In vitro plant regeneration from nodal segments of the alpine wormwood *Artemisia eriantha* Ten. (Asteraceae)

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Abstract. In this study, a plant regeneration system has been established for an endangered species, *Artemisia eriantha* Ten. (Asteraceae). To improve the micropropagation efficiency, frequency of shoot induction, elongation and rooting from nodal explants (plantlets yield), different growth regulators combinations (culture conditions) have been experienced. In different variants of Murashige-Skoog (MS) culture medium, with 3% (w/v) sucrose and 500 mg L^{-1} $CaCO_3$ supplemented medium, rooting shoots and hardened plantlets were produced. MS medium containing 1.8 mg L^{-1} 6-benzylaminopurine (BAP) and 0.2 mg L^{-1} kinetin was the optimum concentration for shoot induction. Obtained shoots elongated best on MS medium with 1.0 mg L^{-1} BAP, 3.0 mg L^{-1} gibberellic acid (GA_3) and rooted in half - strenght MS medium at 0.1 mg L^{-1} indole-3-acetic acid (IAA) at rate of 65%. Antioxidant activity with total phenolic and flavonoid content was screened. The maxim value of the total phenolic content (55.65 µg GAE/g) and the highest value of total flavonoids (128.07 µg ERU/g) show that the rate of these compounds *in vitro* plantlets is reduced comparing with the wild plants.
Key Words: plant regeneration, micropropagation, *Artemisia* nodal stems variety, *in vitro*.

Introduction. The alpine wormwood *Artemisia eriantha* (Ten.) is reported as protected species both at national and European level (through Habitat Directives 92/43/European Economic Community (EEC), annex V included in The IUCN Red List of Threatened Species) (Pace et al 2010; Stevanović 2011). This plant is a subendemic species which grows in rock crevices and on gravel slopes at altitudes between 2000 and 3100 m (Conti et al 2005). Many projects were developed for the protection and the valorization of this high altitude sub-endemic species (Fasciani et al 2017).

A. eriantha belongs to the family Asteraceae, which consists of over 500 species distributed worldwide. *A. eriantha* is an aromatic, hardy, perennial herb with alternate leaves, tubular florets, and small capitula, which usually occur as pendant, racemose, capitate, or paniculate inflorescences, rarely solitary (Mucciarelli & Maffei 2002). The fern-like leaves of many species are covered with white hairs.

The exploitation of this plant in the liqueur industry is justified by the terpenoids and sesquiterpene lactones which give strong aroma and bitter taste (Pace et al 2010). Because of their volatile oils - rich in thujones (Rubiolo et al 2009; Boggia et al 2017), such as terpenoids, flavonoids, coumarins, glycosides, sterols, and polyacetylenes, *Artemisia* spp. can bring benefits to human health, being reported to have antimalaria, cytotoxic, anti-hepatotoxic, antibacterial, antifungal, and antioxidant activities; a potent anti-inflammatory agent, eupatilin 20 is also distinguished in this herb (Jaleel et al 2016).

Therefore, the phytotherapeutical properties such as tonic, antiinflammatory, stimulating, digestive and antispasmodic effects recommend this species for further studies (Vouillamoz et al 2015).

Its threatened status determined us to adopt a conservation strategy through *in vitro* culture. To overcome the prohibited harvest of wild plants and to develop an effective technique for a possible reintroduction of the species in natural environment, alpine wormwood was micropropagated by *in vitro* culture techniques, followed by transfer in field.

Reports concerning micropropagation protocols at European level, focusing on major constituents of *Artemisia* species with phytotherapeutical properties are numerous, mentioning those of Mannan et al (2012), Borzabad et al (2010), and Sudarshana et al (2013), but only a few are carried out on *A. eriantha* species (Pace et al 2009, 2010).

In Romania, studies on *A. eriantha* are on uncharted ground. As a first step in our research field, the purpose of the present study was to exploit the maximum capacity growth of *A. eriantha in vitro* tissues, to different concentrations of hormones from the culture media. Research directions objectives were shoot induction and elongation, rooting and acclimatization.

