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Diversity of endophytic bacteria from *Citrus* and *Inga* trees in the coffee agro-ecosystem and fluctuations by location, shade management and season[1,2,3]

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ABSTRACT

Coffee, *Coffea arabica* L., is planted in diverse agro-ecological environments in the mountains of Puerto Rico (PR). The pathogen, *Xylella fastidiosa* Wells et al., is a xylem-limited phytopathogenic bacterium transmitted by insects that causes coffee leaf scorch, citrus-variegated chlorosis and vascular diseases of many other plant species. The objective of this study was to characterize the fastidious endophytes occupying a similar niche as *X. fastidiosa* in the

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vascular system of *Citrus sinensis* and *Inga vera* trees in low diversity and high diversity shade environments where coffee is grown in PR. Sampling was conducted over two years in four localities: Adjuntas, Jayuya, Las Marías and Yauco during the dry and rainy seasons. *Citrus sinensis* and *Inga vera* were generally the only shade trees in coffee plantations described as "low diversity"; in "high diversity" plantations these same species were present along with *Pithocellobium carbonarium*, *Gliricidia sepium* and *Andira inermis*. Endophyte population (EP) and endophyte diversity (ED) were contrasted using factorial arrangements of shade, locality, season and year. Endophytes were clustered using multivariate analysis. Neither shade nor season had an effect on EP in *Citrus*. In *Inga vera*, EP was about three times higher in the second year compared to the first year regardless of season, location or shade. In both *Citrus* and *Inga*, ED was low. The highest ED was detected in Jayuya and the lowest in Las Marías. Ward's algorithm combined *Citrus* and *Inga* fastidious strains into four and five clusters for Gram-negative and Gram-positive bacteria, respectively. Endophyte diversity of *Citrus* and *Inga* was determined by the plant species, not by location or shade. However, ED was higher in shaded coffee with low diversity of tree species. Thus, effects of shade (low versus high diversity) altered the community structure of endophytes prevalent in the vascular tissue and may impact host plant resistance to pathogens such as *X. fastidiosa.*

Key words: *Xylella fastidiosa*, shaded coffee, endophytes, fastidious bacteria, microbial diversity

RESUMEN

Diversidad de bacterias endófitas de *Citrus* e *Inga* en el agro-ecosistema del café y fluctuaciones por localidad, tipo de sombra y época

El cultivo del café, *Coffea arabica* L., se realiza en un ambiente agroecológico diverso en las montañas de Puerto Rico (PR). La bacteria patógena, *Xylella fastidiosa* Wells et al., está limitada al xilema y es transmitida por insectos que causan la crespera o encorchamiento de la hoja de café, la clorosis variegada de los cítricos y enfermedades vasculares en otras especies. El objetivo de este estudio fue caracterizar las bacterias endófitas de tipo fastidioso con nicho similar a *X. fastidiosa* en *Citrus* e *Inga* en cafetales bajo sombra en PR. *Citrus sinensis* e *Inga vera* se usaron para sombra en fincas de baja y alta diversidad; en esta última, también estaban presentes *Pithocellobium carbonarium*, *Gliricidia sepium* y *Andira inermis*. Durante dos años, en las épocas de secano y de lluvia, se muestrearon cafetales bajo sombra de alta y baja diversidad en cuatro localidades: Adjuntas, Jayuya, Las Marías y Yauco. La población de endófitas (EP) y la diversidad de endófitas (ED) se contrastaron usando arreglos factoriales de sombra, localidad, época y año. Las cepas obtenidas se agruparon usando análisis multivariado de conglomerados. La sombra y la época no mostraron efecto sobre EP en *Citrus*. En *Inga*, EP fue tres veces mayor en el segundo año, sin importar la época, localidad o sombra. La ED fue baja en *Citrus* e *Inga*. La ED más alta se detectó en Jayuya y la más baja en Las Marías. Mediante el análisis de Ward se combinaron las cepas fastidiosas de *Citrus* e *Inga* en conglomerados: cuatro Gram negativas y cinco Gram positivas. En *Citrus e Inga*, ED está determinada por la especie de planta y no por localidad o sombra. Sin embargo, la ED fue más alta en café bajo sombra con baja diversidad de especies de árboles. Por tanto, el efecto de sombra (baja versus alta diversidad), altera la estructura de la comunidad de endófitas prevaleciente en el tejido vascular y podría impactar la resistencia de la planta a patógenos tales como *X. fastidiosa*.

Palabras clave: *Xylella fastidiosa*, café bajo sombra, bacterias endófitas, bacterias de tipo fastidioso, diversidad microbiana

INTRODUCTION

Plants are associated with a wide and diverse range of bacteria, and their interactions can be harmful, neutral or beneficial (Lacava et al., 2004). Endophytic bacteria reside within plant tissues in low population densities (Rosenblueth and Martínez-Romero, 2006). Endophytes can be found in almost all 300,000 species of plants that exist on the planet (Strobel et al., 2004). There are two criteria for determining if a bacterium is endophytic. Tissue potentially containing endophytic bacteria can be isolated after surface sterilization and subsequently plated in a semi-selective culture media; alternatively, microscopy can be used to observe the bacteria in plant internal tissue such as vessel, pith, or cortex (Reinhold-Hurek and Hurek, 1998).

