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In Vitro Development of Gametophyte and Sporophyte in Several Fern Species

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Abstract

The *in vitro* differentiation of the gametophyte and sporophyte of *Pteridophyta*, conducted through the culture, on nutritive media, of the green, incompletely differentiated sporangia and spores, exhibits a variety of peculiarities. Whereas in the case of the spore culture, a single prothallus is usually obtained out of the spore, in the case of the green sporangia (sori) culture, a group (colony) is obtained out of a single explant, a group which is made up of numerous prothalli. The agarized medium ensures a much better physical support for forming larger colonies. The secondary gametophytes can be formed through: the branching out of the prothallic filaments (each ramification representing the intial stage for the development of a new prothallus); the elongation of the cells in the apical area of the gametophyte, (which represent the initial cells of the new prothalli); out of the cells located in the base area of the prothalli, (cells which also function as prothalian initial); out of ramifications of the prothalli. The formation of the sporophyte may be the result of amphimixis, e.g. in *Cystopteris fragilis*, or of apomixis (apogamy) in *Cyrtomium falcatum* and *Phegopteris connectilis*. In the amphimixic species, the embryo is formed after three months from the culture initiation. What is characteristic of the apogamous species is the presence of the tracheides in the gametophyte, and the absence of the root in the first stages of development. The culture of green sporangia on agarized media is recommended, due to the fact that a very large number of gametophytes, and subsequently little plants, are formed.

Keywords: *Pteridophyta*, in vitro, gametophyte, sporophyte, secondary gametophytes, amphimixis, apomixis

Introduction

Characterized by an alternance of generations between a well developed sporophyte, producing spores, and an independent gametophyte, producing gametes, the *Pteridophyta* include over 12.000 species, many of which are ornamental species, medicinal species, species of an ethnobotanical importance, with a role in habitat conservation, etc. The oldest method of researching the morphogenesis of the gametophyte and the sporophyte, namely the culture of pteridospores, is hampered by their low viability, the delicate nature of the gametophyte resulting from the germination of the spores, by numerous factors that influence the germination of the spores, the differentiation of the gametophyte, and implicitly the embryogenesis and the formation of a new plant, among which: the density of the spores cultivated on the medium, the degree of ploidy of the plant, the method of preserving the spores, the temperature necessary for germination, the light, the nourishment conditions, the antheridiogenes, etc.

The culture of tissues in ferns has been utilized as a research instrument for the study of the developing potentialities of the leaf primordia ever since the early 1960's (Torres, 1988). The first successes in the field of the intensive multiplication of plants through *in vitro* techniques are cited around 1970, the fern *Nephrolepis exaltata bostoniensis* being the first plant micropropagated *in vitro* with a commercial purpose (Cachita-Cosma, 1987). According to Pierik (1991), 157 million plants, i.e. 74% out of the total production of micropropagated plants, have been ornamental species. Out of these, approximately 40 million plants have been pot plants. Top of the list, with 17.8 million plants, is the fern *Nephrolepis* (Fernández and Revilla, 2003).

At the present time, a high number of individual researches are oriented towards the propagation of pteridophytes, with a view to conserving their diversity.

Materials and methods

The culture of the spores in Cystopteris fragilis and Phegopteris connectilis

Using the methods of the *in vitro* culture, the culture of pteridospores allows for obtaining a population of spores that are uncontaminated by the spores of other species, as well as the bacterial infection, the infection with fungi, algae and mosses, all of which represent a major problem in the natural environment (Deberg, 1994).

