COMPARATIVE STUDIES REGARDING ULTRASTRUCTURE OF *MARSILEA QUADRIFOLIA* L. (PTERIDOPHYTA) LEAF MESOPHYLL CELLS *IN VIVO* AND *IN VITRO* CULTURE

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A comparative analysis of the ultrastructural peculiarities of *Marsilea quadrifolia* L. mesophyll cells of the leaves belonging to the plants grown in *in vitro* system and in natural habitat are presented in the paper. Cytological observations enabled to remark that the experimental protocol used by us for *in vitro* plant regeneration and clonal multiplication did not affect the normal pattern of plant development. At the ultrastructural level severe alterations of the cells' organelles structure were not observed. Polymorphic aspect of the chloroplast was revealed in *in vitro* culture and this could represent adaptive modifications to culture conditions. In respect of this we consider that this protocol could be used as a successful experimental system for *ex situ* conservation of this threatened species.

Key words: *Marsilea quadrifolia*, ultrastructure, *in vitro*, *in vivo*.

INTRODUCTION

Marsilea quadrifolia L. is one of the threatened species which has protected status both in the Red List and in the international documents such as Bern Convention in the 1st Annex or Habitats Directive of the European Union in the 2nd Annex. It belongs to *Marsilea* Genus, *Marsileaceae* Family, *Hydropteridales Order*, *Filicopsida* Class (CIOCARLAN V., 2000). The species inhabits the aquatic and semi aquatic environments presenting at the surface of the swamp a repent rhizome with multiple branches and many adventitious roots.

In contrast with other pteridophytes, which are ornamental, especially *Cyrtomium falcatum*, *Blechnum spicant* and *Osmunda regalis*, *Marsilea quadrifolia* was not analyzed at the cytological level and the ultrastructural studies are almost inexistent at this species (Soare, Neagu, 2003; Soare *et al.* 2006-2007; Fernandez, Ravilla, 2003).

Also, generally, there was not much interest for studying *in vitro* culture of *Marsilea* genus (Liu, 1984; Lin *et al*, 2005). Lin and Yang (1999) analyzed the effects of the quality of light and of some hormones like ABA on the heterophyllous switch in these species, Laetsch & Brigs (1961), studied the influence of the kinetin

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sporeling ontogeny on *Marsilea vestita*. Our preoccupations in this direction were determined by its status of endangered species. In this frame the establishment of conservation strategy using *in vitro* culture systems, in order to multiply the future repopulation of the native situs, presents a special interest.

Simultaneously, for a better understanding of the developmental processes and of the effects of different experimental conditions of regenerants' development, we consider necessary to do a cytological study of the plant cells from *in vivo* and *in vitro* culture.

In this frame the aim of this paper is to realize a comparative study regarding the fine structure of *Marsilea quadrifolia* mesophyll cells of the leaves *in vitro* and *in vivo* as a base for conservation experiments.

MATERIAL AND METHODS

For the *in vitro* culture samples of *Marsilea quadrifolia* plants have been collected from the swampy area near the Comana village from which explants of somatic tissues of different origins have been taken. Nodal areas on the rhizome and the branching of the leaves presented a high reactivity. The plant prefers a semi liquid culture medium, poor in mineral salts such as Knopp or Murashige-Skoog (1962) basal medium with half salts level, without hormones.

For electron microscopy studies the mesophyll cells of the leaves from both *in vitro* culture as well as from native plants were used. These were processed by a conventional method (Mascorro and Bozola, 2007; Kuo, 2007): pre fixation in a solution of 3% glutaraldehyde in Na cacodylate buffer at 0.2M at pH 7, stored overnight at 4°C and fixed in an unbuffered 1% aqueous solution of OsO_4 overnight at 4°C, rinsed repeatedly in water.

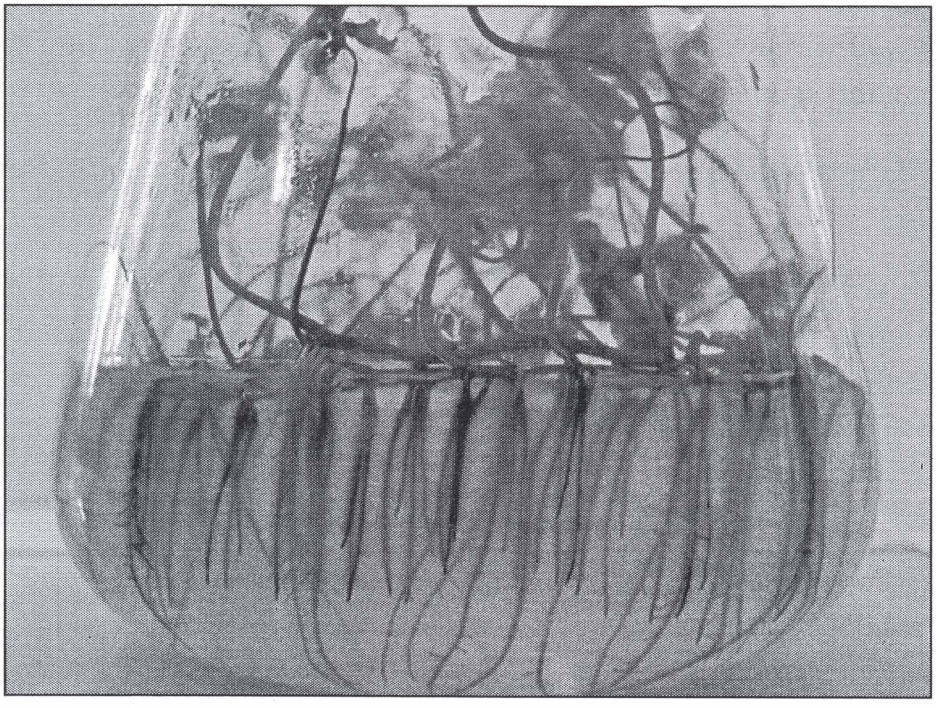
After dehydration by means of a graded series of ethanol and propylene oxide, samples were infiltrated in Epoxy resin. The ultrafine sections were cut on a LKB ultra microtome using a Du Pont diamond knife and after contrastation by Reynold's method (1963) the sections were viewed in an EM-125 (Selemi-Ukraine)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