Bacterial communities are constantly changing due to host nutritional condition, environment and season. It is also known that endophytic bacteria mainly enter the root system (Ryan et al., 2008) and reside internally causing a variety of symptoms (Zinniel et al., 2002). Endophytic and pathogenic bacteria often use similar mechanisms to interact with their plant hosts (Koiv et al., 2015). Numerous endophytic bacteria have been isolated, including the xylem-limited bacterium *Xylella fastidiosa* Wells et al. (1987), a plant parasitic bacteria causing considerable damage to trees worldwide (Purcell and Hopkins, 1996).

Xylella fastidiosa causes epidemics of economic importance in agriculture. Native to the Americas, it has been identified in 27 states of North America (Goldberg, 2007), the Caribbean Basin, throughout Central America, and northwestern and southern South America (Purcell, 2005). The bacterium is well adapted to the conditions of the tropics and subtropics (Purcell and Hopkins, 1996). The recent introduction of a strain of *X. fastidiosa* from ornamental coffee trees of Costa Rica (Purcell et al., 2015) to the Apulian province of Lecce (southeastern Italy) has caused an outbreak of a new disease in olive trees known as "Complesso del Disseccamento Rapido dell 'Olivo" (Saponari et al., 2013; Loconsole et al., 2014; Purcell et al., 2015). In Brazil, *X. fastidiosa* is associated with two diseases: citrus-variegated chlorosis (CVC) (Wickert et al., 2007) and coffee leaf scorch (CLS) (Lima et al., 1998; Miranda et al., 2007; Paradela Filho et al., 1995). In Costa Rica, *X. fastidiosa* caused the disease known as "crespera del cafeto" in coffee (Rodríguez et al., 2001). Bolaños et al. (2015a) quantified the distribution of coffee with apparent symptoms of coffee leaf scorch in Puerto Rico. Only a small proportion of symptomatic plants tested positive

for *X. fastidiosa* in a double antibody sandwich ELISA. Given these somewhat contradictory results, this study also concluded that genetic confirmation is required for positive identification and quantification of *X. fastidiosa* diseases in Puerto Rico.

The vast majority of endophytic bacteria colonize an ecological niche similar to that of phytopathogenic organisms; therefore, some strains have potential as biological control agents (Berg et al., 2005). In our attempt to isolate *X. fastidiosa* in Puerto Rico, a large quantity of bacterial endophytes was isolated from coffee trees during the dry and rainy seasons of 2010 to 2011 and 2011 to 2012 (Bolaños and Zapata, 2011; Bolaños et al., 2015a, b). We found that the location with the highest elevation (Yauco) was disposed to have a higher endophyte population (EP) (log CFU/mL) than at lower elevations, although this difference was not consistent in all seasons and years. There were few differences in endophyte populations from coffee trees under various types of shade (unshaded coffee, coffee shaded by trees with low species diversity, and coffee shaded by trees with high species diversity). In general, the rainy season favored the development of higher populations of endophytes on coffee trees. There were also more endophytes found in samples from branches compared to leaf veins. During the rainy season, many more differences were observed in ED among locations and types of shade. ED tended to be greater in coffee grown under shade compared to unshaded coffee, although this trend was not always consistent. A somewhat larger number of ED was observed in branches compared to leaf samples.

Here, we present the continuation of our previous study, evaluating the effects of location, shade management and season on the EP and ED in leaf veins and branches of the shade trees typically found in coffee agro-ecosystems in Puerto Rico: *Citrus* (predominantly *Citrus sinensis* and *C. aurantifolia*) and *Inga* sp. (predominantly *Inga vera*). The previous study focused on endophytic populations in coffee while the present study includes the common shade species *Citrus* and *Inga* trees often found in coffee agro-ecosystems in Puerto Rico.

MATERIALS AND METHODS

Specifications of the study

The study was conducted on coffee plantations at the following locations of Puerto Rico: Adjuntas (18°09'30", W66°45'27"), Jayuya (N18°09'35"; W66°38'45"), Las Marías (N18°13'14"; W66°01'38") and Yauco (N18°09'57"; W66°49'36"). Elevation varied from 445 m to 947 m above sea level. At each location, three coffee farms were identified,

each with a distinct type of management: (i) shaded coffee with a high diversity of tree species, (ii) shaded coffee with low species diversity, and (iii) unshaded coffee. At each farm five experimental plots (replicates) were defined. Selected plots had a variety of 5- to 6-year-old coffee tree cultivars, all *Coffea arabica*. The tree species encountered in plots of high and low shade tree diversity varied depending on location. Results from the unshaded coffee plots have been previously published (Bolaños et al., 2015b).

Characterization of the plots

A GPS CS60 Garmin (Garmin International, Olathe, KS, USA)[8] was used for geo- referencing of trees in the plots. The shape of the plots varied from elongated to rectangular depending on the arrangement of trees in the field. Sampling was done in the dry (December to May) and rainy (June to November) seasons for two consecutive years (year 1: May 2010 to June 2011; year 2: June 2011 to May 2012). Percentage of canopy was estimated using a GRS densitometer (Geographic Resource Solutions, Arcata, CA, USA). The method for estimation is the line plot transect (Jennings et al., 1999). This is a fast and accurate method for collecting data from a wide variety of quantitative vegetation and ecological characteristics. Irradiation level was determined using a light meter (Extech Instruments, Nashua, NH, USA). The light sensor was placed at a height of 1.5 m; sampling was done on three different days with a low density of cloud cover at noon.