Material and Method

Plant material and culture conditions. An *in vitro* propagation protocol was developed, with the aim of obtaining cloned plants, with some adapted methods described before by Borzabad et al (2010), and Pace et al (2004, 2009, 2010). The *A. eriantha* explant material was excised from original plant, collected and determined at full flowering stage (Ion R., June 2013) from Ceahlau, Cusma Dorobantului. The experiment started on June 2013.

Plant material, consisting of nodal stem internodes were used to initiate *in vitro* cultures. Disinfection was conducted as follows: the 5 mm interdodes segments were washed with tap and sterile distilled water, treated with ethanol 70% for 1 min, and 10% (v/v) of a commercial bleach solution (50 g L^{-1} active chlorine) for 14 min. The basal culture medium was Murashige and Skoog (MS) (Murashige & Skoog 1962) with 8% (w/v) agar as gelling agent, 3% (w/v) sucrose and 500 mg L^{-1} $CaCO_3$.

Inocullum development, elongation and shoots rooting were promoted by adding a number of plant hormones for *A. eriantha* in order to obtain micropropagation, field aclimatisation and repopulation. The pH was adjusted to 5.8. The cultures were transferred to growth room in cool white fluorescent light intensity 2000 lux of 16:8 h photoperiod at 24±1^{o}C. Every 2 weeks the culture medium was replaced with fresh medium throughout the experimental period. The cultures were daily monitored from the *in vitro* culture initiation.

Shoot induction and elongation. To obtain shoot induction from the explants, we added different phytohormones concentrations of cytokinins and auxins, namely 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), thidiazuron (TDZ) at the basal culture medium. After 20 days of culture period, the numer of developing shoots was scored and used to calculate the percentage of explants with shoot induction: shoots/total shoots x 100. The medium was distributed into 250 mL pots with 40 mL fresh culture medium.

Root induction. A total of 20 shoot buds (~3-5 cm length) were separated and subcultured in the rooting medium consisted of half-strength MS salts supplemented with 30 g L^{-1} sucrose and indole-3-acetic acid (IAA) (0.3 -1.5 mg L^{-1}). After a total period of 18 days, root induction was scored. The plantlets were cultured in screw cap culture jars (110 x 60 mm,) containing 30 mL of solidified MS medium. The experiment was repeated three times and 20 explants were cultured per treatment.

Plant acclimatisation. For hardening, 30 regenerated plantlets, 75 days old, were prepared. *A. eriantha* has undergone an intermediate step to prepare for *ex situ* conservation, which consisted of gently removing from the culture vessels of the plantlets with well-developed shoots and roots, washing under running tap water to avoid microbial contamination and passing on liquid variant in jar vessels.

The plantlets were transferred to a sand-soil and pearl-stone (1:1) substrate. Potted plantlets were covered with transparent polyethylene boxes to ensure high humidity, maintained for 2 weeks in a growth room at 22±1^{o}C and watered every 3 days with quarter-strength MS nutrient solution lacking organic constituents. For plant acclimatization, the plastic covers were removed after 2 weeks to lower the relative humidity (70%), while the plants developed an efficient root system and became

photosynthetically active. The conditions in the greenhouse during acclimatization were 25°C temperature, and a 12 h photoperiod. Light was modulated by appropiate whitewash of the greenhouse. After 5 weeks from planting and hardening in the greenhouse, the acclimatized plants were transplanted to pots filled with orchard soil and transferred to the field for evaluation of their morphologic stability and survival.

Determination of total phenolic content and total flavonoid content. From *in vitro* regenerated shoots, polyphenols and flavonoids content of essential oils have been analysed. Total flavonoid content was measured by a modified Zhishen et al (1999) protocol. Methanolic extract of suitable shoots dilluted were mixed with 2 mL/75 µL of 5% Na_2CO_3 and incubated 5 min at room temperature. Then 75 µL of 10% $AlCl_3$ x $6H_2O$ was added. After 6 min of incubation 2 mL of 4% NaOH and 12 mL distilled water were added. The absorbance of the mixture was measured on 510 nm using a UV spectrophotometer (Helios γ Thermo Scientific). Total flavonoid content of extract was expressed as rutin equivalent (ERU) g^{-1} fresh weight.

Statistical analysis. Statistical data were performed using Microsoft Office Excel program. Data were represented as mean±SD. The p values less than 0.05 were considered to be significant.

