# *IN VITRO* CONSERVATION OF *Achillea pyrenaica* Sibth. ex Godr., A PYRENEAN ENDEMIC SPECIES

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**Abstract.** The aim of this paper is to establish an efficient micropropagation protocol for *Achillea pyrenaica* Sibth. ex Godr., an endemic species for the Pyrenees and the mountains of South and Central France. In this regard, the aseptic culture was induced starting from sterilized seeds. In order to obtain an as high as possible multiplication rate and induction of rhizogenesis, there were used 8 culture medium variants with a varied hormone balance. Our results showed that seed sterilization with $H_2O_2$ is suitable for this study, the infection rate being extremely low. Plant growth was stimulated by $GA_3$ addition to culture media. Between the two concentrations of auxin (NAA), the lower one (0.1 mg/l) has proved to be more efficient than the higher one (1 mg/l). Media supplementation with BAP is more favourable than K, since all the studied parameters were increased. The rhizogenesis was improved on media containing IAA, as compared with the one supplemented with NAA. On pre-acclimatization culture medium, the use of activated charcoal leads to an increase of root and shoot development.

**Keywords:** acclimatization, micropropagation, plant growth regulators (PGR), rhizogenesis, seed sterilization.

**Rezumat. Conservarea *in vitro* la *A. pyrenaica* Sibth. ex Godr., specie endemică pentru Munţii Pirinei.** Scopul acestei lucrări este de a stabili un protocol eficient pentru micropropagarea speciei *Achillea pyrenaica* Sibth. ex Godr., specie endemică pentru Munţii Pirinei şi pentru cei din sudul şi centrul Franţei. În acest scop, culturile aseptice au fost iniţiate din seminţe sterilizate. Pentru a obţine o cât mai ridicată rată de micropropagare şi rizogeneză, au fost utilizate 8 variante de mediu de cultură, cu conţinut variat de fitohormoni. Rezultatele obţinute au evidenţiat că sterilizarea seminţelor cu $H_2O_2$ este potrivită pentru acest studiu, rata de infecţie fiind foarte scăzută. Creşterea plantelor a fost stimulată în urma adăugării $GA_3$ în mediul de cultură. Dintre cele două concentraţii de auxină (ANA) cea mai mică (0,1 mg/l) s-a dovedit a fi mai eficientă faţă de cea mare (1 mg/l). Adăugarea în mediul de cultură a citochininei BAP este mai favorabilă faţă de K, deoarece toţi parametrii studiaţi au prezentat valori mai ridicate. Rizogeneza a fost mai ridicată pe mediul cu AIA, faţă de mediul cu ANA. Adăugarea cărbunelui activ in mediul de cultură, în faza de pre-aclimatizare a dus la stimularea procesului de dezvoltare a rădăcinilor şi tulpinilor.

**Cuvinte cheie:** aclimatizare, fitohormoni (PGR), micropropagare, rizogeneneză, sterilizarea seminţelor.

## INTRODUCTION

Two basic strategies regarding plant biodiversity conservation are *in situ* and *ex situ* methods. *In situ* conservation implies the species conservation in their natural habitat, while *ex situ* conservation assumes the preservation and maintenance outside their natural environment (ENGELMANN & ENGELS, 2002). Among *ex situ* conservation methods, *in vitro* techniques are a great interest by allowing the propagation of vegetal material with a high multiplication rate in an aseptic environment (ENGELMAN, 2011). The biotechnology of *in vitro* cultures has a wide range of applications in biodiversity conservation, especially in the case of endemic species or of those vulnerable at different levels (CRISTEA et al., 2010).

*Achillea pyrenaica* Sibth. ex Godr. (Family Asteraceae) is an endemic species for the Pyrenees and for the mountains of South and Central France, and it grows in damp grasslands. It is a perennial species, it has a height of 20-60 cm and it is branched and puberulent above. The leaves are lanceolate, undivided, puberulent and regularly serrate. The corymb has 2-6 capitula with 5 mm white, orbicular ligules (RICHARDSON, 1976). This species is used as a medicinal plant. RIGAT et al., (2007) mentioned that in traditional medicine the inflorescences can be used for gastric analgesic and anti-inflammatory, oral infections, ocular analgesic and antiseptic, for digestion and as a sedative. Beside its medicinal importance, *A. pyrenaica* can be used as an ornamental plant (THE PLANT ENCYCLOPEDIA).

