

IN VIVO AND IN VITRO COMPARATIVE ULTRASTRUCTURAL STUDIES OF *RUSCUS ACULEATUS* L. PHYLLOCLADE CELLS

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A comparative study of the *Ruscus aculeatus* phylloclade cells structure and ultrastructure of plants belonging to the native habitat and *in vitro* culture are presented in the paper in order to establish an adequate protocol for *ex situ* conservation. In our experimental conditions no anomaly of the phylloclade cells ultrastructure has been observed. Some interesting modifications appeared at the plastidial system that are polymorphic as well as at plastoglobuli level that could represent adaptative modifications, but not genetic alterations. The *in vitro* experimental protocol, previously established by us for *in vitro* propagation that requires plants indirect regeneration, via callus, on the MS basal nutritive medium supplemented with 1 mg/l NAA and 5 mg/l BAP may be considered an adequate possibility for medium-term conservation of this species.

Key words: ultrastructure, *Ruscus aculeatus*, phylloclade.

INTRODUCTION

Ruscus aculeatus L. (*Liliaceae* Family), known both as medicinal and ornamental plant, is an endangered angiosperm species protected at national as well as at European level (through Habitat Directive of EU and Bern Convention).

This species is a small evergreen perennial shrub with erect and striated stems, horizontal rhizomes and tough leaf-like branches having assimilatory role, known as phylloclade.

Because of its threatened status, the establishment of a proper conservation strategy presents a special interest. In this frame *in vitro* culture systems have been used by us (Banciu and Brezeanu, 2008).

Although there are numerous reports of micropropagation protocols for many ligneous species, there are only few reports regarding successful micropropagation of *Ruscus aculeatus* because of its *in vitro* no reactivity and of the difficulties to establish the best explant source for primary culture starting (Moyano *et al.* 2006, Balica *et al.* 2005, Ivanova *et al.* 2009).

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Our previous research demonstrated the possibility to *in vitro* plant regeneration using rhizome fragments as explant sources (Banciu and Brezeanu, 2008).

For a correct evaluation of the method efficiency, from a conservative point of view, cytological, biochemical (Banciu *et al.* 2009) and molecular markers were used. In the present paper, comparative cytological (electronmicroscopy) studies of the phylloclade belonging to *in vitro* regenerated plants and from a native habitat were achieved.

MATERIAL AND METHODS

For the *in vitro* culture, samples of *Ruscus aculeatus* plants have been collected from the forest near the Comana village from which explants of somatic tissues of different origins have been taken. Fragments of the rhizome presented a high reactivity. The plant prefers a solid culture medium, such as Murashige-Skoog (1962) basal medium supplemented with NAA and BAP as hormones.

For electron microscopy studies, the phylloclade cells both *in vitro* culture as well as from native plants were used. These were processed by conventional method (Mascorro and Bozola, 2007; Kuo, 2007): pre fixation in a solution of 3% glutaraldehyde in Na cacodylate buffer at 0.2 M at pH 7, stored overnight at 4°C and fixed in an unbuffered 1% aqueous solution of OsO₄ overnight at 4°C, rinsed repeatedly in water.

After dehydration by means of a graded series of ethanol and propylene oxide, samples were infiltrated in Epoxy resin. The ultrathin sections were cut on a LKB ultra microtome using a Du Pont diamond knife and after contrastation by Reynold's method (1963) the sections were viewed in an EM-125 (Selemi-Ukraine) electron microscope.

For light microscopy semifine sections 1–2 μm thick were stained with a solution of 1% toluidine blue in 1% borax (Pickett-Heaps, 1966).

RESULTS AND DISCUSSION

Histological observations. The investigation of the histological structure of *Ruscus aculeatus* phylloclade by semifine cross sections allowed us to observe the same arrangement of the constitutive elements no matter their origin, from both *in vitro* or *in situ* plants (Fig. 1A and B). *In vitro* culture conditions do not seem to induce any modification at the histological level. Epidermis (upper and lower) covered by thick cuticle interrupted by stomata, homogeneous assimilatory parenchyma containing chloroplast, a large parenchyma cell lacking chloroplasts, in the central part and leptocentric vascular bundles, can be observed in both cases (Fig. 1C).