Results and Discussion. Concerning the frequency of bacteria contamination, from 20 initially cuttings, 10 were contaminated. Concentrations over 15% of dichloroisocyanuric acid and longer time of immersion (over 14 minutes) in the commercial bleach solution sterilizing agent were detrimental for the tissues.

We used subculturing at an interval of seven days like an efficient way to avoid contamination and to maintain the micropropagation. Nodal stems explants containing the meristem side have developed. Regarding medium nutrients, MS salts are the most used. For *A. eriantha*, different plant growth regulator combinations for culture initiation and proliferation have been described. Plant growth regulators are a key component of the process that requires optimization, as tissue responses can differ as a function of the explant type. The addition of $CaCO_3$ to the medium was a key factor in starting morphogenetic processes, considering the Pace et al (2004) results according to which the concentration of 500 mg L^{-1} gave an optimal recovery of otherwise chlorotic plantlets, and was used throughout the *in vitro* propagation steps.

Shoot induction and elongation. Effective shoot induction and multiplication was based mainly on hormone types and their proportion. The variants of the experiment took into account two hormones types. According to Roșu (2006), in caulogenesis (shoots development directly from inoculum), cytokinins play an important role and are located at highest concentrations in meristematic regions.

In the present investigation, *A. eriantha* explants formed shoots within 10 days of culture on MS media supplemented with various combinations and concentrations of plant growth regulators (Table 1). In the subsequent 6 days of culture, shoots buds were first observed and most explants showed strong organogenic potential. The number of shoots obtained from the experiments varied depending on the variants used (Tables 1 and 2).

Speed reaction of shoots was improved by 3 mg L^{-1} GA_3 combination with 1 mg L^{-1} BAP. In this context, our results are consistent with those of Borzabad et al (2010). The efficiency of regeneration and average number of shoots per explant increased with increasing concentration of cytokinin (1-1.5). Sudarshana et al (2013) also obtained best shoot induction per explant on media with BAP (0.5-1 mg L^{-1}) and ANA (0.5-1 mg L^{-1}).

The shoot formation decreased when high concentrations of IAA were added to the medium. We obtained best shoot induction and multiplication of shoots (38 shoots/explant) on BAP (1.8 mg L^{-1}) supplemented MS medium in combination with kinetin (0.2 mg L^{-1}). Therefore, among the combination tested, BAP of 0.5 mg L^{-1} and NAA of 0.25 mg L^{-1} induced the highest number of buds per nodal segment.

Hyperhydration of some regenerated shoots was observed. Different phenotypes of shoots were evident (Figure 1).

Table 1

Hormones concentration influence on explants development

Concentration of growth regulator (mg L^{-1})			No. of shoots/explant	Shoot length (cm)	Percent (%) of response
BAP	NAA	k			
0.5	0.1	-	11	2	55
0.5	0.25	-	36	2	75
1	0.5	-	28	3	78
1.5	0.5	-	35	4	80
1.8	-	0.2	38	5.3	82

Table 2

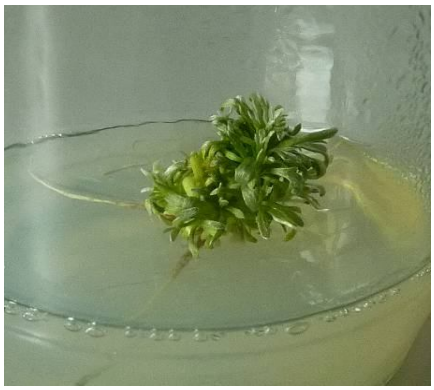
Effects of hormones on explants development

BAP (mg L^{-1})	Gibberellic acid (mg L^{-1})	TDZ (mg L^{-1})	Shoot length (cm)	Percent (%) of response
1	1	-	1.8	36
1	3	-	2.7	45
1	1	0.3	3.2	47
1	3	0.3	4.1	63
1	3	0.5	5.3	70

A 3 cm shoot.

Shoots after 30 d of culture on MS medium with $CaCO_3$ (500 mg L^{-1}) and BAP (1 mg L^{-1}) + gibberellic acid (3 mg L^{-1}) + thidiazuron (0.3 mg L^{-1}) added.

