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Research Article

Differences in Arbuscular Mycorrhizal Fungi among Three Coffee Cultivars in Puerto Rico

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Mycorrhizal symbiosis is important for growth of coffee (*Coffea arabica*), but differences among coffee cultivars in response to mycorrhizal interactions have not been studied. We compared arbuscular mycorrhizal (AM) extraradical hyphae in the soil and diversity of AM fungi among three coffee cultivars, Caturra, Pacas, and Borbón, at three farms in Puerto Rico. Caturra had significantly lower total extraradical AM hyphal length than Pacas and Borbón at all locations. P content did not differ among cultivars. Extraradical hyphal lengths differed significantly among locations. Although the same morphotypes of mycorrhizal fungal spores were present in the rhizosphere of the three cultivars and total spore density did not differ significantly, frequencies of spore morphotypes differed significantly among cultivars. Spore morphotypes were typical of *Glomus* and *Sclerocystis*. Levels of soil nutrients did not explain differences in AM colonzation among cultivars. The cultivar Caturra is a mutant of Borbón and has apparently lost Borbón's capacity to support and benefit from an extensive network of AM hyphae in the soil. Widespread planting of Caturra, which matures earlier and has higher yield if fertilized, may increase dependence on fertilizers.

1. Introduction

Mycorrhizae play an important role in growth and development of wild and cultivated plants. Genetic variation within plant species can influence both the degree of root colonization by mycorrhizal fungi and the response of the plant to mycorrhizal symbiosis [1]. For example, *Citrus* cultivars differed in their degree of dependence on AM fungi from strongly dependent to nondependent [2]. In addition, inbred lines of maize differed in the degree to which they supported AM fungi [3, 4]. Selection of host plant germplasm to maximize productivity and pest resistance or responsiveness to fertilization can affect the ability of the host to sustain or benefit from mycorrhizae [5, 6].

Most classic studies on interactions between cultivars and AM fungi used colonization of mycorrhizal fungi within the root to quantify the fungal part of the symbiosis. However, Miller et al. [7] found that mycorrhizal root colonization was poorly correlated with plant growth, whereas extraradical hyphal length in the soil around plants was a good predictor of plant growth. Extraradical AM hyphal length indicates the amount of contact between AM fungi and soil, where nutrient uptake occurs. Consequently, extraradical hyphal length is recognized as an important aspect of the symbiosis [8, 9] and has become a common measure of AM mycorrhizal colonization [10].

Coffee is a key crop in the economy of many developing countries [11]. In Puerto Rico approximately 38,500 acres of coffee with a market value of $42 million were harvested in 2007 [12]. Caturra, one of the most widely planted coffee cultivars in the Americas, is more nutrient-demanding and requires more fertilization than other cultivars [13–15]. Coffee was shown to be mycorrhizal over a hundred years ago, but its mycorrhizal interactions are still poorly understood [16].

The objective of this study was to determine whether coffee cultivars differ in their interactions with AM fungi. Our hypothesis was that some coffee cultivars (*i.e.*, Caturra) sustain less extraradical hyphae of AM fungi and therefore benefit less from mycorrhizal symbiosis than other cultivars. We compared extraradical hyphal lengths of AM fungi among three coffee cultivars in three plantations. Diversity of AM spore morphotypes in the rhizosphere was compared among cultivars grown at the same location to determine if there were differences in specificity. Soil and leaf chemistry were analyzed to test whether differences in extraradical AM hyphae reflected differences in soil and if they affected plant nutrient status.

2. Materials and Methods

2.1. Study Sites and Soil Samples. Soil samples associated with Caturra, Pacas, and Borbón cultivars were collected from three different sites in Puerto Rico. Samples were collected at Serrallés farm (Ponce), at Rullán farm (Adjuntas), and at Limaní (the UPR Agricultural Experimental Station, Adjuntas). These sites were chosen for uniformity in plant age, environment, and management. *Coffea arabica* plants sampled were 6–10 years old in all farms and cultivars, and all were cultivated as sun coffee. All plots had been last fertilized at least three months before sampling; other agrochemicals were not applied. The Rullán farm only had Caturra and Pacas cultivars, but all three cultivars were present at the other two farms.

