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RESEARCHES CONCERNING THE *IN VITRO* DIFFERENTIATION OF THE FERN *PHEGOPTERIS CONNECTILIS* (MICHX.) WATT

Liliana Cristina Soare[1)], Emilia Vişoiu[2)], M. Andrei [3)]

[1)] University of Pitesti, Faculty of Science, 1 Targul din Vale St., 110040, Pitesti, Arges; Romania, e-mail: soleil_cri@yahoo.com
[2)] National Institute of Research and Development for Bio-Technologies in Horticulture, Stefanesti, Arges; Romania, e-mail: visoiu_e@yahoo.com
[3)] University of Bucuresti, Faculty of Biology, 1-3 Portocalelor St., 060101, Bucuresti; Romania

Abstract. *Phegopteris connectilis* is an apogamous species, endangered in certain regions. The *ex situ* conservation, through the *in vitro* production of the gametophyte, as well as the embryos and the plants, followed by their cryo-stocking, is necessary for ensuring the survival of the endangered species, while at pace with international methodology. The differentiation of the gametophyte is characteristic of the leptosporangiate ferns, passing through the following stages: prothallic filament, prothallic blade, cordate prothallus. The uni- or pluri-serial branchings formed on the gametophytes constitute the initial differentiation stage of a new prothallus. One-cell trichomes are differentiated on the gametophyte, which produce wax, protective against de-hydration. The *in vitro* culture has evinced the fast life cycle of this species, as the apogamous embryo is formed in the course of only seven weeks after culture inception. The first protophylles are formed out of the apogamous embryo and the initial stem cell is differentiated between the first two. In very few cases, however, it was noticed, at the base of the first leaf, the differentiation of the first root; the latter is formed at a stage when the young sporophyte already has two to three protophylles, as rhizogenesis is favoured by the addition of AIA and Kin in the medium. The plants thus obtained can be utilized for re-establishing the natural populations in the regions where the species is endangered.

Key words: *Phegopteris connectilis*, apogamy, *ex situ* conservation

INTRODUCTION

Phegopteris connectilis (Michx.) Watt (2n=90) (*Thelypteridaceae*) is a fern common in shady places, in most areas of Europe, but rather rare in the southern regions of the continent (Tutin et al., 1993). In *Flora of North America and North of Mexico* (Wagner and Smith, 1993) the authors mention that the species is apogamous, and also that it is endangered in certain states. In addition to the *in situ* conservation of the endangered species (considered as fundamental, according to the 8th Article of the Convention on Bio-Diversity Conservation - the *Convention of Biological Diversity* - CBD), the *ex situ* conservation, done via the *in vitro* production of the gametophytes, of the embryos and the small plants, followed by their cryo-stocking, is necessary for ensuring the survival of the genetic diversity of the endangered species (CBD - Art. 9) (Pence, 2002, Cristea and Denaeyer, 2004). Cultures of incompletely differentiated green sporangia (sori), (Henson, 1979) have been set, in order to obtain the *in vitro* gametophyte and the sporophyte of the species *Phegopteris connectilis*. This type of explant presents several advantages, namely: 1) it does not need a great amount of vegetable material, 2) the plant does not have to be sacrificed, as it happens when the rhizome, or the

stem apex are used as explants, 3) the sporophytic tissues that accompany the spores hold an important role in inducing germination.

MATERIALS AND METHODS

The vegetable material was collected, along the Valley of the Vâlsan River in June 2006 (Figure 1). The research technique employed for this material was previously described for other species (Soare et al., 2005). The cultures were realized in the laboratory for *Obtaining and conserving the source of vineyard germoplasm* within the National Institute of Research and Development for Bio-Technologies in Horticulture, Stefanesti, Arges.

Figure 1. *Phegopteris connectilis* (Michx.) Watt - along the Valley of the Vâlsan River (orig.)

