IN VITRO PROPAGATION OF RARE SPECIES *Ruscus aculeatus* L. AND HISTOLOGICAL PECULIARITIES OF THE REGENERANTS

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Abstract. The present study belongs to the international efforts for plant conservation in the areas endangered by human activities. *Ruscus aculeatus* L. is one of the threatened plants (included in all national red list of vascular plants from Romania) that grow in the Natural Park Comana, Southern Romania.

Seedlings and fragments of rhizome, from plants grown in the natural habitat have been used for *in vitro* plant regeneration and multiplication. After successfully rooting and acclimatization of the regenerated plantlets, histological studies have been performed in order to compare the regenerants from *in vitro* cultures with plants from natural habitat. The results indicated that this plant species can be multiplicated, rooted and acclimatized on synthetic medium (MS supplemented with NAA, IBA, kinetin and BAP) with a good efficiency and the regenerants develop a only a few structural modification under vitroculture conditions, with no major consequences for a normal physiology and plant acclimatization.

Keywords: *Ruscus aculeatus* L., *in vitro* culture, morpho-anatomy.

INTRODUCTION

Genus *Ruscus* belongs to family Ruscaceae *sensu stricto* [18] and includes approximately 10 species, the most known being seven as follows: *R. aculeatus* (Butcher's Broom) spread throughout Europe and the Azores, *R. colchicus*, common in Caucasus, *R. hypoglossum* grown in central and south-eastern Europe and Turkey; *R. hypophyllum* is widespread in the Iberian Peninsula and north-west Africa; *R. microglossus* spread to southern Europe, *R. streptophyllum* met in Madeira, and *R. hyrcanus* Stankov & Taliev, endemic in the Talish Mountains, Azerbaijan.

Ruscus aculeatus L. is a rhizomatous evergreen subshrub with flattened, leaf-like cladophylls bearing small pale green flowers followed on female plants by glossy red berries [32]. Species of Mediterranean origin, in Romania vegetates in the south and west, through forests, meadows and open rocky places. The plant propagates by the seed and by rhizome extension [10]. The species is endangered by population reductions due to habitat alteration and also by intense harvesting for floral bouquets. Another cause of population reduction is the lack of seed production due to pollination failure [20]. As a consequence it was added in all Red Lists of vascular plants from Romania being included in different sozological categories: Rare (R) [25], Vulnerable (Vu) [13], and Endangered (En) [8].

Currently, are available a number of studies concerning medicinal properties of this plant, due to secondary metabolic compounds synthesis including some bioactive saponins like ruscogenin and neoruscogenin [16, 19]. The most cited effects of *R. aculeatus* extracts are the cytostatic [23] and the vasoconstrictive [9, 26] effects which made them useful in therapy of cancer, venous insufficiency, diabetes, premenstrual syndrome, etc. [33]. These studies were based on freshly harvested plant material from botanical gardens or natural habitat. In contrast, studies on tissue cultures to obtain bioactive compounds are very few [11, 22]. In Romania pharmacological properties of *Ruscus* extracts were studied by Balica and collaborators [1, 31]. Researches upon preserving *ex situ R. aculeatus* L., by the means of *in vitro* cultures are very few worldwide and almost nonexistent at national level [2, 4].

Current efforts on saving plant species from extinction mainly consist of *in situ* protection of existing specimens and avoid degradation of habitats in which they are still available (e.g. Natural Park Comana). According to bio-economy concept - economic exploitation of living resources [7, 21, 28] - a reliable alternative to intense exploitation of wild flora is *in vitro* regeneration and multiplication for both ornamental and medicinal use [3, 30, 27].

As part of common effort in using biotechnological tools to develop a sustainable bio-economy [5, 6] this project aim to establish an efficient and reproducible regeneration system for *R. aculeatus* from different explants and also to evaluate at morpho-anatomical level the stability of plantlets. The developed protocol allows germplasm conservation in an active vitro-collection, useful for bioactive compounds extraction and also as reserve for repopulation of the natural habitats.

MATERIALS AND METHODS

For micropropagation mature seeds and rhizome fragments were collected from random plants onto selected areas of Natural Park Comana, distant from each other, in order to cover a large area of diversity and to avoid possible negative impacts caused by retrieval of samples from existing population.