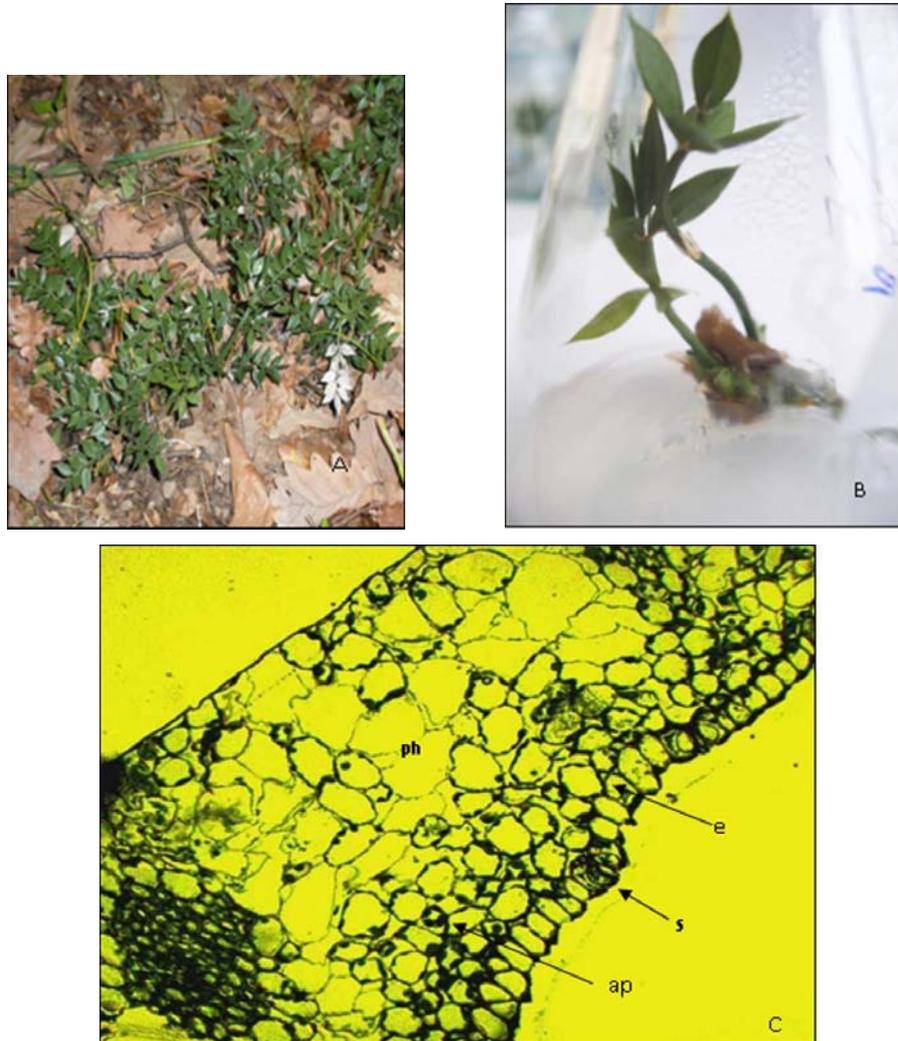


Fig. 1. General morphological view of *Ruscus aculeatus* L. belonging to a natural habitat (A) and from *in vitro* culture (C). Semifine cross section through phylloclade (C) – general view, (oc 10, ob 20), s – stomata, ap – assimilatory parenchyma, ph – parenchyma cells, e – epidermis.

Electronmicroscopical peculiarities of the phylloclade cells belonging to a plant from a native habitat. Electronmicroscopical observations revealed that the main peculiarities of the cells and cellular organelles are the same as those of the mesophyll cells of higher plants described so far by different authors.

The nucleus, oval or round shaped, represents the dominating organelle. It is evident the presence of one or two compact nucleoli and condensed chromatic

material, in the karyoplasm. Frequently, it is in relations of contiguity with other organelles like chloroplast, Golgi bodies or profile of the endoplasmic reticulum (Fig. 2).