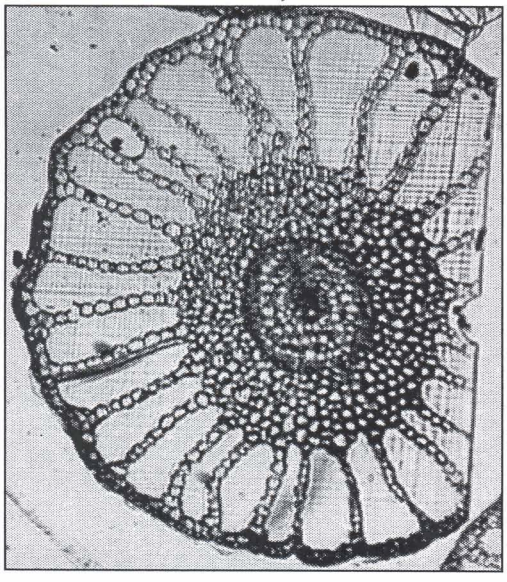
For light microscopy semi fine sections 1-2μm thick were stained with a solution of 1% toluidine blue in 1% borax (Pickett-Heaps, 1966).

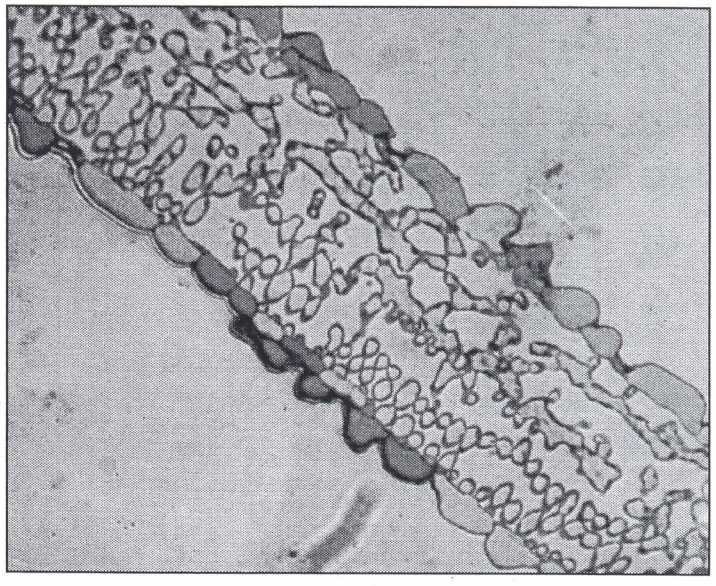
RESULTS AND DISCUSSION

Our previous researches (Banciu *et al.*, 2008) revealed that *Marsilea quadrifolia* is a reactive species to *in vitro* conditions that allow us to obtain numerous regenerants identical from the morpho-anatomical point of view with the donor plant.

As in the native habitat, a repent rhizome with multiple branches and many adventitious roots developed at the surface of the semi liquid nutrient medium. From these rhizomes over the medium level, the aerial leaf with long petioles that resembles a four leaf clover has grown. The new parts of the plant, morphologically identical with the native plant, are formed as individual clonal structures.







a b

Fig. 1. General morphological view of *Marsilea quadrifolia* L. regenerated plants. Semi-fine section through rhizome and leaf (cross section-a: oc.10x, obj 10x; long section b: oc.10x, obj 20x). Semifine sections were stained with a solution of toluidine blue in borax.

Histological analyses on semi fine section also revealed a similar structural architecture of the rhizome and leaves tissues (Fig. 1) both *in vitro* as well as *in situ* conditions.

Comparative analyses of the iso-enzymatic spectra used as biochemical marker showed that certain loci can be used as markers which relieved intra populational heterozygosity or somaclonal variation in plants obtained by *in vitro* systems (Banciu *et al.*, 2009), but not by severe genetic modifications. In order to obtain a general view of the effect of *in vitro* conditions on this species development and propagation a cytological study was imperative.

a) The ultrastructural peculiarities of mesophyll cells of the leaf of native plants

The investigations of structure and ultra structure of *Marsilea quadrifolia* the mesophyll cells of the leaves belonging to the plants developed *in vivo* present general peculiarities of the mesophyll cells of higher plants (Fig. 2).