For faster germination the seeds were immersed in water several hours before inoculation and then sterilized according to previously described methods [27, 29], by a treatment with ethylic alcohol (70%) for

30 seconds followed by two treatments with a product with 5% sodium hypochlorite, commercially named Domestos, for 3 minutes, finalized with 4-6 vigurous washes with sterile distilled water for 2 minutes in order to clean the sterilising agent. Rhizome fragments were sterilized in a similar way, but adding one extra sterilizing agent Mercuric chloride ($HgCl_2$, 1%) in two washes for 2 minutes, after Domestos treatment, to remove high surface contamination. The basal medium used in the experiment consisted in Murashige-Skoog (MS) salts formula [24], with 30 g/l sucrose and Gamborg vitamins [15], solidified with 8 g/l agar and pH adjusted to 5.5. The seeds were inoculated on MS medium, in two variants, MS medium without hormones and MS supplemented with giberelic acid (GA_3) 5 mg/l, in ampoules of 80 mm height and 20 mm in diameter. The ampoules were kept at 22-24^{0}C in dark conditions. Sterilized fragments from rhizome and seedlings (30 days after germination) were aseptically inoculated on Erlenmeyer vessels of 150 ml with regeneration MS medium (R) supplemented with naphthylacetic acid (NAA), and benzylaminopurine (BAP). Well developed shoots were individualized and transferred in Erlenmeyer vessels of 150 ml with rooting media (RR1 and RR2) supplemented with indolebutyric acid (IBA) and kinetin. The cultures were incubated in the culture chamber at 22-24 ^{0}C with 16/8 h illumination periods at 2000 lux light intensity. Growth regulators contents for both regeneration and rooting media can be followed in Table 1.

Table 1. Growth regulators content of media used in *in vitro* culture of *Ruscus aculeatus* L.

Media	Auxin (mg/l)		Citokinin (mg/l)	
	IBA	NAA	Kinetin	BAP
R	-	1.0	-	5.0
RR1	1	-	-	-
RR2	1.8	-	0.022	-

In order to complete the protocol for *ex situ* conservation of the species, the regenerants were subjected to *ex vitro* acclimatization process. Thus they were removed from culture media and the roots were carefully washed with sterile water. The next stage was a two step acclimatization as described by Fira & Clapa [14] by immersion in flasks with sterile tap water at a level that do not exceed the roots and stored at room temperature and light conditions (Fig. 5). Following this procedure, the regenerated plants, were gradually adapted to the specific conditions of humidity and temperature from *ex vitro*. After 7-10 days the plantlets were transferred in pots containing 60% forest soil and 40% perlite, previously sterilized. Plants were exposed to natural light (photoperiod 8/16h) in acclimatization chamber at a temperature of about 24 ^{0}C, with the humidity below 80%.

For the histological comparative studies, fresh tissue samples (stem and phylloclades) were fixed for two weeks in a formalin buffer solution (15 % v/v formalin, 2 % w/v sodium bicarbonate). Dehydration was conducted at room temperature in graded ethanol series (5 to 100 %), followed by clearing with xylene and embedding in paraffin, according to the usual procedures [12]. Serial cross-sections (7-10 µm thick) were cut with a rotary microtome and transferred onto glass slides. The paraffin was removed and the sections were rehydrated and double stained with 1 % w/v night green and carmine alum (2 % w/v carmine, 5 % w/v aluminum potassium sulfate), and finally mounted in synthetic resin (Entallan). Histological observations were made in bright field microscopy, under a Nikon Eclipse E200 microscope, and micrographs were recorded with a Nikon Coolpix 5400 digital camera.

RESULTS

Seed germination was very slow and occurred after 6 months, in dark conditions. On MS hormone free medium the percent of germinated seeds after 6 month was 60%, while the onto MS medium supplemented with GA3 the percent of germinated seeds after the same period of time was 85% (Fig.1).

After 30 days of culture rhizome explants showed a significant morphogenetic potential, which unfortunately was diminished by culture contamination that exceeded 40%. This suggests that some microbial agents achieved symbiosis in parenchyma cells of the rhizome.

