



Isolation of endophytic endospore-forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases

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ABSTRACT

Sixty-nine endospore-forming bacterial endophytes consisting of 15 different species from five genera were isolated from leaves, pods, branches, and flower cushions of *Theobroma cacao* as potential biological control agents. Sixteen isolates had *in vitro* chitinase production. In antagonism studies against cacao pathogens, 42% inhibited *Moniliophthora roreri*, 33% inhibited *Moniliophthora perniciososa*, and 49% inhibited *Phytophthora capsici*. Twenty-five percent of isolates inhibited the growth of both *Moniliophthora* spp., while 22% of isolates inhibited the growth of all three pathogens. Isolates that were chitinolytic and tested negative on *Bacillus cereus* agar were tested with *in planta* studies. All 14 isolates colonized the phyllosphere and internal leaf tissue when introduced with Silwet L-77, regardless of the tissue of origin of the isolate. Eight isolates significantly inhibited *P. capsici* lesion formation ($p = 0.05$) in detached leaf assays when compared to untreated control leaves. ARISA with bacilli specific primers amplified 21 OTUs in field grown cacao leaves, while eubacteria specific primers amplified 58 OTUs. ARISA analysis of treated leaves demonstrated that inundative application of a single bacterial species did not cause a long-term shift of native bacterial communities. This research illustrates the presence of endospore-forming bacterial endophytes in cacao trees, their potential as antagonists of cacao pathogens, and that cacao harbors a range of bacterial endophytes.

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Introduction

Theobroma cacao L. is an economically significant crop, as the seeds are processed into a range of cocoa products. Nearly 30% of the cacao crop is lost to disease annually (Hebbar, 2007). Although fungicide applications can improve yield, they are often too costly for small-holder farmers as well as pose risks to the health of the applicator and the environment. For this reason, researchers have focused on altering the microbial ecology of the cacao tree to suppress diseases. Research has focused testing fungi as potential biological control agents (BCAs). Rubini et al. (2005) demonstrated that endophytic *Gliocladium catenulatum* reduced witches' broom severity in small scale *in planta* studies. Arnold et al. (2003) demonstrated that cacao leaves inoculated with a consortium of seven endophytic fungal species had lower leaf mortality when challenged with a *Phytophthora* sp. than endophyte-free leaves.

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Additional research has demonstrated that the endophytic association of *Trichoderma* spp. activates the expression of cacao expressed sequence tags (ESTs) putatively related to defense genes (Bailey et al., 2006).

Despite the successes reported for fungal endophytes in suppressing cacao diseases, little is known about the presence or potential of bacterial endophytes. Work has demonstrated that actinomycetes are common inhabitants of the cacao rhizosphere (Barreto et al., 2008) and of pod surfaces (Macagnan et al., 2006). Previous work by Melnick et al. (2008) demonstrated that an endophytic *Bacillus* sp. originally isolated from tomato could endophytically colonize cacao leaves for 60+ days and reduce severity of foliar *Phytophthora* disease in detached leaf assays. This research further demonstrated that bacterial endophytes could be introduced into cacao, but the presence of native bacterial endophytes in cacao and their potential for disease suppression still remained unknown.

No published works on the cacao endophytes have utilized culture-independent methods to characterize the cacao beneficial or pathogenic microbial communities, nor have researchers looked at how application of endophytes impacts the native mutualistic microbial communities associated with cacao. The current knowledge of cacao microbial ecology is limited to isolates capable of

growing in a lab. Culture-independent technologies, such as automated ribosomal RNA intergenic spacer analysis (ARISA), have been used in other woody perennials (Lambais et al., 2006) and could be useful in estimating the diversity of all bacteria present in cacao leaves. Additionally, this technology could be used to determine whether application of beneficial bacteria shifts the populations of native microbes.

The objectives of this research were to obtain endospore-forming bacterial endophytes from cacao trees, screen isolates as potential biological control agents of cacao diseases, to determine whether natural endophytes could persist in/on cacao leaves, and to determine whether endophytes were potential antagonists of cacao pathogens *in planta*. An additional objective was to utilize the culture-independent technology of ARISA to assess the bacterial community of cacao leaves and to determine whether application of a single endophyte can shift the bacterial communities of cacao leaves.

2. Materials and methods

2.1. Isolation of endospore-forming bacterial endophytes from cacao tissue

Endophytic endospore-forming bacteria were isolated from several *T. cacao* “Nacional” clones that were previously identified as escaping cacao diseases on the Instituto Nacional Autonomo de Investigaciones Agropecuarias Estacion Experimental Tropical (IN-IAP-EET) in Pichilingue, Ecuador. Cacao tissue was collected and sampled within 4 h. Four leaf disks (3.0 cm) were removed from leaves, disinfected for 5 min in 20% bleach (Clorox), rinsed three times in sterile distilled water, triturated with a mortar and pestle in 3 ml 0.1 M potassium phosphate buffer (pH 7.0), were then transferred to a test tube, and incubated at 75 °C for 15 min. Fifty microliter of the suspension was plated onto tryptic soy agar (TSA, Difco, Franklin Lakes, NJ) and incubated for 48 h at 24 °C. Individual colonies were streaked on TSA.

Pods were surface disinfected with 20% Clorox. The exocarp was aseptically removed and sections (0.42 cm³) of mesoderm were removed. The periderm was aseptically removed from flower cushions and branches and internal sections (0.5 cm³) were sampled. Tissue was placed into test tubes with 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.0) and incubated 75 °C for 15 min. Pod, branch, and flower cushion sections were placed directly on TSA, and incubated for 24–48 h at 24 °C. Individual bacterial colonies were selected and maintained on TSA. Heat treatment selected only endospore-forming bacteria; therefore selective media was not needed. Bacteria were stored on TSA slants for shipping and in 20% glycerol under liquid nitrogen for long term storage.