RESULTS AND DISCUSSIONS

Differentiation of the gametophyte. It starts two weeks after the culture is initiated, and follows the characteristic stages of the gametophyte differentiation in *Leptosporangiatae*, namely the prothallic filament, the prothallic blade, and the ± cordate prothallus (Ehrendorfer, 1999). Chloroplastotomy was evinced in the prothallic chlorocytes, which is an important process in the development of vegetable cells (Pyke, 1999).

The prothalli display secretory one-cell trichomes on the rim, similar to those observed and described in the case of *Cyrtomium falcatum* and *Dryopteris dilatata* (Soare and Andrei, 2005), as well as various other species (Nayar & Chandra, 1963; Nayar & Kaur, 1963, 1964, 1965; Lingle et al., 2004, etc.). The trichomes are differentiated in the apical region of the gametophyte, as a result of an asymmetric division (Alberts et al., 2002). This type of division is also found during the differentiation of the trichomes characteristic to angiosperms sporophyte and stomata (Larkin et al., 1997), etc. The waxlike substance produced by the trichomes is eliminated outwardly, forming a kind of cap covering their tops (Figure 2). Lingle et al. (2004) consider that these secretory trichomes, which are differentiated on the fern gametophyte, partially protect it against de-hydration, being noticed that trichome density augments in water scarcity conditions. For instance, in *Phegopteris,* trichome density on the gametophytes is much lower in the case of the cultures realized on the liquid nutrient medium

Knop (1865). The capability of producing numerous trichomes reminds of one of the briophytes (Renzaglia et al., 2000).

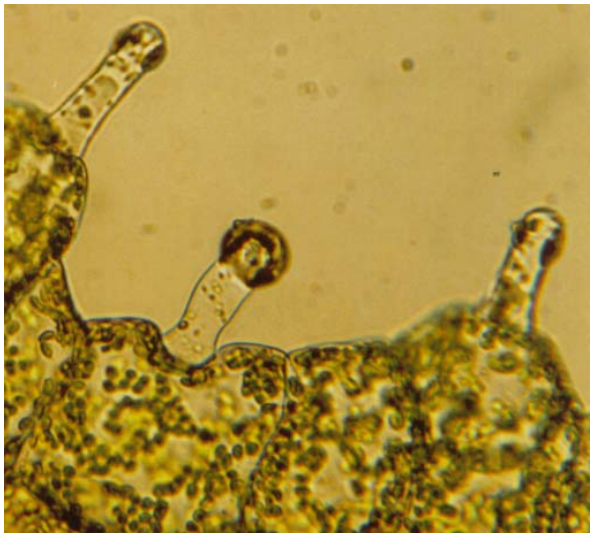

Figure 2. *Phegopteris connectilis* (Michx.) Watt - secretory one-cell trichomes on the rim of the prothallus (oc. 12,5x, ob. 40, orig.)

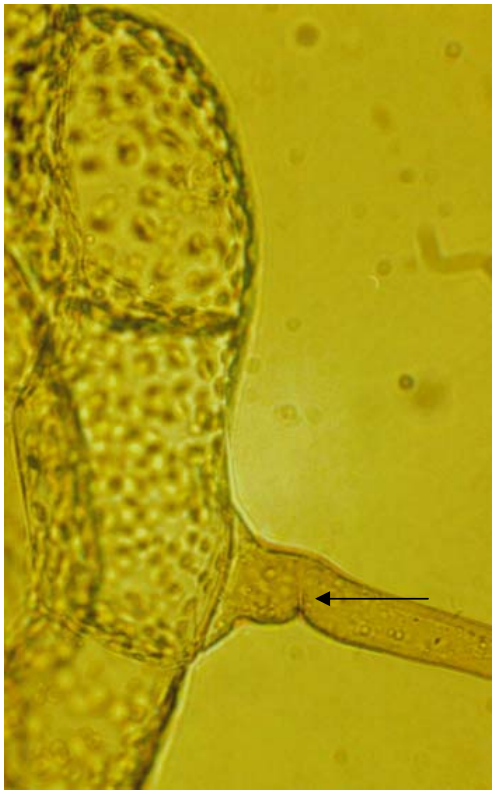

Figure 3. *Phegopteris connectilis* (Michx.) Watt - partially septed rhizoid (shown by the arrow) (oc. 12,5x, ob. 40, orig.).
Figure 4. *Phegopteris connectilis* (Michx.) Watt - a rhizoid that did not separate through a wall from the mother-cell (shown by the arrow) (oc. 12,5x, ob. 40, orig.).

