IN VITRO PROPAGATION OF CRITICALLY ENDANGERED SPECIES *Scilla autumnalis* L. – BIOCHEMICAL ANALYSES OF THE REGENERANTS

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Abstract. The present study belongs to the international efforts for plant conservation from the areas threatened by human activities. The saline soils areas are restricting for agriculture and in some cases for fishery facilities and the plant species are extinct from those areas. *Scilla autumnalis* L. is one of the threatened plants (rare on the national red list of vascular plants from Romania) that grows in the Natural Park Comana, Giurgiu County, South Romania.

Seeds 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 from germinated seeds, biochemical studies have been performed in order to compare the regenerants from *in vitro* cultures with native plants from genetically point of view. Peroxydase and esterase's spectra were the biochemical markers used.

The results indicated that this plant species can be multiplicated, rooted and acclimatized on synthetic medium (MS supplemented with NAA, IBA, IAA, kinetin and BAP) with a good efficiency and the regenerants had no genetic alterations determinated by culture conditions.

Keywords: *Scilla autumnalis* L.*, in vitro* culture, biochemical markers

INTRODUCTION

Scilla genus includes 125 species, widespread in the temperate zone of Eurasia and North Africa. It is part of the family Liliaceae, former Hyacinthaceae.

Species *Scilla autumnalis* L. (*Prospero parathethycum* Speta, Veroff. Int. Clusius-Forsch. Güssing [6]) is a perennial species, with heights of up to 20 cm. It flowers between August-October before the leaf growing. The leaves are linear 1-2 mm wide without bracts. Bulb is ovoid, 13-30 mm long, covered with reddish brown or gray tunics. Strains 1-3 appear in the autumn, 5-20 cm high, cylindrical, often purple tinted. The leaves are in number of 3-8, all basal glabrate, up to 10 cm long and 1-2 mm wide, narrow linear, canaliculated, attenuated peak, appear towards the end of autumn during the flowering or after flowering. The inflorescence has 6-20 (30) flowers, first in corimb shape raceme short cylinder shape oblong, up to 25-70 mm long. Bracts are missing or are very short. Pedicels are erect extended 5-10 mm long, shorter than flowers or up to 2 times longer than them. Flowers are present in numbers 5-25, are odorless, lilac blue or purplish blue. The perigonial leaves are extended stay, 4-5 mm long and are sub obtuse or obtuse, sometimes subacut, with a darker median strip. Stamens filaments are half as perigon or slightly shorter, the flat base, and anthers dark red, contrasting with the purple color of the filament after dehiscence, dark purple, almost black. The ovary is narrow ovoid, with 2 ovules in each lodge, trivial, three-lobed. Capsule dehiscence is before blunt three-lobed sub globular or ovoid, 4-5 mm long and wide dehiscence after extended star-shaped. Two seeds are in each lodge, black or dark brown, sharp-based, appearing non ariliform [5]. The species is widespread in oak forest glade, salt meadows. The plant species is mesophyte, optional halophyte encountered from Comana to Grădiştea on the rail side, together with *Statice gmelini* and *Glycyrrhiza echinata*. It is also found in Alexandria city (Teleorman County) and nearby Mizil town (Prahova County) in south of Romania [18]. The plant is protected in the national Red List of higher plants in Romania [13] as vulnerable/rare; in the Red List edited by Dihoru [7] it has the status of endangered species. The plant is considered to have critically endangered status on the „Red Book of vascular plants in Romania" [6]. The species was identified in the Natural Park Comana near the river Neajlov, the few areas of land that are rich in salt in their natural state, most being used in agriculture or fisheries. For these reasons the species is important for conservation, ornamental and possibly pharmacological, given that an other species of the genus *Scilla* present antioxidant properties with beneficial effects on the digestive system, circulatory and skin [4, 9, 20].

As we know so far, there are no studies concerning the conservations purposes or *in vitro* cultures on *Scilla autumnalis* L. species even on other related species of the genus. This project has the aim to establish a protocol for *ex situ* micropropagation of these species in order to preserve the germplasm in an active collection that can be used in future experiments for medium and long-term conservation and if necessary to repopulate the original habitat.

MATERIALS AND METHODS

Because *Scilla autumnalis* is a monocotyledonous bulbous species, the explants considered to be reactive *in vitro* were the aseptic seedlings obtained by seed germination. Seed samples from statistical randomized plants were picked from salty fields near the railroad and the village Comana in late September.

