CHAPTER FOUR

Divergent communities of arbuscular mycorrhizal fungi on *Terminalia amazonia* and *Urochloa*

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) are an ubiquitous component of most tropical forests (Read 1991), yet their role in forest dynamics, plant community composition, and forest recovery from disturbance remains poorly understood. Perhaps as much as 20% of net carbon fixed by the plant is allocated to the belowground biomass of the AM symbiont in exchange for phosphorus (Jakobsen and Rosendahl 1990) and other poorly-mobile nutrients in soil. Variations in benefit and function across AM plant and fungal taxa are only beginning to be characterized (Fitter 2005).

AM fungi are generally considered to lack host specificity. Under greenhouse conditions, many AM fungal species can be grown in single-species cultures on a handful of host plant species (Smith and Read 1997). However, the few field studies to date using molecular markers to identify AM fungi *in planta* have consistently observed different AM fungal species colonizing co-occurring plant species (e.g., Helgason et al. 2002, Vandenkoornhuyse et al. 2003, but see Stukenbrock and Rosendahl 2005).

Because of the apparently low specificity of the AM symbiosis, multiple researchers have posited the existence of a common mycorrhizal network linking plants and fungi within a community (Francis and Read 1994). Hyphal linkages by AM fungi between plants have been observed both in the greenhouse and field, but the extent to which conspecific and interspecific plants are connected by these hyphae to form a common mycorrhizal network

in the field is not known (Simard and Durall 2004). Experimental evidence from greenhouse studies suggests common mycorrhizal networks can have consequences for outcomes of competition, access to nutrient pools, and maintenance of plant and AM fungal diversity in terrestrial ecosystems (e.g., Grime et al. 1987, Moora and Zobel 1996).

Plants can acquire mycorrhizas either through colonization by AM fungi growing as mycelia from adjacent plants, from germinating spores, or from fragments of mycorrhizal roots or hyphae in the soil (Smith and Read 1997). Several authors have suggested that seedlings are typically colonized by an existing mycorrhizal network (Newman 1988, Kytoviita et al. 2003, van der Heijden 2004). The seedlings could presumably gain the benefits of mycorrhizal symbiosis while having the costs in photosynthate subsidized by established plants (Perry et al. 1989). Transfer experiments show clearly the ability of the fungal symbiont to redirect carbon from the donor plant to hyphae in and surrounding the recipient (Grime et al. 1987, Graves et al. 1997, Lerat et al. 2002).

Historically, determining the identity and distribution of AM fungi occurring as mycorrhizas has been difficult, since AM fungal species are generally only readily distinguishable as spores (Merryweather and Fitter 1998). With the development of molecular markers for AM fungal species, the identity of fungal symbionts and the potential for mycorrhizal networks can now be directly assessed in natural plant populations.

In this study, I used variation in the internal transcribed spacer (ITS) region of fungal ribosomal RNA genes to identify species of AM fungi colonizing the root systems of plants in the field. I compared AM fungal species composition between recently established seedlings of the native tree

species *Terminalia amazonia* (J. F. Gmel.) Exell in reforestation plots in pastures, neighboring individuals of *Urochloa ruziziensis* (R. Germ. & Evrard) Crins and *U. decumbens* (Stapf) R.D. Webster, and individuals of *T. amazonia* found in adjacent forest. *T. amazonia* seedlings in pastures had been inoculated at the time of outplanting with either forest or pasture soil.

I hypothesized that *T. amazonia* planted into pasture would either 1) support an AM fungal community similar to the community found on forest *T. amazonia*, if host specificity is an important determinant of symbiotic partners; or 2) support an AM fungal community similar to the community found on pasture grasses, if the pasture environment or common mycorrhizal network are important determinants of symbiotic partners. If host specificity was important yet forest conversion to pasture had eliminated AM fungal species that associate with *T. amazonia*, I expected *T. amazonia* seedlings inoculated with forest and pasture soil to 3) support divergent AM fungal communities.