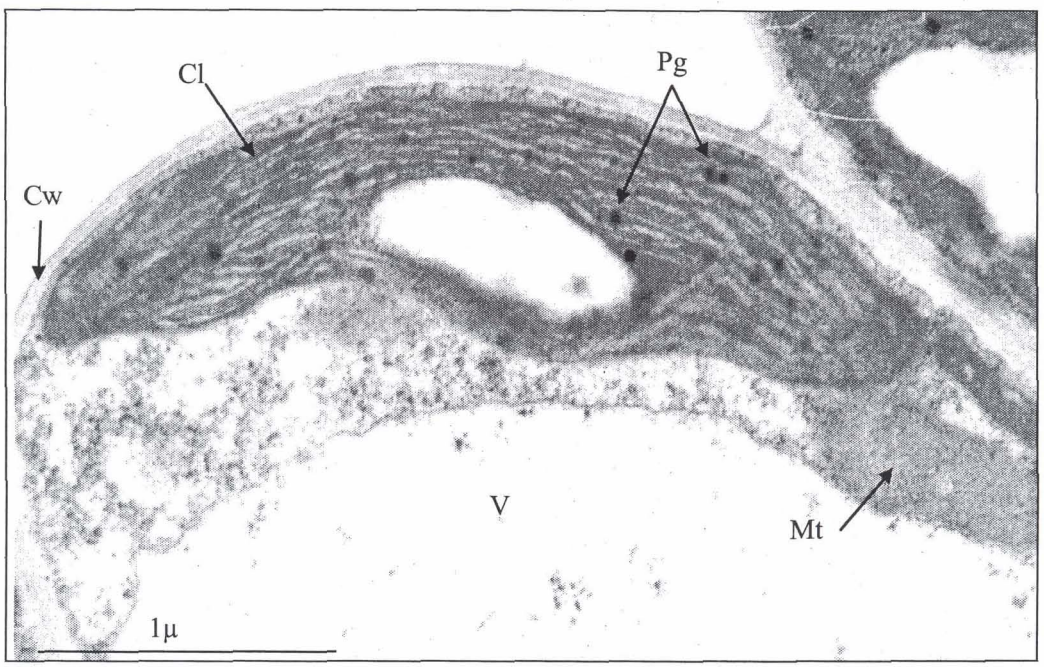


Fig. 2. Ultrastructural peculiarities of the *Marsilea quadrifolia* mesophyll cell growing in native habitat. Aspect of a chloroplast with a weakly differentiated granal system. Cw-cell wall, Cl-chloroplast, V-vacuole, Mt-mitochondrion, Pg-plastoglobule.

Cells contain a large central vacuole and plenty of parietally disposed chloroplasts. Generally, chloroplasts present a weakly differentiated granal system, one or two large amylopherous inclusions (Fig. 2) and a small number of plastoglobuli. Large mitochondria with a typical structure as well as Golgi bodies and endoplasmic reticulum can be observed (Fig. 3).

Frequently mitochondria singly or in groups are located in the cytoplasm, quite often in the nearby of nucleus or chloroplasts and established relations of contiguity with them and Golgi bodies. In cross section through the vascular tissue of the leaf cells manifest typical characteristics of the xylematic, phloematic and parenchymatic tissue cells (Fig. 4). The modifications in thickness of the xylem cell wall were observed.

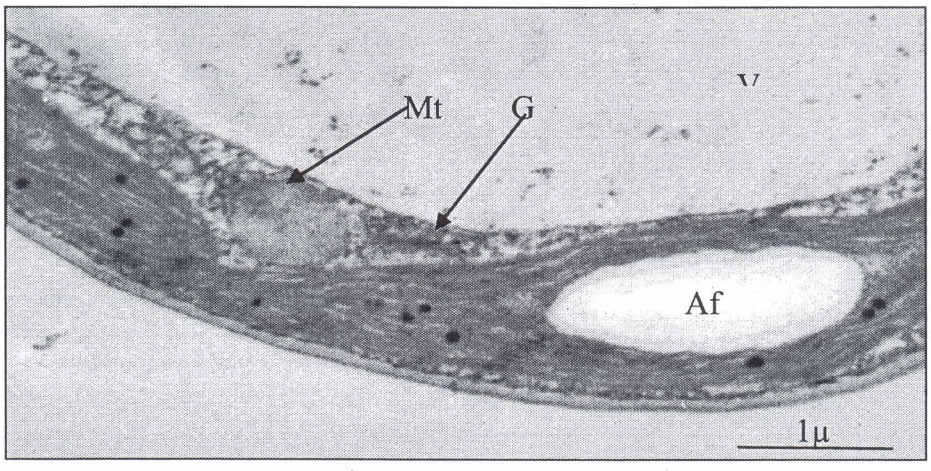


Fig. 3. Sector of a mesophyll cell. A large amyliferous grain can be observed. Af-amyliferous grain, G-Golgi bodies.

