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Biochemical peculiarity of *in vitro* morphogenesis under conservation strategy of *Ruscus aculeatus* L.

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Abstract. The present study is part of the actual concerns in biodiversity conservation of endangered angyosperm species from the Comana Natural Park. *Ruscus aculeatus* L. species is protected both at national and European level (trough Habitats Directive of EU and Bern Convention). The aspects of *in vitro* morphogenesis through all the stages from inoculation, multiplication to rooting and acclimatization have been studied. In order to long term conservation and multiplication or to exploit somaclonal variation induced by *in vitro* technique is required the identification of some biochemical or molecular markers for fast evaluation of regenerants. In this respect, genetic stability or variability of *Ruscus aculeatus* plants collected from three populations and regenerants obtained *in vitro* culture was estimated by electrophoretical methods. Therefore, zymograms of different enzymes as esterase, acid and alkaline phosphatase, glutamate-oxaloacetate transferase, malate dehydrogenase and peroxidase were analyzed. The expression of esterases, phosphatases, malate dehydrogenases displayed changes in correlation with growth condition, while the peroxidases pattern was more stabile in natural population as well as *in vitro* regenerated plantlet.

Keywords: multiplication, regenerants, electrophoresis, isoenzyme, *Ruscus aculeatus*

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Introduction

Ruscus aculeatus species, locally called "Ghimpe" (thorn), belongs to Liliaceae Family, *Ruscus* Genus and is spread in Mediteranean Europe and Africa. The species is important due to multiple uses since the old times. It is used as a decorative plant owing to its perenial green habitus and red fruits. Recently, important studies have been done concerning its pharmacological importance (Balica et al. 2005, Bouskela et al. 1993, Facino et al. 1995). It has proven to be very usefull in the treatment of the venous insufficiency of the lower limbs and microvascular permeability (Bouskela et al. 1994, Parrado &Buzzi 1999).

The plant is a perennial shrub presenting a green erect stem, branched in many small branches, with many rudimentary rigid leaves. They represent stem extensions, each one of them having a sharp thorn at the end. The leaves known as phylloclade are elliptical shape and acuminate apex, having in the center small white-greenish solitary flowers (1-2), with short pedicle flowering in the early

spring. The filaments of the stamen are green or violet. The fruits are red pulpous small berries, with 1-2 seeds. The roots consist in a repent odorless rhizome, with medicinal properties. The plant height is 20-50 (70) cm, branched, forming an oval-pyramid bush (Ciocârlan 2000). The plant is spread in forests, clearings, bushes, open spaces and rocky regions. The characteristic habitat for *Ruscus aculeatus* is: 41.7697 Getic *Pulmonaria mollis - Q.cerris* forest, according to Habitat Directive and 91M0 Pannonian-Balkanic turkey oak-sessile oak woods, according to Natura 2000. The ecosystem type is: 7114 Cerum with *Glechoma-Geum*. The habitat is characterised by altitudes 100-300 m, temperatures around 10.5-9°C, precipitations = 550-700 mm (Doniță et al. 2005).

Ruscus aculeatus is characterized as a rare and endangered species in the national red lists (Oltean et al. 1994, Boșcaiu et al. 1994, Dihoru & Dihoru 1994). At European level it is protected by Habitat Directive, appendix 5. Actually, even the global inventory is not completed, it is estimated that the total number of plant species is around 300.000, a part of these of about 60.000 being threatened (http://www.iucn.org/themes/ssc/our_work/plants/gspc.htm). The species grows in the near-by the populated areas and at a small distance to Bucharest and it is temptating for the ornamental plant pickers. Beside this, the extension of the human facilities are restricting more and more the habitat areas, as the forests are cutting down and the plants are affected by the environmental changes. The plants are exposed to the stress induced by the environmantal factors and responding in different ways (Paucă-Comănescu 2009).