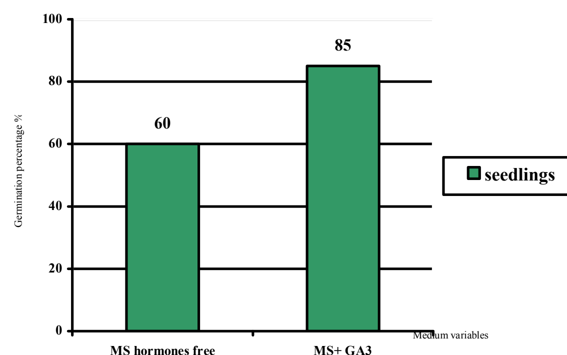

Figure 1. Seed germination *R. aculeatus* L. onto two medium variants within 6 months after inoculation

Shoot regeneration was successfully achieved within 90 days of culture on regeneration media. The morphogenetic potential of the both seedling (Fig. 2) and rhizome (Fig. 4) was expressed in most of explants (Table 2) within 30 days of culture on regeneration media by organogenic callus yield. Morphogenic callus hypertrophies and develops multiple shoot primordia at a very good rate (Fig. 3).

Table 2. Expression of explant reactivity on medium variant R after 90 days of culture

Medium	Results	
	Rhizome fragments	Seedlings
R	80% explants with callus which generated multiple shoots	95% explants with callus which generated shoots

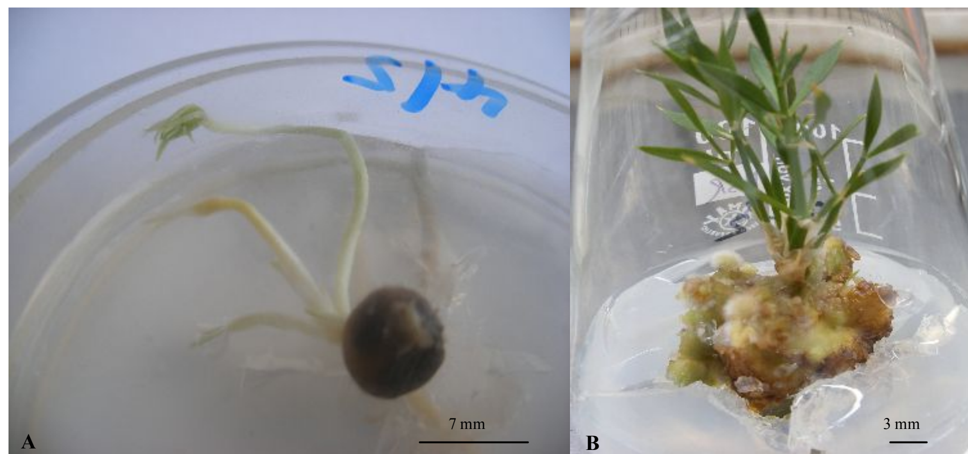

Figure 2. (**A**) Germinated seed; (**B**) organogenic callus and shoots differentiation from seed derived seedlings

Shoots rooted successfully onto rooting media, both considered variants (RR1 and RR2) being efficient in root development. Within 60 days onto rooting media, rooting efficiency was assessed by percent of rooted shoots, which showed that RR2 variant was more effective in root establishment than RR1 variant (Fig. 6).

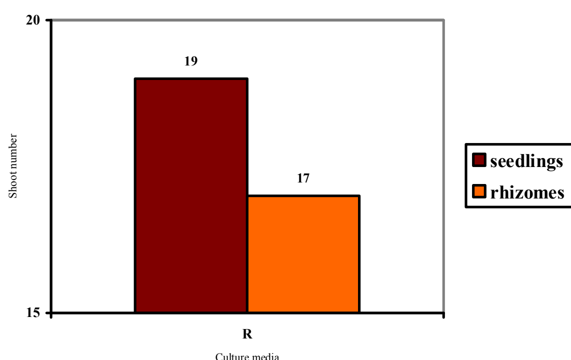

Figure 3. Average number of shoots differentiated by different variations of the average from two types of explants

Plantlets acclimatization was achieved within two weeks with an efficiency of 92%. Micropropagated plants were vigorous intense green and with no obvious phenotypical modifications (Fig. 7).