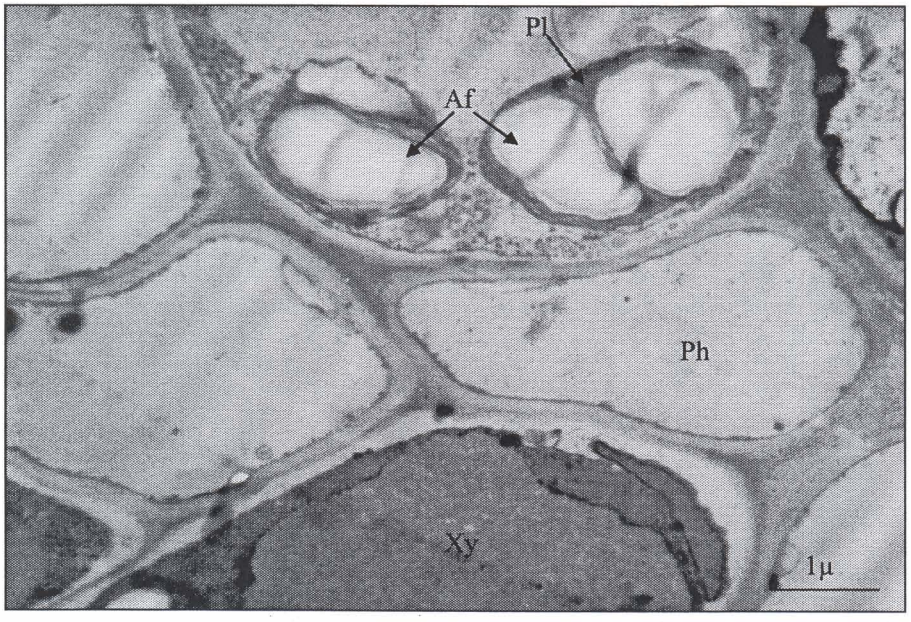


Fig. 4. Cross section through the vascular tissue of the leaf from *Marsilea quadrifolia*. Ph-phloem cell, Xy-xylem cell, Pl-chloroplast, Af-amyliferous grain.

In some cells myelin formations or paramural bodies are very well represented (Fig. 5); they often occupy all vacuolar space and could originate in the tonoplast.

Nucleus round or oval in shape presents two compact nucleoli in the karyoplasm (Fig. 6). One of them possesses a nucleolar organizer with condensed chromatic material to be noticed in the karyoplasm.

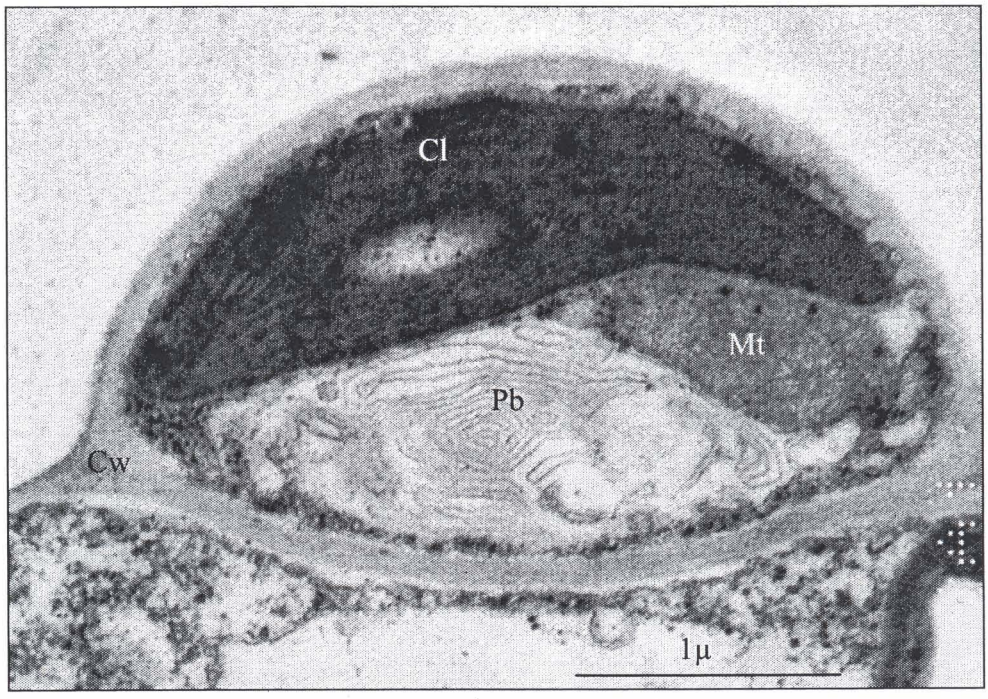


Fig. 5. Section through the leaf tissue of *Marsilea quadrifolia* from native habitat. A large paramural body which occupied all vacuolar space is evident. Cl-chloroplast, Mt-mitochondrion, Cw-cell wall, Pb-paramural bodies.