SITE DESCRIPTION

Fieldwork was conducted at three pairs of adjacent pasture and forest sites at ~850 m of elevation on farms <1 km apart in Siete Colinas, Coto Brus, Costa Rica (8°53' N, 82°57' W; Figure 1.1). Soils are Andepts (Vásquez Morera 1983). The region receives 3000-4000 mm of rainfall yearly, concentrated in a wet season from May-December (Coen 1983). Forest sites were located in two fragments (~2 ha and 7 ha in size) of old-growth Tropical Premontane Wet Forest (Tosi 1969). The largest canopy trees in the forest fragments were *T. amazonia*; their roots extended throughout the forest sites (*personal observation*). In soils from these sites, *T. amazonia* seedlings were shown to require colonization by AM fungi in order to grow (Chapter Three).

Pasture sites were cleared by settlers ca. 1975; they were intermittently burned, but never plowed (José Ramírez and Juan Cascante, *personal communications*; aerial photos). At the time of this study, the pasture sites were dominated by the non-native grass species *Urochloa ruziziensis* (known locally as guinea rastrea). A second non-native grass species, *U. decumbens* (known locally as cumbre) was a co-dominant at the Ramírez site (*unpublished data*). Both species are arbuscular mycorrhizal (Howeler et al. 1986).

METHODS

Establishment of experimental seedlings of T. amazonia in pasture

In July-August 1997, 100 seedlings and 100 stakes (defined below) of *T. amazonia* were planted ~2 m apart in 20 or 25 rows of 8-10 plants each in forest and pasture at each of the three sites (seedlings were part of experiment described in Chapter Two). Seedlings had been previously inoculated with forest or pasture AM fungi by germination in planting boxes filled with pasture or forest soil that was collected from each field site and sown with locally-collected *T. amazonia* seeds in April 1997. Stakes were produced immediately prior to outplanting from one-year-old seedlings grown in a common garden, by pruning each seedling down to ~15 cm each of stem and taproot (Plate 4.1). Stakes were inoculated, and seedlings were reinoculated, with AM fungi by planting in 20 × 20 × 20 cm pits filled with forest or pasture soil; soil came from planting holes for experiment in Chapter Two, and was mixed within, but not across, sites ≤3 d prior to planting.

Plate 4.1 One-year-old seedlings of *Terminalia amazonia* before (left) and after (right) transformation to stakes, which entailed removing all parts except ~15 cm each of stem and taproot.

Plate 4.2 Pasture at Cascante site, Siete Colinas, Coto Brus, Costa Rica at time of root sampling for AM fungi (with Verónica Delgado and Cileny Cascante, June 1999; photo by author).

Sample collection

Root sampling was conducted between 9 May and 14 June 1999 on randomly chosen individuals within a contiguous area of ~800 m^2 at each forest and pasture site. Each sample was collected from a single position on each individual's root system. Individuals sampled within the forest were naturally-occurring and reflected the age-structure of the *T. amazonia* population at these sites (*unpublished data*): most were seedlings (<1 cm in diameter at base) at the time of sampling, with a few saplings (5-20 cm in diameter at base) and emergent trees (>100 cm in diameter at base). At each forest site, 11 individuals of *T. amazonia* were sampled (33 individuals in total). Eight individuals of *Urochloa ruziziensis* were sampled in pasture at each site, and eight of *U. decumbens* were sampled in pasture at the Ramírez site (32 individuals in total). Individuals of *T. amazonia* sampled in the pastures (Plate 4.2) were mostly stakes and seedlings planted two years before. Seven to nine individuals were sampled from each inoculation treatment in each pasture (44 individuals in total). In addition, a root sample was taken from each of three saplings that were the only individuals observed to have regenerated naturally, and which were located at the Ramírez site. Including these saplings, a total of 47 individuals of *T. amazonia* were sampled in pasture. Root samples were dried at room temperature at low humidity and stored at -80 °C.

