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Mycorrhizal status of *Phlebopus bruchii* (Boletaceae). Does it form ectomycorrhizas with *Fagara coco* (Rutaceae)?

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Abstract

Fagara coco (Gill.) Engl, is a native tree found in the mountains of Central Argentina. It generally grows together with the edible bolete *Phlebopus bruchii* (Speg.) Heineman & Rammeloo. This association is considered to be an ectomycorrhizal symbiosis. To test the hypothesis of their co-occurrence as mutualistic partners, we studied the mycorrhizal colonization in roots from either natural stands or inoculated with spores of *P. bruchii* in both, the greenhouse and in pure culture experiments. However, no ectomycorrhizal (ECM) colonization was detected. Instead, roots collected from the field were colonized by arbuscular mycorrhizal (AM) fungi, showing a dual infection of *Arum*- and *Paris*-type morphologies. The percentage of AM mycorrhizal colonization varied according to the season, with the highest occurring in the autumn. Spores of ten Glomalean taxa were isolated from the *F. coco* rhizosphere. Based on our results and previous data, we suggest that the behavior of the genus *Phlebopus* is an exception to the mycorrhizal habit found in most Boletales.

Keywords: Mycorrhizae, *Fagara coco*, *Phlebopus bruchii*, Central Argentina

1. Introduction

Fagara coco (Gill.) Engl., which is a relatively small tree (up to 12 m), is found in South America from Bolivia to Central Argentina, among the mountains of the Yunga and Chaco regions (Demaio et al., 2002). In Argentina, *F. coco* usually grows isolated or in small populations at altitudes of up to 1300 m (Luti et al., 1979). Every year, *F. coco* populations in Central Argentina are visited by mushroom pickers in search of the highly prized and native edible bolete, *Phlebopus bruchii* (Speg.) Heineman & Rammeloo (Deschamps and Moreno, 1999). This mushroom (Fig. 1) was collected and consumed by indigenous people in the past and is nowadays sold sliced and dried by locals in villages and at city markets. As occurs with most boletes, this species is assumed to form ectomycorrhizas (ECM) (Deschamps, 2002). However, this has not yet been demonstrated experimentally. Furthermore, an inherent problem with this assumption is that the host tree, *F. coco*, belongs to the Rutaceae (Sapindales), a family primarily known to have an arbuscular mycorrhizal (AM) habit (Trappe, 1987; Brundrett, 2002).

Similar dubious associations have been proposed, such as those of *Phlebopus beniensis*, a Puerto Rican species suspected to form ectomycorrhizae with the leguminous *Hymenaea courbaril* L. (Miller et al., 2000), and also *P. marginatus* from Australia, presumed to be associated with the eucalyptus species (Bougher, 1995).

Moreover, the genus *Phlebopus* has been suggested to be involved with either non-ECM or facultative ECM species (Singer, 1986). On the other hand, recent molecular data indicate that the nutritional mode found in *Phlebopus* and *Boletinellus* species is an exceptional example of a transition between mutualists and parasites in the homobasidiomycetes (Binder and Bresinsky, 2002). Therefore, considering that the available information on the mycorrhizal status of the species belonging to the genus *Phlebopus* is contradictory, and the lack of knowledge about the relationship between *Fagara coco* and *Phlebopus bruchii*, we proposed to: 1) examine roots of *F. coco* in order to evaluate the seasonal mycorrhizal status in the field; 2) experimentally determine if *P. bruchii* forms ectomycorrhizas with *F. coco*; 3) collect and discuss the scarce information in the literature regarding the mycorrhizal status of the genus *Phlebopus*.

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Figure 1. *Phlebopus bruchii* sporocarps. Scale bar: 1 cm.