Sterilization of spores

This can be done at the same time as the sterilization of the trophosporophylla (Hedge, 1998), either after col-

lecting them and subsequently sterilizing them in packets of filtering paper (Pierick et. al., 1986), or through suspending them in a sterilizing solution, followed by collecting them by means of centrifugation in distilled water (Fernández et. al., 1993). In the case of he sterilization of spores at the same time as the trophosporophylla, the latter are fragmented, hydrated, immersed in 1' ethylic alcohol 80^0, an operation that is followed by washing in sterile distilled water. The leaf fragments are then sterilized with a solution of calcium hypoclorite 6%, prepared before utilization, or with hydrogen peroxide 10% (Fay, 1994; Fernández and Revilla, 2003). The time of exposure to the sterilizing agent ranges between 3' and 12', and is shorter in the case of the material coming from greenhouses, which has a smaller load of germs, and longer in the case of the material coming from the natural environment. In order to diminish the superficial tension, a few drops of Tween 20 are added into the sterilizing agent. After sterilization, the vegetable material washed three times with sterile distilled water is removed into sterile Petri plates, where they are kept till the moment of utilization. To collect the spores, the leaf fragments are left in the Petri plates unil these are delivered out of the sporanges.

Inoculation of spores on the culture medium

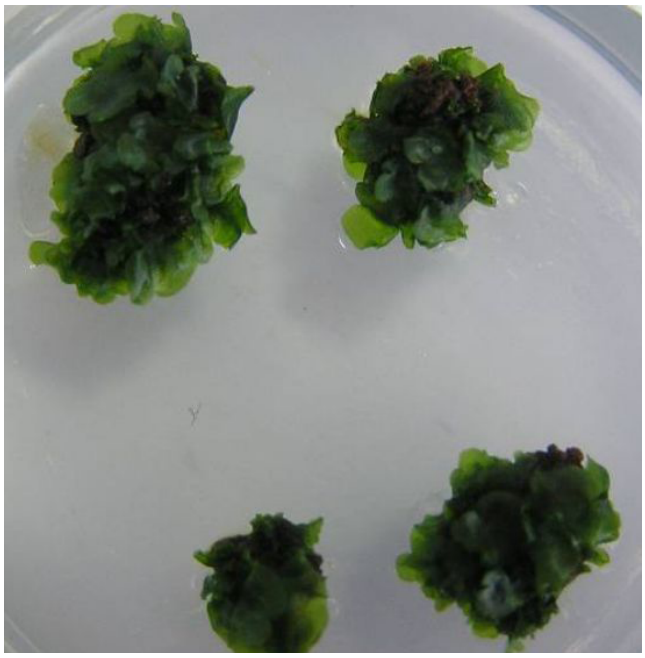
The spores collected were cultivated on the liquid nutritional medium Knop (1865) (Vlădescu, 1934; Fernández and Revilla, 2003) distributed and removed into sterile Petri plates. To diminish evaporation of the water in the nutritional medium, the Petri plates were wrapped in transparent cling film. The culture containers were placed in the growing room, and the culture conditions are as indicated in the speciality papers, namely 25 ± 1^0C, the photoperiod being 16 hours of lights, and 8 hours o darkness (Bertrand et al., 1999; Fernández et al., 1999 etc.).

The culture of the green, incompletely differentiated sporangia, in Asplenium ruta-muraria, Cyrtomium falcatum, Dryopteris dilatata, Osmunda regalis, Phegopteris connectilis, Polypodium vulgare

Out of the numerous type of explants that can be used for the *in vitro* culture in ferns, the green, incompletely differentiated sporangia were utilized to initiate the *in vitro* culture in few species, namely *Dicranopteris linearis* and *Platycerium coronarium* (Henson, 1979), although their use has several advantages, which have already been presented (Soare et. al., 2007).

The sterilization of the green, incompletely differentiated sporangia is conducted very much as in the case of the sterilization of the spores, once with the sterilization of the trophosporophylle.