In this context, our research focused on preserving the germplasm from this species through *in vitro* system, with the aim to establish a germplasm collection, useful in field trials in the natural habitat from Comana, or medium-term conservation and secondary metabolites analysis. Recent research has been developed in order to extract and analyse secondary metabolites from callus and vegetative parts of the plant (Moyano et al. 2006, Palazon et al. 2006), but publications concerning *ex situ* conservation protocols and studies were not identified. There are studies on related species (such as: *Ruscus hypophyllum* L., *Ruscus racemosus* Moench.) concerning *in vitro* propagation or hormones effects during micropropagation (Sumita & Sumitra 1985, Curir et al. 1988, 1989)

A comparative study of samples originated from *in vitro* regenerants and plants from natural habitat was initiated. The electrophoretical analysis provide an indirect method for genome probing by exposing structural variation in enzyme or other protein genome (Sammour 1991, May 1998). A screening of the multiple enzyme system as esterase (EST) acid (ACP) and alkaline phosphatase (AKP), glutamate-oxaloacetate transferase (GOT), malate dehydrogenase (MDH) and peroxidase (POX) was performed for identification of the appropriate markers with conservative expression in different environmental conditions. Also, it was monitored the appearance of differences between regenerates with different origins.

Materials and methods

Samples from three distinct populations of *Ruscus aculeatus* L. were collected from the Natural Park Comana, Giurgiu County, in the areas: "Fântana cu nuc"- Lat. 44°9'28.3'', Long. 26°6'26.7'', alt. 96 m (P1-shoots and P2-fruit), "Padina I" Lat. 44°9'53.7'', Long. 26°7' 28.9'', alt. 61 m (P3-shoots) and "Padina lui Vasile" Lat. 44°9'39.0'', Long. 26°6'34.8'', alt. 65 m (P4-shoots and P5-fruit) as quoted in literature (Paucă-Comănescu et al. 2000; 2001, Tarnavschi et al. 1974).

Starting from rhizome fragments and from seeds, by *in vitro* cultures, the experiments allowed adventive shoots regeneration. Further, these shoots were rooted and acclimated *ex vitro*. The explants were inoculated on multiple culture media based on MS (Murashige & Skoog 1962), supplemented with different concentrations of phytohormons (Banciu & Brezeanu 2008, Balica et al. 2005). Additionally, the culture medium MS with 1 mg/l naphthylacetic acid (NAA) and 5 mg/l 6-benzylaminopurine (BAP) was tested.

For conservation purposes, explants (0.5-1 cm) belonging to the population from "Fântana cu nuc" were aseptically inoculated on differ-

ent culture media as specified in previous methods (Banciu & Brezeanu 2008). The samples were taken from the plylloclades and central rhizome of representative plants for the population. The shoots (P6) were obtained by adventive direct and indirect morphogenesis from rhizome fragments. Also, the roots (P8) and the shoots (P7) of regenerants originated from seeds germination aseptically were used to evaluate genetic stability or variability *in vitro* cultures.

The extraction of soluble cytosolic proteins was done by plantlets grinding in phosphate buffer 0.1 M, pH 7 at 4°C. After centrifugation at 15000 rpm, for 10 min, the supernatant was used for electrophoretic analysis, according to literature (May 1998).

The electrophoretic analysis were carried out by the samples migration at 20mA, duration 2h, in a discontinuous system using a running gel 8% polyacrylamide (PAA), a stacking gel 4% PAA and a buffer Tris-Gly 0.05M, pH 8.3. The samples were loaded into separate cells and then electrophoresed at 10 mA through the stacking gel for 30 min and 20 mA through the separating gel for 90 min. The running marker was bromphenol blue.

The POX activity was detected by the incubation of gels in 0.5 M, acetate buffer pH 5 containing 0.08% benzidine and several drops of H_2O_2. For phosphatase activity, 0.05 M α and β-naphtylphosphate and 0.1% Fast Blue BB and several drops of 0.25 M $MgCl_2$ and 0.5 M $MnCl_2$ solutions were used. The medium reaction was represented by 0.1 M acetate buffer pH 5, for ACP or 0.01 M Tris-citrate buffer pH 8.3, for AKP. For ACP, the gels were presoaked in acetate buffer for 30 min. at 4°C. 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. In the case of glutamate-oxaloacetate transfer the mixture reaction consisted in 49 mM α-ketoglutaric acid, 22 mM apartate acid, 2.6 mM $EDTANa_2$, 1% PVP, 0.1% Fast Blue BB in 0.26 M phosphate buffer, pH 7.4. malate dehydrogenases were relieved using 2 M malic acid and 0.02% NAD as substrates, 0.02% NBT, 0.01% PMS and 0.01% MTT for tetrazolium stain in 0.2 M Tris-HCl buffer pH 8.