2. Materials and Methods

Study area

The study area is located in the province of Cordoba in Central Argentina, at Colanchanga in Colon department (840 m elevation, 31°08′40′′S, 64°21′14′′W). The mean annual temperature is 18.9°C, ranging from –6° to 40°C, with the average precipitation been 949 mm (period 1960–2000) (Gavier and Bucher, 2004). The soils are Entisols, Ustorthent paralitic, which have a moderate to low organic matter content (4.1%) in the A1 horizon, a high percentage of sand and gravel, and neutral to acidic (5.3–6) pH values (Gorgas et al., 2003).

Fagara coco populations and isolated individuals grow in grassy open spaces, or in mixed open forests, but do not form pure stands. The plant community is dominated by the AM host tree species (Wang and Qiu, 2006), such as *Litrhaea ternifolia* (Anacardiaceae), *Schinopsis hankeana* (Anacardiaceae), *Acacia caven*, *Prosopis alba*, *P. chilensis*, *P. nigra* and *Geoffroea decorticans* (Fabaceae), and also shrubs such as *Celtis chinchape* (Celtidaceae), *Condalia microphylla* (Rhamnaceae), *Schinus praecox* (Anacardiaceae), *Heterothalamus alienus* (Compositae) and *Colletia spinosissima* (Rhamnaceae) (Luti et al., 1979). In the study area, five sites were selected containing groups of 3 to 12 *F. coco* individuals.

Sampling methods

The area was visited four times, once during each season of 1995. At each sampling time, five cubic soil samples of 15 cm^3 were obtained by using a spade at a distance varying between 50 cm and 1.5 m from mature trees for each of the five selected sites, giving 100 samples in total. Samples were placed in plastic bags and stored at 4°C until processed in the laboratory. Roots of *F. coco* are characterized by a conspicuous yellow coloration, a soft consistency and a smooth surface, with radical hairs usually occurring in groups and not been evenly distributed. These characteristics make *F. coco* roots easily recognizable, even when other plant roots are present in the samples.

Roots and soil preparation

Roots were cleaned of soil and debris with running tap water, using two mesh sizes (2 mm and 850 µm), and analyzed for the presence of ECM formation under a Zeiss stereomicroscope at 10–40× magnification, following the method of Agerer (1991). Cross-sections of rootlets were examined under a microscope for mantle and Hartig net formation. For AM fungi observation, roots were cleared and stained according to Phillips and Hayman (1970). AM colonization was observed with stereo and compound microscopy and then evaluated based on the presence of vesicles, arbuscules and hyphae within the roots. Black and white photographs were taken with a Zeiss Axiophot microscope (100 ISO).

Quantification of AM colonization

The percentage of *F. coco* roots colonized by AM fungi was determined following the "gridline-intersect method" (Newman, 1966).

Glomalean spore identification

Spores were isolated from 50 g of the collected rhizosphere soil samples. Spores were extracted by wet-sieving (sieves sizes 500, 250 and 38 µm) followed by sucrose density centrifugation (Gerdemann and Nicolson, 1963). The supernatant was washed in water and placed in Petri dishes, from which spores were collected manually under a dissecting microscope. Identification of spores (based on color, size and wall structure) was made on fresh material (Schenk and Perez, 1990; online taxonomic descriptions available at http://invam.caf.wvu.edu/Myc_Info/Taxonomy/species.ht), mounted in water or in either polyvinyl-lacto-glycerol (PVLG) or PVLG + Melzer's reagent (1:1 v:v) in order to differentially stain wall structures of important diagnostic value (Morton, 1998). Only apparently viable spores were used for identification. Spores were photographed using a Zeiss Axiophot microscope (100 ISO).

To provide fresh spores for positive identification of AM fungi species present in field soil samples (Stutz and Morton, 1996), trap cultures were used. A fraction of the original soil samples were diluted with sterilized sand (1:1, v:v) and then transferred to 250 ml plastic pots. Soil samples were used to trap AM fungi by sowing seeds of *Sorghum vulgare* (Hack.) Haines in the soil-sand mixture, and pots were then transferred to a greenhouse (22/18°C

day/night, photoperiod 16/8 h day/night). No fertilizer was used on the trap plants. At the end of the trapping phase, the green parts of the plants were cut off and the pots left to dry in the greenhouse. The dry soil was stored at 8°C before examination of the fungi. The trap culture substrate was first decanted and then sieved as described above.