As a result of the collaboration between the "Alexandru Borza" Botanical Garden (Cluj-Napoca) and the Biological Research Institute (Cluj-Napoca) a number of endangered and/or endemic species for the European flora have been conserved through biotechnology of *in vitro* cultures (CRISTEA et al., 2002; CRISTEA et al., 2004; MICLĂUŞ et al., 2003; MARCU et al., 2006).

Taking into account that there are no available data regarding micropropagation of *A. pyrenaica*, this study was undertaken with the objective of developing an efficient *in vitro* protocol that would maintain and propagate this important species.

## MATERIALS AND METHODS

The plant material used for the aseptic culture initiation is represented by *A. pyrenaica* seeds, obtained from the Alpine Botanical Garden Viote located on Monte Bondone. Seed disinfection assumed a pre-sterilization step by soaking the seeds in 4% $H_2O_2$ for 12 hours. Afterwards, the sterilization itself consisted in submerging the seed for 1 minute in ethylic alcohol (96%), followed by 10% $H_2O_2$ for 17-18 minutes and rinsing in sterile distilled water.

The basal medium (BM) for all variants contained MURASHIGE & SKOOG (1962) micro- and macroelements, thiamine HCl, pyridoxine HCl, and nicotinic acid - 1 mg/l each, myo-inositol 100 mg/l, 2% sucrose, and 0.7% agar (w/v)

(Duchefa Biochemie B. V., Netherlands). According to our purpose, we used 8 culture medium variants with different hormone balance, as seen in table 1.

Table 1. Culture medium variants used for *A. pyrenaica* micropropagation.

| Variants | Plant growth regulators (mg/l) | | | | |
|---|---|---|---|---|---|
| | Auxins | | Cytokinins | | Gibberellins |
| | NAA | IAA | BAP | K | $GA_3$ |
| V1 | - | - | - | - | - |
| V2 | - | - | - | - | 100 |
| V3 | 0.1 | - | - | 1 | - |
| V4 | 1 | - | - | 1 | - |
| V5 | 0,5 | - | 1 | - | - |
| V6 | 0,5 | - | - | 1 | - |
| V7 | - | 1 | 1 | - | - |
| V8 | 1 | - | 1 | - | - |

Gibberellic acid ($GA_3$) is known to have an important role in getting the seeds out of dormancy, therefore this hormone was added to V2 culture medium variant (100 ml/l), comparatively with V1 variant, that does not contain $GA_3$. The PGRs used are represented by auxins - IAA (indole-3-acetic acid) and NAA (α-naphthalene acetic acid), which are known as stimulators of rhizogenesis, and cytokinins - BAP (6-benzylaminopurine) and K (6-furfurylaminopurine), known as stimulators for cellular multiplication and plant neoformation. We studied the influence of two different concentrations of auxin: NAA 0.1 mg/l (V3) and 1mg/l (V4), which were added on a containing kinetin, 1 mg/l. To enhance the micropropagation, the binodal explants were transferred on media containing NAA (0.5 mg/l) and two different cytokinins: BAP 1 mg/l (V5) and K 1 mg/l (V6). In order to optimize the rhizogenesis and to ease the acclimatization process, we compared the influence of two different auxins: IAA 1mg/l (V7) and NAA 1 mg/l (V8), which were introduced on the culture media along with BAP 1 mg/l. Previous to *ex vitro* acclimatization phase, in order to enhance the rhizogenesis, beside the use of V7 and V8 culture media variants with PGRs (Vh), the explants were also transferred on a medium without PGRs, but with activated charcoal (Vc). The microclimatic conditions from the vegetation room were: a temperature of 25±2ºC, a light intensity of 87 μmol/$m^2$/s and a photoperiod of 16 h light/8 hours dark.

