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# *In vitro* propagation of the endangered species Marsilea quadrifolia L. - morphological and biochemical analysis of the regenerates

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## Abstract

*The present study is part of the current concerns with biodiversity conservation of endangered gymnosperm species from The Natural Park of Comana. Marsilea quadrifolia species is protected both at a national and European level (through the Habitats Directive of the EU and the 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 conserve and multiply or to exploit somaclonal variation induced by the in vitro technique identification of some biochemical or molecular markers for fast evaluation of regenerates is required. In this respect, genetic stability or variability of Marsilea quadrifolia regenerates obtained by the in vitro culture was assessed by electrophoretical methods to relieve izoenzymes activity as esterase, acid and alkaline phosphatase, glutamate-oxaloacetate transferase, malate dehydrogenase and peroxidase. In all enzymes systems studied a higher activity correlated with reactivity of this species to in vitro culture conditions was noticed. The expression of esterase, phosphatase, malate dehidrogenase display changes in correlation with growth condition, while the peroxidase pattern is more stable in natural population than in the in vitro regenerated plantlet.*

Keywords: micropropagation, electrophoresis, izoenzime, *Marsilea quadrifolia*

## Introduction

Climatic changes from the last decades, high pollution level of the soils and water and the degradation of the habitat are the main reasons for threatening many plant and animal species, considered now as rare, vulnerable and endangered. Many of them are close to extinction (DIHORU G., DIHORU A., 1993-1994 [3]; OLTEAN M. & al. 1994 [8]).

For these reasons finding efficient procedures for germplasm conservation is an important concern. From the many methods applied now to this purpose, the *in vitro* culture techniques are the largest used now. One of the many arguments to support these technologies on a large scale is the possibility of producing a great number of individuals starting from a small part of inocula, which can be reintroduced in the natural habitat or preserved in cells and tissues cultures banks for future fundamental studies and biotechnological applications.

In this context our research is based on studying the possibilities offered by the *in vitro* techniques for preservation of plant species from the area of The Natural Park of Comana (Giurgiu Department) located at only 30 km far from the capital Bucharest (BOSCAIU & al. 1994 [1]). This protected area is one of the least reserves in the plain region, remacable by high plant diversity, given by the interaction between Argeş and Neajlov rivers with the old part of the Vlasia forest (PAUCA-COMANESCU MIHAELA & al. 2000-2001 [9]).

*Marsilea quadrifolia* L. species taken in study is one of the threatened species that appears as protected both in the Red List and in the international documents such as Bern

Convention in the I$^{st}$ Annex or Habitats Directive of the European Union in the II$^{nd}$ Annex.

*Marsilea quadrifolia* L. species belongs to *Marsilea* Genus, *Marsileaceae* Family, *Hydropteridales Order*, *Filicopsida* Class (CIOCARLAN V., 2000 [2]). 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. From rhizome grows over the water level the aerial leaf with long petioles that resembles a four-leaf clover. At the base of the petiole grows 1-3 sporocarps of kidney shape on a short peduncle (branched or not) attached 1-12 mm above the petiole base.

In order to evaluate regenerants genetic stability or variability several biochemical markers were used. Comparative electrophoresis on samples from *in vitro* regenerated plants, acclimated plants and plants from natural habitat were performed. The electrophoretical analyses provide an indirect method for genome probing by exposing structural variation in enzyme or other protein genome (SAMMOUR, 1991 [12]). The biochemical methods have some disadvantages being profoundly influenced by tissue specificity and developmental stage (SCANADALIOS AND SORENSEN, 1997 [11]). 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.

## Materials and Methods

Samples of *Marsilea quadrifolia* L. plants have been collected from the swampy area near the Comana village from which have been taken explants in order to be inoculated for *in vitro* propagation (TARNAVSCHI I.T, 1974 [10]).

Nodal areas of rhizomes at the branching of the leaves and sporocarps have been used as explants source in order to initiate the cultures (Fig.1). Before inoculation the explants were superficially sterilized as well as the fragments of petiole and leaves using ethylic alcohol (70%) for 30 seconds followed by Domestos (5%) for 7 minutes and rising with sterile distilled water several times. These were inoculated on different culture media (Table 1).

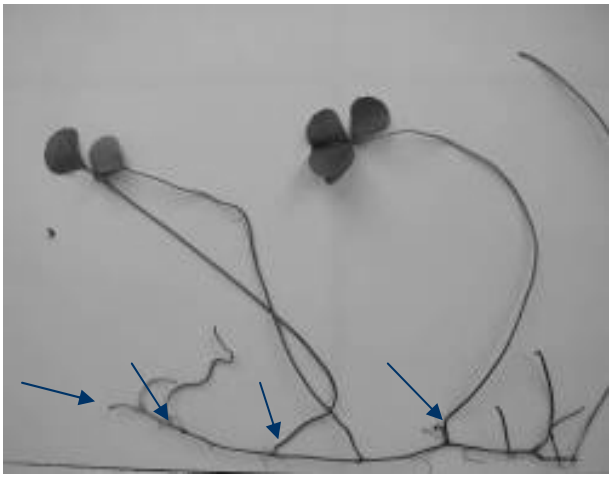

**Figure 1.** The fragment of the *Marsilea quadrifolia* plant from natural habitat used as explant sources. Arrows indicates rhizome areas used as inoculums.