Isolation of cultivable endophytes

In each experimental plot of shaded coffee, one *Inga* tree (usually *Inga vera*) and one *Citrus* tree (predominantly *C. sinensis*, but sometimes *C. aurantifolia*) was selected. Five leaves and three 5- to 6-cm long branches of selected trees were sampled. Samples were transported on ice and processed immediately at the Bacteriology Laboratory of the Department of Agro-environmental Sciences, University of Puerto Rico-Mayagüez. Leaf veins were removed, and branches and leaf veins were washed with diluted liquid soap to remove insects and epiphytic microorganisms with the help of a sterile brush. Samples were surface sterilized for one minute with each solution of 0.05% sodium hypochlorite and 70% ethanol. Samples were washed three times with sterile distilled water to remove disinfectant, then chopped under aseptic conditions in extraction buffer: NaCl, 8 g/L; K_2HPO_4, 1.15 g/L; KH_2PO_4, 0.2

[8]Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

g/L; Tween 20, 0.5 g/L; PVP-40 Polyvinyl prolidone, 6 g/L (Schaad et al., 2001) to prevent oxidation of tissues. Subsequently the samples were placed in liquid PW media (Soytone, 4 g/L; Triptone, 0.5 g/L; K_2HPO_4, 0.6 g/L; KH_2PO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.4 g/L; (NH_4) $2HPO_4$, 0.8 g/L; potato starch, 2 g/L; L-Histidine HCl, 1 g/L; L-glutamine, 4 g/L; bovine serum albumin, 3 g/L; Hemin chloride, 10 mg/L; Phenol, 20 mg/L; cycloheximide, 50 mg/L) for 30 minutes at room temperature and placed in a refrigerator at 4° C until the next day (Schaad et al., 2001). One hundred microliter of plant tissue suspension was placed on a 100 x 15 mm plate with solid medium PW. The plates were incubated at 28° C and bacterial growth was evaluated during one month.

Bacterial colonies were purified by transferring them three consecutive times; a representative colony was chosen according to its frequency, colony shape, margin form, elevation and color. Growth of all strains was also evaluated on Trypticase Soy Agar (TSA) (pancreatic digest of casein, 7.5 g/L; enzymatic digested soybean, 2.5 g/L; sodium chloride, 2.5 g/L; and agar, 7.5 g/L) (Becton Dickinson, MD, USA). Numeric codes were assigned to purified strains.

The values of EP = colony forming units per milliliter (CFU/mL) were determined. Data of EP from veins of leaves and branches were transformed using base 10 logarithm of CFU plus one in order to adjust the errors to the normal curve. The total number of ED = phenotypically distinct bacterial strains per plate was determined.

Phenotypic characterization of isolated endophytic bacterial strains

Fastidious bacteria strains were classified as Gram negative (Gram-), unable to grow on TSA (TSA-) and able to grow on PW (PW+). Bacterial strains were characterized morphologically using data of cell morphology, color change on PW, margin configuration, color and diameter of colonies (Schaad et al., 2001). For the basic biochemical characterization, the following tests were performed: 3% potassium hydroxide (KOH) for indirect evidence of the Gram reaction, catalase and oxidase tests. Fastidious Gram positive (Gram+) bacteria able to grow in PW were isolated, and a spore-staining test was performed with malachite green.

Statistical analysis

Cluster analysis was carried out using InfoStat software (Di Rienzo et al., 2014). This technique was used to reduce the dimensionality of the set of strains to a few manageable groups in order to conduct additional identification. Ward's algorithm (Ward, 1963) was used to group strains with reaction Gram negative and Gram positive. Several clusters were generated by hierarchical cluster analysis and by defining differences according to an arbitrary line at the 50% of the

maximum distance obtained using Gower's distance. Variables used for multivariate analysis were: size (mm), color, margin, configuration and elevation (categorical) of colonies, and catalase and oxidase reactions (binary variables). Categorical variables had more weight when defining dissimilarity.

EP and ED per tree were analyzed using ANOVA. Data for each tree species (*Inga* and *Citrus* trees) were analyzed separately. Data were analyzed using a completely randomized design with five repetitions in a factorial arrangement of two types of shade management (high and low diversity of shade trees) at four locations (Adjuntas, Jayuya, Las Marías, and Yauco). Separate analyses were carried out for the dry and rainy season in each of two years.

Geolocation Information

This study was conducted on coffee plantations in the following locations in Puerto Rico: Adjuntas (18°09'30", W66°45'27"), Jayuya (N18°09'35"; W66°38'45"), Las Marías (N18°13'14"; W66°01'38") and Yauco (N18°09'57"; W66°49'36").

RESULTS

Inga vera and *Citrus* (almost exclusively *Citrus sinensis*, but occasionally *C. aurantifolia*) were generally the only shade trees present on coffee plantations defined as having "low diversity" of tree species. In farms classified as having "high diversity", these same species were present along with species such as *Pithocellobium carbonarium*, *Gliricidia sepium*, *Andira inermis* and other species of *Inga* or *Gliricidia*. The shade tree species found in coffee plantations varied among locations.

In both years and all locations, rainfall was consistently lower in the dry season compared to the rainy season (Table 1). However, relative differences in seasonal rainfall (rainy versus dry season) varied among years and locations. In Adjuntas and Jayuya, during the dry or rainy season, amounts of rainfall were similar in both years. By contrast, rainfall in Las Marías was twice as high in the dry season of year 1 compared to the dry season of year 2. In Yauco, rainfall was substantially lower in both the dry and rainy season of year 2. Thus, by the end of the rainy season of year 2, in both Las Marías and Yauco there was considerably less total rainfall than during the same period in year 1.