Voucher mycorrhizas and AM spores were deposited in the "Museo Botánico de Córdoba", Herbarium (CORD).

Greenhouse inoculation of Fagara coco *seedlings with* Phlebopus bruchii *spores*

A 30% germination rate of *F. coco* seeds was obtained with the following procedure: fresh seeds were treated with concentrated H_2SO_4, for 3 h at room temperature to weaken the thick seed wall. Seeds were then washed in running water for 2 min, before being treated with gibberelic acid (100 ppm) for 18 h at room temperature, and the surface was then sterilized with 30% H_2O_2 for 10 min. Finally the treated seeds were placed in sterilized Petri dishes containing a disc of wet filter paper, at approximately 20–25°C and kept in natural light. The germination rate was reduced to 5% when using four month old seeds.

Seedlings (10–15 days old) were planted in two sets of 25 containers (250 cc capacity), with a mixture of vermiculite and soil (3:1) from the study site. The first treatment, "sterilized-inoculation", was performed using sterilized soil (2 atm, 30 min). After two months, the seedlings were inoculated with a spore solution prepared from *P. bruchii* sporocarps, following the guidelines described by Castellano and Molina (1989). Two further inoculations were performed at intervals of 20 days. The second treatment, "non-sterilized-inoculation", used non-sterilized soil. Seedlings were grown in a greenhouse compartment, under semi-shaded conditions, provided by a shade cloth of 50% density and at a temperature range of between 20–34°C. Water was supplied manually, three times a week in summer and twice a week in fall. After seven months, seedlings were harvested and the roots were processed in the same way as indicated previously.

Phlebopus bruchii *and* Fagara coco *in pure culture*

Hyphal tissue from fresh *Phlebopus bruchii* sporocarps was extracted and grown in sterile Petri dishes (Marx, 1969) containing 10 ml of Melin Norkrans modified culture media (Molina, 1979). Twelve sealed Petri dishes, each containing 3 pieces of fungal tissue each, were placed in an incubator at 26°C, for 15 days. After this period, samples of tissue were extracted for microscopic analysis.

Seedlings were grown as previously described, and then aseptically transferred to glass tubes of 300 × 38 mm (Molina, 1979), containing 110 ml of vermiculite mixed with 10 ml of *Sphagnum* moss, and 70 ml of modified Melin Norkrans nutrient solution (Marx, 1969). This substrate was previously sterilized for 60 min at 1.8 atm. Twenty seedlings were aseptically inoculated under a laminar flow hood with 2 discs of 0.4 cm in diameter, obtained from the fungal cultures previously described, and the tubes were then sealed. Ten control seedlings were not inoculated. Plants were kept at 20°C, and 15 hours of artificial light a day (ca. 10500 lx). After three months of culture, seedlings were extracted from the tubes and analyzed for the presence of ECM morphotypes as previously described.

Data analysis

Testing was performed for the assumptions of normality, the homogeneity of variances and independence of errors. Then, the seasonal variations of the percentage of AM colonization and plant growth were analyzed with a one-way ANOVA using the *Infostat* statistical package (Di Rienzo et al., 2002).

3. Results

Mycorrhizal structures in roots

ECM morphotypes and colonization were absent in all 100 field samples analyzed. *F. coco* feeder roots were thick, yellow colored and abundant in summer and autumn samples. Winter and spring samples were dark-colored with feeder roots appearing old. In some of the samples, *P. bruchii* hyphal strands connecting the sporocarps to the rhizosphere were observed. However, none of these were attached to the *F. coco* roots. Microscopic observation of crush mounted tips and cross sections did not show any evidence of a hyphal mantle or Hartig net structure.

