Mycorrhizal association in gametophytes and sporophytes of the fern *Pteris vittata* (**Pteridaceae**) with *Glomus intraradices*

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Abstract: Ferns, which are usually colonizing different environments and their roots frequently present mycorrhization, have two adult stages in their life cycle, the sporophytic and the gametophytic phase. This paper describes the experimental mycorrhizal association between *Pteris vittata* leptosporangiate fern and a strain of *Glomus intraradices* during the life cycle of the fern, from spore germination to the development of a mature sporophyte. The aim of this study was to compare the colonization pattern of *in vitro* cultures of *G. intraradices* along the fern life cycle with those found in nature. For this, mature spores were obtained from fertile *P. vittata* fronds growing in walls of Buenos Aires city, Argentina. Roots were stained and observed under the light microscope for arbuscular mycorrhizal colonization. Approximately, 75 fern spores were cultured in each pot filled with a sterile substrate and *G. intraradices* (BAFC N° 51.331) as inoculum on the surface. After germination took place, samples were taken every 15 days until the fern cycle was completed. In order to determine colonization dynamics each sample was observed under optical and confocal microscope after staining. Gametophyte was classified as *Adiantum* type. Male and female gametangia were limited to the lower face, mycorrhizal colonization started when they were differentiated and took place through the rhizoids. Spores and vesicles were not found in this cycle stage. *Paris*-type mycorrhizal colonization was established in the midrib and in the embrionary foot. It was colonized by external mycelium. When the first root was developed soil inoculum colonized *de novo* this structure and *Arum*-type colonization was observed. This study proves that the type of colonization is determined by the structure of the host, not by the fungus. Both the gametophyte and embryo foot have determined growth and *Paris*-type colonization, while, sporophyte roots have undetermined growth and *Arum*-type colonization. The structures found *in vitro* cultures were highly similar to those found under natural conditions. Rev. Biol. Trop. 60 (2): 857-865. Epub 2012 June 01.

Key words: *Pteris vittata*, *Glomus intraradices*, Pteridophyta-gametophyte-sporophyte, *Arum* colonization, *Paris* colonization.

Over 90% of terrestrial plant groups have some type of symbiosis with soil fungi, and/or some mycorrhizal form. During the Devonic period, the first plants with roots appeared on land, the ferns belonging to Pteridophyta, Filicales groups, still exist today. They are widely distributed, particularly in tropical environments, and many of them have roots colonized by arbuscular mycorrhizal fungi (AM) (Brundrett 2002). Fern species with fine roots and long absorbent hairs sometimes limit mycorrhizal colonization. This facultative association is considered to be a feature of more evolved ferns (Fernandez *et al.* 2010). Cairney (2000) and Brundrett (2002) suggested that mycorrhizal symbiosis probably enabled plants

to colonize land, conferring advantages such as increased fitness and resistance to drought or pathogenic microorganisms under certain conditions (Smith & Read 1997, Daniell *et al.* 1999, Read 1999).

Preliminary data indicated that the presence of AM fungi at both the sporophyte and the gametophyte stage, stimulates fern growth (Turnau *et al.* 2005). *P. vittata* sporophytes grown in controlled conditions showed an increase in fresh and dry weight shoots when grown in contaminated soil (Trotta *et al.* 2006, Leung *et al.* 2006). In natural conditions (Zhiwei 2000) from 12 different species of *Pteris* (*P. vittata* included) found sporophytic mycorrhization only in *P. setulosocostulata*.

P. vittata is a widely distributed fern in Buenos Aires city, often growing on the substrate that fills hollows in damp walls. Under these stressful conditions, it was observed that the sporophyte was always colonized by arbuscular mycorrhizal fungi.

P. vittata has a chlorophyllous gametophyte of limited growth, the most vulnerable phase of the life cycle (Zhang *et al.* 2008). The appearance of AM fungi *Glomus intraradices* at the gametophyte phase may significantly shorten the period when the small plants are especially susceptible to drought, allowing them to adapt better to the environment (Boullard 1957, 1979, Pirozynski & Malloch 1975).