The experimental design for *in vitro* multiplication and rhizogenesis consisted ~~on~~ in two repetitions for each experiment and on 20 explants for each individual. The results were expressed as the average of replicates ± standard deviation. The statistical analysis was performed by subjecting the experimental data to *t*-test (for testing 2 columns), at a 95% confidence interval, with the aid of GraphPad Prism software (version 5.00 for Windows, GraphPad Software, San Diego) and the graphics were realized with Excel program.

## RESULTS AND DISCUSSIONS

The oxygenated water as a good sterilization agent and also for breaking the seed dormancy was used. The results at 25 days from inoculation highlights that the sterilization procedure was effective, the infection rate was very low, of about 1.8%. The seed germination rate on the 25$^{th}$ day reached a value of 22.9% at light and 25% at dark. In parallel with this experiment, seeds were sown in pots and kept in greenhouse, but the germination rate was significantly reduced.

In figure 1, there are presented the results regarding *in vitro* generated neoplants evolution at 49 days from culture initiation. As it can be seen, for both media variants, there were no infections. Comparing the obtained results, it is obvious the positive effects induced by $GA_3$ on plant growth and development. On media without $GA_3$ there is a ratio of 1:1 between long and short plants, while on V2 medium variant, the ratio is about 3:1.

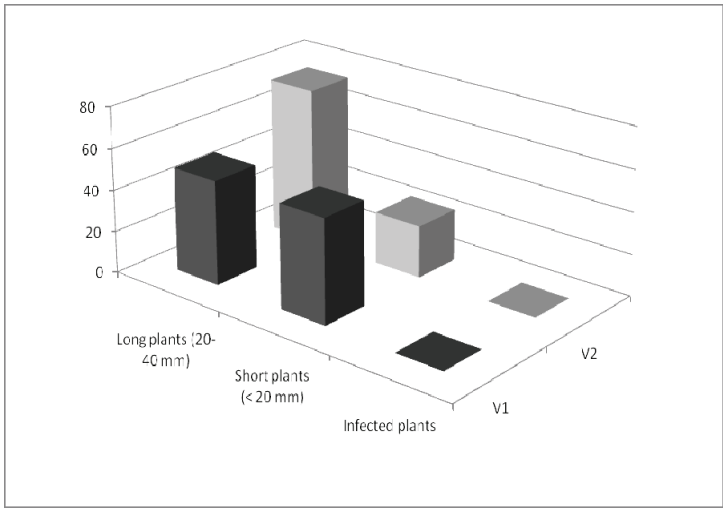



Figure 1. *In vitro* development of *A. pyrenaica* seeds on V1 and V2 culture medium variants, 49 days after sterilization.

The influence of the two different concentration of NAA from V3 and V4 culture medium variants are presented in table 2. The biometric measurements at 51 days from inoculation revealed that at lower concentrations of NAA the length of plants is double than the one obtained at higher concentration. Moreover, the number and length of ramifications were increased at lower concentrations of auxin than at higher ones. That evolution is due to the PGRs balance, the V3 variants having 10 times higher cytokinin concentration comparatively with the auxin concentration. The statistical analysis revealed that the differences between the two media are not statistically significant. At this phase of the *in vitro* culture, the roots were not fully developed, therefore a later analysis, after 98 days of culture was carried out (Fig. 2).

Table 2. *In vitro* multiplication of *A. pyrenaica* explants on V3 and V4 culture media variants, 51 days of cultivation.

| Medium variants | No. of new plants | No. of ramifications | Length of plants (cm) | Length of ramifications (cm) |
|---|---|---|---|---|
| V3 | 6.50 ± 3.1 | 3.4 ± 1.8 | 3.93 ± 2.0 | 0.74 ± 0.5 |
| V4 | 6.33 ± 3.0 | 3 ± 1.6 | 1.83 ± 0.9 | 0.65 ± 0.2 |