A total of six soil samples per cultivar were collected on each farm as follows. Six rows were randomly selected from the area planted to each cultivar. Each sample consisted of five pooled soil cores; each core was 2.5 cm in diameter and 10 cm deep. The first core was taken at the midpoint between two neighboring plants in a row, and the other four were taken 15 cm from the first, forming an X. The soil was collected in plastic bags and transported to the laboratory on ice.

2.2. Soil Analysis. The pH was measured for each sample from Serrallés and Rullán farms using 3.0 g of soil mixed with 3 mL of ultrapure water. Dry to wet weight ratios were estimated using 20 g of fresh soil that was oven dried at 65°C for 7 days. Samples for mineral analysis were air-dried to constant weight and passed through a sieve with 2 mm mesh. Mineral content was determined at the Chemistry Laboratory of the International Institute of Tropical Forestry, USDA Forest Service. Available (extractable) mineral nutrient concentrations were estimated on a V-Beckman Spectraspan direct current plasma-atomic emission spectrometer using USDA IITF analytical lab protocols [17]. Ca and Mg were extracted using 1 M KCl, while extractable P, K, and Mn were extracted using a modified Olsen solution (0.025 N $NaCO_3$, 0.01 M EDTA, 0.01 N NH_4, 0.1 g Super Floc, and distilled water to 2 L [18]). Total N was determined using the modified Kjeldhal method to include nitrate and nitrite [19]. Soil nutrient data were analyzed using one-way ANOVA, followed by Tukey-Kramer HSD tests [20].

2.3. Leaf Collection and Elemental Analysis. To test whether differences in mycorrhizae were correlated with differences in leaf nutrient content, ten leaves were collected from the fourth branch from the bottom of the two plants adjacent to each soil sample. Leaves were air-dried and ground using a Wiley Mill (1 mm opening) [19]. For each sample, 1 g leaf was digested for 16 hr with 8.0 mL of concentrated HNO_3. After 16 hr the solution was heated at 55–65°C for approximately 30 min, let stand until warm and 4.0 mL of H_2O_2 was added. After 1.5 hr, 2.5 mL of HCl was added to the digest and the volume was brought to 100 mL using distilled water. The solution was filtered through Whatman #4 filter paper and analyzed using a Beckman DCP-AES model Spectraspan V plasma spectrophotometer. Leaf nutrient data were analyzed using one-way ANOVA followed by Tukey-Kramer HSD tests [20].

2.4. Extraction of Extraradical Hyphae. The soil cores were broken, homogenized manually and root fragments were removed. Hyphae were extracted as described [21]. 5 g soil was placed in a 600 mL beaker with 250 mL distilled water and 31 mL Graham salt solution (35.7 g/L sodium hexametaphosphate). The suspension was sonicated 5 min, stirred 1 min, and left to soak 12 h at room temperature. After soaking, the suspension was stirred at high speed until the vortex reached the bottom, and then at a reduced speed so the vortex reached halfway to the bottom. Aliquots (6 mL) of suspension were taken from just below the surface halfway to the center of the beaker, and were transferred to a new beaker containing 31 mL Graham salt solution and 250 mL water. The solution was stirred at high speed again to resuspend the hyphae, and two 10 mL aliquots were centrifuged for 8 min at 2200 rpm (1000× g). The pellet was resuspended in 10 mL glycerol (50%) and centrifuged at 600 rpm for 30 sec. The suspension was vacuum-filtered using a Millipore apparatus and the hyphae were collected on a 25 mm diam 0.8 μm cellulose-nitrate filter.

2.5. Estimation of Hyphal Lengths. The filters with hyphae were mounted on glass slides with immersion oil and observed at 400x [21]. A line-intercept method was used to estimate hyphal lengths [22, 23]: six horizontal and six vertical transect lines spaced about 3.5 mm apart were scanned, and the number of intercepts with AM fungal hyphae was recorded. The total filtration area was 227 mm^2.

2.6. Spore Extraction. For the samples from Serrallés farm, AM fungal spores were extracted from 100 g soil by the wet sieving and decanting method described by Pacioni [24]. Spores were trapped on sieves with 120, 90, 70, and 45 μm openings and washed with 10 mL of distilled water. Spores were collected with a spatula, resuspended in 2 mL of water, and maintained at 20°C until counted. A total of 1 mL of each spore suspension was observed by examining 100 aliquots of 10 μL under a microscope at 200x.