Roots of *F. coco* collected from the field showed AM colonization features of both *Arum*- and *Paris*-type, even within a single root. Surface colonization was characterized by the presence of cylindrical or irregular non-septate, ramified external hyphae (1.5–3.2 µm in diam) (Fig. 2A,B). *Arum*-type features were characterized by thin walled, intercellular hyphae (4–8 µm in diam) extended along intercellular spaces (Fig. 2C); oval shaped vesicles of (90) 65–50 × 5–30 (40) µm, were associated with intercellular hyphae (Fig. 2C). Some vesicles matured into thick walled spores (1.6–3.2 µm thick) and remained attached to hyphae (Fig. 1D). However, few intracellular vesicles and arbuscules were observed (Fig. 2E,F respectively). *Paris*-type features were characterized by the presence of intracellular hyphal coils that colonized several contiguous cortical cells (Fig. 2G). No arbusculate-coils were observed.

The percentage of AM colonization was significantly higher in autumn (46%) and lower in spring (13%), whereas summer (28%) and winter (26%) had intermediate values ($F = 5.38$, $P = 0.01$) (Fig. 3).

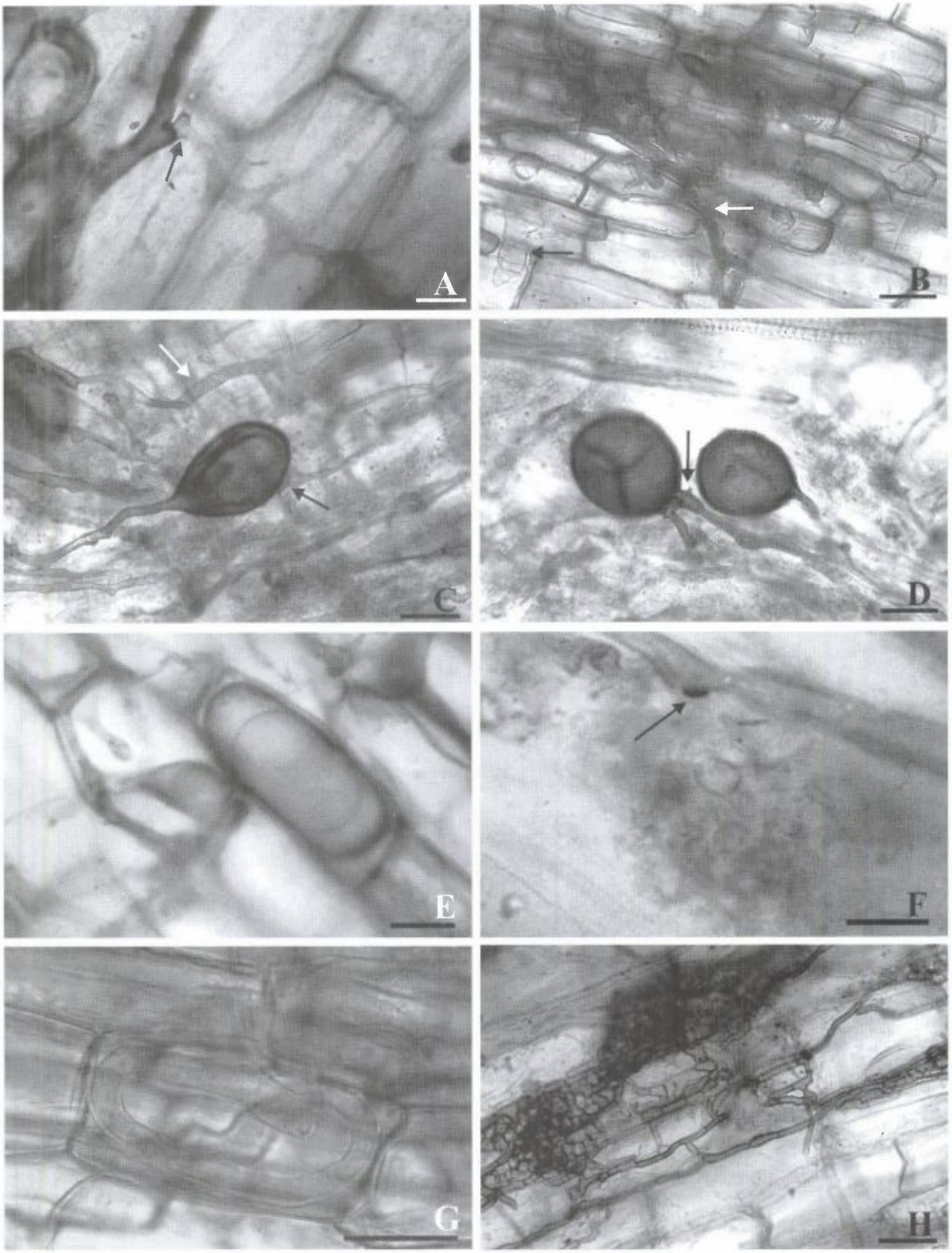

Figure 2. A–H: AM colonization features of field collected roots of *Fagara coco*: A: point of entry (arrow) and hyphal ramification into root cortex; B: external hyphae on root surface (black arrow), radical hair (white arrow); C: intercellular colonization of cortex (white arrow), intercellular vesicle (black arrow); D: vesicles matured into spores, the subtending