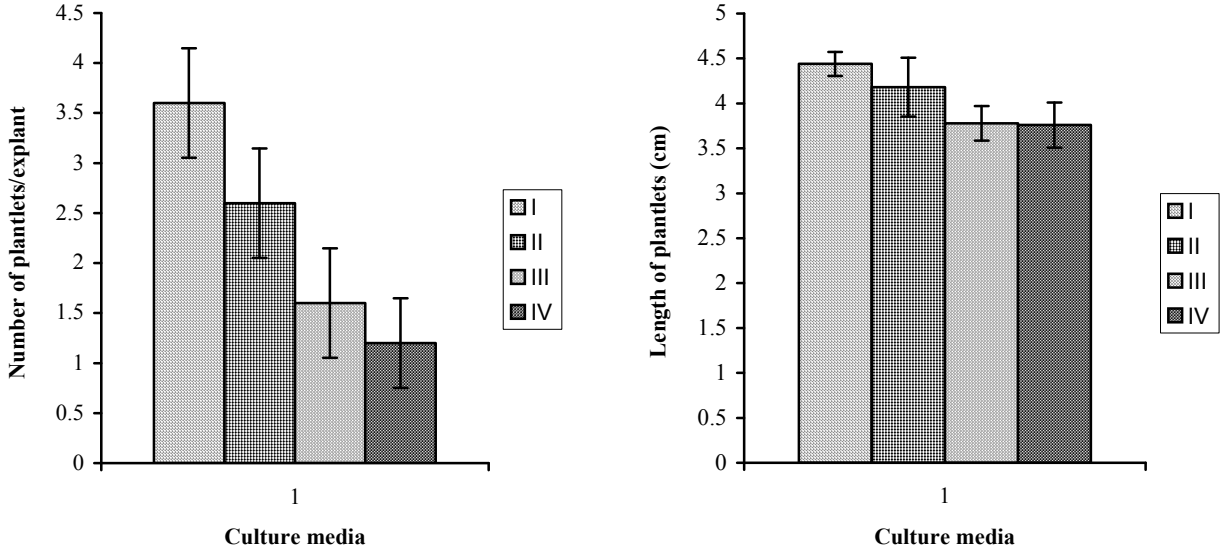

Fig. 2: Plant multiplication of Arnica montana on media supplemented with yeast extract and other chemical compounds (a - number of plantlets/explant, b - length of plantlets).

Root induction was also obtained with good results on the media MS supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 100 mg/l yeast extract (6.6 roots/explant having 4.5 cm length) and MS media supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 300 mg/l yeast extract (6.4 roots/explant having 4.54 cm length). Media MS supplemented with yeast extract and phloroglucinol ensured root induction as well (5.4 roots/explant having 3.38 cm length) (Fig. 3 a,b).

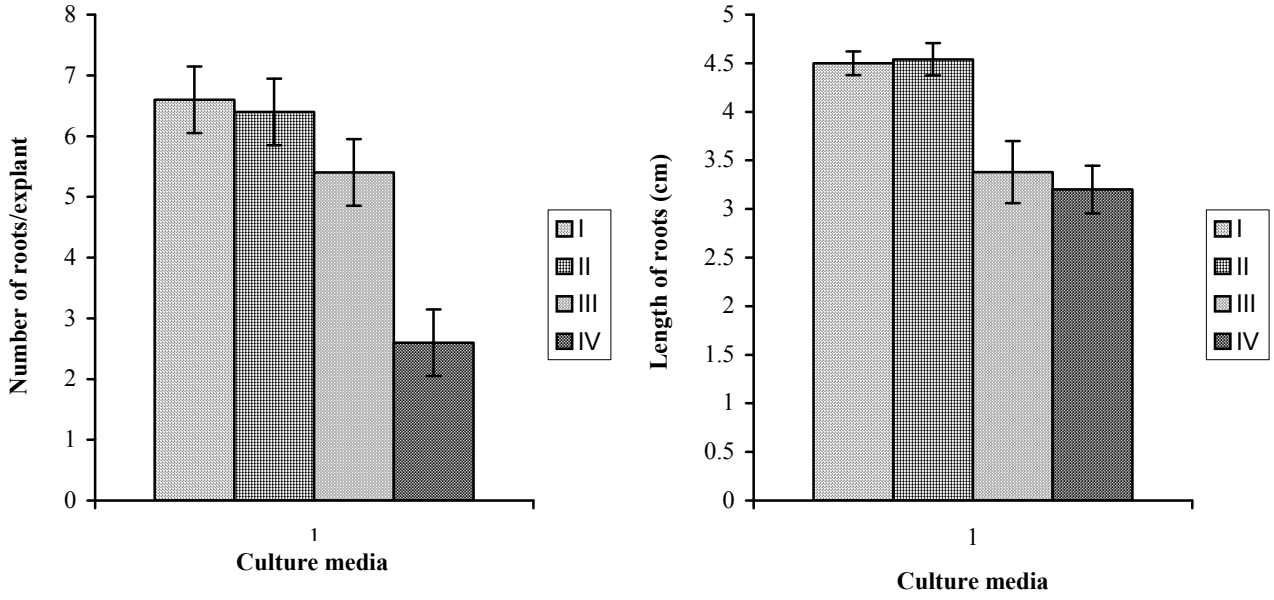

Fig. 3: Plant multiplication of Arnica montana on media supplemented with yeast extract and other chemical compounds (a - number of roots/explant, b - length of roots).