2.2. Species identification

PCR was conducted directly from one day old colonies using 20 µl of master mix (2 µl 10× PCR buffer with 1.5 mM MgCl₂, 1.6 µl dNTP mix (200 µM each), 0.4 µl 530f primer (10 µM), 0.4 µl 1392r primer (10 µM), and 0.2 µl Taq Polymerase (Gene Choice, San Diego, CA) using 16S rDNA universal primers 530f (5'-GTGCCAGCMGCCGCGG) and 1392r (5'-ACGGGCGGTGTGTRC) (Lane, 1991). PCR amplification was conducted using an Eppendorf Mastercycler Personal Thermal Cycler (Eppendorf AG, Hamburg, Germany) with the following cycle: 5 min at 95 °C; followed by 35 cycles of 94 °C for 15 s, 58 °C for 15 s, 72 °C for 15 s; and final extension at 72 °C for 5 min. PCR products were cleaned with ExoSAP-IT (USB Corp., Santa Clara, CA).

Amplicons were sequenced using the 530f and 1392r universal primers using an ABI Hitachi 3730XL DNA Analyzer (Hitachi Ltd.,

Tokyo, Japan) at the Penn State Genomics Core Facility. Sequences from the forward and reverse primer were edited and aligned using Sequencher 4.7 (Gene Codes, Ann Arbor, MI), then the sequences were compared to similar 16s rRNA sequences using Seqmatch of the Ribosomal Database Project (RDP 10.15, <http://rdp.cme.msu.edu/index.jsp>) (Wang et al., 2007). Relatedness of isolates and related bacteria (Ahmed et al., 2007; Anandaraj et al., 2009; Gilbert et al., 2004; Hoffmaster et al., 2004; Jeng et al., 2001; Narisawa et al., 2008) from the GenBank database was inferred using the neighbor-joining method (Saitou and Nei, 1987) of MEGA 4.0 (Kumar et al., 2008) to infer bacterial species.

2.3. Confirmation of heat tolerance by bacterial endophytes

Confirmation of tolerance to heat (assumption of endospore formation) was conducted by growing bacteria in 800 µl of tryptic soy broth (Difco, Franklin Lakes, NJ) in triplicate 1.7 ml vials. Tubes were incubated at 28 °C for 5 days, heated to 75 °C for 15 min, and then spread onto TSA plates. The plates were incubated at room temperature (24 °C) for 24 h and observed for bacterial growth. The experiment was repeated to confirm results.

2.4. *Bacillus cereus* assay

B. cereus HiVeg Agar (HiMedia Laboratories, Mumbai, India) was prepared following manufacturer's directions to select *B. cereus* group cells. Each isolate was spotted onto three replicate plates which were incubated at 28 °C for 24–48 h and plates were observed for a colorimetric change (Mossel et al., 1967). *B. cereus* isolates BP24 and BT8 were used as positive controls. The experiment was repeated to confirm results.

2.5. Chitinase assay

Chitinase production was assessed by visualizing clearing around colonies on chitin nutrient agar (CNA) (Kokalis-Burelle et al., 1992). Plates were incubated in the dark at 28 °C for 7–10 days. *B. cereus* isolates BP24 and BT8, known chitinase producers, were used for positive controls. The experiment was repeated to confirm results.

2.6. *In vitro* antagonism assay

An *in vitro* plate pairing assay was conducted against the cacao pathogens *Phytophthora capsici* (H. Purdy, isolate 73-73, Ecuador), *Monilophthora roreri* (C. Suárez, Ecuador), and *Monilophthora perniciosa* (C. Suárez, Ecuador). Bacteria were streaked between two mycelial plugs of the pathogen (5 cm apart) on triplicate potato dextrose agar (PDA; Difco, Franklin Lakes, NJ) plates. Plates were incubated at 28 °C and radial growth of the pathogen was measured every 1–3 days. Growth rate of the pathogen (mm/day) and final colony diameter were compared using PROC GLM followed by Dunnett's analysis using SAS 9.1 (SAS Institute Inc., Raleigh, NC) and were used to calculate percent inhibition. The experiment was repeated to confirm results.

2.7. Cacao leaf colonization assay

Rooted cuttings of *T. cacao* 'ICS1' (Maximova et al., 2005) were transplanted into a soil mix (two parts fine sand, two parts Perlite, and one part Promix) and maintained in a greenhouse at 60% relative humidity with a photoperiod of 12 h light at 29 ± 3 °C and 12 h dark at 26 ± 3 °C for 3 months. Plants were drip irrigated with 1/10 strength Hoagland's nutrient solution (160 ppm N).

The ability of chitinolytic isolates to colonize cacao leaves was tested in growth chamber (Convion Model PGR16, Winnipeg,

Canada) studies using: *B. cereus* 2506ht 2.1.1, *B. cereus* A2046 1.1.1, *Lysinibacillus sphaericus* A2076 5.1.7, *B. firmus* CCAT1858 2.1.1, *B. cereus* CCAT1858 2.1.2, *B. cereus* CCAT1858, 2.1.3, *B. cereus* CCAT1858 2.1.6, *Lysinibacillus fusiformis* CUR3 2.1.3, *Bacillus subtilis* CUR3 3.1.1, *Bacillus pumilus* EET103ht 1.1.1, *Bacillus* sp. EET Mn 30/10, *B. cereus* UNAP11.2.3, *B. cereus* UVAP 1.2.2, and *B. cereus* UVAP 1.2.3. Chitinolytic bacteria were chosen due to the prevalence of and high losses to the diseases caused by *Moniliophthora* spp. in Central and South America. Bacterial isolates were applied at 1×10^8 CFU/ml with 0.24% Silwet (vol/vol) following the methodology of Melnick et al. (2008) to five replicate plants per treatment. Plants were wrapped in transparent plastic bouquet wrappers and were maintained in a randomized block design at 28 °C with a 12-h photoperiod at 65% RH and 12-h dark period at 65% RH. Irrigation with 1/10 Hoagland's solution occurred at four-hour intervals. Two separate experiments were conducted to accommodate all isolates, due to chamber size and APHIS regulations.

Colonization of cacao leaves was determined at biweekly intervals following the methodology of Melnick et al. (2008). A third experiment was conducted with *L. sphaericus* A2076 5.1.7, *B. cereus* CCAT1858 2.1.2, *B. cereus* CCAT1858, 2.1.3, *B. cereus* CCAT1858 2.1.6, *B. subtilis* CUR3 3.1.1, and *B. pumilus* EET103ht 1.1.1 to determine the distribution of the endophytes through the leaves. Endophytic populations were estimated by disinfecting leaves for 3 min with 10% Clorox with agitation, followed by two rinses in sterile distilled water. Endospore-forming populations were estimated by incubating the triturated supernatant for 15 min at 75 °C before plating. Colony morphology of the introduced bacteria was compared to that of isolated bacteria.