hyphae indicated by the black arrow; E: intracellular development of vesicles; F: terminal arbuscule, point of hyphal penetration (arrow); G: coils infecting adjacent cortex cells; H: dark-septate fungal hyphal colonization and microesclerotia. Scale bar: A, E, F: 10 µm; B, C, D, H: 20 µm; G: 40 µm.

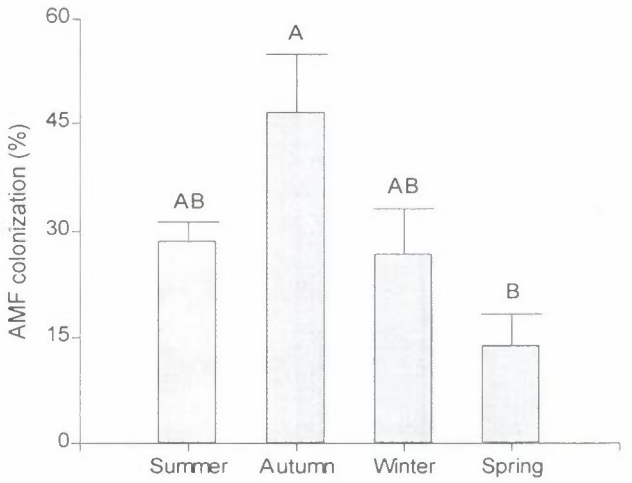

Figure 3. Seasonal variation in percentage of arbuscular mycorrhizal colonization in *Fagara coco*. Error bars indicate +1 SE. Bars with the same letters are not significantly different (Tukey's test, $P<0.05$).

In addition to the AM, we observed colonization by "dark septate fungi", characterized by microsclerotia and septate hyphae (Fig. 2H).

Phlebopus bruchii *tissue culture*

Previously cultured tissue produced visible mycelial growth between 5 and 10 days after isolation. Contaminated Petri dishes were discarded, and then after two weeks, circular colonies were observed of a light brown coloration with developed hyphal strands growing towards the edges. At this time, hyphal samples were taken and analyzed under the microscope, resulting in an effective comparison and match with *P. bruchii* mycelium and sporocarp tissue. Cultured tissue was characterized by hyaline to yellowish, cylindrical, thin walled hyphae, 1.5–4.5 µm in diameter, septate with abundant clamp connections. Rhizomorphs were ochraceous, consisting of cylindrical hyphae, 3–6.5 µm in diameter, parallel to interwoven, with abundant clamp connections and the presence of pigmented wall incrustations.