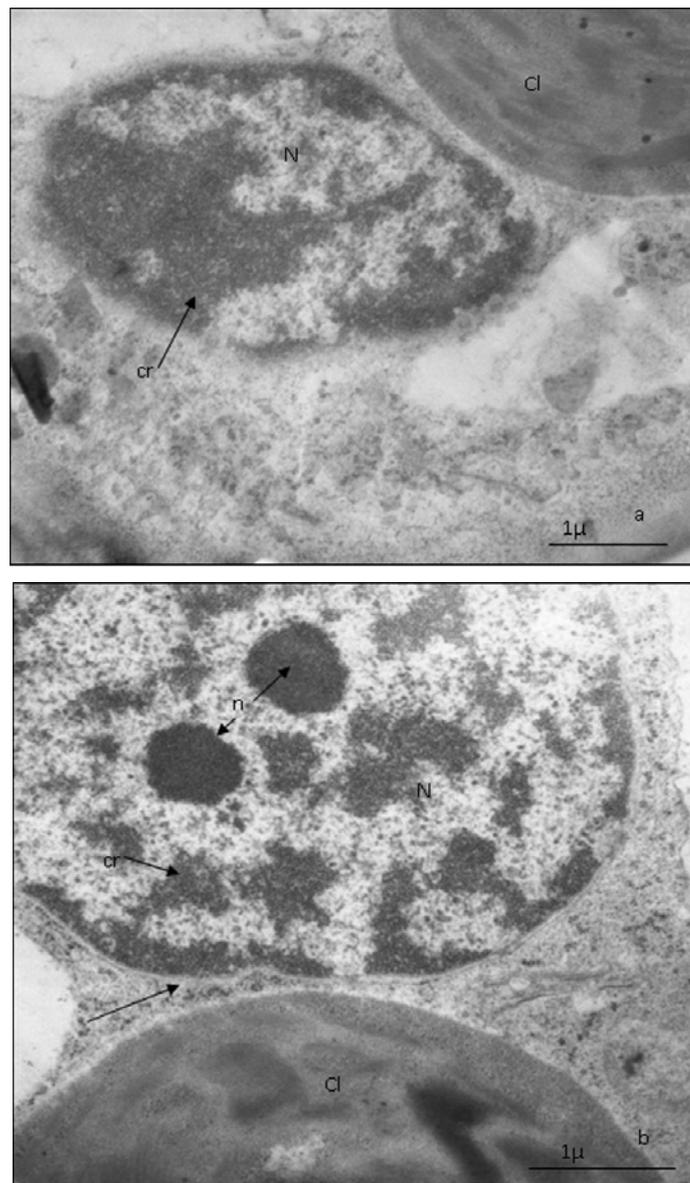


Fig. 2. Aspect of the interphasic nucleus from phylloclade cells belonging to the natural habitat plants (a). Detail (b). N – nucleus, n-nucleolus, Cl – chloroplast, cr – chromatic condensed material. The relations between nucleus and chloroplast can be observed (see arrow).

Numerous chloroplasts with typical grana are present in the parietal cytoplasm. Generally, starch inclusions are absent or very small in size. In plastidial stroma small electron dense plastoglobules randomly spread are present (Fig. 3a).

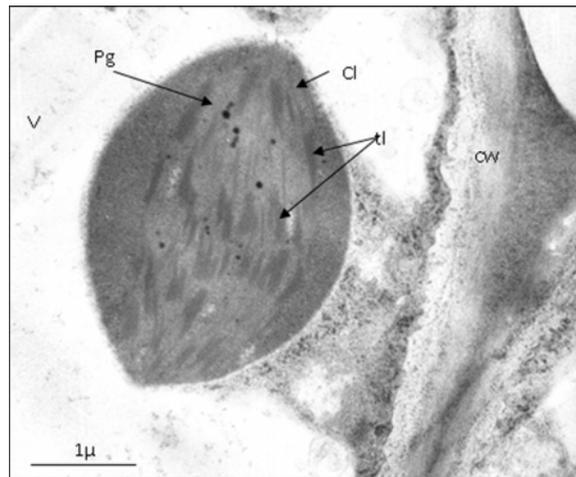


Fig. 3a. Ultrastructural peculiarities of the chloroplast from phylloclade cells belonging to plants from the natural habitat. Cl – chloroplast, Pg – plastoglobule, CW – cell wall, V – vacuome, tl – thylakoidal system.