2.8. Detached leaf assay to determine the ability to suppress disease

The ability of the 14 isolates used in colonization studies to suppress disease was assessed by challenging leaf disks with *P. capsici* isolate 73-73 (Ecuador, Purdy). *P. capsici* zoospores were harvested from 5-day-old cultures grown on 20% V8 agar at 28 °C with a 12 h light cycle, following the protocol of Lawrence (1978). The detached leaf assay was conducted with 5×10^3 zoospores/ml following the methodology of Melnick et al. (2008). Each treatment consisted of 3–5 leaves from four replicate plants, depending on availability of transient young leaves. Disease was determined over time and was used to calculate area under the disease progress curve (AUDPC) (Shanner et al., 1977). Data were statistically analyzed for significance using ANOVA followed by Tukey's HSD ($p = 0.05$) using the SAS 9.1.

2.9. Culture-independent analysis of the microbial communities of cacao leaves

Fifteen, 25 year old randomly distributed 'Nacional' cacao trees with no visible disease were selected for the experiment. Treatments were unsprayed control, *L. sphaericus* A2076 5.1.7 (A20), *B. cereus* CCAT1858 2.1.2 (CT), *B. subtilis* CUR3 3.1.1 (CR), and *B. pumilus* EET103ht 1.1.1 (ET), since they were robust phyllosphere colonists and reduced development of *P. capsici*. Bacteria were grown and prepared as previously stated. Branches of three replicate trees were tagged in the north, east, south, and west cardinal directions and sprayed with the bacterial solution (with 0.24% Silwet L-77) at the onset of the rainy season. No treatments were applied to control branches to estimate the native diversity of cacao leaves. Three months post inoculation, five leaf disks (10.4 cm²) were removed from two leaves from each branch and grouped as sampling across one tree. This time period was chosen to test whether the bacteria could reproduce and redistribute in the cacao canopy. Plugs were disinfected as previously stated and placed into RNA-Later (Applied Biosystems, Foster City, CA) to preserve the tissue for nucleic

acid extraction (Michiels et al., 2003). PCR amplification for ARISA using bacilli specific primers (BacARISA) was conducted following the protocol of Garbeva et al. (2003) using 400 ng of genomic DNA in a 25 µl reaction consisting of 12.5 µl of GoTaq Green Master Mix (Promega Corp., Madison, WI), 1 µl each of primer, and water to 25 µl. PCR amplification for ARISA using eubacterial specific primers (BARSIA) was conducted following Cardinale et al. (2004) with the reaction mix above. The reaction conditions were 95 °C for 3 min; 28 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1.5 min; with a final extension of 72 °C for 10 min. Success of PCR reactions was examined through gel electrophoresis and PCR products were stored at –20 °C.

One microliter fluorescent labeled PCR product was analyzed on an ABI Hitachi 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) with an internal standard at the Penn State Core Genomic Facility, University Park, PA. Fluorograms were analyzed using Genemapper[®] Fragment Analysis Software version 4.0 (Applied Biosystems) to record fragment length and relative abundance of each peak. Unique fragment lengths are reported as operational taxonomic units (OTUs). Relative abundance was inferred by normalizing individual peak fluorescence to total fluorescence to account for variations between runs.

Constrained ordination analysis in CANOCO version 4.5 (Leps and Smilauer, 2003; ter Braak and Smilauer, 2002) was used to determine whether bacterial treatment had a significant impact on the OTUs present in cacao leaves. The input data was the relative abundance of each fragment in the ARISA profiles. Detrended correspondence analysis conducted on the data determined that the data was unimodal (having a beta diversity >4) (Leps and Smilauer, 2003). The axes created by the differing bacterial treatments constrained the ordination of the data. Profiles were analyzed using conical ordination analysis (CCA) using Hill's scaling (Leps and Smilauer, 2003; ter Braak and Smilauer, 2002). Bacterial treatments were assessed with Monte Carlo simulations with 999 iterations (Leps and Smilauer, 2003; ter Braak and Smilauer, 2002). Biplots were created using CanoDraw (ter Braak and Smilauer, 2002).

3. Results

3.1. Isolation and identification of bacterial endophytes

A total of 69 endospore-forming bacteria were isolated from the different cacao clones sampled (see Table 1). Of the isolates obtained, 14.5% were from leaves, 17.4% were from floral cushions, 24.6% were from pods, and 43.5% were from branches. Fifteen different species from five endospore-forming genera were cultured and identified from cacao (Table 1). Multiple species and genera were found to coexist within the same tree as one flower cushion of *T. cacao* clone A2076 was inhabited by three genera of bacteria. Forty-seven isolates were members of either the *Bacillus a* or *Bacillus c* clades. *B. pumilus* and *B. subtilis* were common inhabitants of internal cacao tissues. *B. cereus* group cells comprised 29.0% of isolates. Less common to this collection were *Bacillus flexus*, *Bacillus firmus*, *Bacillus megaterium*, *Solibacillus silvestris*, and *Brevibacillus* sp.

3.2. Screening of bacterial endophytes for antagonistic qualities

One-hundred percent of the isolates were tolerant to heating at 75 °C, suggesting the presence of endospores. Fourteen of the 69 isolates (20.3%) were chitinolytic *in vitro* (Table 1), 56.3% of these isolates being *B. cereus* group cells. Antagonism to pathogenic fungi was detected in *in vitro* plate assays (Table 1). Minimum statistically significant ($p < 0.0001$) growth inhibition of the pathogens

Table 1

Results of characterization of bacterial endophytes for attributes such as putative species identification, Genbank accession of partial 16S gene, endospore-production assumed by heat-tolerance; reaction on *B. cereus* agar; *in vitro* chitinase production; antagonism toward the cacao pathogens *Phytophthora capsici* (PC), *Monilophthora roreri* (MR), and *M. perniciosa* (MP), via *in vitro* plate assays. Bacteria were isolated from surface sterilized cacao tissue from trees escaping diseases at the Instituto Nacional Autonomo de Investigaciones Agropecuarias Estacion Experimental Tropical (INIAP-EET).