Glomalean spores

Ten species were obtained from the samples, six of which were identified and are shown in Fig. 4, namely: *Acaulospora denticulata* Sieverding & Toro; *A. scrobiculata* Trappe; *A. rehmii* Sieverding & Toro; *Entrophospora infrequens* (Hall) Ames & Schneider; *Glomus sinuosum* (Gerd. & Bakshi) Almeida & Schenck and *Scutellospora biornata* Spain, Sieverding & Toro. The unidentified species belonged to the genera *Acaulospora* (1); *Scutellospora* (1) and *Glomus* (2). Although trap cultures provided fresh healthy spores for identification, they did not contain any new species that were not present in the field soil samples. Most AMF (Arbuscular Mycorrhizal Fungi) species identified from field samples also sporulated in trap cultures, indicating that the spores and (or) hyphae were able to initiate new infections in pot cultures.

Inoculation of F. coco *seedlings with* P. bruchii *spores*

Seven month old seedlings, exposed to either sterilized or non-sterilized inoculum, did not show any ectomycorrhizal structures in their roots. In addition, the seedlings appeared healthy, but were small in height ($X = 6$ cm). Some seedlings, 18% and 27% of the individuals inoculated under sterile conditions or in the greenhouse treatments respectively, died before the completion of the experiment. During the analysis of the roots of seedlings, we observed "dark septate fungi" colonization in the second treatment.

Fagara coco *and* P. bruchii *in pure culture*

ECM morphotypes were neither observed under stereo microscope observation, nor in the microscopic preparations of feeder roots. Despite the lack of ECM colonization, hyphae of *P. bruchii* were present in the substrate in 20% of tubes. There were no significant differences in height ($P = 0.1457$).

4. Discussion

In this study, we were unable to confirm the suspected ECM association between *P. bruchii* and *F. coco* (Deschamps, 2002), despite their co-occurrence in Central Argentina.

No ECM symbiosis was present in root samples obtained from sites where *P. bruchii* sporocarps naturally occurred and fructified during the sampling period. In addition, no ECM structures were present in roots of *F. coco* seedlings, following inoculation trials under greenhouse or pure culture conditions. *In vitro* synthesis of mantle and Hartig net structures has been shown to be one of the most reliable methods of verifying ECM formation and symbiotic compatibility (Wiemken, 1999), with results suggesting that *F. coco* and *P. bruchii* establish some kind of interaction other than a typical mycorrhizal relationship, in which the partners are in close contact. There is also evidence that other members of the Boletales develop a close relationship (not mycorrhizal) with non-ectomycorrhizal tree hosts. This particular association might suggest a fungal adaptation to a non-ectomycorrhizal plant community habitat. In such cases, the fungi benefit from exudates produced by the plant, and therefore the