DNA extraction

Root samples were transported from Cornell University to Pennsylvania State University on dry ice and stored at –20 °C for ≤3 mo prior to molecular analysis. A single root fragment was selected at random from each root

sample, weighed, and thawed in the presence of desiccant to avoid water condensation. Root fragments ranged in mass from 2-9 mg.

DNA was extracted from root fragments using the UltraClean Microbial DNA Kit (MoBio Laboratories, Carlsbad, California, USA), with the following changes to the manufacturer's protocol. To grind root fragments, five 2.4-mm dm zirconia beads (Biospec Products, Bartlesville, Oklahoma, USA) were substituted for the garnet beads supplied with the kit. Root fragments were shaken at the highest speed for 10 m (Vortex Genie-2 with adapter, MoBio Laboratories) prior to addition of MicroBead, MD1, and 50 μL of IRS solutions. Root fragments were shaken again for 15 m. Centrifugation times during extraction steps of the protocol were doubled. DNA extracts were kept at –20 °C in manufacturer's storage buffer (MD5) for ≤ 7 d.

Selective amplification of ITS region by nested PCR

The ITS region was amplified by nested polymerase chain reaction (PCR), using primers SSU-Glom1 and LSU-Glom1 (Renker et al. 2003) in the first PCR and fluorescently-labeled universal fungal primers ITS4 and ITS5 (White et al. 1990) in the second. ITS4 was labeled with 5'NED (Applied Biosystems, Foster City, California, USA) and ITS5 with 5'6-FAM (Operon, Alameda, California, USA). While SSU-Glom1 is a general primer compatible with many eukaryotes, LSU-Glom1 appears to be specific to all glomeromycetes for which ITS sequence data are currently available (Renker et al. 2003; *unpublished data*) and also some basal orders in the Basidiomycota (Aphyllophorales, Auriculariales, Cystofilobasidiales, Stereales, Tremellales, and Trichosporonales). Consequently, the first PCR was designed to amplify the ITS region of all glomeromycetes, as well as that of root fungi belonging to

basal orders of the Basidiomycota, while excluding amplification of DNA of other root fungi and plants.

For the first PCR, each reaction occurred in a total volume of 50 μL, containing 0.4 mM dNTPs, 3.5 mM $MgCl_2$, 0.8 μg/μL bovine serum albumen, 2.5 units of TEMPase DNA polymerase (GeneChoice, Frederick, Maryland, USA), TEMPase reaction buffer I, 30 pmol of each primer and 10 μL of genomic DNA extract. Hot start PCR was performed on an MWG Primus 96 thermocycler (MWG Biotech AG, Ebersberg, Germany) for 35 cycles (95°C for 10 m to activate polymerase; four cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 48 s; 31 cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 40 s; a final extension period at 72°C for 10 m). Lid temperature was held constant at 102°C. To confirm amplification, 5 μL of products from the first PCR (PCR1 products) were loaded on a 2% agarose gel, run at 120V for ~45 min, stained with ethidium bromide, and photographed on a UV transilluminator (VWR International, West Chester, Pennsylvania, USA) using the Kodak EDAS 290 digital imaging system and Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, New York, USA).

To reduce further amplification of DNA from non-AM fungi, PCR1 products were digested with the restriction endonuclease *Alu*I (New England Biolabs, Beverly, Massachusetts, USA). The ITS region of most AM fungi lacks an *Alu*I cut site, while the ITS regions of other fungi likely to have been amplified during the first PCR typically contain multiple *Alu*I cut sites (Renker et al. 2003, *unpublished data*). The only glomeromycetes likely to be consistently eliminated by this restriction digest are the Paraglomeraceae and some members of the Archaeosporaceae (Renker et al. 2003; *unpublished data*). Five μL of PCR1 products from each reaction were incubated at 37 °C for 4 h

with 4 units of *Alu*I and the manufacturer's digestion buffer in a total volume of 10 μL.