The inoculation of the green, incompletely differentiated sporangia on the culture medium. This type of explant was cultivated in vitro on the solid nutritional medium 0.5xMS (1962) with no hormones (Henson, 1979; Materi and Cumming, 1991; Fernández et al., 1997). For the species Cyrtomium falcatum and Asplenium ruta-muraria, variants of medium 0.5xMS (1962) were prepared, to which 2 mg% 2.4-D were added to get callus, and afterwards embryos. The pH of the medium was adjusted to 5.6 before the autoclaving (Henson, 1979, Khare et al., 2005), and the sampling of sporangia from the leaf fragments was done by means of a lanceolate needle. The conditions in



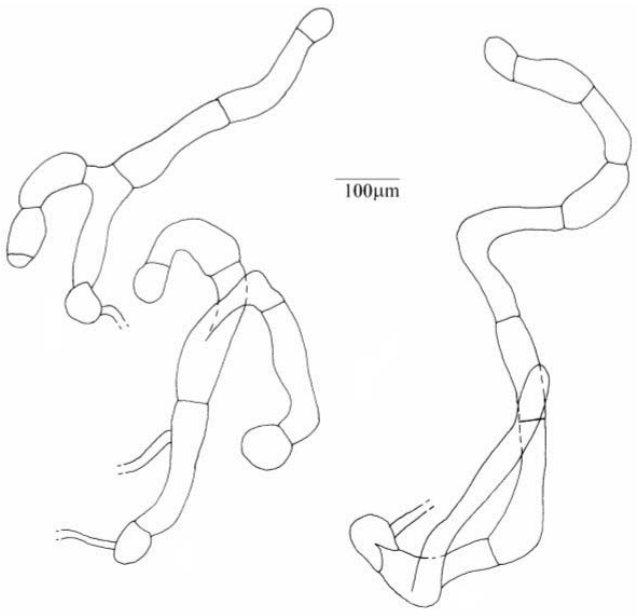


Figure 1 *Osmunda regalis L. – colonies of prothalli obtained from green sporangia (orig)*

Phegopteris connectilis (Michx) Watt – branched out prothallic filaments, obtained from the culture of the spores

the growing chamber are identical to those in the spore culture.

Results and discussion

Differentiation of the gametophyte

In the case of both types of cultures, the differentiation of the gametophyte goes through the characteristic stages, i.e. prothallic filament, prothallic lamella (plate), and cordate prothallus. While in the spore culture, out of a spore a single prothallus is usually obtained, in the green sporangium culture, out of a single explant a whole cluster (colony) is obtained, which is composed of numerous protalli. Although for both types of cultures there occurs the branching out of the prothallic filaments, each ramification representing the intial stage of the formation of a secondary gametophyte, the agarized 0.5xMS medium ensures a much better physical support for the development of larger colonies (Figure 1).

The secondary gametophytes can be formed:

1. through the branching out of the prothallic filaments, each ramification representing the initial stage of devloment of a new prothallus, as for instance in Phegopteris connectilis (Figure1, 5, 7), *Cyrtomium falcatum* (Soare et al., 2005);

2. the elongation of the cells in the apical area of the gametophyte, which represent the initial cells of the new prothalli in *Cyrtomium falcatum* (Figure 2), in the case of the variant with the 2.4-D medium;

3. out of the cells to be found in the base area of the prothalli, cells that also function as prothallic initials in *Dryopteris dilatata* (Figure 2j),

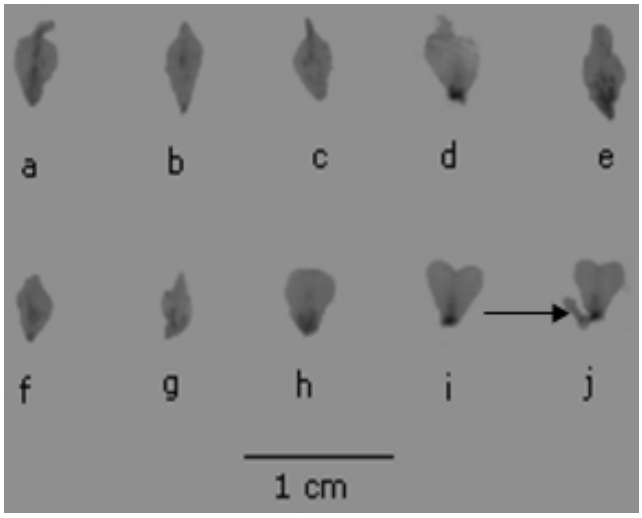


Figure 2 *Dryopteris dilatata* (Hoffm.) A. Gray – various forms of the prothalli: a-g elongated prothalli, h-j cordated prothalli, the last one having a secondary prothallus (the arrow) attached to it, resulted out of a ramification (orig.)