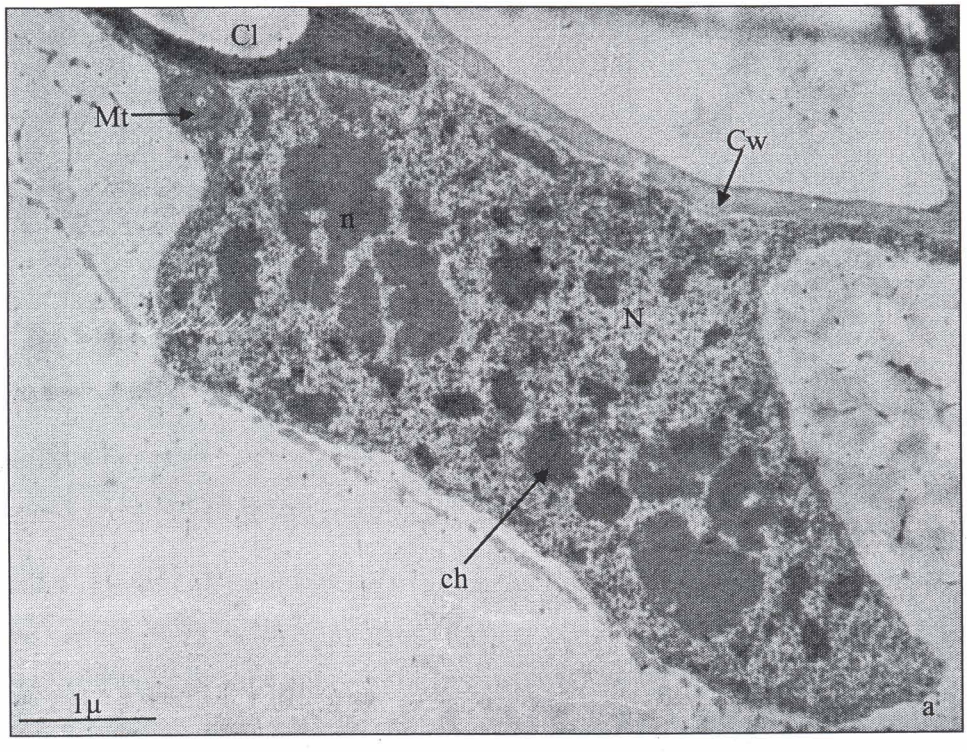


Fig. 6a

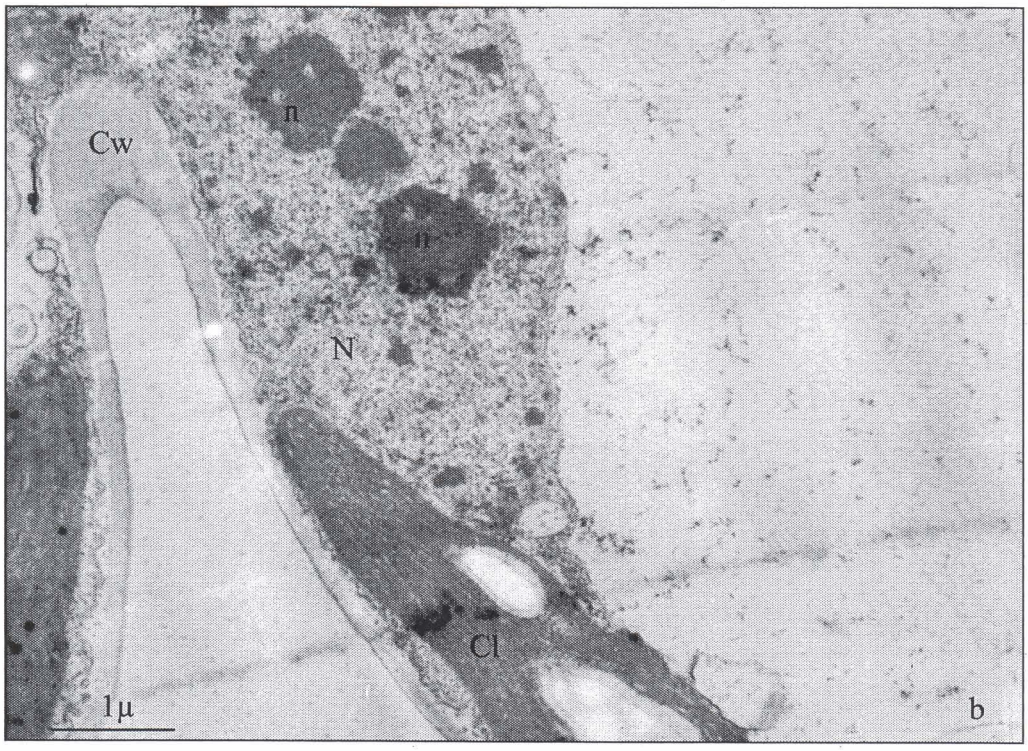


Fig. 6 a&b. Ultrastructural peculiarities of the nucleus. The presence of the nucleolus and chromatic material condensed in the karyoplasm is to be noticed. n-nucleolus, ch-chromatic material, N-nucleus, Cw-cell wall, Cl-chloroplast, Mt-mitochondrion.

b) The ultrastructural characteristics of the mesophyll cells of the leaves of *in vitro* regenerants

Electron microscopical observations of mesophyll belonging to regenerants from *in vitro* culture present from many points of view a similar structure with those from the native plants, which demonstrates that our experiment *in vitro* conditions does not induce morphological alterations at the ultrastructural level.

Characteristically, plastids are polymorphic. Part of them are smaller with weakly differentiated granal system, small starch grains, without plastoglobuli (Fig. 7). A second category of plastids present a typical structure with a small number of large thylakoids grouped in the middle part of the organelle (Fig. 8). A third category presents a characteristic inner architecture of the lamellae system with thylakoids grouped in granas and intergranas. Plastidial stroma is electron opaque and plastoglobuli are not obvious (Fig. 7a). This polymorphic aspect is a frequent phenomenon in tissue culture both in the callus and in the regenerated plant. They could be determined by culture conditions.

The histological observations correlated with electron-microscopical studies on callus tissue of other plant species support the idea that the particular aspects of the plastids in cells from different zones of callus are determined by two factors: a differentiated level of nutrient supply through the callus mass and of light irradiation intensity in different areas of this callus tissue (Brezeanu, 1991).