Depending on visibility in the agarose gel, *AluI* digest products were diluted 15- to 50-fold prior to the second PCR. The same protocol was followed for the second PCR as for the first, except the amounts of polymerase and DNA template used for each reaction were halved. Products of the second PCR (PCR2 products) were visualized as described for the first PCR. Of 130 reactions conducted with root fragments, only one failed to yield amplification products. Amplification products were never observed in negative controls (PCR1 and PCR2 with sterile water substituted for DNA template).

PCR2 products were purified using the UltraClean PCR Clean-Up Kit (MoBio Laboratories) and following the manufacturer's protocol, except purified DNA was stored in 50 μL of sterile water rather than elution buffer. Purified PCR2 products were stored for ≤3 d at 4 °C.

Terminal restriction fragment length polymorphism analysis

Two 5-μL aliquots of purified PCR2 products from each reaction were digested separately with 2 units of *Hin*fI and 1 unit of *Dpn*II (New England Biolabs) and the manufacturer's digestion buffers in a total volume of 10 μL at 37 °C for 4 h. Each completed digest was diluted 15- to 50-fold in sterile water, depending on the visibility of the unpurified PCR2 products in an agarose gel, and stored overnight at 4 °C. One μL each of restriction digest products and purified PCR2 products from each reaction were submitted for automated sequence-length analysis by capillary gel electrophoresis (Nucleic Acid Facility, Pennsylvania State University, University Park, Pennsylvania, USA). Lengths of terminal restriction fragments and PCR2 products (Figure

4.1) were verified using Genescan Analysis Software, Version 3.7 (Applied Biosystems). To assess the variability in AM fungal types present within each root sample, the ITS region was amplified and digested for 1-2 additional root fragments for each of ten individual plants.

The identities of AM fungi detected in roots were determined by comparing lengths of TRFs and PCR2 products with lengths obtained from three sources for known AM fungal species. First, lengths of PCR2 products and TRFs were determined for 49 spores from 18 species (1-10 spores per species) collected from field samples and trap cultures from these field sites. Spores from these samples had been previously identified by the author, using morphological characteristics, at the International Culture Collection of (Vesicular) Arbuscular Mycorrhizas (INVAM, West Virginia University, Morgantown, West Virginia. USA). Second, lengths of PCR2 products and TRFs (*Hinf*I only) were determined for 131 spore samples from ~35 species in a study using the same methods at field sites in Zimbabwe (Y. Lekberg, *unpublished manuscript*). Lastly, virtual lengths of PCR2 products and TRFs were determined by the author for 435 ITS sequences derived from spores of 37 species and acquired through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank).

Phylotypes from roots were considered to match each other and to match knowns if they differed by <3 base pairs (bp) in length for three of four data points (Figure 4.1). This criterion was established based on variation in lengths of PCR2 products and restriction fragments between spores within a single species in the knowns databases (*personal observation*). Most root fragments contained multiple unknowns. Phylotypes for these samples were