In order to assess the results, a specific software (Quantity One - a software produced by BIORAD that scans the gel and analyses the differences between the intensity) was used for numeric quantification of the variations (Table

Table 1 The numeric quantification of EST isoenzymes and activity estimation of each isoesterase expressed in *Ruscus aculeatus*

Probe-band	Relative front (pixels)	Peak Int (pixels)	Average Int (pixels)	Trace Int x mm	Relative Qty
P1-2	0.079	77.86	72.99	1004.205	5.7
P1-3	0.159	86.72	80.59	1506.811	8.5
P2-2	**0.076**	**125.57**	**94.88**	**2343.000**	**10.6**
P2-3	**0165**	**195.57**	**142.55**	**4324.704**	**19.5**
P2-4	**0.275**	**145.43**	**129.64**	**3155.548**	**14.2**
P2-5	0.318	116.14	94.88	1573.136	7.1
P2-7	0.718	67.43	62.92	1420.683	6.4
P2-9	**0.819**	**63.86**	**61.79**	**1504.192**	**6.8**
P3-2	0.092	62.00	60.05	826.255	5.1
P3-3	0.175	72.86	66.73	1765.603	10.8
P4-1	0.023	69.00	63.33	1362.933	8.0
P4-3	0.159	70.28	64.17	1992.234	11.6
P4-6	0.653	64.14	62.21	1448.550	8.5
P4-8	0.790	64.00	62.19	1404.054	8.2
P5-2	**0.065**	**118.72**	**88.68**	**2252.383**	**10.3**
P5-3	**0.161**	**167.57**	**138.19**	**3266.370**	**15.0**
P5-4	**0.269**	**133.28**	**122.94**	**3252.812**	**14.9**
P5-9	**0.813**	**75.43**	**66.87**	**1698.374**	**7.8**
P6-3	0.147	67.28	64.39	1590.019	9.2
P6-10	0.900	73.43	70.61	1793.522	10.2
P7-3	0.147	67.28	64.39	1590.019	9.2
P7-10	0.900	73.43	70.61	1793.522	10.2

1). The enzyme analysis were realized using this software, which quantified the differences in bands intensity and presence of the isoforms counting the pixels on the image of the gel.

Results and discussion

Morphological aspects of *in vitro* regeneration

The plant species responds in different ways to *in vitro* conditions depending on the culture media composition and the type of explant used for inoculation. From the vegetative parts of plant, only the rhizome fragments responded to *in vitro* cultures, generating multiple shoots and roots. But, this type of explants presents the disadvantage of an intense internal contamination with pathogens (i.e. fungi). The seeds were aseptically inoculated on MS medium (Murashige & Skoog 1962) and it germinated in 4 months in 80% cases, being largely free of contaminates.

Previous results on shoots regeneration from preformed meristems on the rhizome fragments (Banciu & Brezeanu 2008) showed a high multiplication rate on the basal medium MS, supplemented with 0.1 mg/l NAA and 1.0 mg/l BAP.

Shoots and roots from seeds which germinated on MS medium were further used for multiplication and acclimatization (Fig. 1B). In this case, the multiplication of the shoots was slow, taking about 6 months on MS basal medium supplemented with 2 mg/l BAP and 0.2 mg/l indolylbutiric acid (IBA), as recommended in literature (Balica et al. 2005).

For increasing of the regenerating rates it was further tested a new media composition, which proved to induce a faster multiplication of the shoots (as described in Materials and Methods). Using this composition, the shoots have been indirectly regenerated. The resulting plantlets could be used later in repopulation trials experiments (Fig. 1A).

Isoenzymes expression

For evaluation of the genetic stability, there were initiated analysis of enzymes spectra in the shoots grown *in vitro* and *in vivo* conditions. The biochemical methods have some disadvantages being influenced by tissue specificity and developmental stage (Scandalios & Sorensen 1977).

The first biochemical analyses followed the determination of the isoenzymes pattern (POX, EST, ACP, AKP, MDH and GOT) variations in natural population and *in vitro* regenerates.