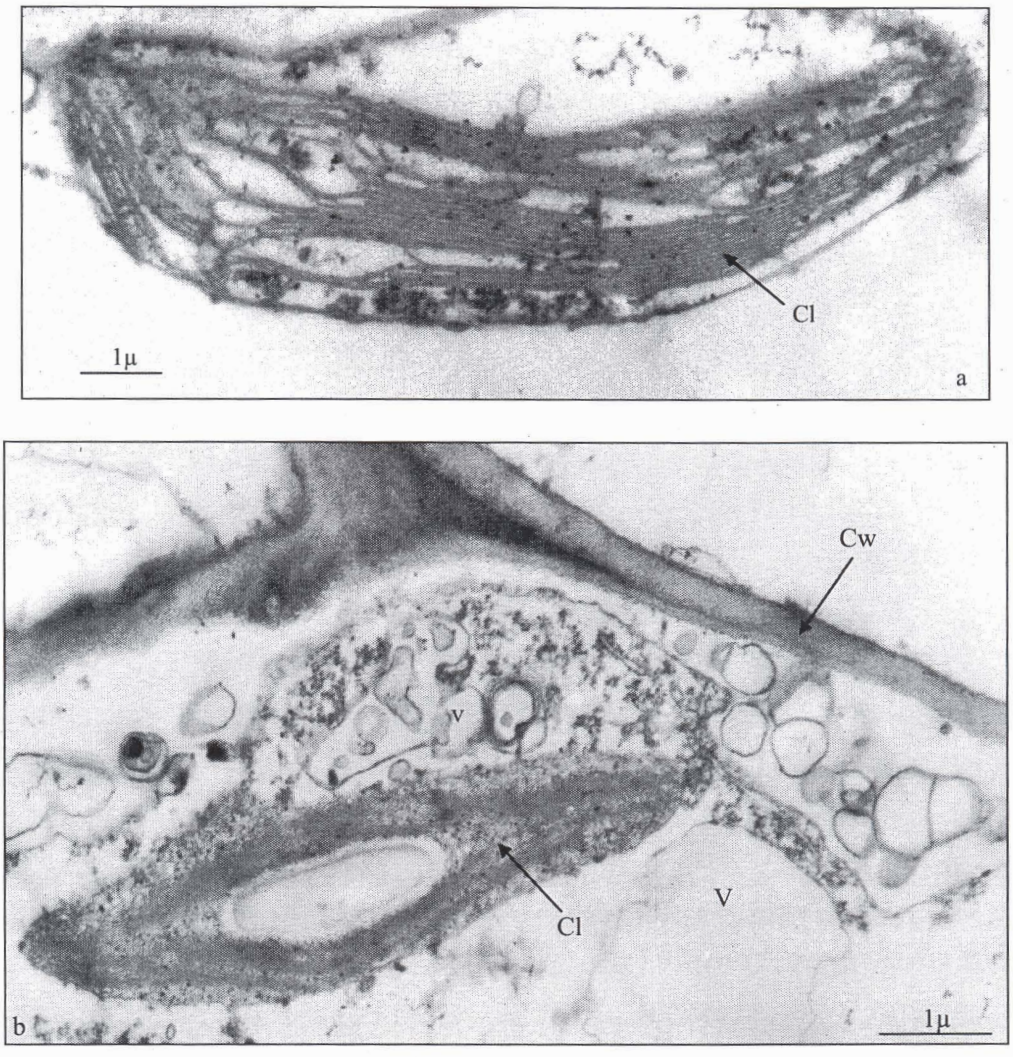


Fig. 7 a&b. Ultrastructural characteristics of the chloroplasts from leaf cells of *Marsilea quadrifolia* growing in *in vitro* culture. Cw-cell wall, Cl-chloroplast, V-vacuole, v-vesicles.

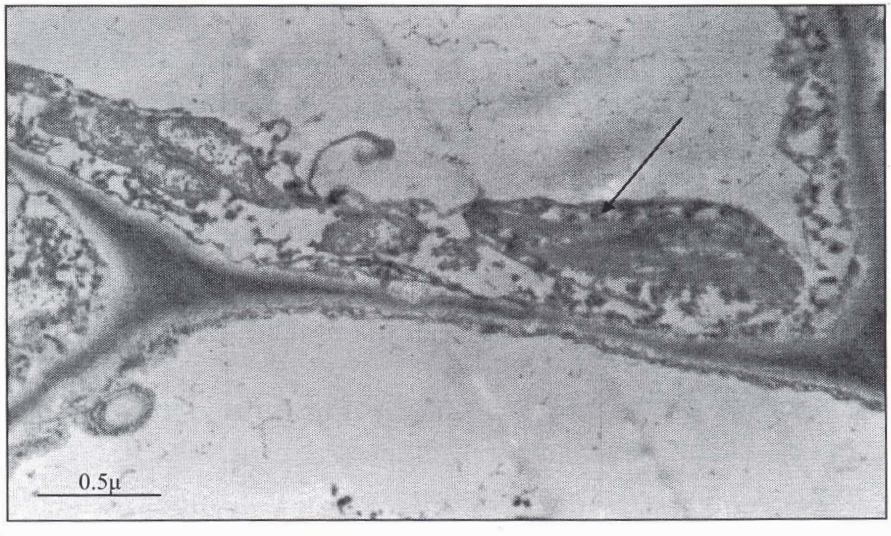


Fig. 8. Chloroplast with atypical structure. It presents a small number of large thylakoids grouped in the middle part of the organelles (see arrow).

Plastid polymorphism which is frequently present in *in vitro* cultures appeared like a complex phenomenon which could be determined by biotic and abiotic factors. In our experiment we consider these modifications the results of some adaptive processes determined by specific conditions of culture and development by *in vitro* systems. Typical mitochondria as well as Golgi bodies with a small number of cisterns and numerous vesicles as well as the profile of endoplasmic reticulum are present in some cells. This consists of long cisterns lying parallel to the cell wall (Fig. 9).