Isolate ID ^a	Cacao tissue	Species ID	GenBank Accession	Heat tolerance ^b	<i>Cereus</i> agar	Chitinase Prdtn	PC ^c	MR	MP
1 103.hm(2)	Leaf	<i>Bacillus subtilis</i>	HQ262955	+	–	–	67.8%	100.0%	67.5%
2 103.hm 2.1.1	Branch	<i>Bacillus pumilus</i>	HQ262956	+	–	–	50.0%	77.2%	67.5%
3 103.ht 2.1.1	Branch	<i>Bacillus subtilis</i>	HQ262957	+	–	–	69.7%	100.0%	81.8%
4 103ht 2.1.2	Branch	<i>Bacillus pumilus</i>	HQ262958	+	–	–	n.s.	n.s.	49.4%
5 2073 2.1.1	Branch	<i>Bacillus subtilis</i>	HQ262959	+	–	–	73.1%	100.0%	78.6%
6 2073 2.1.2	Branch	<i>Lysinibacillus fusiformis</i>	HQ262960	+	–	–	n.s.	n.s.	57.1%
7 2073 2.1.3	Branch	<i>Bacillus amyloliquefaciens</i>	HQ262961	+	–	–	75.0%	100.0%	83.8%
8 2506tallo 1.1.1	Pod	<i>Bacillus</i> sp.	HQ262962	+	+	–	38.5%	n.s.	n.s.
9 2506hm 2.1.1	Branch	<i>Bacillus pumilus</i>	HQ262963	+	–	–	n.s.	n.s.	n.s.
10 2506hm2.1.1(2)	Branch	<i>Bacillus cereus</i>	HQ262964	+	–	–	51.9%	100.0%	n.s.
11 2506ht2.1.1	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262965	+	+	–	35.6%	56.1%	n.s.
12 A2046 1.1.1	Pod	<i>Bacillus cereus</i>	HQ262966	+	+	+	n.s.	28.6%	n.s.
13 A2076 1.1.1	Pod	<i>Bacillus megaterium</i>	HQ262967	+	–	–	n.s.	n.s.	n.s.
14 A2076 3.1.1	Leaf	<i>Lysinibacillus sphaericus</i>	HQ262968	+	–	–	n.s.	n.s.	n.s.
15 A2076 3.2.1	Leaf	<i>Bacillus pumilus</i>	HQ262969	+	–	–	43.8%	61.9%	96.3%
16 A2076 5.1.1	Flower cushion	<i>Bacillus pumilus</i>	HQ262970	+	–	–	63.0%	85.2%	41.6%
17 A2076 5.1.2	Flower cushion	<i>Lysinibacillus sphaericus</i>	HQ262971	+	–	–	n.s.	n.s.	n.s.
18 A2076 5.1.3	Flower cushion	<i>Lysinibacillus sphaericus</i>	HQ262972	+	–	–	n.s.	n.s.	n.s.
19 A2076 5.1.4	Flower cushion	<i>Bacillus cereus</i>	HQ262973	+	–	–	42.3%	n.s.	n.s.
20 A2076 5.1.5	Flower cushion	<i>Lysinibacillus sphaericus</i>	HQ262974	+	–	–	38.7%	n.s.	46.4%
21 A2076 5.1.6	Flower cushion	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262975	+	+	–	n.s.	n.s.	n.s.
22 A2076 5.1.7	Flower cushion	<i>Lysinibacillus sphaericus</i>	HQ262976	+	–	+	n.s.	n.s.	n.s.
23 A2076 5.1.8	Flower cushion	<i>Solibacillus silvestris</i>	HQ262977	+	–	–	41.9%	n.s.	n.s.
24 CCAT1858 1.1.1	Pod	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262978	+	–	–	50.0%	30.4%	n.s.
25 CCAT1858 2.1.1	Branch	<i>Bacillus firmus</i>	HQ262979	+	–	+	n.s.	n.s.	n.s.
26 CCAT1858 2.1.2	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262980	+	–	+	n.s.	n.s.	n.s.
27 CCAT1858 2.1.3	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262981	+	+	+	n.s.	70.4%	n.s.
28 CCAT1858 2.1.4	Branch	<i>Paenibacillus</i> sp.	HQ262982	+	–	–	n.s.	n.s.	n.s.
29 CCAT1858 2.1.5	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262983	+	+	–	41.8%	41.3%	n.s.
30 CCAT1858 2.1.6	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262984	+	+	+	n.s.	n.s.	n.s.
31 CCAT1858 2.1.7	Branch	<i>Bacillus cereus</i>	HQ262985	+	+	–	n.s.	n.s.	n.s.
32 CCAT4688 2.1.1	Branch	<i>Paenibacillus alvei</i>	HQ262986	+	–	–	43.8%	n.s.	n.s.
33 CUR3 1.1.1	Pod	<i>Bacillus subtilis</i>	HQ262987	+	–	–	79.3%	40.4%	60.4%
34 CUR3 1.1.2	Pod	<i>Bacillus subtilis</i>	HQ262988	+	–	–	n.s.	n.s.	n.s.
35 CUR3 2.1.1	Branch	<i>Bacillus pumilus</i>	HQ262989	+	–	–	30.7%	n.s.	38.3%
36 CUR3 2.1.2	Branch	<i>Lysinibacillus sphaericus</i>	HQ262990	+	–	–	n.s.	n.s.	n.s.
37 CUR3 2.1.3	Branch	<i>Lysinibacillus fusiformis</i>	HQ262991	+	–	+	n.s.	41.9%	n.s.
38 CUR3 2.1.4	Branch	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ262992	+	–	–	39.9%	n.s.	n.s.
39 CUR3 2.2.1	Branch	<i>Bacillus pumilus</i>	HQ262993	+	–	–	43.3%	n.s.	n.s.
40 CUR3 2.2.2	Branch	<i>Solibacillus silvestris</i>	HQ262994	+	–	–	n.s.	n.s.	63.7%
41 CUR3 3.1.1	Leaf	<i>Bacillus subtilis</i>	HQ262995	+	–	+	n.s.	n.s.	n.s.
42 EB1922 1.1.1	Pod	<i>Bacillus pumilus</i>	HQ262996	+	–	–	n.s.	91.6%	51.3%
43 EB1922 1.1.2	Pod	<i>Bacillus megaterium</i>	HQ262997	+	–	–	n.s.	n.s.	n.s.
44 EB1922 1.1.3	Pod	<i>Bacillus subtilis</i>	HQ262998	+	–	–	77.9%	100.0%	53.2%
45 EB1922 5.1.1	Flower cushion	<i>Bacillus flexus</i>	HQ262999	+	–	–	n.s.	n.s.	n.s.
46 EB1922 5.1.2	Flower cushion	<i>Bacillus cereus</i>	HQ263000	+	–	–	n.s.	n.s.	n.s.
47 EET103Tallo1.1.1	Leaf	<i>Bacillus pumilus</i>	HQ263001	+	–	–	n.s.	n.s.	n.s.
48 EET103ht 1.1.1	Leaf	<i>Bacillus pumilus</i>	HQ263002	+	+	+	33.7%	53.3%	n.s.
49 EET103.ht 2.1.1	Branch	<i>Bacillus cereus</i>	HQ263003	+	–	–	42.3%	53.3%	n.s.
50 EET103Mm	Pod	<i>Bacillus pumilus</i>	HQ263004	+	–	–	n.s.	n.s.	50.0%
51 EET103Mm 30/10	Pod	<i>Bacillus</i> sp.	HQ263005	+	–	+	44.2%	n.s.	n.s.
52 EET103Tallo 3-10	Branch	<i>Bacillus mycoides</i>	HQ263006	+	+	–	n.s.	n.s.	42.9%
53 ET62 2.1.1	Branch	<i>Bacillus amyloliquefaciens</i>	HQ263007	+	–	–	64.4%	96.4%	92.9%
54 ET62 2.1.5	Branch	<i>Bacillus subtilis</i>	HQ263008	+	–	–	82.7%	86.8%	85.7%
55 ET62 2.1.6	Branch	<i>Bacillus subtilis</i>	HQ263009	+	–	–	72.1%	86.8%	89.3%
56 ET62 2.1.7	Branch	<i>Bacillus amyloliquefaciens</i>	HQ263010	+	–	–	79.0%	94.7%	89.3%
57 ET62 5.1.1	Flower cushion	<i>Bacillus mycoides</i>	HQ263011	+	+	–	n.s.	n.s.	71.4%
58 ET62 5.1.3	Flower cushion	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ263012	+	+	–	50.0%	n.s.	n.s.
59 L25H64 2.1.1	Branch	<i>Bacillus cereus</i>	HQ263013	+	+	–	30.6%	72.4%	n.s.
60 L25H64 2.1.3	Branch	<i>Brevibacillus brevis</i>	HQ263014	+	–	–	n.s.	n.s.	53.6%
61 SCA 1.1.1	Pod	<i>Bacillus megaterium</i>	HQ263015	+	+	–	n.s.	n.s.	n.s.
62 SCA(5) 1.1.1	Pod	<i>Bacillus megaterium</i>	HQ263016	+	+	–	n.s.	n.s.	n.s.
63 SPEC541 1.1.1	Pod	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ263017	+	+	–	61.3%	100.0%	86.4%
64 UNAP1 1.1.1	Pod	<i>Bacillus subtilis</i>	HQ263018	+	–	–	58.1%	37.5%	60.7%
65 UNAP1 1.2.2	Pod	<i>Bacillus pumilus</i>	HQ263019	+	–	–	41.9%	n.s.	n.s.
66 UNAP1 1.2.3	Pod	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ263020	+	+	+	n.s.	n.s.	n.s.
67 UVAP 1.1.1	Pod	<i>Paenibacillus</i> sp.	HQ263021	+	–	–	n.s.	n.s.	n.s.
68 UVAP 1.2.2	Pod	<i>Bacillus cereus</i>	HQ263022	+	–	+	n.s.	n.s.	n.s.
69 UVAP 1.2.3	Pod	<i>Bacillus cereus</i> or <i>thuringiensis</i>	HQ263023	+	–	+	n.s.	n.s.	n.s.