For *in vitro* culture of the explants various media having multiple characteristics both rich in mineral salts such as the MS (MURASHIGE & SKOOG, 1962 [7]) or poor in minerals (Knopp) have been used. Cultures were kept in a growth chamber at a temperature of 24±2°C with a 16h photoperiod illumination with fluorescent tubes at 3000 lux intensity of light.

The reactivity of explants was evaluated after a week from inoculation and after a month of culture. The plantlet obtained were rooted and then acclimated in order to be readapted to their natural environment.

Biochemical analyses followed the determination of the izoenzime pattern (POX, EST, ACP, AKP, MDH and GOT) variations in natural population and in vitro regenerates. Also, it was monitorized appearance of differences between regenerates on different culture media or in acclimatization conditions.

The extraction of soluble cytosolic proteins was performed by plantlet grinding in phosphate buffer 0.1 M, pH 7 at $4^0$C. After centrifugation at 15000 rpm for 10 min, the supernatant was used for electrophoretic analysis.

The electrophoretic analyses were carry out by the samples migration at 20mA, 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. Samples were loaded into each well 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 0.5M, 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 0.1M 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' at $4^0$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 transferase the mixture reaction consisted in α-ketoglutaric acid, apartate acid, $EDTANa_2$, PVP, Fast Blue BB in phosphate buffer, pH 7.4.

Malate dehydrogenases were relieved using malic acid and NAD as substrates, NBT, PMS and MTT for tetrazolium stain in 0.2 M Tris-HCl buffer.

## Results and Discussions

### 1. Some morphological aspects of *Marsilea quadrifolia* L. *in vitro* regenerated

The reactivity of different types of explants on various culture media was tested in order to establish experimental protocol for *in vitro* multiplication and conservation of this species.

**Table 1.** The *in vitro* reactivity of *Marsilea quadrifolia* species considering the explant type, growing factors and their concentration in culture media

| Explant type | MS without hormones | MS at half salts level and 4 g/l agar | MS + 0.57 μM IAA | MS + 0.53 μM NAA | Knopp liquid | Knopp with 4 g/l agar | Knopp with 8 g/l agar |
|---|---|---|---|---|---|---|---|
| Nodal part of rhizom | - | ++ | - | - | ± | ++ | + |
| Sporocarps | - | - | - | - | - | - | - |
| Spores | - | - | - | - | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Petiol fragments | - | - | - | - | - | - | - |

Legend:
- \- no regenerative response;
- ± the explants survived but without regeneration processes;
- \+ poor regeneration rate;
- ++ high regeneration rate.

The studies on different fern species revealed the positive effects of the poor mineral salts culture media on micro multiplication possible due to similar consistence of natural habitat (LAETSCH WM, BRIGGS WR. 1961 [4]). Therefore the successful results were obtained on *Marsilea quadrifolia* using Knopp medium (BAI-LING LIN, WEN-JEN YANG, 1999 [5]). However, several reports mentioned aseptic culture established from sporocarps, in our experimental data only nodal parts of rhizome with preformed axillary meristems proved regenerative capacities (LIU B-LL, 1984 [6]). The rhizome fragments elongated and in the nodal areas roots and new leaves developed (Figure 2). In our experimental conditions the *in vitro* multiplication was realized by direct adventitious morphogenesis. Comparative analyses of the plant reactivity to *in vitro* conditions revealed the semi liquid culture medium (4g/L agar) was preferred and media as basal Knopp and MS with half mineral salts level, represented optimal conditions. The acclimatization at the *ex vitro* conditions was performed on sterile aquatic soil taken from the natural habitat in four weeks from inoculation. The new parts of plant morphological identically with the native plant are formed as individual clonal structures. Thus we would suppose that plants can be identically with native plant and from genetic point of view too. In these frame biochemical methods for establish of eventually variations induced by in vitro culture conditions have been used, by monitorized the activity of some izoenzime systems as EST, GOT, POX, MDH, ACP and AKP.

**Figure 2.** *In vitro* multiplication of *Marsilea quadrifolia*

## 2. Biochemical characterization of *Marsilea quadrifolia* regenerants

For somaclonal variation the individual izoenzime pattern of regenerated plants was compared (ex. group III). The grade of heterozygoty in natural population was estimated by distinction in izoenzime pattern between individuals (ex. group II). The evaluation of the effects *in vitro* culture condition on zymogram was achieved by similarly and dissimilarly presents between *in vitro* regenerated plants, acclimated plants and explants of native plants.