4. out of the ramifications of he prothalli in *Polypodium vulgare* (Figure 3).

Due to this process of vegetative reproduction of the gametophyte, frequently noticed *in vitro*, a large amount of material results, a fact that is also reported by Fernández et al. (1999). The excellent capacity of *in vitro* multiplication of the gametophyte can however influence the number of the sporophytes formed, as was noticed in *Asplenium*, *Dryopteris*, *Osmunda*, etc., as the absence of the sporophyte presupposes a nutritional competition between the two generations. This presupposition is supported by the fact that, when the multiplication of the gametophyte is blocked in *Osmunda*, the production of sporophytes increases. (Fernández et al., 1999).

The differentiation of the sporophyte

The formation of the sporophyte can take place through amphimixis or apogamy. The production of the sporophyte in a sexual way implies the fusion of the gametes formed on either the same prothallus, or different prothalli (Klekowski and Lloyd, 1968); he presence of water is necessary for the displacement of the male gametes. The antheridia are differentiated before the archegonia, thus increasing the chances of fecundation between the antherozoids and the oospheres on different prothalli, and so maintainig a high level of the of the potential of genetic variability. This in fact gives the possibility for the fecundation of the oospheres with the antherozoids of other species within the same genus, and the result is the formation of a hybrid. As a matter of fact, recent studies conducted on the cromosomes of several genera of ferns have shown that the history of the fern evolution is an intricate network of hybridizations (Camus et al., 1992). Even in the case when the oosphere and the antherozoids are formed on the same gametophyte, the zygote is frequently formed through the merging of the gametes generated by different gametophytes. The time difference between the formation of the reproductive male and female structures is often enhanced by the antheridiogenes released by the gametophyes that are in the female stage, which induce the gametophytes nearby to remain in the male stage (Holsinger, 2000). The experiments of growth with isolated prothalli and pairs of prothalli have shown that, on average, 56% of the isolated prothalli are successful in forming the sporophyte and the percentage is much larger in the case of the

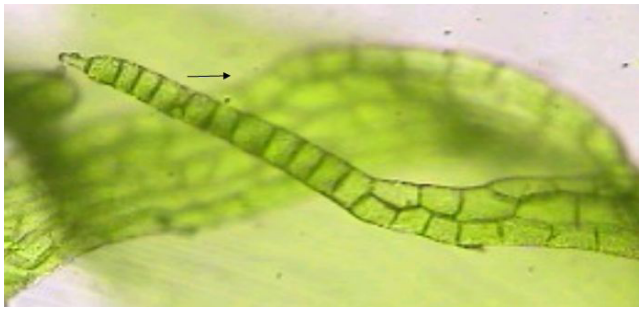


Figure 3 *Polypodium vulgare* L. – ramification of the prothallus (arrow) (oc. 10x, ob. 10)(orig.)

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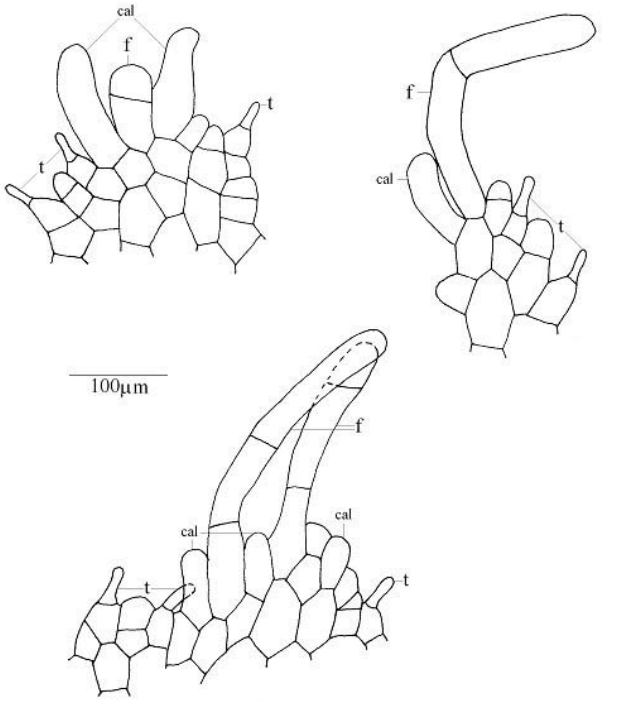