^a The letters in the isolate ID indicate the cacao clone the isolate was isolated from, i.e. CCAT1858 1.1.1 is from a selection of clone CCAT-1858 at INIAP-EET in Pichilingue, Ecuador.

^b “+” indicate a positive result with “–” indicates a negative result.

^c Percentages listed under pathogens indicate statistically significant percentage of inhibition of the pathogen growth (mm/day) compared to the growth of unchallenged control at $p < 0.0001$ via the Dunnett's test. The designation n.s. indicates that statistically significant pathogen inhibition was not achieved.

by antagonistic isolates was: 30.7% for *P. capsici*, 28.6% of *M. roleri*, and 38.3% for *M. pernicioso*. Of the isolates tested, 37.7% were antagonistic to *M. roleri*, 36.2% were antagonistic to *M. pernicioso*, and 49.3% were antagonistic to *P. capsici*. Both *Moniliophthora* spp. were inhibited by 24.6% of isolates, while 39.1% inhibited *M. roleri* but not *M. pernicioso* and 40.6% inhibited *M. pernicioso* but not *M. roleri*. All three pathogens were inhibited by 21.7% of the isolates.

One-hundred percent of *Bacillus amyloliquefaciens* isolates inhibited the growth of all pathogens, while 0% of *B. firmus* and *B. megaterium* isolates inhibited pathogen growth. Eighty percent of *B. subtilis* isolates inhibited the growth of all three pathogens,

while the remaining 20% did not inhibit growth of the three pathogens. Only one *B. cereus* isolate SPEC541 1.1.1 inhibited the growth of all the pathogens tested and this was the only *B. cereus* to inhibit the growth of *M. pernicioso*.