There are two main morphological types of arbuscular mycorrhizas (AM), the *Arum*-type and the *Paris*-type and a continuum between them. They are responsible of the P plant nutrition (van Aarle *et al.* 2005). In the *Arum*-type, fungi form intercellular hyphae between the cortical cells and intracellular arbuscules within them. The *Paris*-type is characterized by extensive intracellular hyphal coils and arbusculate coils in the root cortex. In the *Paris*-type the intercellular phase of colonization is almost absent. With very few exceptions, members of single plant species formed only one type of colonization. It is often accepted that AM morphology is controlled by plant identity (Smith & Smith 1997). The aim of this study is to determine the relationship between the life cycle of *Pteris vittata* and the colonization of the arbuscular mycorrhizal fungi *G. intraradices*.

MATERIALS AND METHODS

Collection of plant material: Approximately 100 specimens of *P. vittata* L. at different stages of sporophyte development were collected from hollows filled of saline substrate on damp walls of Buenos Aires city, and analysed for root colonization. Sampled sporophytes ranged in height from few millimetres up to 6cm. A number of 20 samples were attached to the gametophyte.

Cultivation of plant material: Spores were obtained from fertile *P. vittata* fronds and kept in dry, covered containers until they were used. Thirty 50mL pots were filled with a sterile mixture of perlite:peat:soil 5:2:1V/V, and 500mg of *G. intraradices* inoculum placed on the surface of each pot, containing approximately 75 fern spores on top.

The mycorrhizal strain used in this experiment is a pure culture identified as *G. intraradices*, originated from a pastureland from Buenos Aires province in Argentina, grown in association with white clover (*Trifolium repens*). Spores have been preserved as herbarium material (BAFC N° 51.331). The culture was replicated, and part of it kept in our collection under the name Strain GB1 (Banco de Glomeromycota *In vitro* (BGIV) http://www.bgiv.com.ar/strains/glomus-intraradices/gb1).

Pots were watered to field capacity and kept in a humid chamber (relative humidity 100%) at 27-30°C and a regime of 16 hours light/8 hours darkness. As from germination, samples were taken every 15 days until the fern cycle was complete, in order to determine colonization dynamics.

Microscopy analyses: The cultured and field material was cleared and stained using the method of Phillips & Hayman (1970) as follows: fresh roots were heated in a 10% KOH solution at 90ºC for 15 minutes, washed in tap

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water and immersed in 20 vol (H_2O_2) for 10 minutes until bleached. Then, they were rinsed in tap water to remove H_2O_2, acidified in 0.1N HCl, and stained with 0.05% Trypan Blue solution for 20 minutes at 90ºC. Dye excess was removed in clear 85% lactic acid. Root segments were mounted on slides in 85% Lactic acid. Observation and microphotography were assessed using a Nikon Opthot-2 microscope fitted with a digital Coolpix 950 camera.

For a more accurate observation of root colonization, confocal microscopy was used. Root samples were fixed at least 12 hours in 50% ethanol. Roots were cleared by heating them in 5% KOH (w/v) at 90ºC, roots were washed with tap water, and then acidified with 0.1N HCl for 5-10 minutes. Roots were stained with 0.01% acid fuchsin (w/v) in solution of acid-glycerin-water (875mL lactic acid, 63mL glycerin, 63mL water) for one hour at 55ºC. Dye excess was removed in 100% glycerin. Observations and microphotography were conducted using an Olympus FV300 confocal scanning HeNe green laser microscope and with excitation at 543nm (Argon laser). The lenses used included Zeiss UplanFI 20x/0.5 and UplanApo 40x/1.0 water immersion objectives. Images were captured and processed using Photoshop v 5.5 software Adobe Systems, San Jose, CA, USA (Peterson *et al.* 2004).