Detached from the fruits, the seeds were sterilized according to the methodology described in literature, by a treatment with ethylic alcohol (70%) for 30 seconds and two treatments with a product with 5% sodium hypochlorite, commercially named Domestos, each of 3 minutes, finalized with 4-6 washes of 2 minutes with sterile distilled water in order to clean the

sterilising agent [15]. Sterilization was done to remove coarse and microbial contaminants on the surface tissue and to hydrate and stimulate the endosperm faster germination. The basal medium used in the experiment consisted in Murashige-Skoog salts formula [11], with 30 g/l sucrose and B5 vitamins, solidified with 8 g/l agar and pH adjusted to 5.5, generically named by us MS. The seeds were inoculated on two MS media (named S1 and S2), one of them supplemented with giberelic acid (GA_3) 5 mg/l, in ampoules of 80 mm height and 20 mm in diameter with 5 ml of medium. After 30 days, the seedlings of the *Scilla autumnalis* L. were transferred on Erlenmeyer vessels of 150 ml with 50 ml of multiplication MS media supplemented with the auxins: 1-naphtilacetic acid (NAA), indole-3-acetic acid (IAA), β indolilbutiric acid (IBA), N^6-benzyladenine (BAP) as described in Table no. 1 (media named S3 and S4). We have used the most common hormones used for the purpose of the stage (germination, multiplication or rooting) according to the literature cited [4, 3]. The shoots were transferred in Erlenmeyer vessels of 150 ml with 50 ml of rooting media culture (named S5-S7). The cultures were incubated in the culture chamber at 22-24 ^{0}C with 16/8 h illumination periods at 2000 lux light intensity.

Table 1. Variations of nutrient media used in *in vitro* culture of species *Scilla autumnalis* L.

Media	Basal medium MS					
	Phytohormonal supplement (mg/l)					
	NAA	IAA	IBA	BAP	Kinetin	GA_3
S1	-	-	-	-	-	-
S2	-	-	-	-	-	5
S3	1.0	-	-	5.0	-	-
S4	-	1.0	-	-	2.0	-
S5	-	1.0	-	-	-	-
S6	-	1.8	-	-	0.022	-
S7	-	-	1.0	-	-	-

In order to complete *ex situ* conservation of the species taken in the study, the regenerants were subjected to *in vitro* acclimatization process. Thus they were removed from culture medium and the roots carefully washed to not attract microbial contamination. The next stage was immersion in tubes with sterile tap water so that its level does not exceed the roots and stored at room temperature and light conditions for 7-10 days. Following this procedure, *Scilla autumnalis* L. regenerated plant species, were gradually adapted to the specific atmospheric conditions of temperature and humidity *ex vitro*. Then they were planted in pots containing substrate in a specific composition with 50% soil forest and 50% perlite, previously sterilized at 121^{o}C for 30 min in order to help the plantlets to accommodate easier with the new conditions as other authors recommends [15]. Plants were exposed to summertime natural light (photoperiod 8/16h) acclimation chamber at a temperature of about 24 ^{0}C, with the humidity below 80%.

The statistical calculations were done using specific software (ANOVA) that helps us to emphasize the degree of signification of the results. In all the stages of the *in vitro* culture we have used 10 explants.

In any action for *ex situ* conservation of endangered plant species, a great importance presents to practical applicability if experimental protocols used are reproducible and whether the regenerants obtained are uniform in morpho-phyisiologically and genetically terms. For this reason it is important to determine the initially genotype preservation, after attending conservation processes. Methods of changes detection are numerous and based both on conventional morpho-anatomical, but also modern ones as biochemical and molecular marker analysis [10].

Biochemical analysis were based on the use of specific markers and were concerned with possible changes in the izoenzymatic spectra of the main types of enzymes sensitive to changing of environmental conditions [12].

The experiments started with the extraction of soluble proteins cytosol, made by grinding in 0.1 M phosphate buffer, pH 7 at 40 ^{0}C. After centrifugation fraction at 15,000 rpm for 10 minutes, the supernatant was used for electrophoretic analysis.