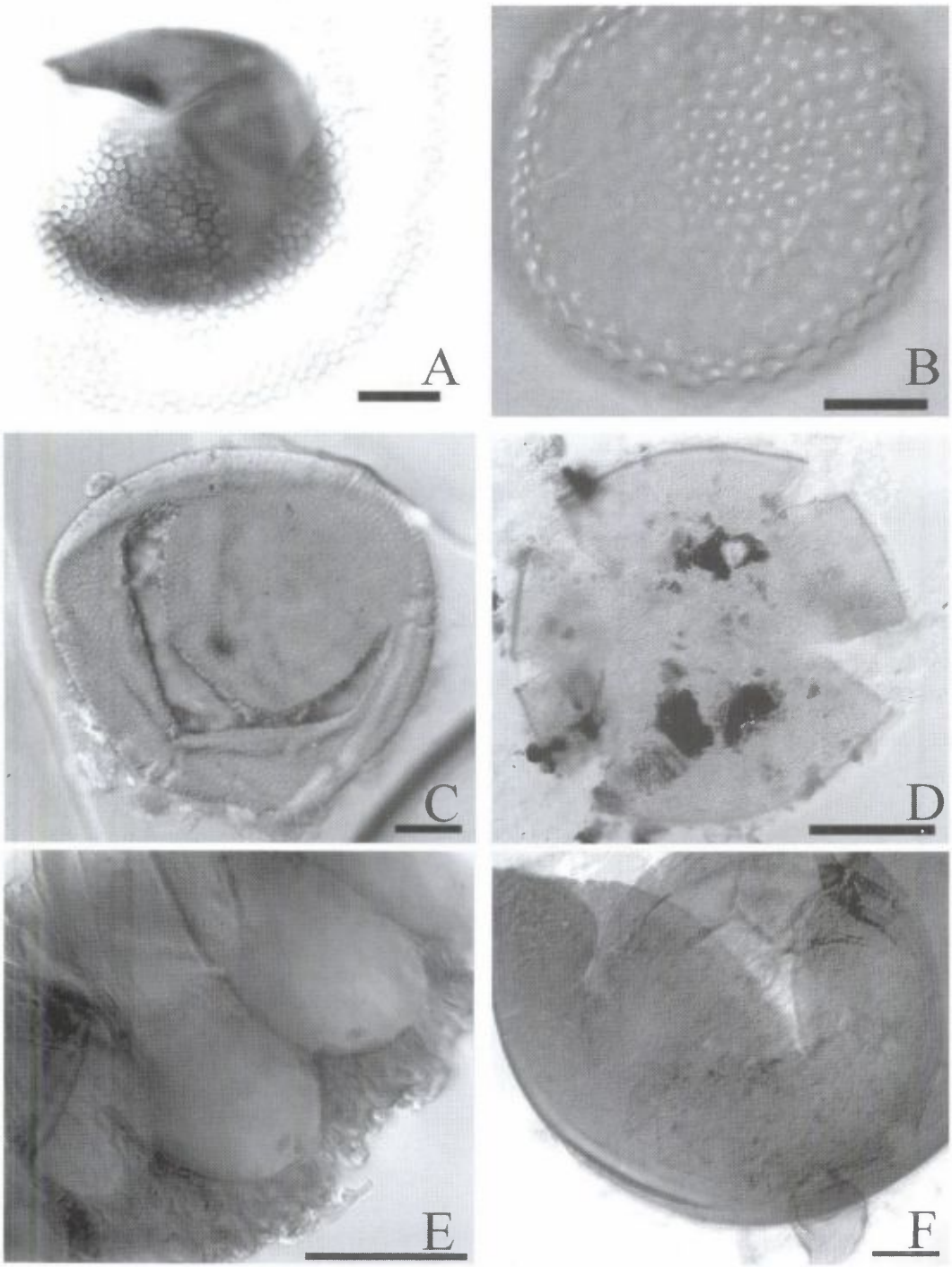

Figure 4. A–F: AM fungal spores: A: *Acaulospora denticulata*, B: *A. scrobiculata*; C: *A. rehmii*; D: *Entrophospora infrequens*; E: *Glomus sinuosum*, detail of sporocarp peridium; F: *Scutellospora biornata*. Scale bar: A, C: 20 µm; B: 30 µm; D: 40 µm; E, F: 50 µm.

fungus can frequently alter the root system morphology as in the case of *Boletinus meruloides* (Schwein.) Murrill and *Fraxinus americana* L. (Gruhn et al., 1992). In that particular study, it was observed that production of indolae-3-acetic acid (IAA) by the fungus was able to enhance tree growth, and this explains why the fungus is consistently found near *F. americana* in the field (Cotter, 1987). However, Cotter and Miller (1985) were unable to

demonstrate that *B. meruloides* could form ectomycorrhizae in pure culture synthesis with *Fraxinus*.