The one-cell rhizoids are differentiated, like the trichomes, as a result of the asymmetric division of a chlorocyte (Alberts et al., 2002). It presents a dilated base, and, in certain cases, the wall displays incomplete septs (Figure 3), similar to those met in the rhizoids of a number of bryophytes. In certain cases, the rhizoids fail to separate through a wall from the mother-cell, thus becoming similar to the radicular hairs (Figure 4).

Some cells on the gametophyte rim, situated towards the base region, start mitotic division, generating uni- or pluri-serial branchings (Figure 5), which can constitute the initial stage in the differentiation of a new gametophyte, a process frequently noticed *in vitro* (Fernández and Revilla, 2003).

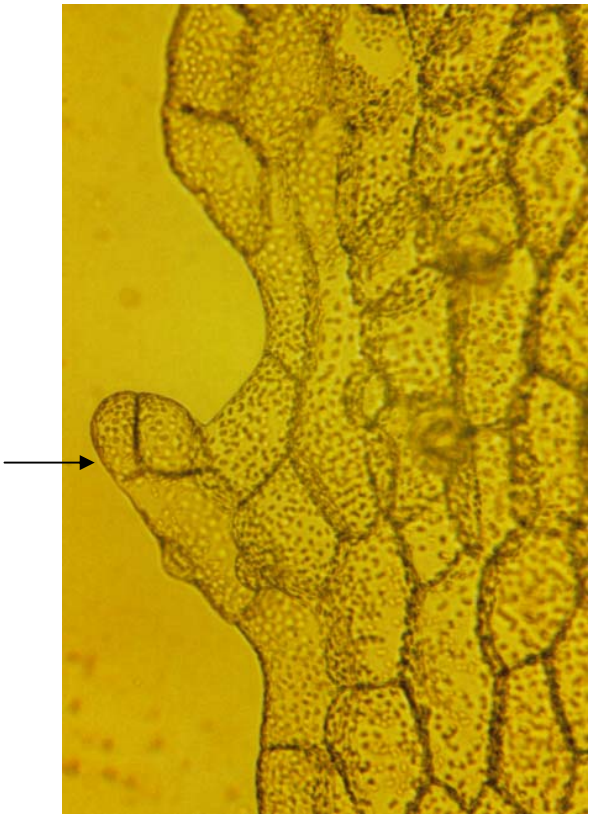

Figure 5. *Phegopteris connectilis* (Michx.) Watt - formation of a branching (shown by the arrow) on the rim of the prothallus (oc. 12,5x, ob. 25, orig.)

Differentiation of the sporophyte. After only seven weeks from the initiation of the culture, apogamous embryos have already been formed on the prothalli, in the manner described by Vladesco (1934) in *Pellaea viridis*, Nayar and Bajpai (1964) in species of *Notholaena* and *Pellaea*, Fernández et al. (1996) in *Dryopteris affinis* ssp. *affinis*, etc. Although antheridia and archegonia are formed on the prothalli, fecundation does not take place, and the apogamous embryo is formed directly out of the prothallic tissue, through the multiplication of a number of cells situated behind the gametophyte meristem. As noticed by Vladesco (1934) in *Pellaea viridis*, in certain cases, the necks of several archegonia can be observed on the apogamous embryo. Unlike other species of leptosporangiates, where the embryo is formed after circa three months after the initiation of the culture, as for instance *Dryopteris affinis* ssp. *affinis*, or after 6 to 8 months, in *Asplenium nidus*, the apogamous

embryo in *Phegopteris connectilis* is already formed after seven weeks from the initiation of the culture, as the life cycle of this species is faster, being close to that of the species *Woodwardia virginica*, in which the sporophyte is formed within one month (Fernández and Revilla, 2003).