Histological comparative studies of samples from natural habitat and micropropagated plants showed that there are no notable structural differences. Since dehydration is the main problem of the acclimatization of the regenerated plants, the observations were made mainly upon structures that maintain water balance: epidermal cuticle, cell walls thickness, position and function of stomata and tissues development in vascular bundles. Micropropagated plants has, in both stem and phylloclade, a well defined epidermis with cells with external walls obvious thickened and covered by a thin layer of cuticle; stomata are functional and positioned at the same level with epidermal cells as a characteristic for mesophytes (Fig. 10); vascular bundles are proper developed and with all the structural peculiarities of monocotyledonous species (Fig. 9). Although the general plan of structure is the same, in samples micropropagated were noted

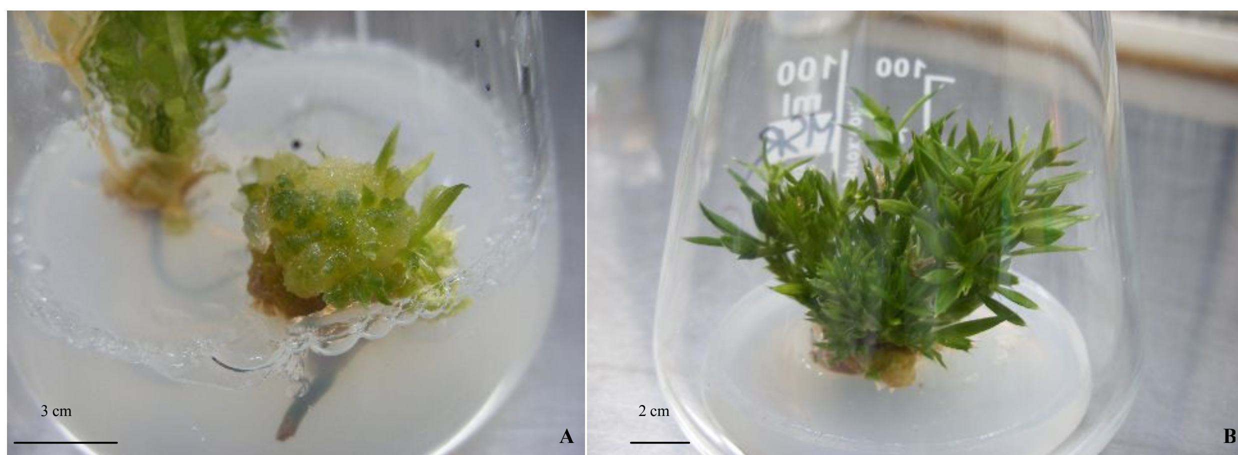

Figure 4. Indirect morphogenesis from rhizome derived explants: (**A**) Organogenic Callus; (**B**) Multiple shoot regeneration

Figure 5. *R. aculeatus* rooted plants

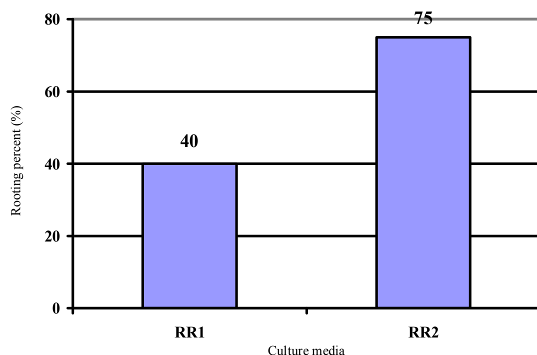

Figure 6. The percentage of rooting (%) of *R. aculeatus* shoots after 60 days after inoculation on rooting media variant

Figure 7. *R. aculeatus* acclimatized plants

some small differences as follows: phylloclade ground parenchyma is less developed and veins are less prominent (Fig. 8).

DISCUSSIONS

The Natural Park Comana belongs to the protected national areas with particularly richness in flora and fauna, therefore taxonomical and ecological studies of rare species from this region and developing methods for *in situ* and/or *ex situ* conservation of those that are endangered, are prioritarily. The research conducted for the *ex situ* conservation of *R. aculeatus* are of a special interest, as part of the concern for achieving responsibilities for Romania to implement the European Strategy for Biodiversity Protection.