RESULTS

Experimental life cycle of *P. vittata* showed that spores germinated after eight days and they gave rise to a few cell filaments, and a rhizoid able to differentiate. Gametophyte development (Fig. 1A) was of *Adiantum*-type (Nayar & Kaur 1969, Martinez 2010), characterized by a meristematic apical cell that divided laterally giving rise to new cells, which formed a notch. The apical cell divided transversally into further meristematic cells, which began to divide rapidly on another plane, producing a central zone made up of several cell layers with rhizoids and two wings. Male and female gametangia were limited to the lower face and to the ventral surface below the notch, mainly
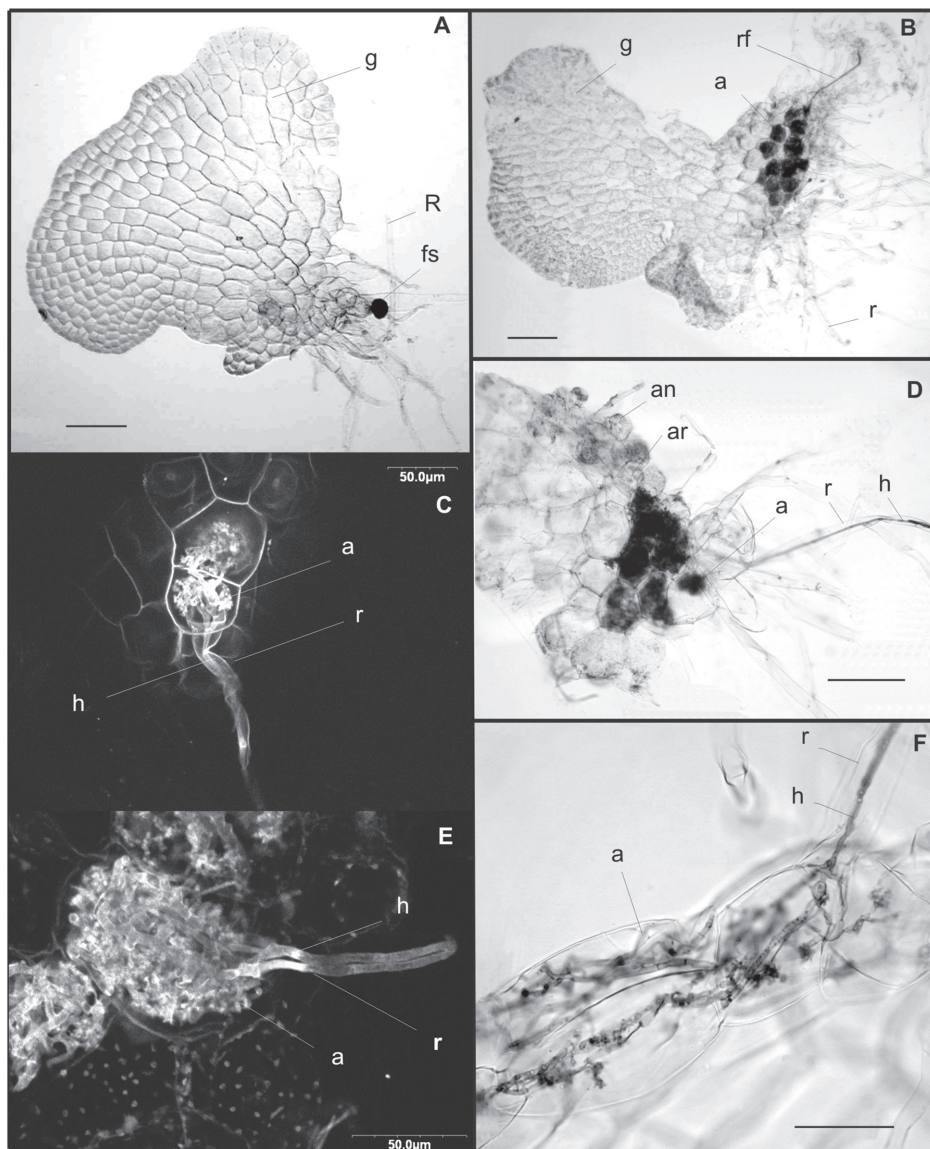
between the rhizoids (Fig. 1D). About 75% of the gametophytes examined were colonized by *G. intraradices*, of sporophytes arising from embryo development.