The first leaf of the sporophyte is differentiated out of the apogamous embryo, which displays, in apical position, one initial characteristic cell. One-cell trichomes (Plate I, Figure 3) are formed on its rim are, as well as uniserial pluricellular ones (Figure 6), the latter representing the initial stage of the palea differentiation. The lamina of the first leaf is trilobate, having a nervation of a dichotomic type (Plate I, Figure 1). The rim of the lamina is mono-stratified, as the cells contain few chloroplasts. The initials of the trichomes are differentiated from the marginal cells, following a series of asymmetric divisions. The stomata can be observed on the inferior face of the lamina.

The apogamous sporophyte with one to two leaves very rarely displays a root; it differentiates in a growing number upon transplanting the material obtained into a rooting medium that contains 1.8 mg/l AIA and 0.22 mg/l Kin. Subsequently, the apogamous embryo differentiates a second leaf, and the initial of the stem is differentiated between the two leaves.

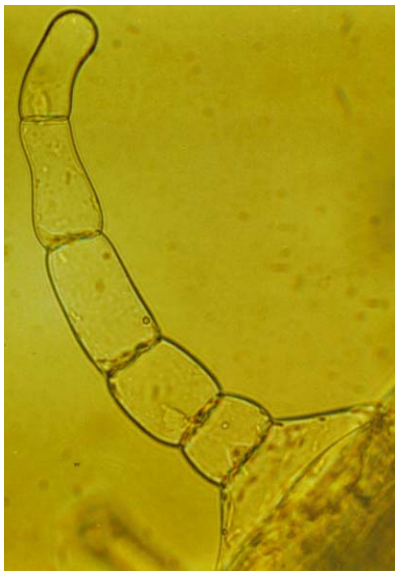

Figure 6. *Phegopteris connectilis* (Michx.) Watt - uniserial pluricellular trichomes on the juvenile leaves of the sporophyte (oc. 12,5x, ob. 40, orig.)
Figure 7. *Phegopteris connectilis* (Michx.) Watt - apogamous sporophytes formed on the prothalli, three months after the initiation of the culture (orig.)

As in the case of other apogamous species (Nayar and Bajpai, 1964), the following juvenile leaves are formed out of the initials lying close to the stem apex, the only element responsible for generating the growing axis of the post-embryo plant: the embryo is uni-polarly structured (Groff and Kaplan, 1988). The protophylles are developed, the sines between the incisions get deeper, and numerous subulate one-cell trichomes are differentiated (Plate I, Figure 4) on their rims.

In order to be acclimatized, the small plants in the large groups (Figure 7) were individualized and transferred to a fresh medium, and later on into pots with sterile sand, covered with glass bowls meant to keep a high humidity. In a later stage of the acclimatization, the small plants were transplanted into pots whose substratum was

constituted of compost (30%), forest earth (30%), peat (15%), sand (15%) and perlite (10%) (Figure 8).

The plants thus obtained can be used for re-establishing or replenishing the natural populations in the areas where the species is endangered (Cristea and Denaeyer, 2004).