3.3. Colonization of cacao leaves and suppression of *P. capsici* in planta

All 14 isolates, chosen based upon chitinolytic ability, were capable of long term colonization (≥ 55 days) of cacao foliage when sprayed onto plants with 0.24% Silwet L-77 (Fig. 1). Control plants were colonized (Fig. 1), but at population levels 1.5–4 log CFU/cm² lower than treated leaves. Most isolates were capable of

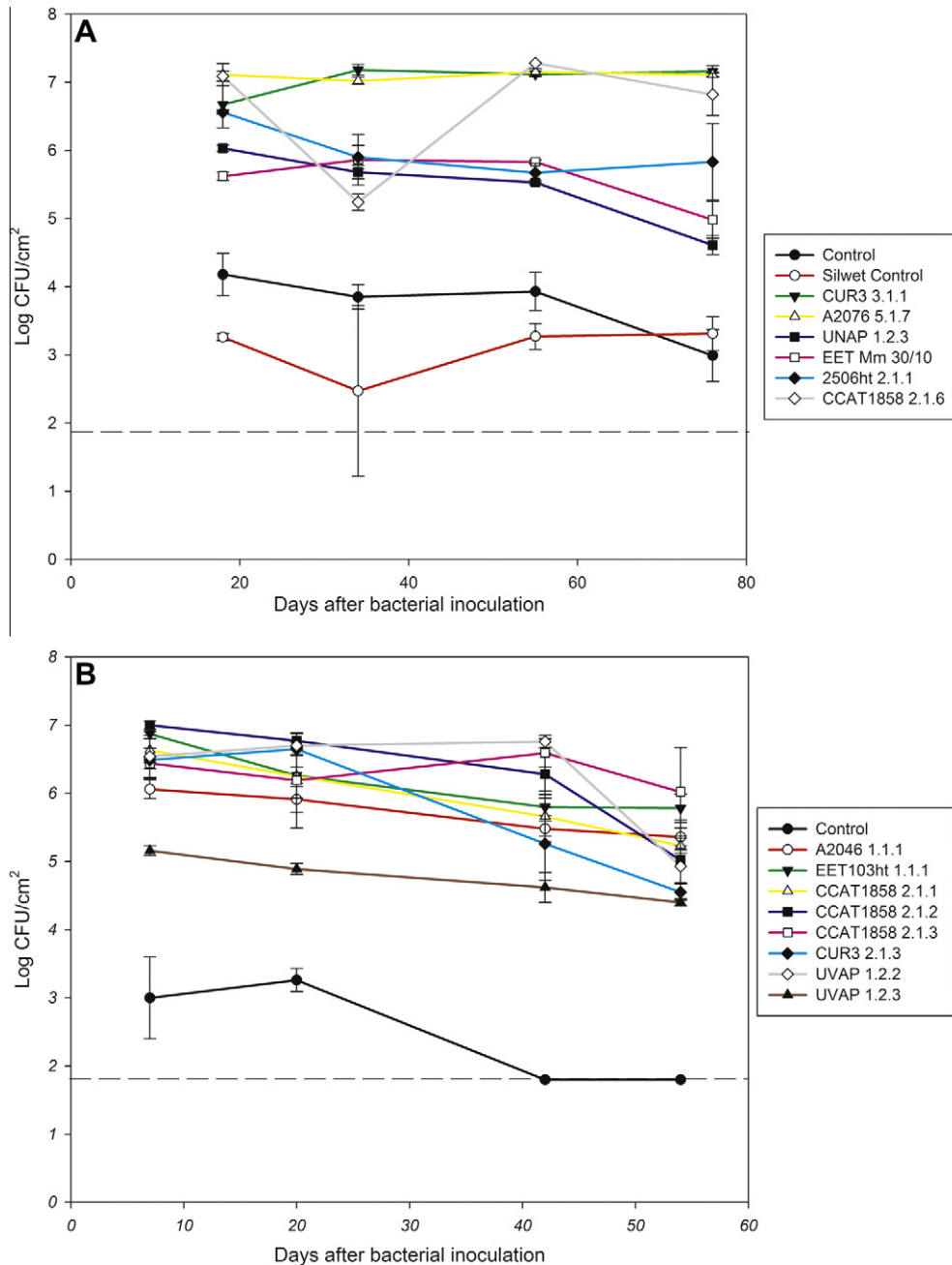


Fig. 1. Mean bacterial colonization of mature green cacao leaves sprayed with either 0.24% Silwet (vol/vol) or 0.24% Silwet + log 8.0 CFU/ml bacterial endophyte. Control represents leaves that were never sprayed and Silwet control represents leaves that were sprayed with 0.24% Silwet L-77 in 0.1 M potassium phosphate buffer without bacteria. The remaining treatments represent chitinolytic cacao bacterial endophytes applied at log 8.0 CFU/ml with 0.24% Silwet. Bars extending from the means represent standard errors of that mean. The dashed line indicates the minimum detection level of the experiment. (A) In experiment one, colonization was measured at 18, 24, 55, and 76 days after application of the bacteria and (B) in experiment two, colonization levels were assessed on 7, 20, 42, and 54 days after application of the bacteria.

long-term colonization at relatively high population levels (log 5.0–7.0 CFU/cm²) (Fig. 1). Population levels were not dependent on the bacterial species or the tissue the isolate originated from, but were variable amongst isolates.

In the confirmation study, total colonization (Fig. 2A) was 4.5–6.8 CFU/cm². Isolates colonized the endophytic portions of cacao leaves (Fig. 2B), but at lower population levels than total colonization (0.5–1.2 log CFU/cm² depending on the comparison). Control leaves were endophytically colonized, but at lower populations (1.5–3.5 logs) than treated leaves and had a rapid drop in internal colonization between 21 and 30 dpi (Fig. 2B). Endospores of the five isolates were detected in the endophytic portions of cacao leaves (Fig. 3). The population levels of endophytic endospores in control plants were below detectable levels (1.8 log CFU/cm²). Several isolates had fewer endospores in the internal portions than the detection threshold levels at 12 days after inoculation, but these populations recovered by 21 days.

Fifty-seven percent of the chitinolytic isolates suppressed disease in the detached leaf assay (*L. sphaericus* A2076 5.1.7, *Bacillus firmus* CCAT1858 2.1.1, *B. cereus* CCAT1858 2.1.2, *B. cereus* CCAT1858 2.1.3, *B. cereus* CCAT1858 2.1.6, *L. fusiformis* CUR3

2.1.3, *B. subtilis* CUR3 3.1.1, and *B. pumilus* EET103ht 1.1.1) (Table 2). *B. cereus* 2506ht 2.1.1 and *Bacillus* EET103 Mm 30/10 suppressed the growth of *P. capsici* *in vitro* (Table 1), but not *in planta* (Table 2). Leaves colonized with *Bacillus* EET103 Mm30/10, *B. cereus* UNAP1 1.2.3, and *B. cereus* UVAP 1.2.3 had AUDPCs that were 40–55% higher than controls. Of the isolates that suppressed *P. capsici* in detached leaf assays, only EET103ht 1.1.1 inhibited the growth of *P. capsici* in the dual plate assay. Based upon high levels of endophytic colonization and consistent disease suppression results, the six best bacterial colonists (A2076 5.1.7, CCAT 2.1.2, CCAT 2.1.3, CCAT 2.1.6, CUR3 3.1.1, and EET103ht 1.1.1) were used in subsequent studies to further characterize of colonization of cacao leaves.