The first stages of prothallus development lack mycorrhizal colonization even when rhizoids have developed (Fig. 1A). Gametophyte colonization only began when gametangia were differentiated (Fig. 1D). Gametangial differentiation was separated in the time (protandric gametophyte). Colonization took place through the rhizoids (Fig. 1B-F), on which an *appresorium* formed and penetrated the cell. On reaching the basal cells of the midrib, the hypha grew forming coils and spread through cells (*Paris*-type), taking up ¾ of the midrib (Fig. 1B, D). The arbuscules, being very small, grew from these coils (Fig. 1C, E, F). There was little, if any, intercellular growth. Arbuscules were ephemeral, and in most cells we observed amorphous, blue-stained material. Spores and vesicles were not found in this phase of the cycle. Gametophyte wings and growth apex were uncolonized, probably because the wings were unstratified.

When the embryo developed, the foot that penetrated the gametophyte also showed *Paris*-type colonization (Fig. 2A). This colonization did not originate from the colonized gametophyte cells, but rather from external mycelium (Fig. 2B), as zones remain separated by uncolonized cells, the foot had determinate growth and enabled the young sporophyte to obtain nutrients from the gametophyte (Fig. 2B).

Right from the start, there was *Arum*-type colonization of the first sporophyte root (Fig. 2D). Penetration points, intercellular arbuscules and intercellular hyphae could be seen (Fig. 2C-E). There was no colonization through the gametophyte. The sporophyte was colonized by inoculum in the medium (Fig. 1F).

The substrate accompanying the samples was analyzed, resulting in a salt imbalanced substrate with the following characteristics: pH 7.6 (1:2.5 H_2O), C.E. dS/m 3.59, C_t(W. Black) 24.98g/kg, Nt (Kjeldahl) 1.92g/kg, P (K y B) 9.3mg/kg, CIC (Ac. NH_4 pH7 µDest.) 18.4cmolc/kg, Ca^{2+} (Ac. NH_4 pH7

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Fig. 1. *Pteris vittata*. A) Young gametophyte without mycorrhiza. B) Mature, colonized gametophyte. C) Confocal laser scanning microscopy of colonization through a rhizoid. D) Confocal laser scanning microscopy of distribution of differentiated gametangia (archegonium and antheridium) in relation to the colonized zone. E) Colonization through a rhizoid and arbuscule (*Paris*-type). F) Arbuscule. g: gametophyte, r: rhizoid, Pt: *Paris*-type arbuscule, h: hyphae, fs: fern spore, ar: archegonium, an: antheridium, a: arbuscule, rf: rhizoid with fungal colonization. Bars: A: 100μ, B: 100μ, C: 100μ, D: 100μ, E: 50μ, F: 30μ.

A.A) 30.5cmolc/kg, Mg^{2+} (Ac NH_4 pH7 A. A) 1.7cmolc/kg, Na^+ (Ac NH_4 pH7 E/A.A) 4.2 cmolc/kg, K^+ (Ac. NH4 pH7 E/A.A) 1.7 cmolc/kg.