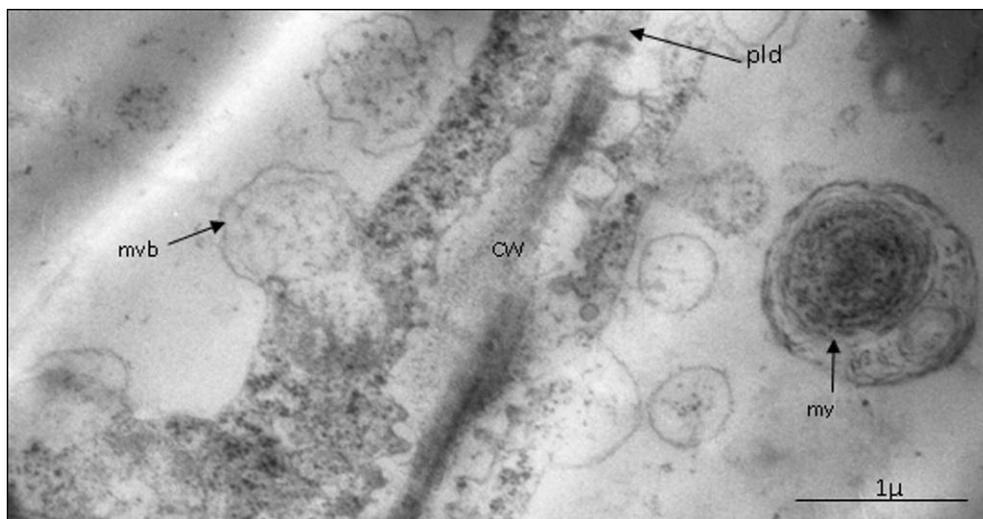


Fig. 3b. Sector of the *Ruscus aculeatus* phylloclade cell belonging to plants from the natural habitat. Numerous plasmodesmata (pld) crossing the cell wall (PC) as myelinic figures (my) and multivesicular bodies (mvb) can be observed.

Mitochondria with a typical inner structure were observed single or in group nearby the chloroplast or/and the nucleus. The thin cell wall is frequently crossed by plasmodesmata, a membranous channel that connects the neighboring cells anatomically and physiologically to form a functional intercellular communication network that supports cellular specialization in symplasmic transport (Fig. 3b).

Numerous multivesicular and paramural bodies derived from tonoplast as well as from plasmalemma (plasmalomasomes) can be remarked in many cells (Fig. 3b). It was suggested by many authors that they are involved in cyclic cytomembrane processes and in cell wall biosynthesis particularly (Anghel *et al.* 1981).

Ultrastructural aspects of the phylloclade cells belonging to in vitro regenerated plants. As we observed by histological analyses, *in vitro* conditions did not produce severe perturbations of the normal pattern of cellular organisation.

Some interesting modifications appeared at the plastidial system level which is polymorphic. Most of the chloroplasts are ellipsoidal in shape with a weakly differentiated granal system without starch grains and containing numerous small electrondense plastoglobules (Fig. 4).

A second category includes plastids with a disorganised lamellar system, contains numerous larger electrondense plastoglobules and few starch grains (Fig. 5a). A third category is represented by chloroplasts with a normal inner lamellar system but weakly differentiated grana and numerous large electronopaque plastoglobules which occupy the most part of the plastidial stroma (Fig. 5b). Small electrondense plastoglobules are not observed in them.

Early electronmicroscopic studies (Greenwood 1963) revealed the presence of “osmiophilic globuli” (plastoglobules) inside of chloroplasts as well as other plastid types, but their specific functions are unknown. Their size and number generally vary during plastid development and differentiation and strongly increase during stress conditions at the same time with thylakoid membrane degradation. The mechanism and the role of stress factors in modulation of plastoglobule size and number are not understood completely.

Recent publications (Brehelin *et al.* 2007) strongly suggest that plastoglobules actively participate not only in stress responses but in numerous secondary metabolism pathways too. They are not merely a “passive storage” component but rather versatile particles. In chloroplasts they were proposed to serve as reservoirs for α -tocopherol, plastoquinone and triacylglycerols but they were also proposed to play a role in the removal of protein catabolites as part of thylakoid turnover (Smith *et al.* 2000). In chromoplasts they are the main storage and remodeling site for carotenoids (Deruere *et al.* 1994).