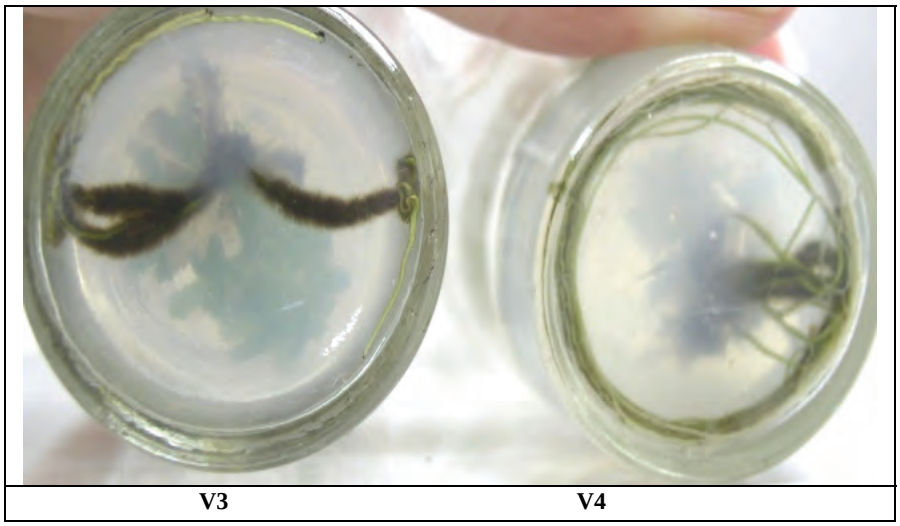



Figure 2. (original) *In vitro* rhizogenesis on V3 and V4, after 98 days of culture.

A next step in our study consisted in observing the influence of two different cytokinins – BAP (V5) and K (V6) - on *A. pyrenaica* multiplication rate and rhizogenesis, using nodal inocula. The development after 30 days of culture on V5 and V6 culture medium variants can be seen in Figs. 3a, b. Data of a subsequently analysis, respectively after 70 days of culture, are presented in table 3. Regarding the development of the nodal inocula it can be noticed that the number of neoplants is enhanced on V5 media as compared with V6. Moreover, BAP had a better influence on multiplication rate, plant growth and root number than K. The results obtained on two media are not significantly different in statistical terms.

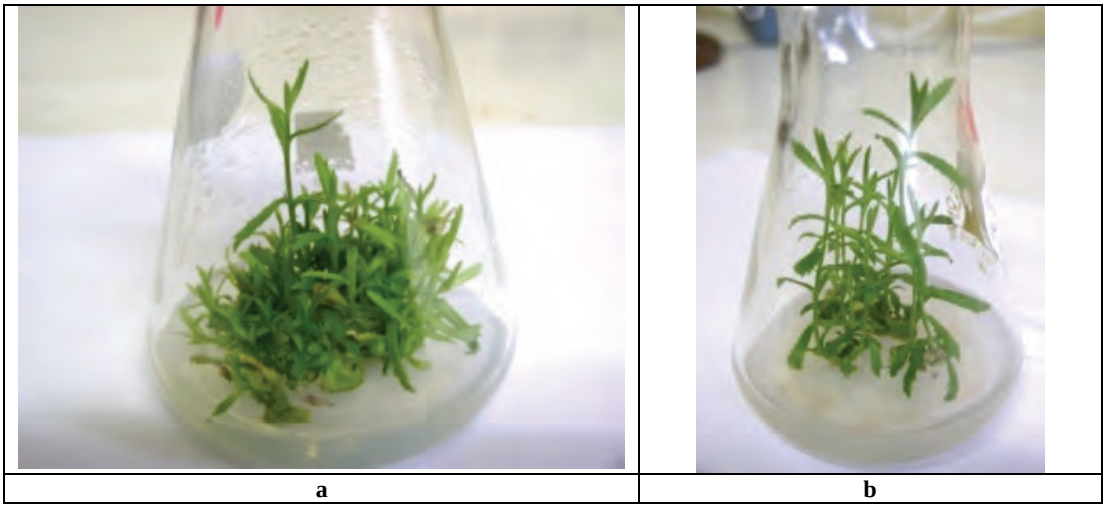



Figure 3. (original) *In vitro* multiplication of. *A. pyrenaica* on V5 and V6 culture medium, after 30 days of culture. a) Nodal explant on V5 medium variant. b) Nodal explant on V6 medium variant.