Electrophoretic analysis were based on the principle of migration in electric field of 20 mA for 2 hours in the batch system, in a migration of polyacrylamide gel (PAA) concentration 8%, a 4% polyacrylamide stacking gel and a buffer 0.05M Tris-Gly, pH 8.3. Samples were inoculated into wells of stacking gel and subjected to electrophoretic migration process with a voltage of 10 mA through the stacking gel for 30 minutes and then at a voltage of 20 mA through the separating gel for 90 minutes. As a marker for highlighting the migration front was used bromfenol blue. We have used an electrophoresis system from Biometra. When the migration process was completed, the gel was subjected to emphasize specific steps for coloring enzyme spectrum of interest [16, 19].

The highlight of peroxidase activity (POX) was performed by incubating the gel in 0.5M acetate buffer, pH 5 containing 0.08% benzidine and a few drops of H_2O_2.

The esterase bands were developed in 0.1 M phosphate buffer pH 6.5 containing 0.2 % α and β-naphtylacetate as substrate and 0.05 % Fast Blue RR as dye.

RESULTS

After sampling and identifying them as belonging to the species *Scilla autumnalis* L. we started the *in vitro* culture of plant material.

Two variants of the same culture media for seed germination were tested for *Scilla autumnalis* species, namely hormone-free MS and MS supplemented with GA_3 (5 mg/l). Making a comparison between the two media (95% vs. 65%) it is obviously that the seeds germinated in a higher percentage on MS+GA_3, after 30 days (Fig. 1).

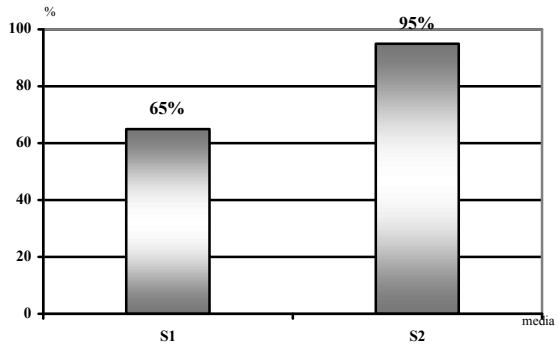

Figure 1. Seed germination rate on *Scilla autumnalis* L. species after 30 days.

Vitroplants obtained from aseptic seed germination were inoculated on MS multiplication media supplemented with 1 mg/l NAA and 5 mg/l BAP and respectively on MS with 1 mg/l IAA and 2 mg/l kinetin (named S3 and S4).

The seed germination explants proved to be very reactive, generating a big number of shoots in a short time of culture (Fig. 2). Once separated and transferred into individual vessels they started to form small bulbs and then roots at the base.

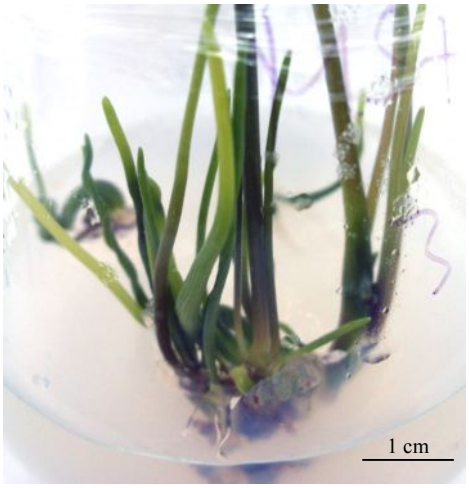

Figure 2. Multiple shooting of *S. autumnalis* L. plants obtained by aseptic germination of seeds on MS with 1 mg/l NAA and 5 mg/l BAP (S3).

The capacity of bulb fragments to survive in artificial conditions, to multiply and regenerate plants depended on a variety of autonomous factors, endo and exogenous like endogenous hormone levels and the general culture conditions (exogenous phytohormons, light and temperature) [2].

Subcultivation of individual plants obtained by seed germination on MS medium supplemented with 1 mg/l NAA and 5 mg/l BAP used for multiplication (S3), has stimulated the differentiation of primary and new caulinary shoots possible by multiple axillary shooting the buds on basal disc. Shoots developed in aseptic conditions conditions were detached and transferred to rooting media (Fig. 3).

Figure 3. Developing roots on the explants of *Scilla autumnalis* L. on MS supplemented with IAA 1.8 mg/l and kinetin 0.022 mg/l (S6).

In vitro evolution of *Scilla autumnalis* L. species showed the influence of hormonal supplements added to the medium on the development of shoots from each explant.