Except for Las Marías in 2010-2011, mean daily temperature was consistently lower in the dry season compared to the rainy season in all locations (Table 1). Average daily temperature differences were as high as 10° C in certain locations. Thus, the coffee region of western Puerto

TABLE 1.— *Average precipitation and temperature during 2010-2012 in four experimental locations in coffee growing areas of Puerto Rico. Taken from Bolaños et al. (2015a).*

		Year 1: 2010-2011		Year 2: 2011-2012	
Location	Season[1]	Temperature °C	Precipitation (mm)[2]	Temperature °C	Precipitation (mm)[2]
Adjuntas	Dry	20.8[3]	700	20.8[3]	820
	Rainy	22.5[3]	1,950	22.6[3]	1,600
Jayuya	Dry	20.8[4]	720[5]	20.3[6]	720
	Rainy	25.5	1,200[5]	29.3[7]	1,200
Las Marías	Dry	23.5[4]	810	20.7[6]	410
	Rainy	22.9	1,970	30.4[7]	1,770
Yauco	Dry	17.7[4]	910	19.0[6]	610
	Rainy	N/A[8]	1,720	29.5[7]	1,110

[1]Dry season December-May; rainy season June-November
[2]Precipitation not recorded in Jayuya. Value taken from the Southeast Regional Climate Center (SERCC).
[3]Temperature not recorded in Adjuntas. Values taken from: http://weather.com/climate/annual-Climo-USPR0001.
[4]Mean of February to March 2011. Data were taken with log tag recorders in the experimental plots.
[5]Precipitation not recorded. Value presented is the average dry season precipitation recorded in Jayuya from 1939 to 2002.
[6]Mean of December 2011 and January to May 2012. Data were taken with log tag recorders in the experimental plots.
[7]Mean from November to June 2011. Data taken with logtag recorders in the experimental plots.
[8]N/A data not available

Rico can be characterized as having a hot rainy season and a much cooler dry season. Rainy season temperatures were especially high in Jayuya, Las Marías and Yauco in 2011-2012.

Citrus

Over the two years of this study, leaves and branches sampled from *Citrus* trees had an average EP of 170 and 168 CFU/mL (media untransformed from log CFU/mL), respectively. Although there were some significant differences in EP found on *Citrus* trees at various locations (in the rainy season of year 1 and both seasons of year 2), there was no consistent effect of location on the number of endophytes (Table 2). When results did vary among locations, these differences were observed in samples from *Citrus* branches, not in leaf veins. Neither shade management (low versus a high diversity of shade trees) nor season (dry versus rainy) influenced the number of endophytes on *Citrus* trees in coffee plantations. Overall, a similar EP number was observed in both years of the study.

The ED was very low in *Citrus* trees sampled in this study. At least one strain was isolated from each of the experimental units analyzed.

TABLE 2.—*Gram-negative endophyte population (EP) (log CFU/mL) in* Citrus *spp. used as shade in coffee plots sampled during the dry and rainy seasons at four locations in Puerto Rico: Means, significance of F tests, and Fisher's least significant difference (F-LSD).*

	Gram-negative endophyte population (log CFU/mL) on *Citrus* spp.[1]							
	2010-2011				2011-2012			
	Dry season		Rainy season		Dry season		Rainy Season	
Effect	Leaves	Branches	Leaves	Branches	Leaves	Branches	Leaves	Branches
Location (L)								
Adjuntas	0.61	1.07	0.64	0.89 b[2]	1.36	0.79	1.41	1.31 ab
Jayuya	1.00	0.83	1.26	0.27 c	1.53	0.97	1.66	2.01 a
Las Marías	1.73	1.74	0.63	0.83 b	1.02	1.50	0.59	1.20 ab
Yauco	1.08	0.74	1.85	2.10 a	0.59	0.72	1.12	0.97 b
F test[3]	NS	NS	NS	*	NS	*	NS	*
F-LSD[4]	n/a[5]	n/a	n/a	0.30	n/a	n/a[2]	n/a	0.84
Shade (S)								
Low diversity	1.31	1.26	1.11	0.91	1.03	0.90	1.31	1.30
High diversity	0.90	0.93	1.07	1.14	1.22	1.08	1.08	1.44
F test[3]	NS	NS	NS	NS	NS	NS	NS	NS
F-LSD[4]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Overall mean	1.105	1.095	1.090	1.025	1.125	0.990	1.195	1.370
L x S F test	NS	NS	NS	NS	NS	*	NS	NS

[1]Usually *Citrus sinensis*, but occasionally *C. aurantifolia*.
[2]Means followed by the same letter in the same column, are not different at $\alpha = 0.05$.
[3]NS, * = F tests not significant (NS) or significant (*) at $\alpha = 0.05$.
[4]F-LSD = Fisher's least significant difference at $\alpha = 0.05$. F-LSD was not used in cases where the F test was not significant.
[5]n/a: Means of main effects were not compared due to a non-significant F test or, in the case of a significant F-test, due to the presence of a non-ordered interaction.

On average, only a single strain (mean = 0.95) was present (Table 3). In the dry season of 2011-2012 there was a significantly greater number of ED in *Citrus* leaves collected in plantations with a low diversity of shade trees, but this trend was not observed in any other year or season.

Inga

On *Inga* trees' (generally *Inga vera*) leaves and branches, in both the dry and rainy seasons, there were about three times as many EP present in the second year of the study (1.54 log CFU/mL) compared to the first year (0.58 log CFU/mL) (Table 4). Greater numbers of EP were consistently observed in year 2 no matter the location or shade.