Spores were classified into seven morphotypes based on size, color, and shape: yellow globose, yellow ovoid/ellipsoid, brown globose, brown ovoid/ellipsoid, brown elongated with narrow base (i.e., *Sclerocystis*), white ovoid to ellipsoid, and white globose [25]. The white ovoid/ellipsoid and white globose morphotypes were included in the analysis of total spores per cultivar, but not in individual statistical analyses because they seemed to be immature spores of the other morphotypes.

2.7. Data Analysis. Data for hyphal lengths, soil nutrient concentrations, and leaf elements were normally distributed. Differences among cultivars and replicates (farms) were analyzed by 2-way unbalanced ANOVA [26]. Frequencies of spore types in the three cultivars were analyzed by chi-square tests for independence.

3. Results

3.1. Extraradical Hyphal Length. Mean hyphal length was lowest in Caturra at all three farms, and highest in Borbón on the two farms where it was grown (Figure 1). For the three sites combined, differences among cultivars in mean total extraradical hyphal length were significant ($P < 0.0001$); differences were also significant within each site (Serrallés, ANOVA, $P < 0.001$; Limaní, $P < 0.001$; and Rullán, $P < 0.05$). Total mean hyphal lengths in each cultivar were similar at Serrallés and Limaní, but were three to four orders of magnitude lower at Rullán (Figure 1).

3.2. Spore Abundance and Classification. Most AMF examined had yellow, brown, or white globose spores greater than 54 μm, characteristic of *Glomus* species [25, 27]. All of the *Glomus* spore morphotypes were recovered from soil beneath each of the three coffee cultivars. Brown, elongated spores (typical of *Sclerocystis* sp.) were also found in Borbón and Pacas, but not in Caturra (Figure 2). Frequencies of spore morphotypes varied significantly among cultivars ($X^2 = 129$; $P < 0.0001$). Caturra had the highest counts for five of the morphotypes, but not white globose spores (Figure 2). There was no significant difference in the density of spores (combined) among the three cultivars ($P > 0.18$; Borbón = 172.0, Caturra = 62.0, Pacas = 78.2 spores/g soil). Five spores with typical *Glomus* morphology were assigned to *Glomus* group A as defined by Schwarzott et al. [28] on the basis of nuclear ribosomal ITS sequences, grouping with *G. sinuosum* and *G. manihotis* (data not shown).

3.3. Soil Chemical Analyses. Soil pH values were similar among cultivars at Serrallés (pH 5.0) whereas the pH ranged from 4.4 under Caturra to 5.5 under Pacas at Rullán. Mg was the only soil mineral nutrient measured that differed significantly among cultivars ($P = 0.006$) (Table 1); Mg concentrations were highest in soil under Caturra at all three sites and lowest in Pacas at Limaní and Rullán. Significant differences were found among sites for all mineral nutrients ($P < 0.0001$ for P, Mg and Mn; $P = 0.04$ for Ca). Serrallés had approximately double the concentration of Olsen total P as the other two sites, while Rullán had the highest concentrations of soil Mg and Mn. Soil C and N content differed significantly among cultivars ($P < 0.002$ for C and $P < 0.005$ for N) (Table 1) and also among sites ($P < 0.001$) (two-way ANOVA); there was no interaction between cultivar and site. At both Limaní and Serrallés, soil from Caturra had the highest percentages of C and N and soil from Borbón the lowest (Table 1); however, C and N levels in all cultivars were sufficient to ensure that they were not limiting factors for plant growth. Serrallés had the highest percentages of soil C and N and Rullán the lowest for all the three cultivars.