Figure 4 *Cyrtomium falcatum* (L. fil.) C. Presl – lamellar prothalli with elongated cells (cal) and filaments (f) in the apical area, obtained through the culture of green sporangia on the medium with 2.4-D; t–trichome (orig.)

pairs of prothalli, which indicates the fact that a crossed fecundation, between the gametes on different prothalli, is produced (Suter et al., 2000).

In the species cultivated on the liquid medium Knop (1865), such as *Cystopteris fragilis*, which differentiates, on the prothalli, both the male gametangia, namely the antheridia, where the male gametes are formed (Figure 5a), and the female gametangia, namely the archegonia (Figure 5b), where the female gamete is formed, fecundation occurs and the zygote is formed, after approximately three months from the initiation of the culture.

The embryo formed out of the zygote will leave the archegonial envelope, and its first leaf can be noticed (Figure 6 a, b), a moment when the young sporophyte becomes able to feed by itself (Sakamaki and Ino, 1999). The embryo stem displays a characteristic initial cell (Figure 6 c, d), and the stem apex is responsible for generating the plant's growing axis, as the embryo of ferns has a one-pole structure (Groff and Kaplan, 1988).

Approximately 10% of the ferns proper and an unknown proportion of other pteridophytes have a life cycle of an apogamous type (Sheffield and Bell, 1987), as their sporophyte is formed out of common gametophyte cells. The lack of water on the prothalli represents the reason why the sporophyte is formed in a sexuated way, because the antherozoids cannot move towards the archegons to reach the oosphere, and obligatory apogamy is an adaptation to the xerophytic habitats (Wagner and Smith, 1993; Fernández şi Revilla, 2003).

As Duncan (1941) stated, in the case of certain species, the embryo is formed apogamously out of necessity, with no archegons, as for instance in *Cyrtomium falcatum,* or they can be present, as for instance in *Phegopteris connectilis*, although the antheridia are generally present and produce viable a antherozoids. In such cases, the last premeiotic division of the sporophyte is not complete, so that restitution nuclei with a twofold number of cromosomes are formed. These cells, namely the mother-cells of the spores, divide meiotically, resulting spores which have the same umber of cromosomes as in the cells of the sporophyte. This type of non-reductional (somatic) apogamy was discovered as early as 1874 (BAra and GhiorghiTA, 1980).