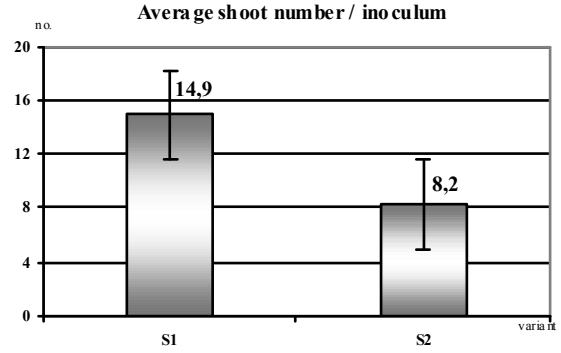

Figure 4. Average numbers of shoots/explant (with standard deviation) regenerated at the species *Scilla autumnalis* L. on S1 and S2 culture media.

Graphic values are expressed as mean values ± standard deviation. Concerning the fact that the two samples from the Figure 4 are independent, we have tested the average differences signification level using "Independent Sample T-test'. t-test for equality of means shows that the average of the two samples in statistically different on a level less that 0.001 (Table 2), then we can reject the null hypothesis (that is, we can conclude that the two groups of samples can be distinguished by average number of shoots/explant).

Table 2. The statistical result concerning the mean number of shoots/ explants

Statistical calculations	Media variant	
	S1	S2
$\bar{x} \pm S\ \bar{x}$	14.9±0.172	8.2±0.079
s	2.99	2.57
Variance	8.98	6.62
t test	< 0.001	

Note: $\bar{x} \pm S\ \bar{x}$ (average ± standard deviation of the average), s (standard deviation),

Basal medium MS supplemented with 1 mg/l NAA and 5 mg/l BAP stimulated the regeneration of shoots from preformed meristeme (Fig. 4). Due to the concentrations of cytokine and a low concentration of auxin, the culture medium proved to be sufficiently effective to stimulate clonally multiplication and obtain a significant number of adventitious shoots. In this experimental phase, it was necessary to transfer the detached shoots on special culture media to stimulate the development of adventitious roots and the production of plants capable of being acclimatized. On most effective culture medium described in the variant of rooting (S6) consists of MS, supplemented with 1.8 mg/l IAA and 0.022 mg/l kinetin) the shoots developed a rich and efficient root system, achieving a perfect connection between the root vascular system and the caulinar one (Fig. 3 and 5).

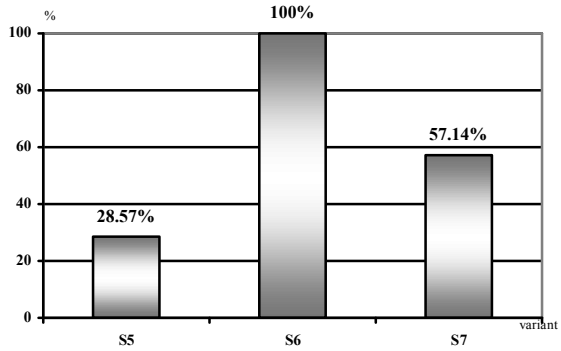

Figure 5. Rates of shoot rooting in the case of the species *Scilla autumnalis* L.

This step consisted in gradual adaptation vitroplants from *in vitro* culture to conditions of acclimatization room, in the similar conditions to those of nature. Environmental differences to which is necessary to accommodate are non-sterile atmosphere, natural light, lower humidity and changing atmosphere [15].

Acclimatization method used is recommended by the authors in specialized works as optimal for a high percentage of plants accommodated to the new substrate [8, 14].

Acclimatization of individuals belonging to the species that are rooted in the study was conducted on specific substrates, imitating as much as possible the natural conditions of the habitat and providing the nutrients necessary to ensure the greatest degree of acclimatization.

For the regenerants of *Scilla autumnalis* L. species (Fig. 6) the acclimatization was done on solid substrate with relatively high percentage of perlite (50%) to ensure gas exchange to the roots, but also to maintain humidity at an optimum level.

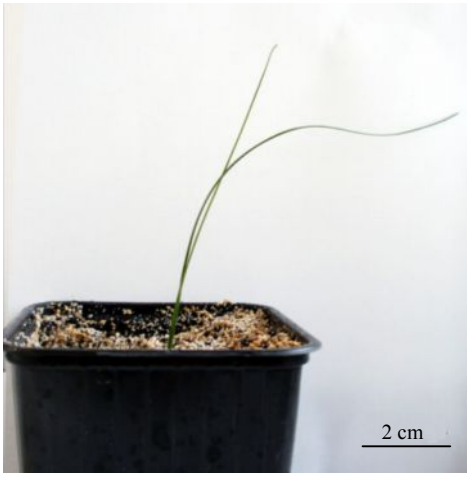

Figure 6. Acclimatization to pots of *Scilla autumnalis* L. plants grown on S6 culture medium.