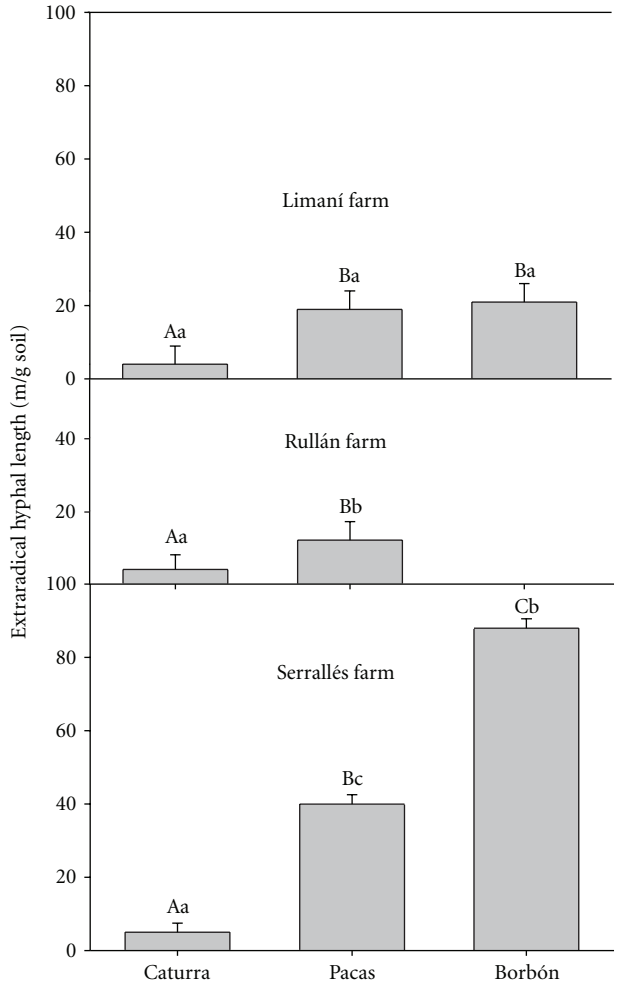

Figure 1: Extraradical hyphal length per gram of soil associated with coffee cultivars at three farms in Puerto Rico. Cultivars within each location were significantly different. Capital letters represent comparisons within locations and small letters differences within each cultivar among locations; bars with different letters are significantly different. Error bars show +1 s.d.

3.4. Leaf Chemical Analysis. All nutrients analyzed in leaves showed significant differences among cultivars at Limaní ($P < 0.02$, Table 2); all but P also differed significantly at Rullán. Leaves of Borbón were significantly higher in P than

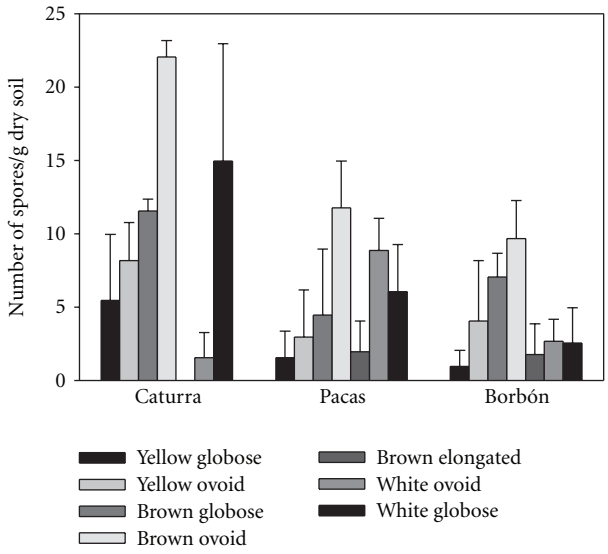

Figure 2: Abundance (number g^{-1}) by morphotypes of AM fungal spores associated with coffee cultivars at Serrallés farm. For each cultivar the sequence of bar represents seven spore morphotypes: yellow globose, yellow ovoid/ellipsoid, brown globose, brown ovoid/ellipsoid, brown elongated with narrow base (i.e., *Sclerocystis*), white ovoid/ellipsoid, and white globose. Chi-square tests for independence showed significant difference in morphotype distribution among cultivars ($X^2 = 129$; $P < 0.0001$). Error bars show +1 s.d.

leaves of Caturra and Pacas at Limaní (Table 2). These differences support the hypothesis that Caturra is deficient in AM interactions compared to Borbón; they are not attributable to soil nutrient levels because differences in soil P were not significant. Other nutrients were also lower in leaves of Caturra; for instance, Ca and Mn were significantly higher in Pacas at Limaní. However, AM symbiosis is not important in their uptake as it is for P [29]. Since other soil minerals (including P) did not differ significantly among cultivars, differences among cultivars in AM extraradical hyphal length and in leaf nutrient concentrations are not attributable to levels of these minerals.