The evaluation of the effects *in vitro* culture conditions was assessed by similarly and dissimilarly in isoenzymes expression, present

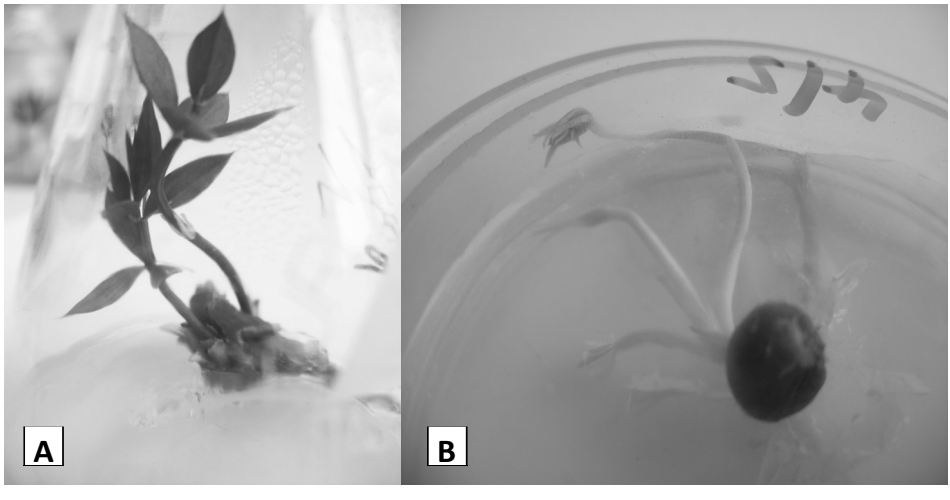

Figure 1 Shoots regenerated from rhizome explants by *in vitro* cultures (A) and seed of *Ruscus aculeatus* L. after 2 weeks from germination (B).

between *in vitro* regenerated plants and explants of native plants, from the same population. The same test was performed over the three Comana populations.

Taking into account the type of tissues collected and explants used for regeneration, the analyses of each tested enzyme were done by comparing similar bands from successive samples, as far as we suppose that each isoenzyme could have a specific role, responding in a specific way to *in vitro* culture conditions.

The GOT pattern showed that the samples from native populations P1 had more intense activity, comparing with P3 and P4, but all of them presented activity in the three loci (Fig. 2 A). There are also differences in intensity, comparing the samples from fruits from two populations (P2 and P5), the first population presenting higher activity, as well as one extra band (L2), in comparison with the third population. The samples from natural habitat presented a more intense GOT activity than those from *in vitro* cultures.

The MDH pattern showed that only the samples from fruits (P2 and P5) expressed isoform L1 and had the highest enzyme activity (Fig. 2 B). The native populations did not display differences in isoenzyme patterns. Comparing the samples from natural population with those from *in vitro* cultures, it was obviously that the MDH isoenzymes exhibited structural modifications (i.e. a decrease of migration front).

The stress induced by *in vitro* culture did not affected POX pattern (Fig. 2C), although this enzyme expression is usually sensitive to artificial conditions. However, the samples from the three native populations from nature present differences in band intensity and the first population (P1), expressed one extra band (L4), in comparison with the other two populations (P3 and P4). The low intensity of bands from samples of *in vitro* cultures (P6 and P7), comparing with those from native plant (P1) showed a high activity in natural conditions. The intensity of the fruit samples in POX electrophoresis was lower than the shoots samples in the first population (P1), but higher than shoots from the third population (P4), probably caused by the differences in the degree of maturity of fruit and seeds.

The EST pattern of regenerated plants was relatively similar with differences only in intensity of bands. The intense migration strips were those from fruits sampled from two populations (P2 and P5). The differences between P2 and P5 showed a different distribution of allele in populations (Markova et al. 1998). The activity of esterase in natural habitat was more intense than that in the shoots from *in vitro* cultures (Table 1).

In ACP spectra was detected one extra band with high expression in fruit samples from populations 1 and 3 (P2 and P5), in compari-