Most gametophytes of *P. vittata* that developed in hollows in damp walls were mycothallic (Fig. 3A). Fern gametophytes and roots of sporophytes were strongly colonized (Fig.
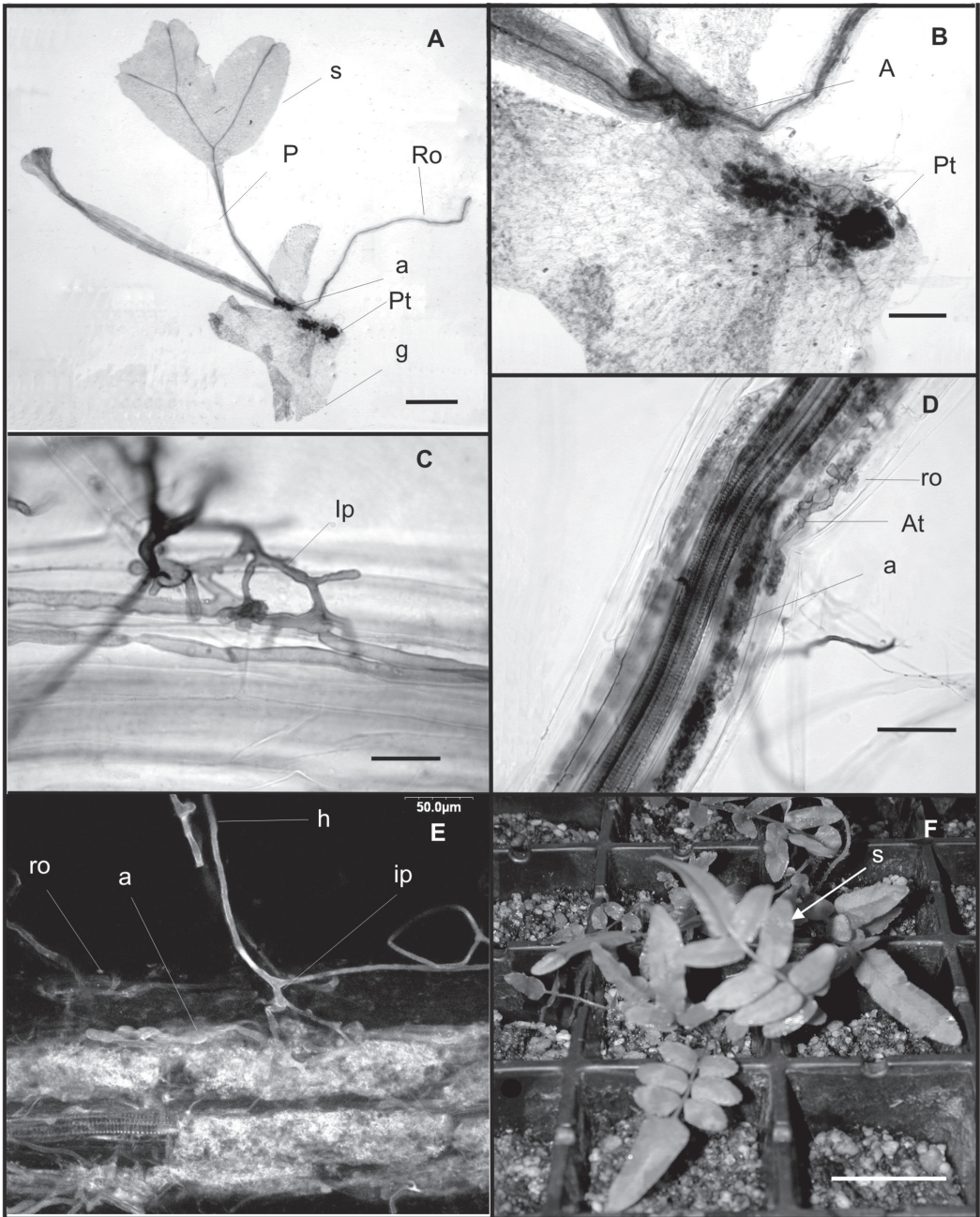
Fig. 2. *Pteris vittata.* A) - B) Gametophyte and young sporophyte with colonization of *G. intraradices*. C) Infection point on the surface of the sporophyte root. D) Sporophyte root with arbuscules. E) Confocal laser scanning microscopy of infection point on the surface of the sporophyte root. F) Pots with mature sporophyte. a: arbuscules, g: gametophyte, p: petiole, Pt: *Paris*-type colonization, ro: root, s: sporophyte, ip: infection point, At: *Arum*-type colonization. Bars: A: 600μ, B: 200μ, C: 40μ, D: 50μ, E: 8mm, F: 16mm.