3.4. Multivariate analysis of ARISA profiles via ordination analysis

PCR of rRNA using group specific primers indicated that cacao leaves harbored both bacilli (Fig. 4) and a broader array of eubacteria (Fig. 5). In terms of bacilli, 21 unique bacterial OTUs (as determined by fragment length) were present in all sampled leaves, with 16–23S intergenic spacer region length varying from 321 to

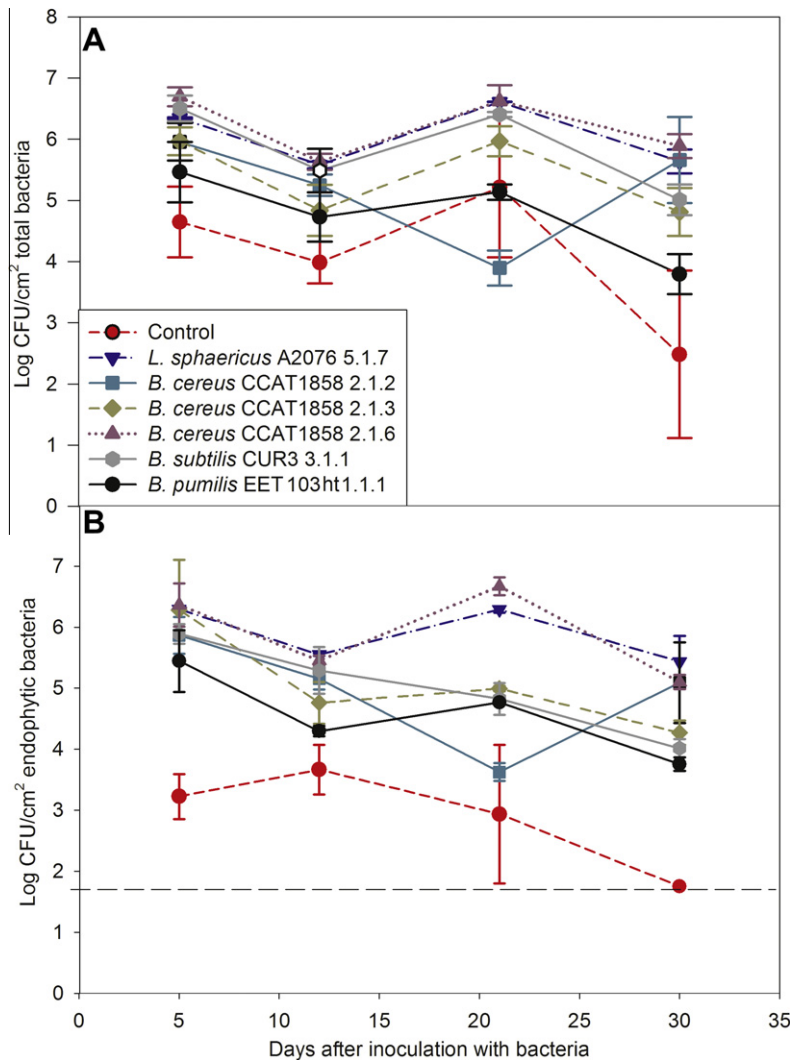


Fig. 2. Mean total and endophytic colonization of mature cacao leaves at 5, 12, 21, and 30 days after inoculation with bacterial endophytes. (A) represents total colonization of leaves in the epiphytic and endophytic portions of the leaf and (B) represents colonization by vegetative cells and endospores in the endophytic portions of the leaves. Application of bacteria to cacao leaves occurred at day 0 when plants were sprayed with 0.24% Silwet L-77 (vol/vol) + log 8.0 CFU/ml bacterial suspension. Control plants were treated with 0.24% Silwet in 0.1 M potassium phosphate buffer. Bars extending from the means represent the standard error of that mean. The horizontal dashed line indicates the minimum detection level of the experiment (log 1.8 CFU/cm²). Population from control plants were below the minimum detectable level, so could not be included on the graphs.

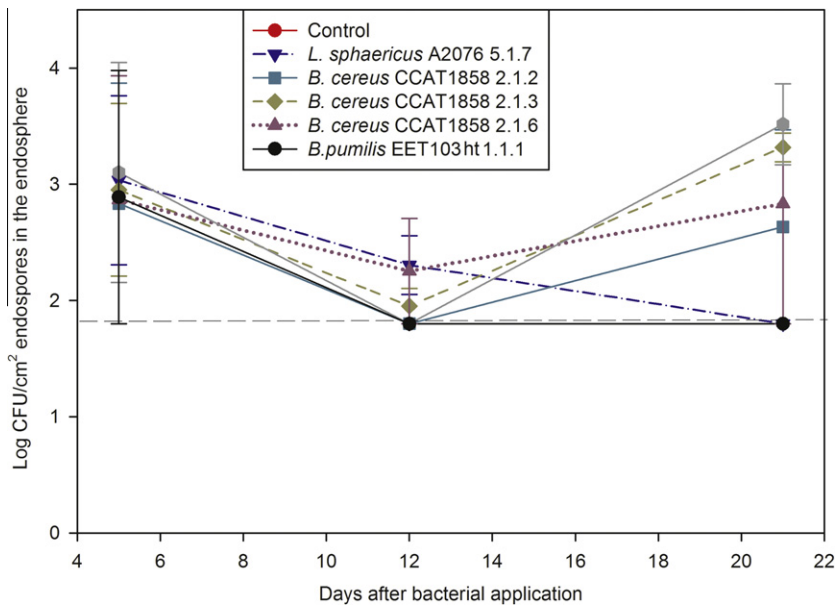


Fig. 3. Mean endospores colonizing the endosphere of mature cacao leaves at 5, 12, and 21 days after inoculation with bacterial endophytes. Application of bacteria to cacao leaves occurred at day 0 when plants were sprayed with 0.24% Silwet L-77 (vol/vol) + log 8.0 CFU/ml bacterial suspension. Control plants were treated with 0.24% Silwet in 0.1 M potassium phosphate buffer. Bars extending from the means represent the standard error of that mean. The horizontal dashed line indicates the minimum detection level of the experiment (log 1.8 CFU/cm²). Population of control plants were below the detectable level.