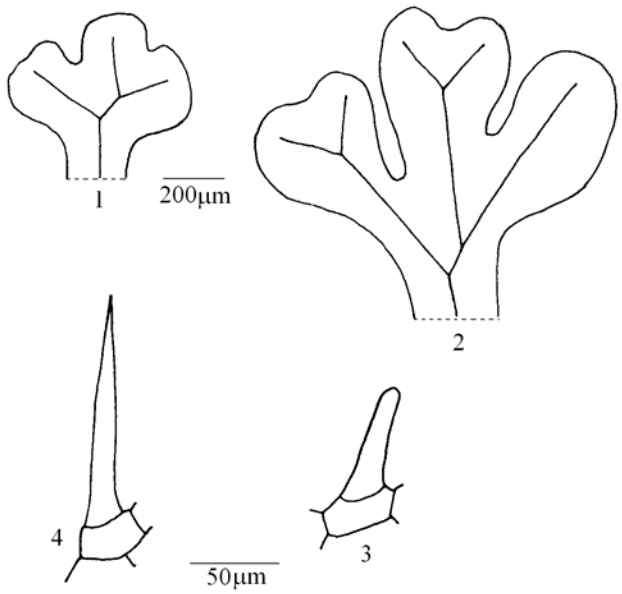

Plate I
Figure 1-2. *Phegopteris connectilis* (Michx.) Watt - juvenile leaves of the apogamous sporophyte (orig.)
Figure 3. *Phegopteris connectilis* (Michx.) Watt - one-cell trichomes on the juvenile leaves of the apogamous sporophyte (orig.)
Figure 4. *Phegopteris connectilis* (Michx.) Watt - subulate trichomes on the juvenile leaves of the apogamous sporophyte (orig.)

Figure 8. *Phegopteris connectilis* (Michx.) Watt – acclimatized plant (orig.)

CONCLUSIONS

The differentiation of the gametophyte is characteristic of the leptosporangiate ferns, passing through the following stages: prothallic filament, prothallic blade, cordate prothallus.

One-cell trichomes are differentiated onn the gametophyte which produce wax, protective against de-hydration. Apart from the characteristic one-cell rhizoids, rhizoids are formed on the gametophytes, similar to the radicular hairs, which are not separated through a wall from the mother-cell. The uni- or pluri-serial branchings formed on the gametophytes constitute the initial stage of the differentiation of a new gametophyte, a process frequently observed *in vitro*. In this species, the fairly fast life cycle is noticeable: the apogamous embryo is formed after only seven weeks from the initiation of the culture. The first protophylles are formed out of the apogamous embryo, and the initial stem cell is differentiated between the first two. In very few cases, however, the differentiation of the first root was noticed, at the base of the first leaf; a first root which is formed in the stage where the young sporophyte already has two to three protophylles, as rhizogenesis is favoured by the addition of AIA and Kin in the medium. The embryos obtained can be utilized for cryo-stocking, in keeping with the international methodology. Moreover, the material obtained can be used for replenishing the natural populations in the regions where the species is endangered.

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REZUMAT

CERCETĂRI PRIVIND DIFERENȚIEREA *IN VITRO* A FERIGII *PHEGOPTERIS CONNECTILIS* (MICHX.) WATT

Phegopteris connectilis este o specie apogamă, periclitată în anumite regiuni. Conservarea *ex situ*, prin obținerea *in vitro* a gametofitului, embrionilor şi plantulelor urmată de criostocarea acestora, conform metodologiei internaționale este necesară pentru asigurarea supraviețuirii speciilor periclitate. Diferențierea gametofitului este caracteristică pentru ferigile leptosporangiate, trecând prin stadiile de: filament protalian, lamă protaliană, protal cordat. Ramificațiile uni- sau pluriseriate formate pe gametofit constituie stadiul inițial al diferențierii unui nou protal. Pe gametofit se diferențiază trihomi unicelulari care produc ceară, cu rol în protejarea acestuia împotriva deshidratării. Cultura *in vitro* a evidențiat ciclul de viață rapid al acestei specii, embrionul apogam fiind format după numai şapte săptămâni de la inițierea culturii. Din embrionul apogam se formează primele protofile, între primele două diferențiindu-se celula inițială a tulpinii. În foarte puține cazuri, la baza primei frunze s-a observat diferențierea primei rădăcini, aceasta formându-se în stadiul în care tânărul sporofit are deja 2-3 protofile, rizogeneza fiind favorizată de adăugarea de AIA şi Kin în mediu. Plantele obținute pot fi utilizate pentru refacerea populațiilor naturale, în zonele în care specia este periclitată.