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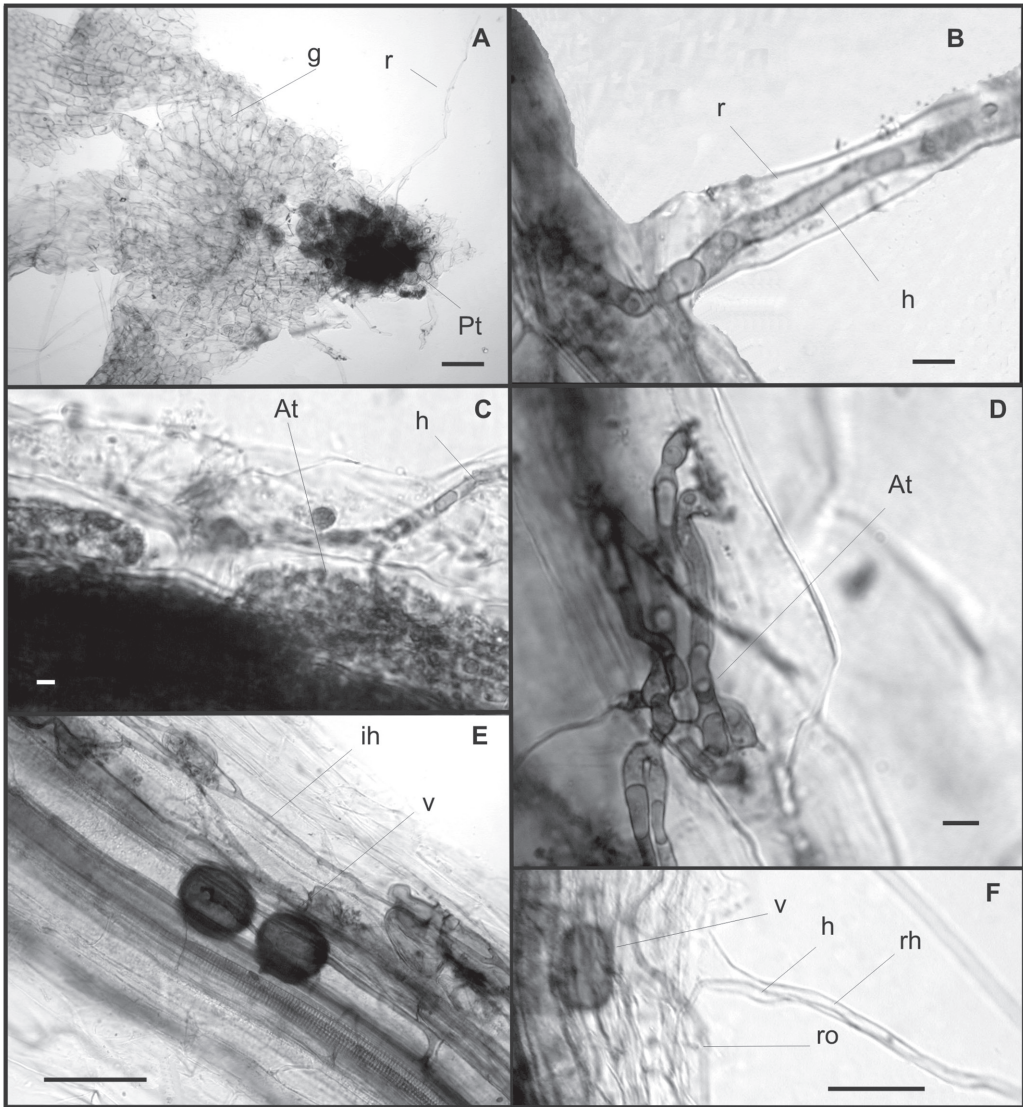
Fig. 3. *Pteris vittata* (wild). A) *Paris*-type colonized gametophyte. B) Penetration through a rhizoid. C) Root with arbuscule. D) *Arum*-type colonization, arbuscule in formation. E) Colonized root with intercellular hyphae and vesicles. F) Colonization through root hair. At: *Arum*-type colonization, g: gametophyte, h: hyphae, ih: intercellular hyphae rh: root hair, Pt: *Paris*-type colonization, r: rhizoid, ro: root, v: vesicle. Bars: A: 100μ, B: 10μ, C: 10μ, D: 10μ, E: 50μ, F: 50μ.