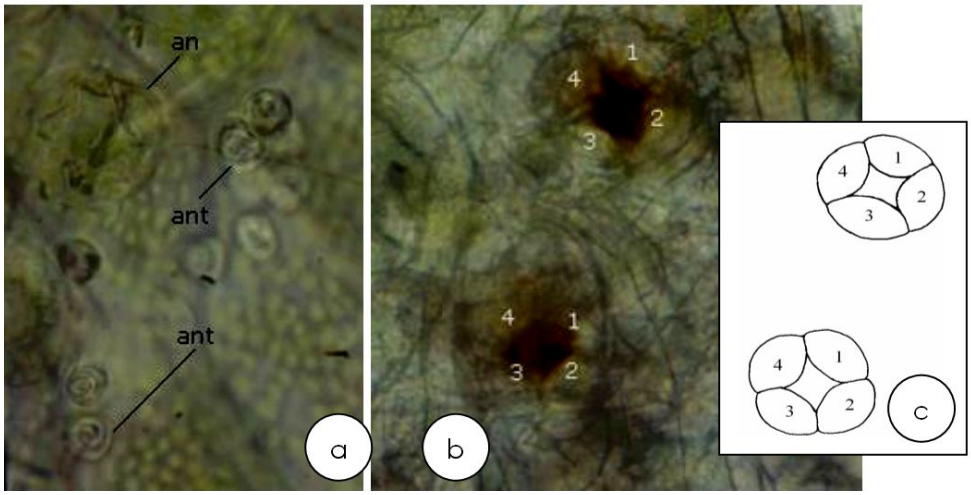

Figure 5 *Cystopteris fragilis* – a: antheridium (an) out of which the anterozoids (ant) were released onto the surface of the prothallus (oc. 16x, ob. 63) (orig.); b, c: the ventral part of the open archegonia, seen apically; 1-4 the four rows of cells in the neck (oc. 16x, ob. 40) (orig.).

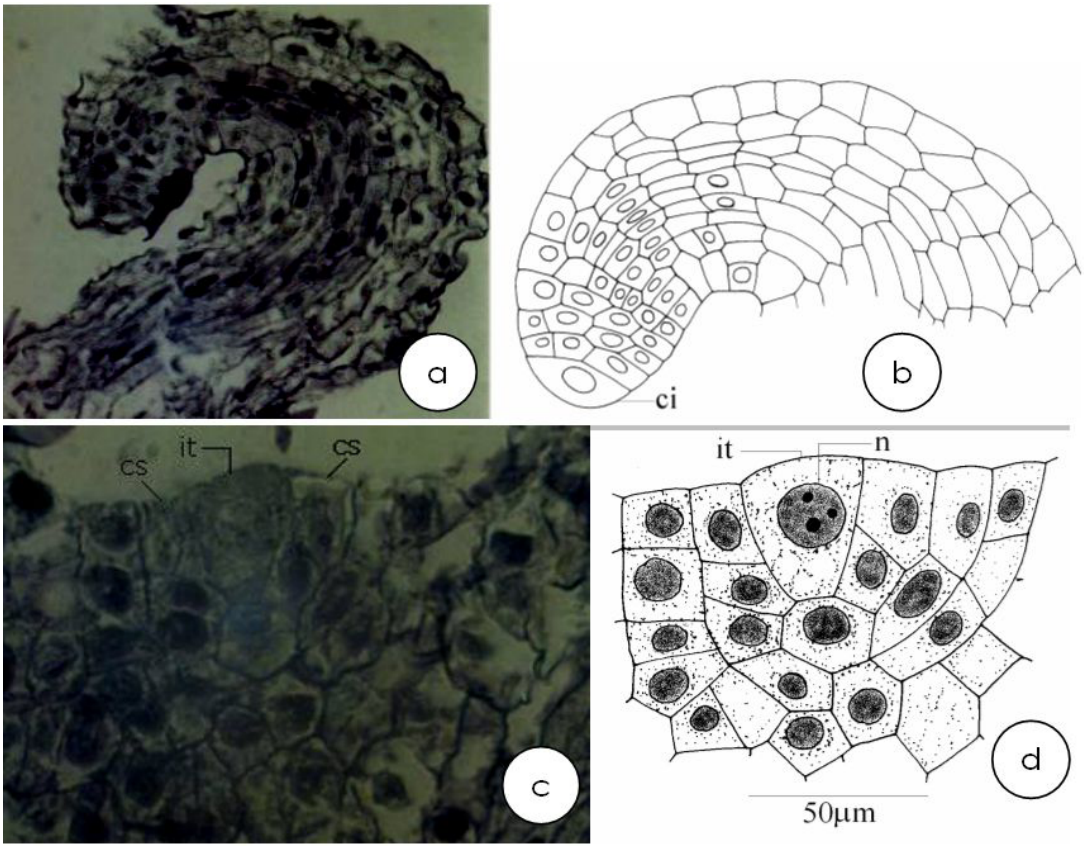

Figure 6 *Cystopteris fragilis* – a, b: the first leaf of the embryo, seen in a longitudinal-median cross-section (the embryo has left the archegonial envelope (oc.12,5 x, ob. 10)(orig.), it – the initial cell of the stem; c, d: the initial of the stem (it) seen in a longitudial-median section, through an embryo that has broken through the archegonial envelope: cs-segment cells, n-the nucleus of the initial cell (oc. 12,5x, ob. 25) (orig)