Table 3. Micropropagation of *A. pyrenaica* on V5 and V6 culture medium variants, after 70 days of culture.

| Medium variants | No of plants | No of ramifications | Plant length (cm) | No of roots | Root length (cm) |
|---|---|---|---|---|---|
| V5 | 9.75 ± 3.6 | 3.85 ± 2.0 | 1.43 ± 0.6 | 7.62 ± 2.72 | 5.79 ± 2.4 |
| V6 | 2.25 ± 0.9 | 3.01 ± 1.3 | 0.93 ± 0.3 | 6.75 ± 2.34 | 5.99 ± 1.6 |

The results obtained comparing the influence of two different auxins, respectively IAA (V7) and NAA (V8) are presented in figures 4a, b, c, d (after 59 days of culture) and in table 4 (after 97 days of culture). The statistical analysis obtained on the V7 and V8 medium variants did not show any significant difference, but on media supplemented with IAA the rhizogenesis process is enhanced, approximately 71.4% of plants exhibit a high number of thin roots, while within those propagated on media with NAA only 53.3% showed elongated roots, but visible thinner.

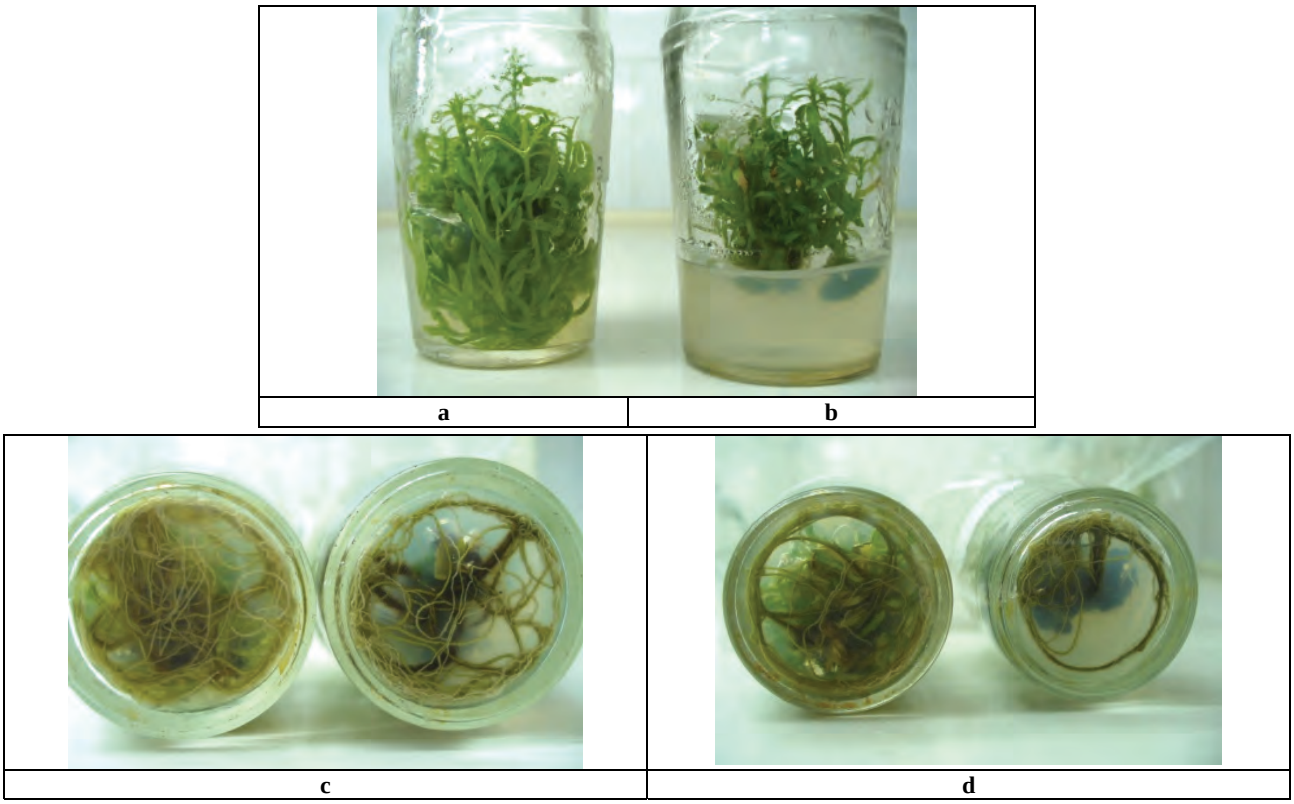
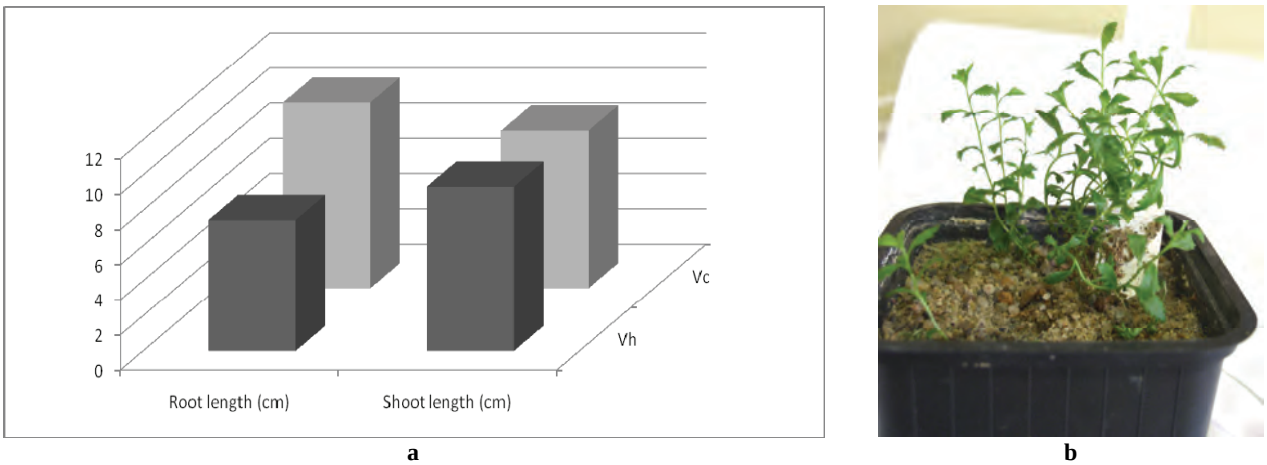