As in *Citrus*, the ED was very low in *Inga* trees (Table 5). During the dry season of 2010-2011, there were statistical differences by locality for *Inga* leaves. The highest value of ED was detected in Jayuya and the lowest in Las Marías. During the rainy season, in leaves and branches of *Inga* statistical differences were detected for locality and shade. The highest ED was detected in the low diversity plots, and in Jayuya for leaves and branches (Table 5).

A total of 454 strains were isolated from *Citrus* and *Inga* trees (6% Gram negative, and 94% Gram positive). Twenty-nine of these endophytes were isolated from *Inga* and *Citrus* and showed fastidious growth (Gram -, PW+, and TSA-); 34% of these strains were found on *Citrus* and 66% on *Inga* trees. Ward's algorithm from these strains yielded four major groups (Figure 1). These clusters serve as a basis for breaking up the whole group of endophytes into smaller groups according to similarities and distances, and help to establish diversity within the main group to reduce the size for identification purposes.

Cluster one contains all catalase negative strains with oxidase positive and negative reactions. Clusters two and three grouped strains with catalase positive and oxidase negative reactions. Cluster four grouped strains with catalase positive and oxidase positive reactions. Percentages of 28.6%; 60%; 27.3% and 33.3% corresponded to *Citrus* strains in clusters one to four, respectively.

Gram-positive strains isolated from *Citrus* and *Inga* were represented by 425 strains (24% from *Citrus* trees, and 76% from *Inga* trees). Strains from *Citrus* were grouped by oxidase and catalase reactions. The major configuration was catalase positive and oxidase negative with 78% of the strains, followed by the configurations catalase positive oxidase positive (11%), catalase negative oxidase positive (6%), and catalase negative oxidase negative (5%). The same characteristics were present in *Inga* strains with the major configuration in catalase positive and oxidase negative (76%), catalase positive oxidase positive

TABLE 3.—*Gram-negative endophyte diversity (ED) per tree in* Citrus *spp. used for shade in coffee plots sampled during the dry and rainy seasons at four locations in Puerto Rico: Means, significance of F tests, and Fisher's least significant difference (F-LSD).*

	Gram-negative endophyte diversity per *Citrus* spp.[1] tree							
	2010-2011				2011-2012			
	Dry season		Rainy season		Dry season		Rainy season	
Effect	Leaves	Branches	Leaves	Branches	Leaves	Branches	Leaves	Branches
Location (L)								
Adjuntas	0.70	1.40	1.00	1.10	0.40	0.80	1.20	1.30
Jayuya	0.90	0.90	1.10	1.20	1.20	0.90	0.80	0.20
Las Marías	0.70	0.90	0.80	0.40	0.90	1.40	0.40	1.20
Yauco	0.60	0.60	1.00	1.20	1.10	0.90	1.40	1.90
F test[2]	NS	NS	NS	NS	NS	NS	NS	NS
F-LSD[3]	n/a[4]	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Shade (S)								
Low	0.70	1.05	0.85	1.00	1.20 a[5]	1.10	0.90	1.45
High diversity	0.75	0.85	1.10	0.95	0.60 b	0.90	1.00	0.85
F test	NS	NS	NS	NS	*	NS	NS	NS
F- LSD	n/a	n/a	n/a	n/a	0.50	n/a	n/a	n/a
Overall mean	0.725	0.950	0.975	0.975	0.900	1.000	0.950	1.150
L x S	NS	NS	NS	NS	NS	NS	NS	NS
F test	0.70	1.40	1.00	1.10	0.40	0.80	1.20	1.30

[1]Usually *Citrus sinensis*, but occasionally *C. aurantifolia*.

[2]NS, * = F test not significant (NS) and significant (*) at $\alpha = 0.05$.

[3]F-LSD = Fisher's least significant difference at $\alpha = 0.05$. F-LSD was not used in cases where the F test was not significant.

[4]n/a: Means of main effects were not compared due to a non-significant F-test or, in the case of a significant F-test, due to the presence of a non-ordered interaction.

[5]Means followed by the same letter in the same column, are not different at $\alpha = 0.05$.

TABLE 4.—*Gram-negative endophyte population (EP) (log CFU/mL) in* Inga vera *used as shade in coffee plots sampled during the dry and rainy seasons at four locations in Puerto Rico: Means, significance of F tests, and Fisher's least significant difference (F-LSD).*

	Gram-negative endophyte population (log CFU/mL) on *Inga vera*							
	2010-2011				2011-2012			
	Dry season		Rainy season		Dry season		Rainy Season	
Effect	Leaves	Branches	Leaves	Branches	Leaves	Branches	Leaves	Branches
Location (L)								
Adjuntas	0.26 c[1]	0.21	0.35	0.92	1.02	1.03	1.56	1.85
Jayuya	0.77 a	0.52	0.68	1.18	1.42	1.99	1.93	1.31
Las Marías	0.33 c	0.35	0.68	0.91	1.37	1.25	2.14	2.66
Yauco	0.49 b	0.59	0.55	0.56	0.64	1.00	1.56	1.94
F test[2]	*	NS	NS	NS	NS	*	NS	*
F-LSD[3]	0.11	n/a[4]	n/a	n/a	n/a	n/a[2]	n/a	n/a
Shade (S)								
Low	0.31 b	0.45	0.73	1.23a	1.16	1.24	1.86	2.14
High	0.61 a	0.39	0.40	0.55b	1.06	1.39	1.74	1.74
F test	*	NS	NS	**	NS	NS	NS	NS
F-LSD	0.21	n/a	n/a	0.28	n/a	n/a	n/a	n/a
Overall mean	0.460	0.420	0.565	0.890	1.110	1.315	1.800	1.940
LxS								
F test	NS	NS	NS	*	NS	**	NS	**