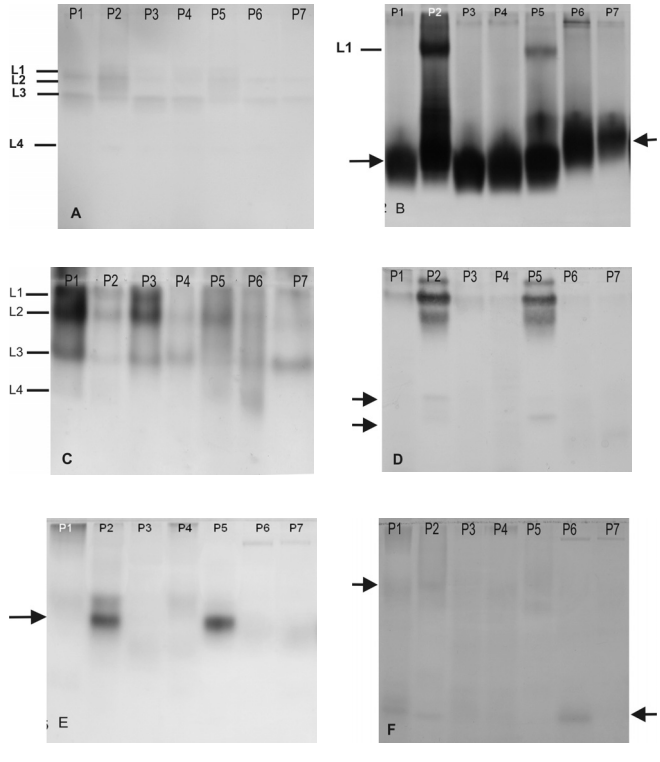

Figure 2 The isoenzyme patterns of GOT (A), MDH (B), POX (C), EST (D), ACP (E) and AKP (F) in *Ruscus aculeatus* L

son with samples from shoots grown in the natural habitat. This could suggest that the phosphate metabolism is more intense in fruits than in vegetative parts, as other authors stated (Efron 1970).

Comparing the AKP spectra of *in vitro* cultures with those from natural habitat, the regenerated shoots presented more intense bands in fast migration zone, while shoots from natural populations expressed multiple isoforms in slow migration zone. This could be explained by variations of the growing conditions imposed by the using of multiple means of adapting the metabolism (Wendel & Weeden 1989).

Conclusions

The *Ruscus aculeatus* species is relative refractory to *in vitro* environment, owing to the high endogenous contamination degree, but also because of the slow morphogenetic response. The research succeeded to establish a protocol for multiplication of shoots in this species, although the biotechnological potential became unlimited.

Preliminary analysis of electrophoretical profiles in the six isoenzymes systems, in natural populations, did not show significant differences between the individuals belonging to these three populations. The eventual differences in isoenzymes patterns could be associated with allele spread in those populations. The samples from *in vitro* cultures (P6-P7) usually present lower band intensity, comparing with those from the native population. In *in vitro* culture, spectra variations could be due to genetic variability of the seeds used and the *in vitro* regeneration method.

The studies on *Ruscus aculeatus* species are useful in the efforts of genetic biodiversity conservation on national and international level, according to the Strategy of IUCN, untill 2010 at least 10% of the threatened species (http://www.iucn.org/themes/ssc/our_work/plants/gspc.htm). Beside this, the micropropagation technology using multiple sources of inoculum is important in ellaborating new drugs in medicine. Future studies will analyze the possibility of cryoconservation of this species and viability tests post-conservation.

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References

Banciu C., Brezeanu A., 2008. Potențialul regenerativ *in vitro* al explantelor de țesut somatic, de diferite origini la *Ruscus aculeatus* L. Biotehnologii vegetale pentru secolul XXI, Lucrările celui de-al XVII-lea Simpozion Național de Culturi de Țesuturi și Celule Vegetale, Ed. Risoprint, Cluj-Napoca, pp 62-68.

Balica Deliu, C., Tămaș M., 2005. Biotehnologii aplicate la specia *Ruscus aculeatus* L. (Liliaceae), Hameiul și plantele medicinale, 25(1-2):163-166.

Boșcaiu N., Coldea G., Horeanu C., 1994. Lista roșie a plantelor vasculare dispărute, periclitate, vulnerabile și rare din Flora României, Ocrotirea Naturii și Mediului Inconjurător 38(1): 45-56.

Bouskela E., Cyrino F., Marcelon G., 1993. Effects of *Ruscus* extract on the internal diameter of arterioles and venules of the hamster cheek pouch microcirculation. J. Cardiovasc. Pharmacol. 22: 221-222.

Bouskela E., Cyrino, F., Marcelon, G., 1994. Possible mechanisms for the inhibitory effect of *Ruscus* extract on increased microvascular permeability induced by histamine in hamster cheek pouch. J. Cardiovasc. Pharmcol. 24: 281-285.

Ciocârlan V., 2000. Flora ilustrată a României. Editura Ceres, Bucuresti, 916p.

Curir P., Damiano C., Esposito P., Ruffoni B., 1988. *In vitro* propagation of *Ruscus racemosus* Moench. Acta Hort. (ISHS) 226: 217-222.