Figure 4. (original) *In vitro* propagation of *A. pyrenaica*, on V7 and V8 culture medium, after 59 days of culture. Multiplication on V7 medium (a) and on V8 medium (b). Rhizogenesis on V7 medium (c) and on V8 medium (d).

Table 4. Multiplication and rhizogenesis on V7 and V8 culture medium variants, containing two different auxins, after 97 days of culture.

| Medium variants | Root number | Root length (cm) | No of ramifications | Ramifications length |
|---|---|---|---|---|
| V7 | 12.20 ± 3.7 | 61.64 ± 21.3 | 8.01 ± 1.8 | 29.14 ± 15.2 |
| V8 | 7.96 ± 3.9 | 65.76 ± 32.6 | 10.20 ± 2.5 | 31.11 ± 11.2 |

Regarding the culture on media with phytohormones (Vh) or without phytohormones, but with activated charcoal (Vc), data statistical analysis revealed that rhizogenesis was improved on media containing charcoal, while shoot length was higher on media supplemented with phytohormones (Fig. 5a). Before planting outdoor the *in vitro* obtained plants, there were cultured in greenhouse, in sand and soil (1/1) mixture (Fig. 5b).




a b

Figure 5. (a) Pre-acclimatization phase on a medium containing phytohormones (Vh) and on media without phytohormones, but with activated charcoal (Vc); (b). (original) *Ex vitro* acclimatized *A. pyrenaica* plants, before planting outdoor.

An essential step in achieving a high efficiency in *in vitro* cultures of plant tissues is to establish a proper method of sterilization. Seeds represent an easy and safe material for culture initiation (HOLOBIUC et al., 2009). The sterilization method applied in this study was reported as a highly efficient for other species of the *Dianthu*s genus, for *Centaurea reichenbachii* DC. and *Aquilegia nigricans* Baumg. ssp. *subscaposa*) Borb. Soó (CRISTEA et al., 2004). The germination rate of *A. pyrenaica* did not present high values, but plants generated *in vitro* allowed a good micropropagation of this species.