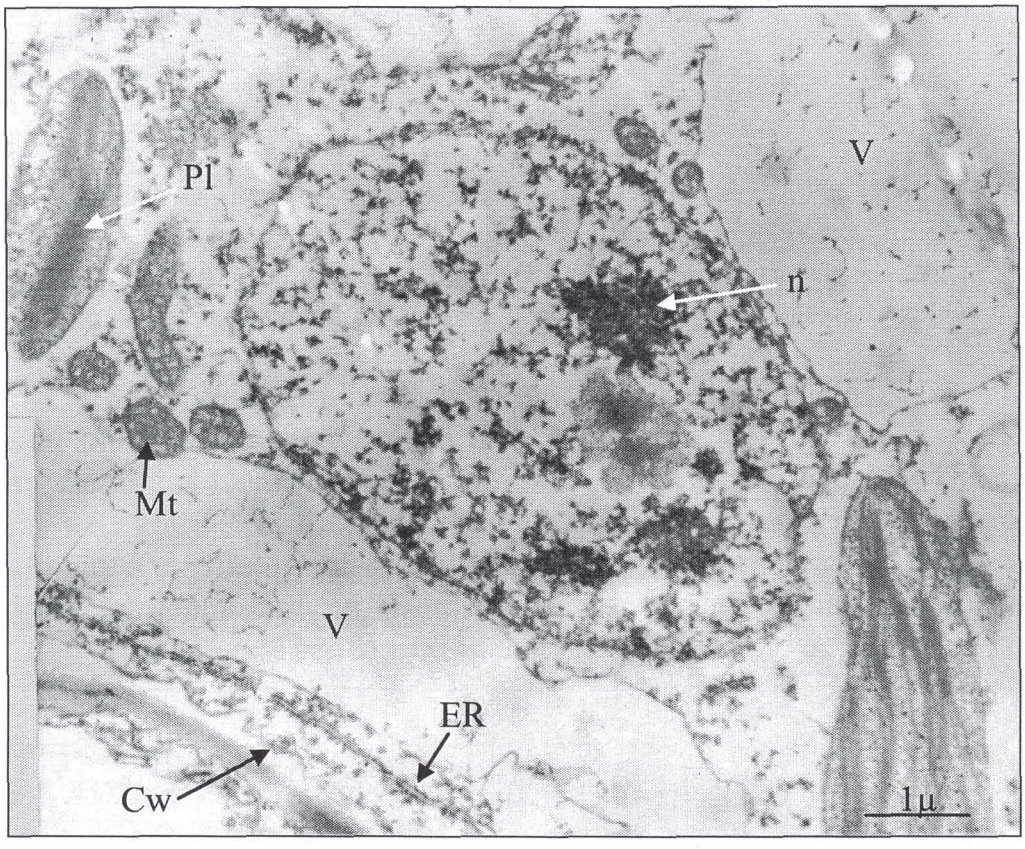


Fig. 9. Sector of the *Marsilea quadrifolia* leaf cell from growing in *in vitro* plants. N-nucleus, n-nucleolus, Pl-plastid with atypical structure (see arrow), Mt-mitochondrion, Cw-cell wall, V-vacuole, ER-endoplasmic reticulum.

Significant morphological modifications did not appear at nucleus level. It was oval in shape and in some cells the chromatic material was finely dispersed in the karyoplasms of some cells, but larger condensed chromatic masses were also frequent in many other cells. Two small nucleoli with characteristic granular fibrillar structure are revealed (Figs. 9-10).

In plant cells from *ex vitro* conditions and in the parietal cytoplasm numerous vesicles of different sizes as well as multivesicular bodies, paramural bodies (plasmalomasomes) are frequent (Figs. 7b and 12).

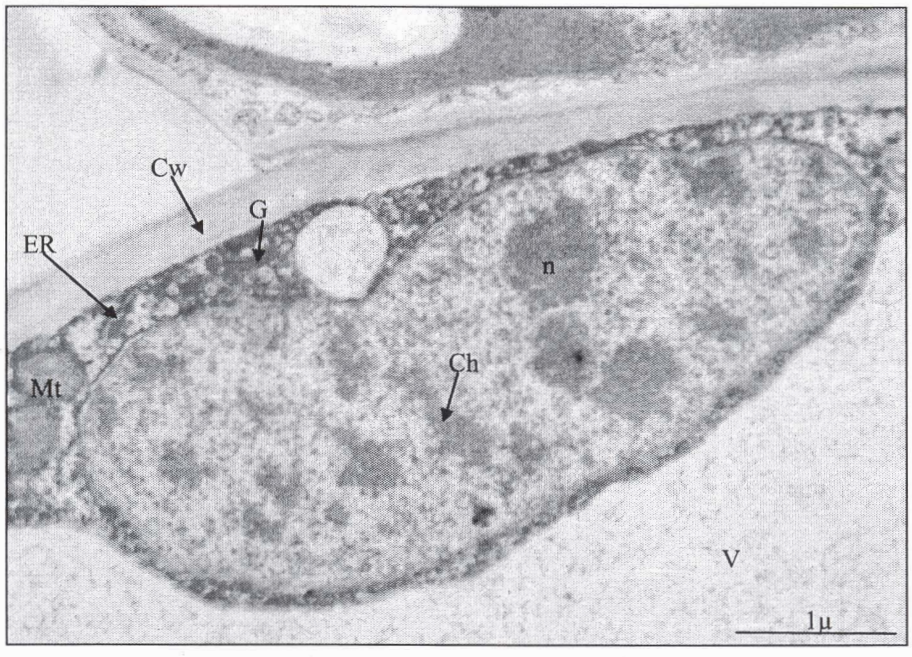


Fig. 10. The characteristics of the nucleus in *in vitro* conditions. ER-endoplasmic reticulum, G-Golgi bodies, Cw-cell wall, V-vacuome, n-nucleolus, Ch-chromatin.