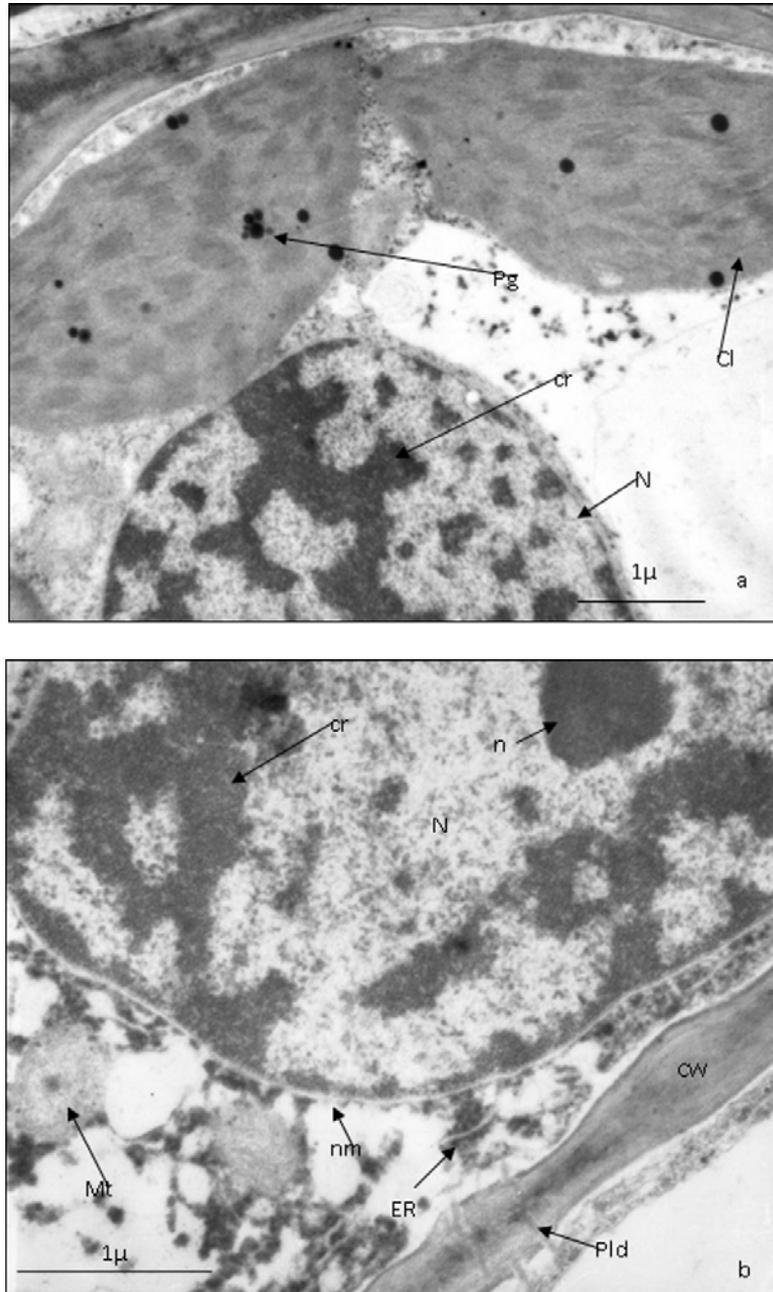


Fig. 4. Sector of the *R. aculeatus* phylloclade cell from *in vitro* plant regenerated (a). Detail (b) presenting a sector of the cell with nucleus (N), nucleolus (n), nuclear membrane (nm), chromatin (cr), endoplasmic reticulum (ER), mitochondrion (Mt), plasmodesmata (Pld).