4. Discussion

4.1. Variation in Mycorrhizal Associations among Cultivars. Many common *Coffea arabica* cultivars were derived from Typica and Borbón [15]; the cultivars Caturra and Pacas are mutants of Borbón. Thus the three cultivars used in this study are very closely related. Although Caturra yields well and produces high quality coffee, it requires more fertilization than Pacas or Borbón [15]. *Coffea arabica* is usually dependent on arbuscular mycorrhizal fungi [16, 30]. We found that extraradical hyphal lengths were significantly lower in Caturra than in other cultivars at all three sites (Caturra < Pacas < Borbón), showing for the first time that coffee cultivars differ quantitatively in their mycorrhizal symbiosis. It appears that genetic changes associated with the derivation of the Caturra and Pacas cultivars from Borbón reduced their abilities to support extraradical hyphal production by AM fungi.

Variation among cultivars and genotypes in dependence on AM fungi has been found in many crop plants [2, 3, 31–35]. Breeding and selection programs often include fertilization regimes that might be expected to select against ability to form mycorrhizae [5, 6]. Similarly, breeding for resistance to diseases might be expected to select for resistance against mycorrhizal fungi [36, 37]. Several studies have supported this idea, showing that new cultivars have less extensive mycorrhizal colonization than older cultivars. In winter wheat, breeding for high productivity with heavy fertilization may have been responsible for the loss of dependency on AM fungi [38]. In breadfruit, recently derived cultivars showed lower AM colonization than older cultivars and wild populations [39].

In contrast, in other cases new cultivars showed higher mycorrhizal colonization than older cultivars. Cultivated varieties of tomato were found to be more responsive to AM fungi than wild accessions [32, 33]; a similar relationship was found in oats [40]. In an extensive comparison of hundreds of lines of maize, newer hybrids had significantly higher percent mycorrhizal colonization than older inbred lines and landraces [4]. Most of these reports measured internal colonization of fine roots, rather than the extraradical hyphal length used in this study, so choice of method does not explain the contradictory results of the studies cited above. In a comparison of mycorrhizal colonization of marigold cultivars (*Tagetes patula* and *T. tenuifolia*), the cultivars, fungus-cultivar combinations with higher percent root colonization were usually higher in extraradical hyphal length and internal hyphal length as well [41].

The mechanism by which Caturra, Borbón, and Pacas differ in AM extraradical hyphae is also unclear. Variation in plant responses to arbuscular mycorrhizal fungi may result from differences in the extent of fine root development [31, 42]. Caturra might allocate less photosynthate to AM fungi, which might limit their ability to grow into the soil and absorb nutrients, as has been found for other plants [43, 44]. Differences could also reflect other inherent characteristics of the cultivars, their rhizospheres, or differences in availability of soil nutrients, as explained below.

4.2. Differences among Coffee Cultivars in Mycorrhizal Fungi. Another explanation for the differences in hyphal lengths among cultivars could be different AM fungi associated with each cultivar. To assess this hypothesis we estimated numbers and diversity of spores in the rhizosphere of the three cultivars. Although we were not able to identify species, the morphotypes we found appear to belong to species of *Glomus* and *Sclerocystis* [25, 27], genera that have previously been reported from coffee [16, 45, 46]. All the spore morphotypes (except *Sclerocystis*) were present in all three cultivars, but they were differentially abundant among the cultivars. Differences in relative abundance were not obvious and may not be biologically significant. A preliminary phylogeny based on nuclear ribosomal DNA sequences suggested that the *Glomus* present was related to

Table 1: Soil mineral concentrations at three coffee farms in Puerto Rico. Values are concentrations of extractable nutrients. Mean values are followed by standard deviations in parentheses. The first letter following each mean shows LSD differences among cultivars within a location, while the second shows differences among locations within the same cultivar. Values for the same element followed by the same letter were not significantly different ($P < 0.05$).