Until 2006 there are no reports on the successful micropropagation of *R. aculeatus*, partly because of the difficulties in establishing primary explants [22]. Moyano and collaborators studies showed that a good explants source is immature embryos, shoot and rhizome buds, and organogenic calli derived from mature tissues (cladode, stem and rhizome). Although the micropropagation protocol previously reported [22] seems to be efficient and reproducible, explants like immature embryos and bud meristems are difficult to handle and mature tissues like cladode and stems are not always responsive. In a preliminary study [2] we have tested the regenerative potential of various explants from mature organs of *R.aculeatus* (cladode, stem and rhizome) and we have found that the most responsive explant type is rhizome fragments. The same study suggest that the regenerative potential of the explants is stimulated and sustained by a combination of low auxin (0,1mg/l NAA) and high cytokinin (1mg/l BAP) content in culture media. These results lead us to choose for the present study as explants source rhizome fragments but also seedlings from in vitro germinated seeds, which are known to be very reactive. As growth regulators we used the same combination but in higher content (1mg/l NAA and 5 mg/l BAP) which proved to be much more efficient in shoot regeneration compared with those previously described, in terms of multiple shoot regeneration (17-19 shoots/explants compared with 3-5 shoots/explant). The present study is in accordance with Moyano findings regarding rhizome ability to develop abundant organogenic calli [22] but the growth regulators which proved to be efficient in the process were different, in both type and concentration. We found that NAA in combination with BAP is very efficient in organogenic calli development and Moyano report a combination of 0.5 mg /l 2,4-dichlorophenoxyacetic acid (2,4D) and 1 mg/l kinetin, which suggest that the organogenic potential of rhizome explants can be stimulated by a wide range of auxin and cytokinin combination, if the balance favoring cytokinin is kept. The presented protocol showed that seedlings obtained by aseptic seed germination are also a very good source of primary explants. Although the regeneration rate is very high when seedling derived calli is used, the main inconvenience of this regeneration pattern is the log time taken for seed germination (up to 6 month), even germination stimulators were used (supplements with GA3). The efficiency of the presented micropropagation protocol is sustained by the histological investigations which shows that vitroculture condition did not result in important morpho-anatomical alterations in regenerated plants, with no consequences for a normal physiology and plant adaptation to *ex vitro* conditions.