3A, E). Thin fungal mycelium with extraradical hyphae of 0.8-1.3μm, hyphae developing within the gametophyte often forming complex coils (*Paris*-type arbuscules) filling the gametophyte cells (Fig. 3A), strongly stained in trypan blue, were visible either in the basal part of the rhizoids or within cells of the *Adiantum*-type gametophyte, where the rhizoids were initiated. The mycelium was also observed within the elongated part of the rhizoids (Fig. 3B). The fungus was spreading from one cell to another without the development of the intercellular phase (Fig. 3A). The colonization *Arum*-type of sporophyte roots was observed

by the mycelium originating from the extraradical hyphal net (Fig. 3D-F). Vesicles of 3-7μm diameter were found in root cortical cells. (Fig. 3E-F).

From the field material collected, the samples presenting gametophyte (n=20), showed a *Paris*-type colonization occurring through the rhizoids in contact with the inoculum (Fig. 3A, B). A large amount of extra-radical mycelium can be seen penetrating root hairs in the sporophytes, forming intercellular and intracellular hyphae, arbuscules and vesicles (Fig. 3C-F) similar to the ones found in the sporophytes cultured with *G. intraradices*.

DISCUSSION

Mycorrhizal dependence of a plant species is one of its constitutive features, allowing plant classification as facultative or obligately mycotrophic. Facultative mycotrophs are those that can grow without mycorrhizae in relatively fertile natural soils. Previous studies, found a low mycorrhization level in *Pteridophyte* sporophytes, particularly in *P. vittata* roots (Zhiwei 2000). On the contrary our results showed a high level of fern roots colonization, supporting the previous hypothesis that the amount of available nutrients in the substrate regulates this process (Janos 1993, 2007, Brundrett 2002), leading to the notion that symbiosis may be necessary when *P. vittata* is growing under highly stressful conditions (wild *P. vittata*) or under low experimental soil fertility (cultured *P. vittata*) (Hajiboland *et al.* 2010). This symbiotic relationship between plant and fungi allowed completing its life cycle in different substrates, enabling the estimation of the colonization at all stages of the fern life cycle. To our knowledge, this is the first time the experimental AMF development in fern sporophytes and gametophytes under culture is reported.

Both gametophytes and sporophytes can form symbioses with the same Glomeromycota fungi that are common symbionts of phanerogamic plants. Previous studies showed that gametophytes of different genera as: *Pteridium*, *Histiopteris*, *Todea*, *Cyathea*, *Asplenium*,

Blechnum and *Schizaea pusilla* grown in natural environments were colonized by arbuscular mycorrhiza when they were found in places where nutrients were unavailable (Cooper 1976, Swatzell *et al.* 1996). Although, as it was shown in this study, the development of the symbiosis between the gametophytes and the Glomalean fungi is not obligatory, we observed that the sporophyte was usually colonized by *Glomus intraradices*, leading to the notion that sporophytes could be colonized by other mycorrhizal fungus.

Our observation showed *Paris*-type colonization in cells of the gametophyte and sporophyte foot, while the mycelium never colonized the sporophyte through the junction between the gametophyte and the sporophyte, in line with Turnau (2005) and Reyes-Jaramillo *et al.* (2008) reports. Previous studies found hyphal coils in the gametophyte, near the penetration point considering this structure as an adaptation of the fungus to the limited growth of the gametophyte and the embryo foot (Schmid & Oberwinkler 1995). Gametophyte colonization is restricted to the basal zone of the midrib. Fungus colonization always occurs through the rhizoids after male gametangia differentiation. Previous experiments have shown that the gametophytes synthesize antheridiogen (Döpp 1950), which is a gibberellin-like compound (Wynne *et al.* 1998, Banks 1999). This change in the phytohormonal balance (Shaul-Keinan *et al.* 2002), enabling the hypha to penetrate the rhizoid and colonize the gametophyte, suggests a biochemical regulation of the mycorrhizal colonization.