[1]Means followed by the same letter in the same column, are not different at $\alpha = 0.05$.
[2]NS, *, ** = F tests not significant (NS) or significant (*) at $\alpha = 0.05$, or significant (**) at $\alpha = 0.01$
[3]F-LSD = Fisher's least significant difference at $\alpha = 0.05$. F-LSD was not used in cases where the F test was not significant.
[4]n/a: Means of main effects were not compared due to a non-significant F-test or, in the case of a significant F-test, due to the presence of a non-ordered interaction.

TABLE 5.—*Gram-negative endophyte diversity (ED) per tree in* Inga vera *used for shade in coffee plots sampled during the dry and rainy seasons at four locations in Puerto Rico: Means, significance of F tests, and Fisher's least significant difference (F-LSD).*

	Gram-negative endophyte diversity per *Inga vera* tree							
	2010-2011				2011-2012			
	Dry season		Rainy season		Dry season		Rainy Season	
Effect	Leaves	Branches	Leaves	Branches	Leaves	Branches	Leaves	Branches
Location (L)								
Adjuntas	0.60 ab[1]	0.90	1.00	1.30	0.70	0.80	0.50 b	1.10 b
Jayuya	1.20 a	1.60	0.90	0.80	1.10	1.20	1.50 a	1.80 a
Las Marías	0.20 b	1.20	1.50	1.10	1.80	1.50	0.50 b	0.70 b
Yauco	0.70 ab	0.60	1.00	1.10	0.80	0.70	0.70 ab	0.60 b
F test[2]	*	NS	NS	NS	NS	NS	*	*
F-LSD[3]	0.64	n/a[4]	n/a	n/a	n/a	n/a	0.80	0.69
Shade (S)								
Low	0.85	0.95	1.05	1.20	1.05	1.35 a	1.10 a	1.30 a
High	0.50	1.20	1.15	0.95	1.15	0.75 b	0.50 b	0.80 b
F test	NS	NS	NS	NS	NS	NS	*	*
F-LSD	n/a	n/a	n/a	n/a	n/a	n/a	0.57	0.48
Overall mean	0.675	1.075	1.100	1.075	1.100	1.050	0.800	1.050
L x S F test	NS	NS	NS	NS	NS	*	NS	NS

[1]Means followed by the same letter in the same column, are not different at $\alpha = 0.05$.
[2]NS, * = F test not significant (NS) or significant (*) at $\alpha = 0.05$.
[3]F-LSD = Fisher's least significant difference at $\alpha = 0.05$. F-LSD was not used in cases where the F test was not significant.
[4]n/a: Means of main effects were not compared due to a non-significant F test or, in the case of a significant F test, due to the presence of a non-ordered interaction.

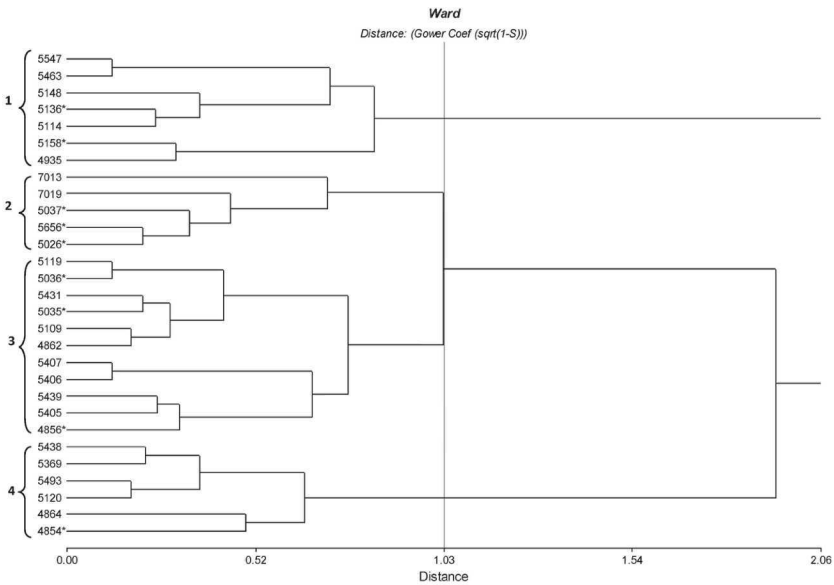

FIGURE 1. Dendrogram showing four clusters of Gram-negative fastidious endophytes (TSA-, and PW+) isolated from *Inga* (*Inga vera*) and *Citrus* (*Citrus sinensis*) used as shade trees in coffee agro-ecosystems sampled in Puerto Rico. Cluster analysis was carried out using Gower distance, and Ward's agglomeration method (Cophenetic correlation: 0.616). Values at the left represent numeric codes assigned to purified strains. Strains followed by an asterisk were isolated from *Citrus sinensis*; those without an asterisk were isolated from *Inga vera*.

(10%), catalase negative oxidase positive (8%), and catalase negative oxidase negative (6%).