Biochemical aspects of the regenerates have been analyzed by each type of enzyme tested, comparing similar bands from successive samples. Each izoenzime has a specific sensibility to *in vitro* culture factors respond in a specific way.

The **esterases (EST)** patterns of regenerated plants are relatively similar with differences only in intensity of bands. Comparing with originate plants the regenerated ones express new isoforms in fast migrating zone (L4 and L5 loci). The absence of bands in fast migration zone of primary plant shows that these isoforms are expressed in intensively growth phases (acclimatization and regeneration). Thus the isoenzymes from L4 and L5 loci cannot be sensitive markers for estimation somaclonal variation.

Concerning the second group of samples from the natural habitat, the bands in the fast migration zone have also lower activity. Variations in L4 locus indicate presence of heterozygote in originate population.

Group I samples corresponding to acclimated plants, from the *in vitro* culture, shows higher enzyme activity of all expressed izoesterases. Probably these plants have an active metabolism of hydrolases, in connection with adaptation to natural substrates. The *in vitro* regenerated and acclimated plants present a higher intensity of the bands in slow migration area unlike those from the natural habitat.

Therefore, the three groups of samples used in esterases analysis showed differences in intensity or band position, proving that the growing conditions influence plant metabolism.

The **peroxidase**s **(POX)** expression is similar for all tested plants. It is known that POX pattern is very sensitive to medium conditions. Apparently, the stress induced by *in vitro* culture not affected this izoenzymes expression. In this case, differences in POX pattern can be considered as marker for somaclonal variation due to a wide range of izoforms and the stability of expression.

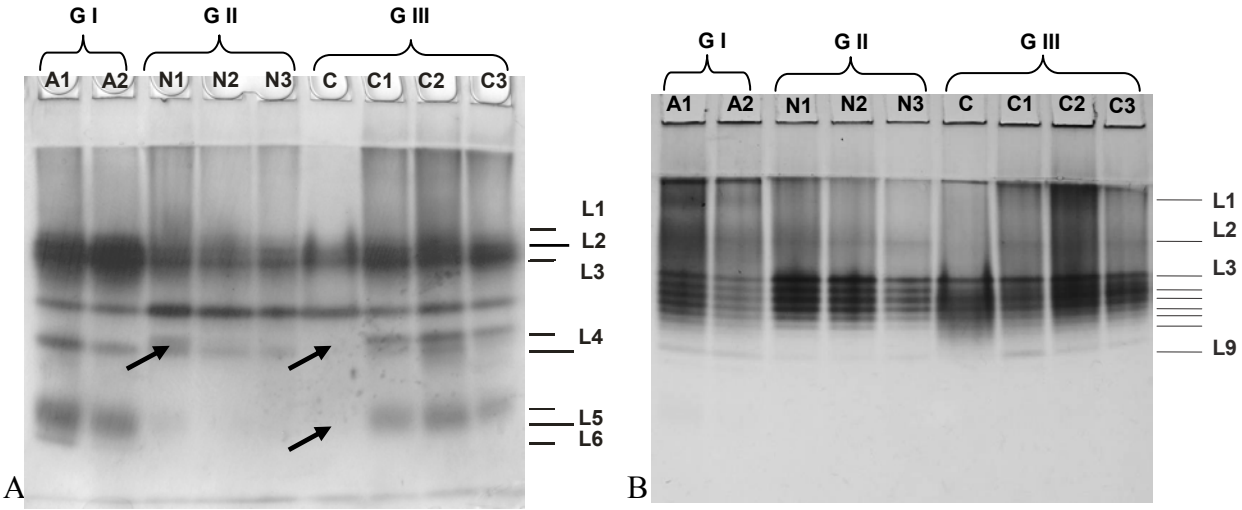


**Figure 3.** The electrophoretic spectra of the EST (A) and POX (B) izoenzymes in G I: plants acclimated to soil; G II: plants from the natural habitat; G III: *in vitro* regenerated plants: C- primary plant; C1-Cn- regenerated plants

The **phosphatases** patterns are similar with the observation that the band with **AKP** activity from fast migration zone are more intense in plants proceed from *in vitro* cultures. Also this observation shows a more active metabolism of hydrolases in artificial growth conditions.

The expression of **ACP** is similar with situation describes for AKP. The bands corresponding L2, L3 and L4 loci is more intense in GI and GIII group, which consists in probes from *in vitro* cultures.