Farm	Cultivar	P (mg/g)	Mn (mg/g)	Mg (mg/g)	C (%)	N (%)
Limaní	Borbón	$0.46^{Aa}(\pm 0.10)$	$0.47^{Aa}(\pm 0.29)$	$1.96^{Aa}(\pm 0.48)$	$2.36^{Aa}(\pm 0.11)$	$0.21^{Aa}(\pm 0.01)$
	Caturra	$0.49^{Aa}(\pm 0.13)$	$0.22^{Aa}(\pm 0.06)$	$1.75^{Aa}(\pm 0.53)$	$2.65^{Aa}(\pm 0.48)$	$0.22^{Aa}(\pm 0.03)$
	Pacas	$0.43^{Aa}(\pm 0.10)$	$0.83^{Ba}(\pm 0.83)$	$0.45^{Ba}(\pm 0.08)$	$2.00^{Ba}(\pm 0.19)$	$0.18^{Aa}(\pm 0.00)$
Serrallés	Borbón	$0.93^{Ab}(\pm 0.51)$	$0.10^{Aa}(\pm 0.02)$	$0.49^{Ab}(\pm 0.15)$	$2.53^{Aa}(\pm 0.42)$	$0.23^{Aa}(\pm 0.03)$
	Caturra	$0.89^{Ab}(\pm 0.31)$	$0.13^{Aa}(\pm 0.02)$	$0.56^{Ab}(\pm 0.07)$	$4.03^{Bb}(\pm 0.23)$	$0.19^{Aa}(\pm 0.17)$
	Pacas	$0.86^{Ab}(\pm 0.18)$	$0.12^{Ab}(\pm 0.02)$	$0.50^{Aa}(\pm 0.06)$	$3.46^{Bb}(\pm 0.19)$	$0.31^{Bbc}(\pm 0.03)$
Rullán	Caturra	$0.55^{Aa}(\pm 0.28)$	$1.08^{Aa}(\pm 0.65)$	$1.43^{Ab}(\pm 0.32)$	$6.33^{Ac}(\pm 1.66)$	$1.55^{Ac}(\pm 0.90)$
	Pacas	$0.49^{Aa}(\pm 0.15)$	$1.29^{Ab}(\pm 0.37)$	$1.13^{Ac}(\pm 0.28)$	$4.50^{Bb}(\pm 1.48)$	$2.28^{Bc}(\pm 0.68)$

Table 2: Mean leaf element concentrations in three coffee cultivars at two farms in Puerto Rico. Values in parentheses are standard deviations. The first letter following each mean shows LSD differences among cultivars within a location, while the second shows differences among locations within the same cultivar. Values followed by the same letter were not significantly different ($P < 0.05$).

Farm	Coffee cultivar	Ca (mg/g)	P (mg/g)	Mn (mg/g)	Mg (mg/g)
Limaní	Borbón	$9.25^{A}(\pm 0.75)$	$2.09^{A}(\pm 0.27)$	$0.24^{A}(\pm 0.11)$	$3.55^{A}(\pm 0.40)$
	Caturra	$10.37^{Aa}(\pm 2.74)$	$1.72^{Ba}(\pm 0.21)$	$0.35^{Aa}(\pm 0.08)$	$3.75^{Aa}(\pm 0.37)$
	Pacas	$15.37^{Ba}(\pm 1.90)$	$1.60^{Ba}(\pm 0.84)$	$0.58^{Ba}(\pm 0.28)$	$2.48^{Ba}(\pm 0.21)$
Rullán	Caturra	$12.83^{Ab}(\pm 2.42)$	$1.47^{Aa}(\pm 0.49)$	$0.40^{Aa}(\pm 0.15)$	$3.90^{Aa}(\pm 0.73)$
	Pacas	$10.39^{Bb}(\pm 0.75)$	$1.66^{Aa}(\pm 0.20)$	$0.21^{Bb}(\pm 0.07)$	$3.16^{Bb}(\pm 0.32)$

G. sinuosum and *G. manihotis*, as expected [16] (data not shown). Thus there was no evidence that differences between cultivars in mycorrhizal hyphal length reflected differences in specificity for AM fungi. Although AM fungi are usually not host specific, Diaz Medina et al. [45] reported differences in coffee responses to different species of *Glomus*.