Cluster analysis for Gram-positive fastidious strains isolated from *Citrus* and *Inga* grouped 33 strains into five groups (Cophenetic correlation 0.69) (Figure 2). The number of strains from each host was similar (51.5% from *Citrus* trees and 48.5% from *Inga* trees). This contrasted greatly with non-fastidious Gram-positive endophytic bacteria where twice as many strains were found in *Citrus* as opposed to *Inga* trees. Cluster one contains all catalase positive and oxidase negative and positive reactions; cluster two contains all catalase positive with oxidase negative; cluster three contains catalase positive and negative with oxidase negative; cluster four contains catalase positive with oxidase negative; cluster five contains catalase positive with oxidase negative and positive reactions. Percentages of 42.8%, 75%, 57.1%, 60% and 33.3% corresponded to *Citrus* strains in clusters one to five, respectively.

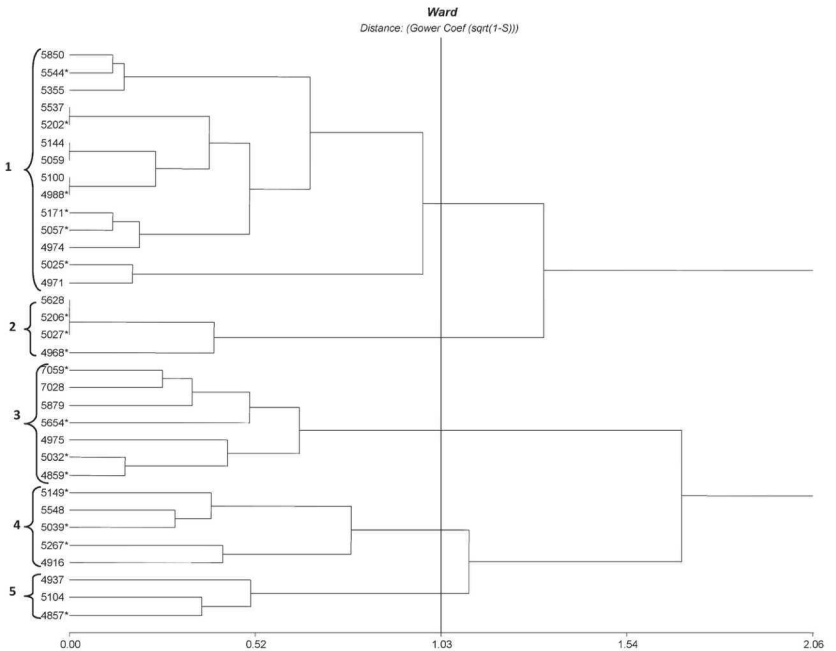

FIGURE 2. Dendrogram showing five clusters of Gram-positive fastidious endophytes (TSA- and PW+) isolated from *Inga* (*Inga vera*) and *Citrus* (*Citrus sinensis*) used as shade trees in coffee agro-ecosystems sampled in Puerto Rico. Cluster analysis was carried out using Gower distance, and Ward's agglomeration method (Cophenetic correlation: 0.616). Values at the left represent numeric codes assigned to purified strains. Strains followed by an asterisk were isolated from *Citrus sinensis*; those without an asterisk were isolated from *Inga vera*. Incubation time was included in the analysis.

DISCUSSION

A total of 454 bacterial strains were isolated from *Citrus* and *Inga* trees. The fastidious group was represented by 29 Gram negative (6.38%) and 33 Gram positive (8.41%). Fastidious strains isolated from *Citrus* and *Inga* represented 51.5% and 48.5%, respectively. The most frequent chemotaxonomic configuration of the non-fastidious group (392 strains, 86%) was: round morphology, smooth and flat margin, Gram positive, catalase positive, oxidase negative and the ability to grow on TSA. Fastidious and non-fastidious bacteria have not been identified. Numeric codes were assigned to each strain (culture collection of bacteria belonging to the Bacteriology Laboratory, Agroenvironmental Sciences Department, University of Puerto Rico-Mayagüez).

Cluster analysis from fastidious strains provides valuable information for identifying the profiles of the different groups. Clusters showed

no pattern of association by locality, shade, or season but showed high variability of origin of strains. Hence, cluster analysis is a powerful tool to associate strains from different origins and plant species (Figures 1 and 2). The fastidious Gram negative and Gram positive showed four and five clusters, respectively, which were representative of different major physiological groupings or characteristics (Figures 1 and 2).

In both years, higher EP values were observed in branches versus leaf veins of *Citrus* ($P < 0.0001$), and *Inga* ($P < 0.018$). For *Inga*, the overall means were 869 CFU/mL (mean transformed of log CFU/mL) in branches and 360 in leaf veins. By contrast, the overall means for *Citrus* were 170 CFU/mL in leaf veins and 168 CFU/mL for branches. These values are within the range obtained by Kobayashi and Palumbo (2007) for endophytic bacteria in alfalfa, sweet corn, sugar beet, cotton and potato with values in the range 10^2 to 10^5 CFU. Averages found were higher than those reported by Rivera (2006) in Puerto Rico, who found an overall average of 102 endophytic bacterial isolates in *Citrus* sp. The EP found in *Citrus* leaves versus branches was consistently similar (49% vs. 51%, respectively), comparable to results previously reported in coffee by Bolaños et al. (2015b). This trend was different for *Inga*, with higher EP numbers isolated from *Inga* leaves versus branches.