Shoots produced from stem internodes on MS + BAP (1 mg L^{-1}) + gibberellic acid (3 mg L^{-1}) + calcium carbonate (500 mg L^{-1}).



Shoots obtained on MS elongation medium + $CaCO_3$ (500 mg L^{-1}) - BAP (1 mg L^{-1}) + gibberellic acid (3 mg L^{-1}) + IAA (0.5 mg L^{-1}).

Figure 1. Different phenotypes of shoots regenerated.

Rooting and acclimatization. When investigated the effect of IAA concentration on root induction, we observed that the optimal concentration was 0.1% and the percent of response was 65% (Table 3, Figure 2). A low concentration of IAA in the medium was beneficial and essential of the initiation of roots. Our results concerning rooting are in

agreement with that of Pace et al (2004). Roots had differentiated in 12 days. Additionally, the development of numerous lateral roots were also observed.

Table 3

The influence of IAA on rooting system

Concentration of IAA (mg L^{-1})	*Percent (%) of response*	*Mean root length (cm)*
0.1	65	12
0.5	58	5
1	43	3
1.5	35	2

Figure 2. Root system (original).

After 12 days, regenerated plants were transferred in polycups containing a mixture of sand, soil and pearl-stone in proportion of 1:1:1 (Figure 3). The *Artemisia* pot was covered with a transparent case; over time, this was removed, in the early days a few minutes, until the time came to hours, then days. Phytotron inside served as a acclimatization medium.

Plantlets rooting system development of on MS liquid half strength medium with 0.5 mg L^{-1} IAA - preacclimatization stage.

Acclimatized *Artemisia eriantha*.

Figure 3. Acclimatization stages (original).

After 6 weeks of laboratory and greenhouse acclimatization, the survival rate was > 65% and all plantlets showing strong leaf development and stem elongation. Furthermore, it seems the process of shoot and root formation is regulated by a fine balance between

particular types of growth regulators. These results are in agreement with the report of Borzabad et al (2010) regarding rooting and acclimatization who stated that a low concentration of IAA in the medium was beneficial and essential for the rooting of shoots.

The same author found that root morphogenesis for *Artemisia vulgaris* occurred in 10-12 days of culturing with highest root induction (87%) on MS medium containing 0.5 mg L^{-1} IAA. All the regenerated plantlets appeared morphologically normal and grew well. Furthermore, the plantlets in the greenhouse grew in height and were vigurous with dark green leaves and shoots of sufficient thickness. The survival percentage was high and the acclimatized plantlets did not show symptoms of nutrient defficiency or excess.

In order to exploit the biotechnological potential of *A. eriantha* fresh plant extracts, we determined the total phenolic compounds and total flavonoids content (Table 4).

The maxim value of the total phenolic content (55.65 µg GAE/g) and the highest value of total flavonoids (128.07 µg ERU/g) show that the rate of these compounds *in vitro* plantlets is reduced comparing with the wild plants.

Table 4

Total phenolic and flavonoids contents of *A. eriantha* plant extract (mean ± SD)

Sample	*Polyphenols µg GAE/g fresh weight*	*Flavonoids µg ERU/g fresh weight*
In vitro shoots	55.65±14.61	128.07±23.08

Conclusions. From the results obtained it is concluded that an efficient propagation protocol has been established for *Artemisia eriantha*. The results of the experiments carried out for the purpose of tissue regeneration revealed that the exogenous contribution of growth regulators (auxins and cytokines) is an essential requirement for the induction of adventitious shoots.

The optimization of the plant growth regulator conditions has been obtained; an alternative approach has been demonstrated. To summarize, higher BAP (1.8 mg L^{-1}) and lower IAA (0.1 mg L^{-1}) concentrations gave better response for shoots induction and rooting, and also 22^{o}C temperature was found to be optimum. The results clearly indicate that the selection of appropiate plant growth regulators can significantly regulate the morphogenetic potential of *A. eriantha* explants. Regeneration protocol provides plants suitable for biochemical future studies of this species that is rare in nature because it lives in a very selective environment (2200 m above sea level). All the subculture media tested in this study were able to support the growth of the species. The tissue culture procedure using nodal segments was considered suitable for the induction of plantlets and can be efficiently used for rapid multiplication of *A. eriantha*.

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