The characteristic thing for the apogamous pteridophytes is the differentiation of the tracheides in the gametophyte, as in the case of a number of pteridophytes with subterranean gametophytes, as for istance *Psilotum nudum* (Bierhorst, 1971), a character that is considered as primitive. Another characteristic trait is the absence of the root until the stage where the little plant has 2-3 leaves (Soare and Neagu, 2003), which contradicts (disproves the theory of the phyllorize, supported by Chauveaud (1911, 1921), Vladescu (1934), while confirming the fact that the fern embryo does not have a two-pole structure, as in the spermatophytes, but a one-pole (Groff and Kplan, 1988), with the first root being an endogenous, laterally cauligenous formation (Ehrendorfer, 1999). Since the apogamous sporophytes obtained in *Cyrtomium falcatum* and *Phegopteris connectilis* does not display a root in the one-leaf stage, the tracheides differentiated in the prothallus have the role of conducing to the first leaf the substances needed for photosynthesis, in view of the well-known fact that, after they exhibit the sporophyte, it is capable of achieving photosynthesis (Sakamaki and Ino, 1999), so that the sporophyte of the pteridophytes is no longer a parasitic sporophyte like that of the bryophytes (Kenrick, 2000).

Adding 2.4-D to the culture medium in *Asplenium ruta-muraria* and *Cyrtomium falcatum* does not determine the formation of callus, but only the elongation of the cells in the apical area of the gametophyte. This can be accounted for through the presence in the spores of the abscisic acid (ABA), which has an antiauxinic action.

Taking into account the fact that in the case of the *in vitro* culture a large number of gametophytes are formed, on which an apogamous is differentiated, out of a small

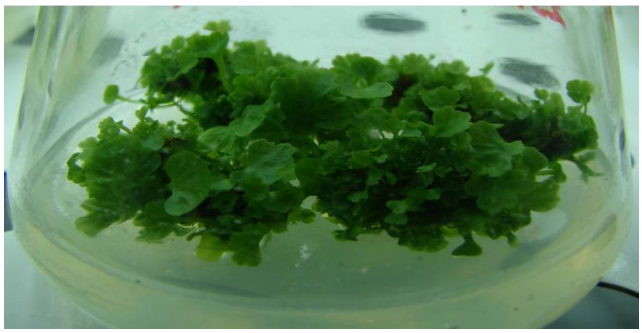

Figure 7 *Cyrtomium falcatum* (L. fil.) C. Presl – apogamous sporophytes (orig.)

number of explants a large number of little plants are obtained (Figure 7).

Conclusions

The *in vitro* culture in *Pteridophyta* is currently used for the multiplication of the ornamental and medicinal species, but also for the endangered ones, with a view to preserving them *ex situ*. In the case of the endangered species, establishing the experimental protocol is recommended in related species. While, in the spore culture, only one prothallus is, as a rule, obtained from a single spore, in the case of the green sporangia culture, from a single explant a colony is obtained, formed of numerous prothalli, on account of the fact that, in various manners, the secondary gametophytes are formed. The excellent *in vitro* multiplication capacity of the gametophyte can however influence the formation of the sporophyte as was noticed in *Asplenium ruta-muraria*, *Dryopteris dilatata*, *Osmunda regalis*; the absence of the sporophyte is the result of the nutritional competition between the two generations. The formation of the sporophyte can be the result of amphimixis, as for instance in *Cystopteris fragilis*, or of apomixis (apogamy), in *Cyrtomium falcatum* and *Phegopteris connectilis*. The characteristic trait of the apogamous species is the presence of the tracheids in the gametophyte, and the absence of the root in their first stages of development. We recommend the culture of green sporangia on agarized media, due to the formation of a very large number of gametophytes, and, subsequently, little plants.

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