During the rainy season of the first year, the EP from branches of *Inga* was twice as high in low diversity plots than in high diversity. These data were similar to those obtained with the potential leafhopper vector *Caribovia coffeacola* by Brodbeck et al. (2011) and Brodbeck et al. (2015). On coffee farms where *Inga* is the only species planted for shade, populations of *C. caribovia* may increase significantly. *Inga* trees are the preferred host by *C. coffeacola*. Brodbeck et al. (2015) and Andersen (2005) found that the abundance of *C. coffeacola* was extremely high in seedlings of *Inga* sp., with fluctuations of 25 to 40 individuals per seedling.

In Brazil, Lacava et al. (2004) found nine common endophytic genera isolated from *Citrus*: *Bacillus pumilus*, *Curtobacterium flaccumfaciens*, *Enterobacter cloacae*, *Methylobacterium extorquens*, *M. mesophilicum*, *Nocardia* sp., *Pantoea agglomerans*, *Streptomyces* sp. and *Xanthomonas campestris*. In Puerto Rico, Zapata et al. (2011) frequently found eight species in *Citrus*: *Bacillus cereus*, *Bacillus coagulans*, *M. mesophylicum*, *Pseudomonas putida*, *Pseudomonas warneri*, *Staphylococcus*, *Stenotrophomonas maltophilia*, and *X. axonopodis* pv. *vasculorum*. The community of endophytic bacteria isolated from *Citrus* in this study is likely to include the species mentioned above.

Vega et al. (2005) found a total of 87 strains of cultivable endophytic isolates belonging to 19 genera from leaves and fruits of coffee from

Colombia, Hawaii, and Mexico. They found predominance of the genera *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*. In Brazil, Nunes (2004) reported a total of 252 strains, including those belonging to species *Bacillus lentimorbus*, *B. megaterium*, *B. subtilis*, *B. cereus*, *Pseudomonas* sp., *Pantoea* sp., *Stenotrophomonas maltophilia*, *Kluyvera cryocrescens*, *Escherichia coli*, *Enterobacter* sp., *Serratia* sp. and *Kocuria kristinae*.

In Puerto Rico, Zapata et al. (2011) found 20 species in coffee: *B. cereus*, *B. coagulans*, *B. pumilus*, *Citrubacter farmer*, *Curtobacterium pusilum*, *Erwinia stewartii* ss. *stewartii*, *Kokuria kristinae*, *K. varians*, *Methylobacterium mesophylicum*, *Microbacterium chocolatum*, *Micromonas paracarbonacea*, *Pantoea dispersa*, *P. aglomeranss* (*Erwinia herbicola*), *Pseudomonas amyloderamosa*, *Psycrobacter inmobilis*, *Staphylococcus aureus*, *S. epidermidis*, *S. simulans*, *Stenotrophomonas* and *Xanthomonas axonopodis* pv. *vasculorum*. Mariño-Cárdenas and Zapata (2009) reported 178 different bacterial strains isolated in the coffee agro-ecosystem from sharpshooter leafhoppers as potential vectors of *Xylella fastidiosa* belonging to the genus: *Acinetobacter*, *Bacillus*, *Brevundimonas*, *Chryseobacterium*, *Clavibacter*, *Curtobacterium*, *Curtobacterium*, *Kluyvera*, *Kokuria*, *Microbacterium*, *Micrococcus*, *Nocardia*, *Paenibacillus*, *Pantoea*, *Pasteurella*, *Pectobacterium*, *Providencia*, *Pseudomonas*, *Rhizobium* and *Vibrio*. Some of these genera could be related to those obtained in this study that involve the cultivation of coffee.

There were significant differences ($\alpha = 0.05$) by season between *Citrus* and *Inga* trees with the greatest EP in the rainy versus the dry season. This is consistent with the findings of Irizarry (2010) whose study on the isolation of endophytic bacteria from *Coccoloba uvifera* in Cabo Rojo, Puerto Rico, indicated a higher percentage of colonization of endophytic bacteria in the rainy versus the dry season. The effects of location and shade treatment were more variable with a tendency of lower ED in trees with high diversity versus low. The results of this study indicate that EP levels were high, while the ED per tree was low. This behavior was consistent in *Citrus* and *Inga* trees.

Plants are normally associated with a broad and diverse population of microorganisms in the ecosystem. Their interactions could be beneficial, neutral or harmful. This study highlights that coffee agro-ecosystem structure (selection and diversity of shade species) alters the community structure of endophytic bacteria. This may have implications for disease resistance to pathogens such as *X. fastidiosa*. Lacava et al. (2004) found that growth of *X. fastidiosa* was stimulated by *Methylobacterium extorquens* and inhibited by *Curtobacterium flaccumfaciens*,

suggesting that resistance to *X. fastidiosa* is certainly possible. Zapata et al. (2011) described for the first time endophytes from the vascular tissue of coffee and citrus in Puerto Rico. They found bacteria previously reported as synergistic (*Methylobacterium extorquens*) and antagonistic (*M. mesophylicum* and *Curtobacterium flacumfacciens*) to *X. fastidiosa*. Ardanov et al. (2012), working with *Methylobacterium*, showed that plant disease resistance could be altered by manipulation of host endophytic communities.

High diversity of shade trees in coffee agro-ecosystems has been shown in other systems, such as shaded coffee, to provide the proper environment for the arboreal spiders that help to control the coffee berry borer insect (Hajian et al., 2014). Knowledge of the endophytic community composition is of great value for the development of technologies and strategies for agricultural management (Pérez et al., 2016).

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