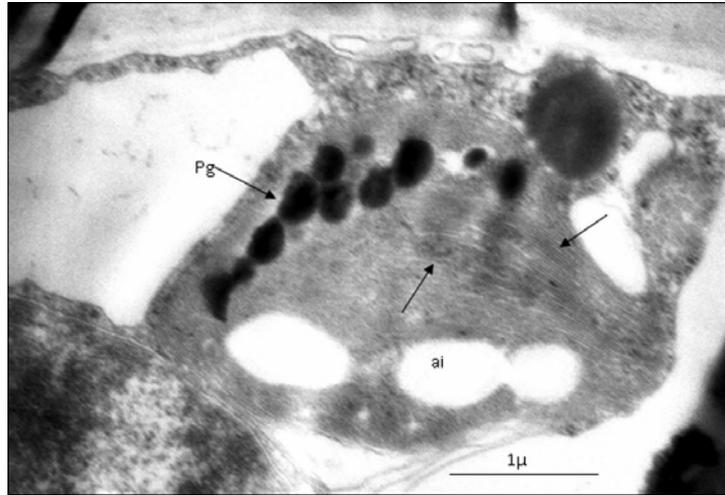


Fig. 5a. Chloroplast with weak differentiated granal system (see arrow) and numerous large electron-dense plastoglobules (Pg) in the plastidial stroma, close to the nucleus, in the phylloclade cell of *R. aculeatus in vitro* regenerated plants. ai – amyiferous inclusions.

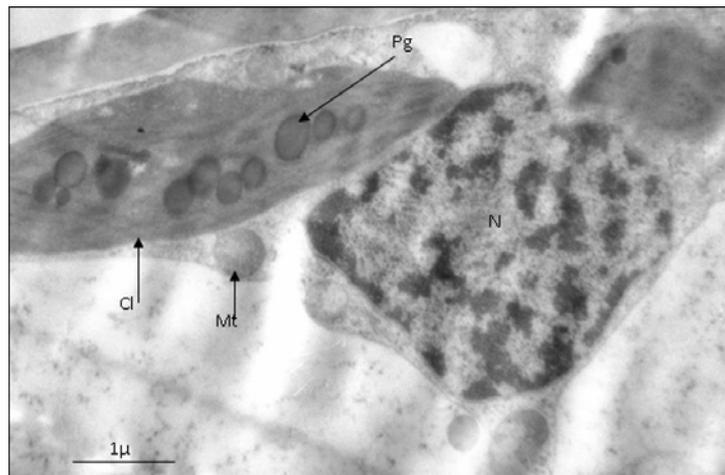


Fig. 5b. Chloroplast (Cl) with characteristic electron-opaque plastoglobules (Pg) in the phylloclade cell from the *in vitro* regenerated plants. Mt – mitochondrion, N – nucleus.

Currently, a rapid growing body of evidence suggests an active role for plastoglobules in stress response pathways which all suggest that plastoglobules are a metabolic intersection between different plastid components (Brehelin *et al.* 2007). In our research we consider that it may be possible that these two kinds of plastoglobules are related with the specific type and nature of substances accumulated in them during *in vitro* culture growth.

The polymorphic aspect of the plastides described by us in phylloclade cells of the *in vitro* growing *Ruscus aculeatus* plants is a frequent phenomenon in tissue culture both in the callus and in the regenerated plant cells (Brezeanu 1991, Brezeanu and Banciu 2009). This appeared like a complex phenomenon which could be determined by the specific *in vitro* system which, in fact, represents a stress factor. Generally, it is known that plastids are very sensitive to any stress factor. The data presented by us pointed out that our *in vitro* experimental conditions do not induce severe alterations of the plant cells structure and functions. In this context it is important to mention that nonsignificant morphological modifications appeared at the other major organelles level, like nucleus, nucleoli, or mitochondria as well as senescence or necrosis phenomenon.

These observations can be correlated with biochemical studies regarding the evaluation of the effects of *in vitro* conditions on the isoenzymes pattern variations in natural population and in *in vitro* regenerants (Banciu *et al.* 2009). Analyses of electrophoretical profiles in the six isoenzymes (POX, EST, MDH, GOT, AKP, ACP), in natural populations comparative with *in vitro* regenerated plants did not reveal significant differences. These aspects were remarked by us in case of other species (*Marsilea quadrifolia*) too that could represent adaptative modifications to *in vitro* conditions (Brezeanu and Banciu, 2009). Our results allowed to appreciate that the experimental protocol used represents an efficient micropropagation method, that can be successfully applied in medium-term conservation strategy of *Ruscus aculeatus* species.