In contrast to the mycorrhizal habits of most Boletales, it has been previously suggested that the genus *Phlebopus* comprises either non-ECM or facultative ECM species (Singer, 1986; Bougher, 1995). An example of the second group is *P. sudanicus* Har. & Pat., an African species, which under experimental conditions formed an ECM mantle with exotic species of *Acacia* and *Pinus caribaea* (Thoen and Duccouso, 1989), but did not form ECM with native tree species. *Phlebopus bruchii* had a different behavior to *P. sudanicus*, and did not form ECM symbiosis with *Pinus elliottii* seedlings under greenhouse conditions (Nouhra, 1998). It has been also demonstrated that artificial stimulation of the dichotomous branching in pine roots (Turner, 1962), or mantle development in a mycorrhizal host, are not sufficient to accurately determine the ecological role for a fungus species (Gruhn et al., 1992).

Other species within the genus *Phlebopus* were able to develop peculiar root associations (not mycorrhizal) with parasitizing insects, in one case linking the ash tree (*Fraxinus* sp.) roots to *Boletinellus meruloides* and aphids (Brundrett and Kendrick, 1987), in another involving a complicated case of symbiosis, epibiosis and parasitism with *P. tropicus*, *Citrus* plants and the Pseudococcideae *Pseudococcus comstocki* (Singer, 1986).

Most recently, the nutritional mode found in the *Phlebopus* and *Boletinellus* species was considered to be an exceptional example of a transition between mutualists and parasites in the homobasidiomycetes (Binder and Bresinsky, 2002). These authors placed both genera in the new Family Boletinellaceae, on the basis of the molecular data, morphological characters, similar nutritional requirements and habitat preferences. However, based upon the above observations, the ectomycorrhizal symbiosis seems to be the exception and not the rule within *Phlebopus* species.

Fagara coco, as well as many other Rutaceae species (Graham and Sylvertsen, 1985; Graham, 1986), has also shown AM colonization. A recent analysis of another *Fagara* species (*F. heitzii*), has shown an arbuscular mycorrhizal status (Wang and Qiu, 2006). In the present study, we have reported the presence of the simultaneous development of both types of AM colonization, the *Arum*- and *Paris*-types. In some samples, these two types of colonization were present and well represented within the same root system. The co-occurrence of both morphologies of AM has also been observed by Mayumi et al. (2005) and Bonfante-Fasolo and Fontana (1985), in association with different host species.

The Glomalean taxa, *Acaulospora scrobiculata*, *A. denticulata, Entrophospora infrequens, Scutellospora biornata* and *Glomus sinuosum* have been previously reported in Central Argentina (Lugo, 1995; Menéndez et al., 2001; Lugo and Cabello, 2002), while *A. rehmii* is known in Northern Argentina. Further studies are necessary in order to elucidate which species colonize *F. coco*. It is already known that the presence of *Arum*- and *Paris*-type colonization is not solely dependent on plant families or root structure, but also strongly influenced by the colonizing fungus (Dickson, 2004).

Seasonal variations in AM colonization have been frequently reported in the literature (Sanders and Fitter, 1992; DeMars and Boerner, 1995; Lugo et al., 2003). The highest mycorrhizal colonization rates occurring in roots of *F. coco* during summer and autumn, and being this tendency also observed for herbaceous species in the region, probably influenced by the high levels of soil moisture present at these times (Lugo et al., 2003). Other results suggest that the patterns of mycorrhizal colonization are host dependent and may be related to the physiology and phenology of host plants (Smith and Read, 1997).

In conclusion, as would be expected for a member of Rutaceae (Brundrett, 2002; Trappe, 1987), our results showed that *F. coco* is colonized by AM fungi in the field. However we found no evidence that F. *coco* forms an ECM association with *P. bruchii*, neither for field observations nor for synthesis studies.

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