The results obtained so far have allowed the development of effective processes and reproducible *in vitro* regeneration of the species, based on their short-term conservation. We have into account two future aspects, namely:

- Repopulation of plant original habitats, which will be done by transferring them into the natural environment, where plants can readjust their metabolism in terms of nature.

- Continue to long-term conservation, by cryopreservation, operation that once successfully completed, allows a reduction efforts sub cultivation and genetic stability assessment, known as that in liquid nitrogen, plant specimens have the metabolism slowed to a minimum, keeping all the individual characteristics.

Determination of optimal conditions for the short and medium term conservation by *in vitro* micro multiplication, of endangered species, can not be achieved without a complex analysis of regeneration and development.

In this context regenerative material from *in vitro* cultivation must be subject to cytological, biochemical and molecular analysis meant to highlight the extent that experimental conditions did not affect the normal pattern of their development or possible genetic variations occurred [1, 3].

Combined results of these tests will allow selection of optimal experimental procedures.

Biochemical analyses performed in *Scilla autumnalis* L. species have shown that both peroxydase and esterase enzyme activities from native plant samples are lower than those from *in vitro* cultures. Peroxydase electrophoresis of the individuals from different seeds shows that *in vitro* samples present in addition two isoforms (loci 2 and 3) and the one at locus 1 presents an increased intensity compared with the natural habitat samples. The differences are due to the stress induced by *in vitro* culture (Fig. 7 left). The esterase electrophoretic bands of samples from different seeds regenerants are less intense but more numerous compared with samples from natural habitat,

due to the heterozigoty of individuals (Fig. 7 right). The extra bands from locus 9 are present only on two regenerants of three. The samples from *in vitro* germplasm differ in expression of isoforms due to different alleles in individuals grown from seeds. In the case of the second group of gels, we have used different samples of *in vitro* culture regenerants from the same individual. In this case is observed that the isoform bands are identical for both peroxydase (Fig. 8 right) and esterase (Fig. 8 left). Thus, it might be possible that all the individuals could have the same genetic system for those two enzymes and they use the same isoforms in their metabolism.

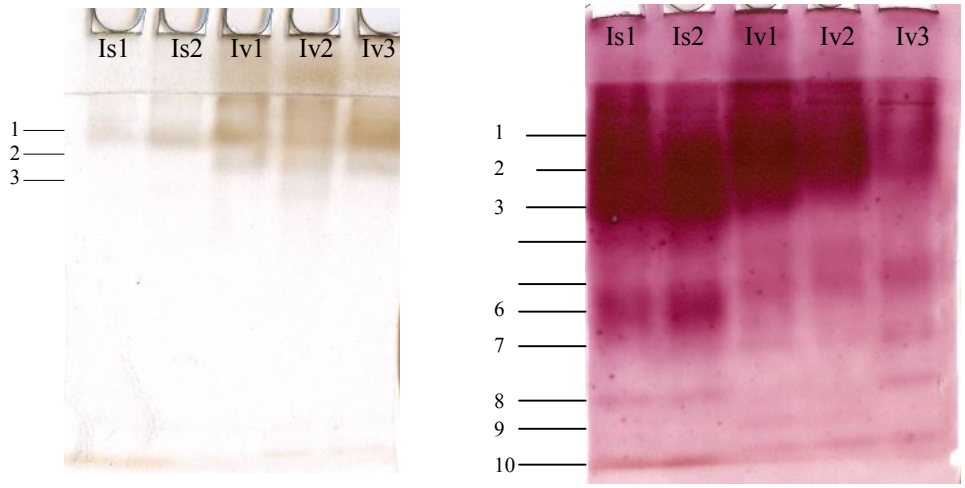

Figure 7. Acrylamide gel electrophoresis of peroxydase (left) and the esterase (right) in samples of natural habitat (Is 1-2) and *in vitro* culture (iv 1-3) of *Scilla autumnalis* L. species