As it is known the natural maize extract has valuable properties and has been successfully used for other plants regeneration and micropropagation as *Leontopodium alpinum* [1,8], several flowery species [9]. The phloroglucinol was used for micropropagation of *Leontopodium alpinum* [1], *Frittilaria imperialis* [9]. Adenine sulfate was used for germination of somatic embryos of *Rosa hibryda* cv. *Landora* [6].

The semisolid media stimulated the roots induction and their growth that ensure the successful acclimatization of plantlets. To our knowledge, this is the first report on the use of semisolid media supplemented with natural yeast extracts or phloroglucinol and adenine sulfate for *Arnica montana* micropropagation.

In the protocol described here, we improved the multiplication of this plant species. Micropropagation of endangered plants from shoot tip cultures is limited because of difficulty to obtain a large number of explants. Thus the micropropagation from stem explant from in vitro regenerated plantlets can be an alternative to shoot tip culture. Plant regeneration of *Arnica montana* from leaf or root explants is very difficult, firs the callus was induced and then after organogenesis, the number of plantlets was low. The in vitro culture can be initiated from aseptic seedlings obtained through in vitro seed germination as well. Hence, in vitro multiplication using natural maize extract or others chemical compounds as phloroglucinol or adenine sulfate may be recommended for the clonal propagation of *Arnica montana*, that is important for preservation of this plant species.

Conclusions

In vitro multiplication of *Arnica montana* on media supplemented with natural explants in combination or not with growth regulators is generally low. The best media for multiplication were MS supplemented with 1.0 mg/l BA + 100 mg/l yeast extract, where 2.66 plantlets/explant have been obtained.

Root induction was obtained only on media ED2, EY1, and EY2.

Plant multiplication and root induction were improved by using semisolid MS media supplemented with 1.0 mg/l BA + 1.0 mg/l AIB and 100-300 mg/l yeast extract.

In summary, using the above protocol, it will be possible to obtain in average 4 plantlets per nodal explant in one month.

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ROLUL EXTRACTELOR NATURALE ASUPRA MULTIPLICĂRII IN VITRO LA *ARNICA MONTANA* L.

(Rezumat)

Multiplicarea masivă şi rizogeneza in vitro la *Arnica montana* este dificil de realizat pe medii de cultură suplimentate cu concentraţii moderate de reglatori de creştere. Există numeroase studii privind aceste aspecte, însă numărul de plantule/explant obţinute in vitro este relativ mic

(2-3 plantule). Studiile privind utilizarea extractelor naturale, care au dat rezultate foarte bune în multiplicarea şi rizogeneza in vitro la alte specii, nu au avut aceleaşi rezultate şi la *Arnica montana.*

În această lucrare s-a urmărit studiul rolului extractelor naturale de porumb, dovleac, malţ şi drojdii asupra proceselor de multiplicare şi rizogeneză in vitro la *Arnica montana,* în vederea îmbunătăţirii tehnicilor de multiplicare existente, precum şi pentru înlocuirea unor componenţi ai mediului de cultură cu extracte naturale, în vederea reducerii preţului de cost al multiplicării in vitro. În acest sens s-au realizat două serii de experimente. În prima etapă s-a testat rolul extractelor naturale, în combinaţie sau nu cu BA şi AIB. S-a constatat că indiferent de natura extractului natural utilizat, multiplicarea este foarte redusă, cel mai mare număr de plantule/explant (2,66) obţinându-se pe mediul EY1. Pe mediile ED1, ED2, EM3, EP1, EP2, Ep3 şi EY3, nu s-a obţinut multiplicare, iar pe mediile ED3 şi EM2, explantele s-au necrozat. Creşterea plantulelor este relativ bună, cele mai bune rezultate sa-u înregistrat pe mediile EM3, EY1 şi EY2, unde plantulele au atins în medie 3,05-3,53 cm lungime. Pe mediile de cultură suplimentate cu extracte naturale, rizogeneza a fost indusă doar pe mediile ED2, unde s-a obţinut cel mai mare număr de rădăcini/explant (6 având 3,96 cm lungime), EY1 (2,66 rădăcini/explant având în medie 1,16 cm lungime) şi EY2 (1,66 rădăcini/explant având în medie 1,06 cm lungime).

În următoarea etapă s-a urmărit îmbunătăţirea multiplicării in vitro prin utilizarea mediilor de cultură semisolide suplimentate cu extract de drojdii (deoarece acesta a dat cele mai bune rezultate în experimentul anterior) în combinaţie cu reglatori de creştere, floroglucinol sau sulfat de adenină. S-a constatat că mediile semisolide au stimulat atât multiplicarea, cât şi rizogeneza. Cele mai bune rezultate s-au obţinut pe mediile suplimentate cu 1,0 mg/l BA + 1,0 mg/l AIB şi 100 mg/l extract de drojdii, unde s-au format 3,6 plantule/explant având 4,44 cm lungime precum şi un sistem radicular bine dezvoltat (6,6 rădăcini/explant având 4,5 cm lungime). Creşterea plantulelor şi rizogeneza sunt bune şi pe celelalte medii de cultură, mai puţin pe cele ce conţin sulfat de adenină.

Se poate astfel concluziona că multiplicarea masivă la *Arnica montana* este dificil de realizat, pentru aceasta fiind necesare medii de cultură complexe care să conţină în mod obligatoriu concentraţii moderate de reglatori de creştere precum şi extracte naturale.