Curir P., Termini A., Ruffoni B., Marchesini A., 1989. NAA effect on phenolase activity in *Ruscus racemosus* Moench during in vitro propagation. Acta Hort. (ISHS) 251: 135-140.

Dihoru G., Dihoru A., 1993-1994. Plante rare, periclitate și endemice în Flora României - Lista roșie. Acta. Bot. Hort. Bucurestiensis, 173-197.

Doniță N., Popescu A., Paucă-Comănescu M., Mihăilescu S., Biriș I.-A., 2005. Habitatele din Romania, Ed. Tehnică Silvică, Bucureți, 496p.

Efron Y., 1970. Tissue specific variation in the isozyme pattern of the AP1 Acid phosphatase in maize, Genetics 65: 575-583.

Facino R., Carini M, Stefani R, 1995. Anti-elastase and anti-hyaluronidase activities of saponins and sapogenins from *Hedera helix, Aesculus hippocastanum* and *Ruscus aculeatus*: factors contributing to their efficacy in the treatment of venous insufficiency. Arch. Pharm. (Weinheim) 328:721-724.

Markova M., Vodenicharova M., Stoilova T., Cholakova

N., 1998. Application of seed esterase isoenzymes in testing hybridity of tomato. Biologia Plantarum 41 (2): 265-269.

May B., 1998. Starch gel electrophoresis of allozymes. In Hoelzel A.r.(ed) Molecular Genetic Analysis of Populations - A practical approach, Oxford Univeristy Press, pp. 1-28.

Moyano E., Montero M., Bonfill M., Cusido R., Palazon J., Pinol M., 2006. *In vitro* micropopagation of *Ruscus aculeatus*, Biologia Plantarum 50 (3): 441-443.

Murashige-Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture; Physiol. Plant. 15: 473-479.

Oltean M., Negrean G., Popescu A., Dihoru G., Sanda V., Mihăilescu S., 1994. Lista roşie a plantelor superioare din România. Studii, sinteze, documentaţii de ecologie, Acad. Rom., Inst. de Biol. 1: 1-52.

Palazon J., Moyano E., Bonfill M., Osuna L.T., Cusido R., Pinol T., 2006. Effect of organogenesis on steroidal saponin biosynthesis in calli cultures of *Ruscus aculeatus*. Fitoterapia 77(3): 216-220.

Parrado F, Buzzi A.,1999. A study of the efficacy and tolerability of a preparation containing *Ruscus aculeatus* in the treatment of chronic venous insufficiency of the lower limbs. Clinical Drug Investigation 18 (4): 255-261.

Paucă-Comănescu M., Negrean G., Paspaleva M., Talpeanu M., Doniţă N., Bândiu C., Onete M., 2000-2001. Padurea Fântânele (Comana) - arie de conservare a biodiversitatii native a pădurilor şi zonelor umede din Câmpia Română, Ocrotirea Naturii şi Mediului Înconjurător 44-45: 15-27.

Paucă-Comănescu 2009. Toleranţa planatelor vasculare la stres - factor de modelare cenotică. In: Anonymouss (ed.) Adaptarea la stres: Condiţie de supravieţuire şi/sau factor de biodiversitate. Ed. Academiei Romane, pp. 175-194.

Tarnavschi I.T., Paucă A., Andrei M., Cristurean I., Ionescu V., Lungu L., Nedelcu G.A., Petria El., Popescu A., Rădulescu D., Rădulescu-Mitroi N., Şerbănescu-Jitaru, G.,1974. La flore du complexe Comana. Acta Botanica Horti Bucurestiensis 239-287.

Scandalios Jg., Sorenson J.K., 1977. Isozymes in plant tissue culture. In Reinert J., Bajaj Y.P.S. (eds) Applied and fundamental aspects of plant cell, tissue and organ culture. Springer Verlag, Berlin, pp. 719-730.

Sammour R.H., 1991. Using electrophoretic techniques in varietal identification, biosystematic analysis, phylogenetic relations and genetic resources management, Journal of Islamic Academy of Science 4 (3): 221-226.

Sumita J., Sumitra S., 1985. *In vitro* regeneration of *Ruscus hypophyllum* L. plants, Plant Cell, Tissue and Organ Culture, 5(1): 79-87.

Wendel J.F., Weeden N.F., 1989. Visualisation and interpretation of plant isozymes. In Soltis, D.E., Soltis P.S. (eds.) Isozymes in plant biology. Dioscorides Press, Oregon, pp. 5-45.