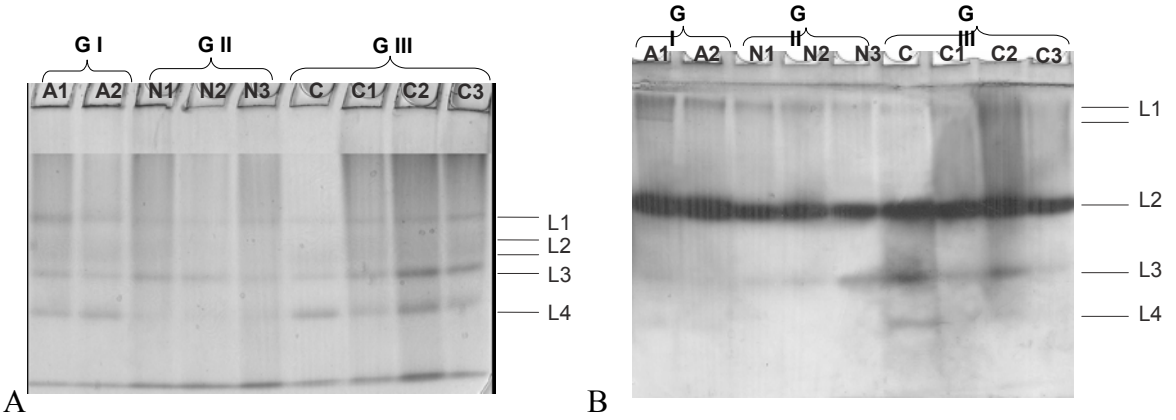


**Figure 4.** The electrophoretic spectra of alkaline (A) and acid (B) phosphatases in G I: plants acclimated to soil; G II: plants from the natural habitat; G III: *in vitro* regenerated plants: C- primary plant; C1-Cn- regenerated plants

The only one locus for GOT was detected on gels with low activity in *ex vitro* samples and more intensity in the acclimated plants. As opposed to GOT, the MDH pattern present more variation in izoenzyme expression. L3 locus show presence of heterozygote in acclimated plants, while L4 locus not discriminate between *in vitro* regenerated plants. Also, the expression of MDH izoenzymes is more decrease in natural medium.

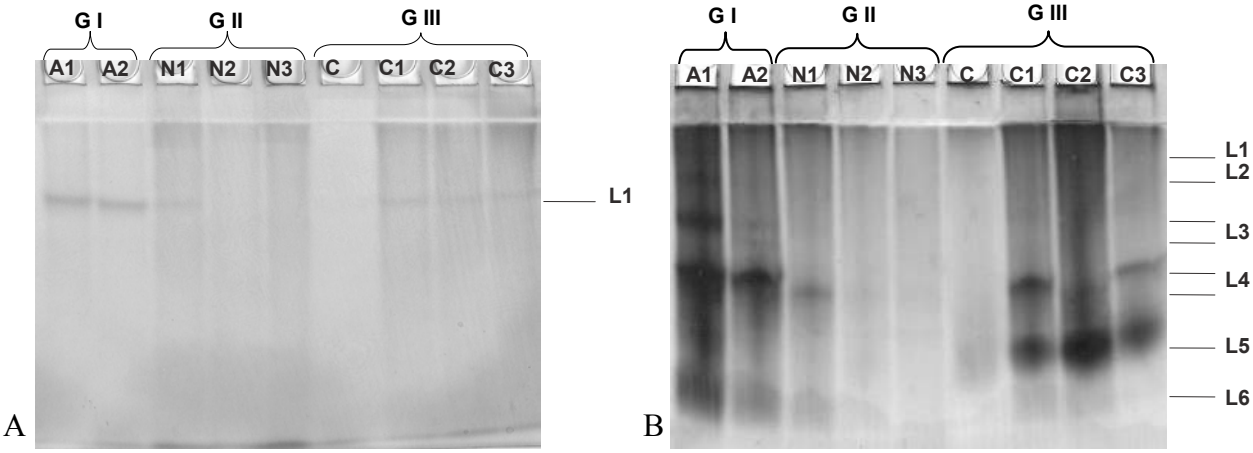


**Figure 5**. The electrophoretic spectra of GOT (A) and MDH (B) in G I: plants acclimated to soil; G II: plants from the natural habitat; G III: *in vitro* regenerated plants: C- primary plant; C1-Cn- regenerated plants.

## Conclusions

- *Marsilea quadrifolia* L. is a reactive species for *in vitro* cultures. Explants of rhizome represent optimal source of inoculums.In our experiments sporocarps did not germinate;
- The plant prefers semiliquid culture media, poor in mineral salts (such as Knopp and ½ MS) without hormons;
- Comparative analysis of the izoenzimatic spectra proved that *in vitro* culture regenerated plants present a more intense catalytic activity than those of native plants.
- The differences at the level of the isozymes from the 6 types of studied enzymes show that certain loci can be used as markers which relieved intrapopulation heterozygoty or somaclonal variation in plant obtained by *in vitro* system.

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