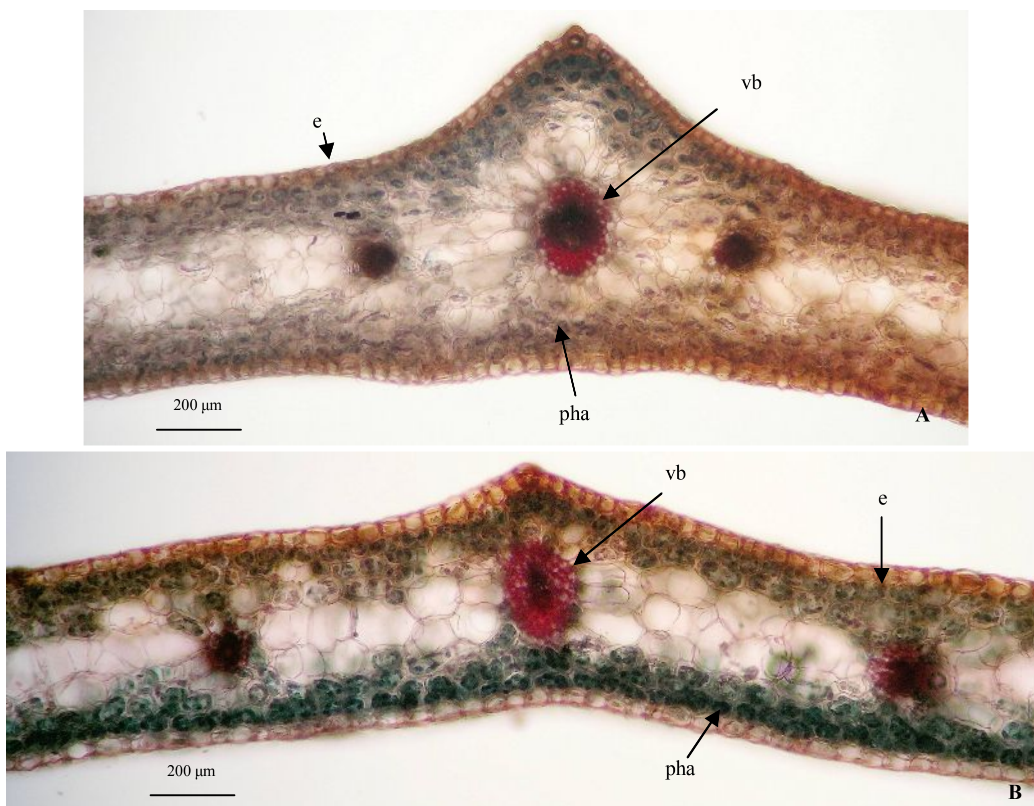

Figure 8. Cross-sections through comparative phylloclades from *R. aculeatus* from the natural habitat (**A**) and from *in vitro* culture (**B**), e-epidermis, vb-vascular bundle, pha- assimilator parenchyma

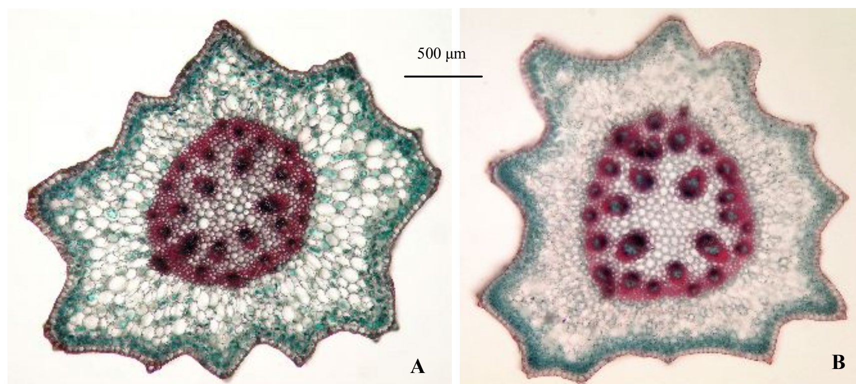

Figure 9. Comparative cross-sections through the stem of *R. aculeatus* derived from plants in their natural habitat (**A**) and *in vitro* culture (**B**)

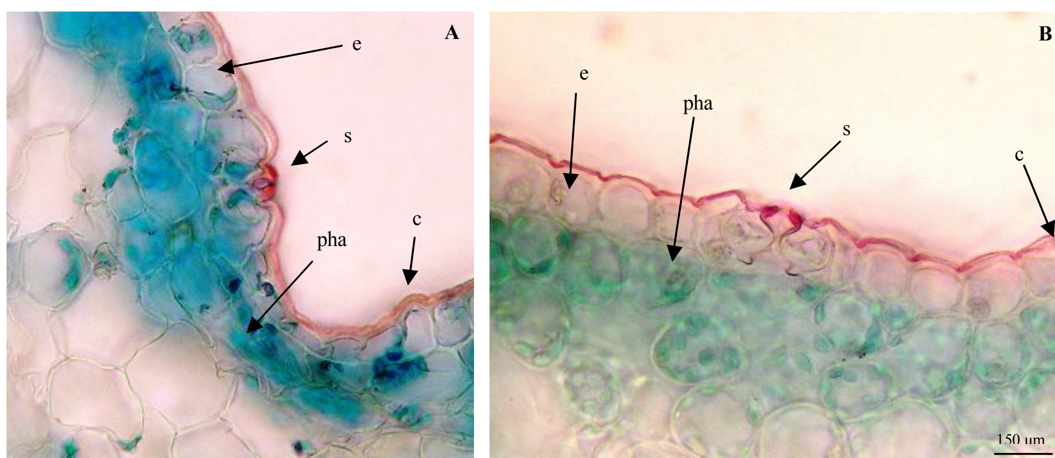

Figure 10. Detail of *R. aculeatus* phylloclade epidermal cells and stomata in transverse cross section of samples from natural habitat (**A**) and from *in vitro* culture (**B**); e-epidermis, s-stomata, c-layer of cuticle, pha-assimilator parenchyma

As a conclusion the micropropagation protocol presented is reproducible and efficient generating a high number of individuals that can be used in further studies such as: biotechnological applications (primary source of secondary metabolites of pharmacological interest), repopulation of natural habitat or for germplasm conservation (long-term storage by organogenic calli cryopreservation). In the same time, this is an original experimental model that can be adapted and applied to other endangered species.

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