CONCLUSIONS

– Our electronmicroscopical studies on the *Ruscus aculeatus* L. phylloclade cells revealed similarities in general cellular architecture with mesophyll cells of other higher plants reported by different authors.

– The experimental protocol used by us in case of *in vitro* micropropagation of *Ruscus aculeatus* plant species did not affect cells structure and function which allowed us to consider it an adequate possibility for medium-term conservation strategy.

Acknowledgements. We express our gratitude to our collaborators in Electron Microscopy: Viorica Stan and Alexandru Branzan.

REFERENCES

1. Anghel I., Brezeanu A., Toma N., 1981, *Ultrastructura celulei vegetale*, Editura Academiei RSR, 205 p.
2. Balica G., Deliu C., Tămaș M., 2005, Biotehnologii aplicate la specia *Ruscus aculeatus* L. (Liliaceae), *Hameiul și plantele medicinale*, nr. 1-2, Ed. Academic Press, Cluj-Napoca, pp. 25-26.

3. Banciu C., Brezeanu A., 2008, Potențialul regenerativ *in vitro* al explantelor de țesut somatic, de diferite origini la *Ruscus aculeatus* L., în vol. *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.
4. Banciu C., Mitoi M.E., Brezeanu A., 2009, Biochemical peculiarities of *in vitro* morphogenesis under conservation strategy of *Ruscus aculeatus* L., *Annals of Forest Research*, **52**, pp. 27-35;
5. Brehelin C., Kessler F., van Wijk Klaas J., 2007, Plastoglobules: versatile lipoprotein-particles in plastids. *Trends in Plant Science-Review*, **12**, (6), pp. 260-266.
6. Brezeanu Aurelia, Banciu C., 2009, Comparative studies regarding ultrastructure of *Marsilea quadrifolia* L. (Pteridophyta) leaf mesophyll cells *in vivo* and *in vitro* culture, *Romanian Journal of Biology – Plant Biology*, Ed. Academiei Române, București, **54**, (1), pp. 13-24.
7. Brezeanu A., 1991, Ultrastructural peculiarities of the plastidial system in *Vitis vinifera* L., Cells from callus culture, *Revue Roumaine de Biologie, Biologie Vegetale*, **36**, (1-2): 45-48.
8. Deruère J., Römer S., d'Harlingue A., Backhaus R.A., Kuntz M., Camara B., 1994, Fibril assembly and carotenoid overaccumulation in chromoplasts; a model for supra molecular lipoproteins structures, *Plant Cell*, **6**, pp. 119-133.
9. Greenwood A.D., Leech R.M., Williams J.P., 1963, The osmiophylic globules of chloroplasts. I. Osmiophylic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba* L., *Biochim. Biophys. Acta*, **78**, 148-162.
10. Ivanova, T., Gussev, Ch., Bosseva, Y., Stoeva, T., 2009, Storability of micropropagated *Ruscus aculeatus* L. (*Liliaceae*) plants, *5th Balkan Botanical Congress*, Belgrade, Serbia, 07-11 Sept. 2009.
11. Kuo J., 2007, Processing Plant tissues for ultrastructural study, Electron Microscopy, *Methods and Protocols*, Second Ed. Humana Press, 35-47.
12. Mascorro J.A., Bozzola J.J., 2007, Processing Biological Tissues for Ultrastructural Study Electron Microscopy, *Methods and Protocols*, second edition, Ed. by J. Kero, Humana Press, pp. 19-35.
13. Moyano, E., Montero M., Bonfill M., Cusido R., Palazon J., Pinol M., 2006, *In vitro* micropropagation of *Ruscus aculeatus*, *Biologia Plantarum*, **50**, (3), pp. 441-443.
14. Pickett-Heaps D.J., 1966, Incorporation of radioactivity in wheat xylem walls, *Planta*, **74**: 1-14.
15. Smith M.D, Licatalossi D.D., Thompson J.E., 2000, Co association of cytochrome (catabolites and plastid-lipid-associated protein with chloroplast lipid particles), *Plant. Physiol.*, **124**; pp. 211-222.