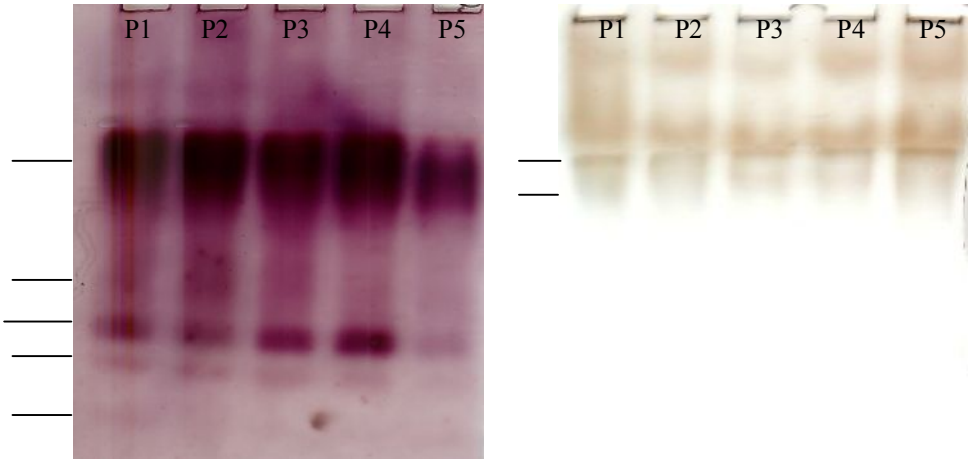

Figure 8. Acrylamide gel electrophoresis of esterase (left) and peroxydase (right) samples from the same individual *in vitro* regenerants (P1-P5) of *Scilla autumnalis* L. species.

DISCUSSIONS

The researches conducted for the *ex situ* conservation of wild *Scilla autumnalis* L., are of a special interest, as part of the concern for achieving responsibilities for Romania to implement the European Strategy for Biodiversity Protection [17].

The Natural Park Comana is one of the protected areas, particularly rich in flora and therefore the correct identification and characterization studies of the endangered species in this region and to develop protocols for medium and long term conservation are particularly needed. For this reason we had the support of the experimented taxonomist Dr. Gavril Negrean.

The *Scilla autumnalis* L. species is listed in all national red lists and has protective status in the Comana Natural Park. The plant proved a satisfactory *in vitro* reactivity, similar with other related species [4]. In this plant species case, for micropropagation purposes we used as explant source seedlings obtained by aseptic seed germination presenting a very high efficiency. The species proved to prefer solid culture media rich in nutrients and supplemented with significant amounts of growth factors. To propagate the species, 7 variants of modified MS basal medium were tested. According to the results obtained, the optimum protocol for species micropropagation includes seed germination on MS mediun supplemented with 5 mg/l GA3, shoot multiplication on MS medium supplemented with 1 mg/l NAA and 5 mg/l BAP as it was used on other species as well [3]. For the rooting process we recommend subcultivation on MS modified with the addition of 1.8 mg/l IBA and 0.022 mg/l kinetin as other scientists recommended in literature [3]. The acclimatization should use the optimum combination of perlite and forest soil in the rapport of 1:1.

Acclimatization of vitroplants belonging to the

species studied was based on a single protocol. The microplants were first immersed in tubes with water, from which they were transferred to pots with substrate adapted to specific requirements. Average of acclimation has reached 92%. This method helps the plants to gradually adapt to the atmospheric conditions, increasing the survival rate comparing with the traditional method that transfers the plantlets directly into the soil [8].

Other authors consider that the peroxydase has a specific sensibility to the growing environment, then it should be one of the first enzymes that may suffer genetic alteration during the stressful conditions [16]. Analyzing the isoforms of the peroxydase and esterase on samples of *Scilla autumnalis* L. species we have observed an increased enzyme activity *in vitro* when compared with the samples in their natural habitat. The samples from *in vitro* regenerated seedlings showed differences in number and band position, probably because of the expression of different alleles in individuals. Samples from the *in vitro* multiplied regenerants from the same individual have identical bands, suporting to a certain extent that the micropropagation method did not affect the genetic pattern of the enzymes [19].

We mention that our approach on biochemical level has a degree of national news for the studied species.

Protocols developed by us for short-term *ex situ* conservation of wild *Scilla autumnalis* L. are original experimental models that can be adapted and applied to other endangered species.

By the end of growth cycle: inoculation and multiplication-rooting-acclimation are opened opportunities to obtain individuals in large numbers that can be used in subsequent studies from three directions: biotechnological applications (extraction of secondary metabolites of pharmacological interest), repopulation of natural habitat and long-term storage by cryopreservation.

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