4.3. Soil and Leaf Chemistry. Concentrations of P in soil can affect the interaction between mycorrhizal fungi and plants. High P concentration has been shown to inhibit AM fungal colonization of roots [29, 47]. In coffee high P concentrations in the soil have been shown to decrease AM colonization, which in turn decreases Zn uptake [48]. However, we found no significant differences among cultivars in soil P at any site, so P availability is not the cause of differences among cultivars in leaf P content or AM hyphal length. Olsen extractable soil P levels in our study sites (430 to 930 μg/g soil) were higher than those previously reported to inhibit AM symbiosis. For example, Parádi et al. [49] reported a decrease in AM fungal colonization when phosphorus increased from 1.7 to 32.7 μg P/g soil. The high levels of soil P at our sites may have contributed to the low extraradical hyphal lengths of AM fungi (0.1 mm/g soil). These amounts of hyphae were low compared to many previous studies (i.e., those found by Miller et al. [7] for tall grass prairie and pasture).

Although P concentrations in soil did not vary among cultivars within sites, leaf P concentrations were significantly higher in Borbón than in Caturra. The greater extent of extraradical hyphae in Borbón than in Caturra may have caused this difference in leaf P concentrations by facilitating P uptake. P concentration in plant tissue was correlated with their levels of mycorrhizal colonization in coffee [48] as well as other plants [29, 47].

4.4. Extraradical Hyphal Length as a Measure of Mycorrhizal Association. Extraradical hyphal length in the soil is directly related to root colonization, though colonization rates can be a poor predictor of extraradical hyphal length [7, 10, 50]. As the plant allocates photosynthates to the fungus, it promotes hyphal growth inside and outside of the root, and the extension of the extraradical hyphae results in greater nutrient transfer from soil to the plant.

5. Conclusions

In this study we found significant differences in AM fungal extraradical hyphal lengths among coffee cultivars within all three sites, and these differences were not directly attributable to soil nutrient concentrations. The results of this study can help to explain differences in cultivar responses to fertilization practices, and have implications for crop management.

Coffee researchers and growers in Puerto Rico have long observed that Caturra has a yield comparable to Borbón if fertilized, but a much lower yield than Borbón without fertilization [13, 14]. Our data are consistent with this observed difference in responses of Caturra and Borbón to fertilization, that is, the limited extraradical mycorrhizal hyphae of Caturra may make it less efficient in taking up nutrients from the soil. In this respect, Borbón and Pacas are less costly to manage than Caturra because they require

less fertilization. Selection of coffee cultivars for their ability to associate with AM fungi should be considered as an alternative to selection for responsiveness to fertilization, both in choosing cultivars and in developing new ones. Such development of cultivars able to take advantage the soil microbiota to grow in nutrient-poor soils with lower inputs has been called the second green revolution [51].

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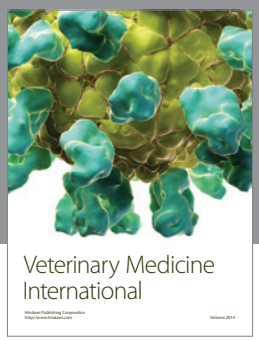

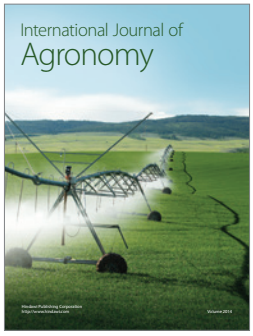

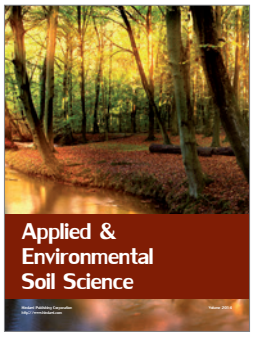

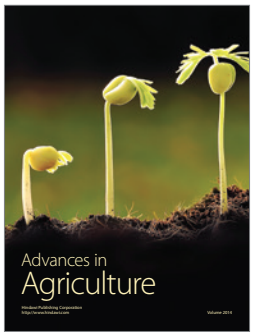

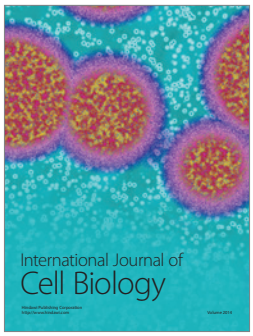

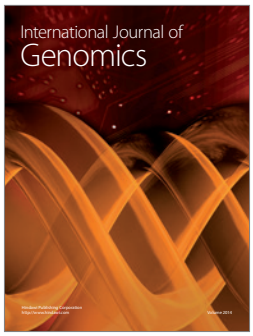

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