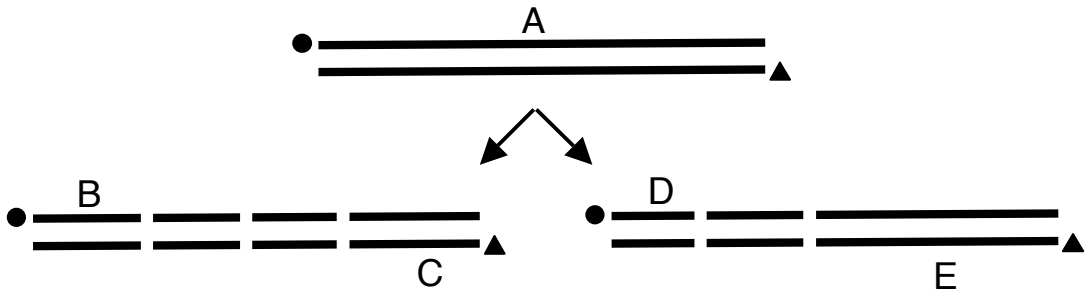

Figure 4.1 Diagram of lengths of DNA used to distinguish fungi in plant roots. Only sequences linked to fluorescent tags (● and ▲) were detectable. Each phylotype was defined by four data points: its PCR2 product length (A), and lengths of terminal fragments after digestion with *Hinf*I (B and C) and with *Dpn*II (E). The length of 5' terminal fragment D did not vary. In this example, two hypothetical cut sites are shown for each restriction enzyme.

determined by comparing samples with multiple phylotypes to samples with single phylotypes. In a handful of cases, phylotypes were inferred through the consistent co-occurrence of data points. Two programs assisted in matching phylotypes, the T-RFLP Phylogenetic Assignment Tool (PAT; Kent et al. 2003; http://trflp.limnology.wisc.edu/) and the Good-Enough RFLP Matcher (GERM, Dickie et al. 2003). AM fungal taxa were found to have consistent differences in lengths of PCR2 products and TRFs (Y. Lekberg, *unpublished manuscript*). Consequently, even AM fungal phylotypes that did not match known species could still be recognized as belonging to a particular genus.

Because not all contaminant fungi were eliminated by the *Alu*I digest (Renker et al. 2004), each phylotype was also compared with a database of non-AM fungi. Approximately 40% of amplified sequences more closely matched non-AM fungi than known AM fungi and were excluded from further analysis (*data not shown*). Only 12 amplicons representing five phylotypes did not resemble either known AM or non-AM fungi; they were also excluded from further analysis.

Statistical analyses

Phylotype accumulation curves and phylotype richness estimates were calculated using EstimateS version 7.5 (Colwell 2005). Similarity of phylotype composition between root fragments was also calculated in EstimateS, using the Sørensen Similarity Index (SSI):

$$SSI = 2q/(a + b)$$

where q = the number of shared phylotypes between two root fragments, a = the number of phylotypes in one root fragment, and b = the number of phylotypes in the other (Magurran 1988). Statistical tests were conducted in JMP version 3 (SAS Institute 1994).

Differences in the AM fungal communities colonizing the four plant types were evaluated with indicator species analysis (Dufrêne and Legendre 1997), using PC-ORD (McCune and Mefford 1997) to calculate indicator values for each phylotype:

$$IV_{kj} = 100\ (RA_{kj} \times RF_{kj}),$$

where RA = relative abundance, RF = relative frequency, k = plant type and j = phylotype. The null hypothesis that the highest indicator value (IV_{max}) for each phylotype was no larger than would be expected by chance was tested by a Monte Carlo method with 1000 randomizations (McCune and Grace 2002).

RESULTS

Summary of AM fungi detected in roots

In total, 168 AM fungal amplicons representing 41 phylotypes were detected in the 115 plants sampled (Table 4.1). Of these phylotypes, 31 were characterized as distinct phylotype groups (corresponding approximately to species) –based on comparisons with databases of known species– while 10 appeared to be variants within these groups. Thirteen of the 41 phylotypes (32%) were detected only once. The phylotype groups (henceforth referred to as phylotypes) were distributed among five of the six AM fungal groups for which ITS sequence data are available: *Glomus* (21), *Acaulospora/Entrophospora* (6), *Scutellospora* (2), *Gigaspora* (1), and *Archaeospora* (1); as expected with this technique, no members of the genus *Paraglomus* were detected. There was no difference among plant types (forest *T. amazonia*, pasture *T. amazonia* exposed to forest soil, pasture *T. amazonia* not exposed to forest soil, and *Urochloa*) in genera detected at the Cascante and Delgado sites ($\chi^2_6 = 6.395$; $P = 0.3804$). At the Ramírez site,