PGRs represent a critical culture medium component for the established type of culture (PĂUNESCU, 2009). The addition of $GA_3$ to culture media induced an increase of plant length and a stimulation of the germination process. $GA_3$ plays an important role in promotion and maintenance of germination and it was effective in breaking seed dormancy in different plants including trees as *Myrica rubra* (Lour.) Siebold. & Zucc. (CHEN et al., 2008), leafy spurge (FOLEY & CHAO, 2008) and eggplant (GISBERT et al., 2009). The most widely used PGR are the auxins and the cytokinins and their ration within culture media is essential for *in vitro* cultures (PĂUNESCU, 2009). The repeated transfers on culture medium variants with different hormonal composition stimulate multiplication and rhysogenesis. Thus it was obtained the maximum multiplication rates for *Dianthus petraeus* Waldst. & Kit. ssp. *simonkaianus* (Péterfi) Tutin, on a medium with BAP and IAA, 1 mg/l each, with 110 neoplantlets/inoculum and for *Dianthus glacialis* Haenke ssp. *gelidus* (Schoot, Nyman / Kotschy) on a culture medium with BAP (1 mg/l) and NAA (0.1 mg/l), with 55 neoplantlets/inoculum. A good multiplication rate was also obtained for *Dianthus spiculifolius* Schur (40 neoplantlets/inoculum) using the latter medium cited above (CRISTEA et al., 2006). The study of *Dianthus pyrenaicus* Pourr. revealed that the multiplication rate and rhizogenesis were enhanced at low auxin concentration (MARCU et al., 2006). BAKER & WETZSTEIN (2004) stated that higher concentration of auxin induces the higher level of degradative metabolites in tissues thus blocking the regeneration process. According to BISWAS et al., (2011) lower concentrations of auxins in combination with higher concentrations of cytokinin enhance the rate of shoot proliferation. Regarding the differential response to 2 types of cytokinins, our results show that BAP had a better influence on *A. pyrenaica* cultures as compared with BUAH et al. (2010) that attributed such response to BAP higher stability in *in vitro* cultures, therefore it persists longer in the medium. Our previous study on *Dianthus pyrenaicus* showed similar results (MARCU et al., 2006). Of the two types of auxin supplemented, the response of the explants was higher for IAA. The superior effects of IAA on root elongation as compared to NAA might be attributed to several factors, such as its preferential uptake, transport, metabolization and subsequent gene activation (MULLER, 2000).

The addition of active charcoal to culture media has a beneficial effect on *A. pyrenaica* rhizogenesis, while medium supplemented with phytohormones slightly enhanced shoot length. The use of charcoal in culture medium can either stimulate or inhibit growth *in vitro*. The main induced effects can be summarized in: providing a dark environment in the medium, adsorption of certain inhibitory substances in culture, produced by either media or explants, adsorption of PGRs and other organic compounds and the release of substances naturally present in or adsorbed by activated charcoal, which are beneficial to growth of *in vitro* culture (PAN & STADEN, 1998). In parallel with this experiment it was conducted another one on *Dianthus pyrenaicus*, and the results showed that the best rhizogenesis was obtained on media with phytohormones (MARCU et al., 2006). This results highlight that evolution of *in vitro* culture is influenced both by species and culture environment.

## CONCLUSIONS

The present study describes *A. pyrenaica* behavior in *in vitro* culture in close correlation to hormone balance of 8 different culture medium variants. The seed sterilization procedure applied in this study has proved to be highly efficient, the percent of infected seeds being very low. Regarding *in vitro* multiplication, our results revealed that addition of $GA_3$ to media lead to an increase of plant growth. Low concentration of NAA had a better effect in rhizogenesis, on plant length and on ramification number. Out of the two types of used cytokinins, BAP increased all the studied parameters, thereby its application in *in vitro* culture is more favourable than the use of K. Explants developed on media with IAA showed an improved rhizogenesis as compared with those on media with NAA. On the pre-acclimatization phase, the use of activated charcoal had positive effects on root growth and developments, as well as on the shoot growth.

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Received: 21 March, 2014
Accepted: 29 April, 2014