Reyes-Jaramillo *et al.* (2008) found sporophyte root does not become colonized from the gametophyte; this finding is in agreement with our observations. In *P. vittata* the embryo foot established a close association with the gametophyte from which it takes nutrients for its growth, but was not colonized by the intracellular mycelium growing in the gametophyte. A plant tissue with several layers of uncolonized cells separated structures: embryo foot/gametophyte. Sporophyte colonization took place after development of root hairs. There were

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intercellular hyphae in the subepidermic tissue, parallel to the root surface, which formed intracellular branches that gave rise to arbuscules (*Arum*-type). This type of colonization has also been found in *Gleichenia bifida* (Schmid & Oberwinkler 1995). We report the simultaneous development of colonization units of *Paris*-type and *Arum*-type mycorrhizae with the same fungus in different stages of the life cycle concluding that the morphology of arbuscular mycorrhizae, (*Arum* versus *Paris*-types), is solely under the control of *P. vittata* genome during its life cycle.

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RESUMEN

Los helechos presentan dos etapas en su ciclo de vida, una fase esporofítica y una gametofítica. Estos por lo general pueden colonizar diferentes ambientes y frecuentemente presentan raíces micorrizadas. Este estudio describe la asociación experimental entre *Pteris vittata,* un helecho leptosporangiado y una cepa de *Glomus intraradices* durante el ciclo de vida del helecho, desde la germinación de las esporas hasta el desarrollo del esporofito maduro. El objetivo de este estudio fue comparar los patrones de colonización de *G. intraradices* a lo largo de todo el ciclo de vida del helecho con los tipos encontrados en la naturaleza. Las esporas maduras fueron obtenidas de frondes fértiles de *P. vittata* que crecen sobre las paredes de la ciudad de Buenos Aires, Argentina. Las raíces se tiñeron y fueron observadas bajo microscopio óptico para el estudio de la colonización micorrízica. Aproximadamente 75 esporas de helecho se cultivaron en macetas con un sustrato estéril y con un inóculo de *G. intraradices* (N° 51.331 BAFC) en la superficie. Después de la germinación, se tomaron muestras cada 15 días hasta que se completó el ciclo de vida del helecho. Con el fin de determinar la dinámica de la colonización, cada muestra se observó con el microscopio óptico y el microscopio de confocal luego de la tinción correspondiente. El gametofito fue clasificado como del tipo *"Adiantum"*. Los gametangios femeninos y masculinos se desarrollaron en la cara inferior del mismo. La micorrización comenzó cuando los gametangios estaban ya diferenciados y la colonización se produjo a través de los rizoides. Las esporas y las vesículas no se encontraron en esta fase del ciclo. La micorrizacion tipo *Paris* se observó sobre la línea de la nervadura central. El pie del esporofito fue colonizado por el micelio externo. Cuando la raíz se desarrolló, se colonizó *"de novo"*, y se observó una colonización de tipo *Arum*. Este estudio demuestra que el tipo de colonización está determinado por la estructura del helecho y no por el hongo. Tanto el gametofito como el pie del embrión tienen crecimiento definido y colonización tipo *Paris*, mientras que las raíces del esporofito presentan un crecimiento indeterminado y una colonización tipo *Arum*. Las estructuras que se encontraron bajo cultivo coinciden con las que se encontraron en condiciones naturales.

Palabras clave: *Pteris vittata*, *Glomus intraradices*, Pteridophyta-gametofito, esporofito, colonización tipo *Arum*, colonización tipo *Paris*.

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