Generally, plasmalomasomes permit a substantial increase of plasma membrane surface. These formations could be involved in intra and intercellular transport of substances and it is possible to represent a mechanism of "enzymatic stock" control during cell wall biosynthesis (Anghel *et al.* 1981). In some cells deposits of electron dense formations localized along tonoplast membrane are evident and demonstrated a possible biosynthesis process and translocation from dictyosomes through vesicles of some metabolites (Fig.11).

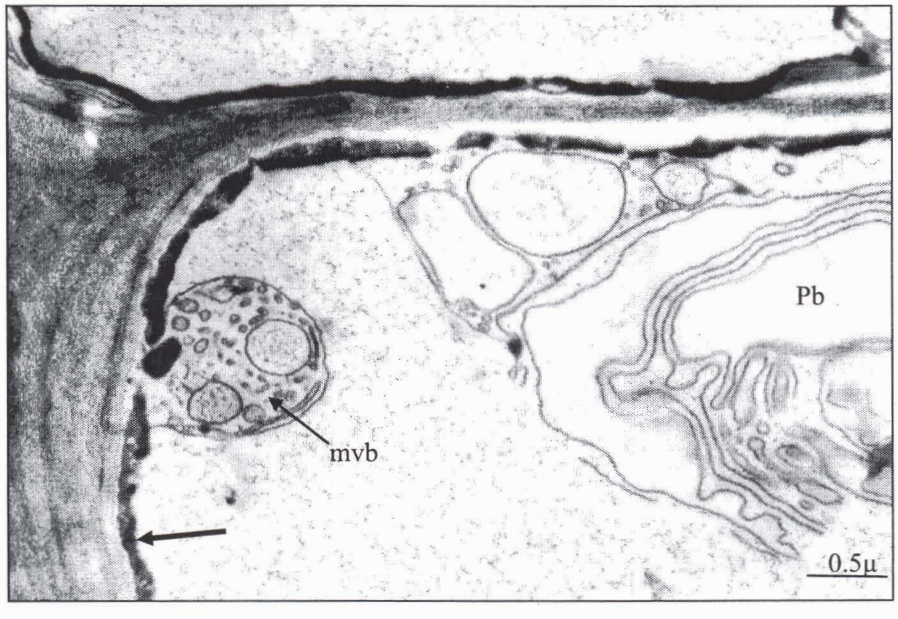


Fig. 11. Multivesicular bodies (mvb) and paramural bodies (Pb). Deposits of electron dense formations are located along tonoplast membrane (see arrow).

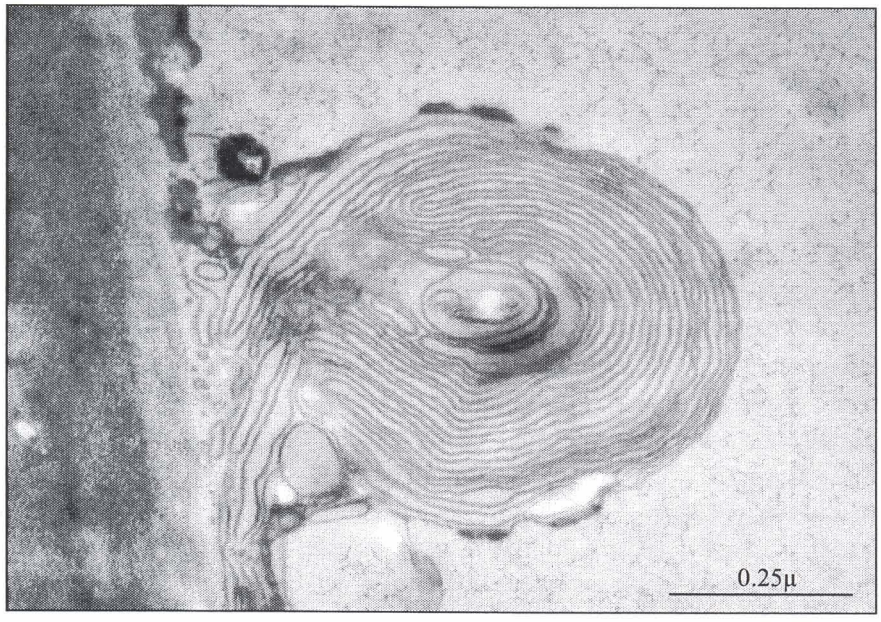


Fig. 12. Plasmalomasomes in a leaf cell of *Marsilea quadrifolia* L plants growing in *in vitro* conditions.

CONCLUSIONS

Our cytological observations permitted to conclude that the experimental protocol used by the researchers for *Marsilea quadrifolia* L. *in vitro* plant regeneration and multiplication did not affect the normal pattern of plant development and can be used as experimental system for *ex situ* conservation of this species.

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