Table 2

Results of detached leaf assay for cacao leaves colonized with bacterial endophytes and challenged with *P. capsici*. Bacteria were applied to the plants as a 1×10^8 CFU/ml bacterial suspension with 0.24% Silwet (v/v). Immature green leaves (not present at bacterial application) were removed from the plants when present and challenged with 10 μ l droplets containing *P. capsici* zoospores at 5×10^3 CFU/ml. The percent area of the lesion that was necrotic was measured every 8–12 h for 52 h, and then used to calculate area under the disease progress curve (AUDPC). AUDPC of control indicates disease severity on leaves of untreated control plants, while AUDPC of treated leaves indicates disease severity of leaves colonized with chitinolytic endophytes. The variation in the AUDPC of the control is due to separate challenges throughout the course experimentation.

Isolate ID	AUDPC of control	AUDPC of treated leaves ^a	Inhibition of <i>P. capsici</i> ^b
2506ht2.1.1	3120	3775	35.60%
A2046 1.1.1	2143	2287	0%
A2076 5.1.7	916	313*	0%
CCAT1858 2.1.1	2143	1123*	0%
CCAT1858 2.1.2	2143	943*	0%
CCAT1858 2.1.3	924	353*	0%
CCAT1858 2.1.6	3120	2418*	0%
CUR3 2.1.3	915	339*	0%
CUR3 3.1.1	3120	2150*	0%
EET103ht 1.1.1	923	282*	33.70%
EET103Mm 30/10	923	1429	44.20%
UNAP1 1.2.3	2113	3000	0%
UVAP 1.2.2	1826	1892	0%
UVAP 1.2.3	2143	3000	0%

^a Asterisks (*) indicate that the AUDPC of treated leaves was significantly ($\alpha = 0.05$) less than of the control for the experiment when compared using Tukey's HSD.

^b Percentage of inhibition of the *P. capsici* growth in dual plate assays.

796 bp. Larger fragments could not be accurately resolved on the genetic analyzer, so were not included in analysis. In terms of total bacteria, 58 unique bacterial OTUs were present in all treated leaves, with the 16–23S intergenic spacer region varying from 223 to 797 bp. In *B. pumilus* EET103ht1.1.1 treated leaves, *B. pumilus* (OTU434 through BacARISA of the pure culture and OTU259 through BARISA) was not the most abundant fragment amplified. Similar results were found for the other bacterial isolates.

CCA did not show clustering by treatment; therefore inundative application of the four bacteria did not significantly shift the microbial community of either bacilli ($p = 0.0662$, Fig. 4) or of total eubacteria ($p = 0.1724$, Fig. 5) inhabiting cacao leaves at three months after application. In terms of bacilli, the first axis of the biplot explains 38% of the variance in the microbial community (Fig. 4), while the second axis explains 29% of the variance. The first axis of the CCA biplot of eubacterial community explains 27% of the

variance in the community composition (Fig. 5), while the second axis explains 25%. Community composition was not significantly different between leaves treated with the four bacteria or untreated control leaves.

4. Discussion

This research demonstrated that cacao is not only home to fungal endophytes, but also bacterial endophytes that inhabited all the tissues sampled. Branches were the source of 31/69 isolates (Table 1), but no comparison on diversity of bacterial endophytes in different cacao tissue can be made as the number of samples varied between tissues. It should be noted that a larger number of leaves were sampled than branches, but only six bacterial endophytes were isolated from these leaves (Table 1). As seen in previous work,

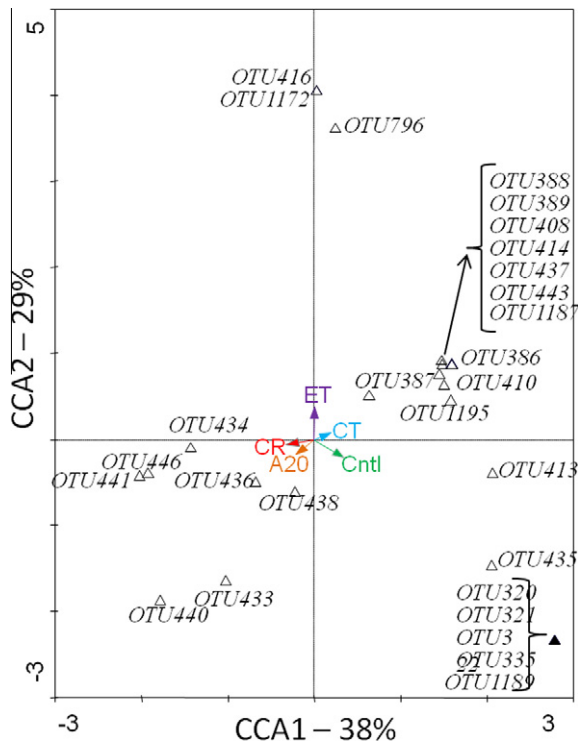


Fig. 4. CCA biplot showing the affect of applying different BCAs on community structure of endospore-forming bacteria (bacilli) of cacao leaves, as determine by ARISA profile. Bacteria were applied to cacao foliage at 1×10^8 CFU/cm² with 0.2% Silwet. Treatments (indicated by vectors) were untreated control (Cntl), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Microbial OTUs are signified by Δ and OTU# signifies the length of ARISA fragment. The first two axes explain 67% of the relationship between the bacterial community and treatment. This plot indicates that there is no significant difference ($p = 0.0662$) in the community structures of bacilli in leaves receiving treatments with different BCAs.

applied bacterial endophytes were capable of persisting for 68+ days in cacao leaves (Melnick et al., 2008). The adverse nature of the sterilization and varied sampling techniques of the tissues may have influenced the number of isolates recovered from the different tissues. The endospore-forming bacteria isolated in this study likely exist in and on cacao trees with actinomycetes (Barreto et al., 2008; Macagnan et al., 2006), enterobacteria (Torres et al., 2008), and a diverse range of fungal endophytes (Arnold et al., 2003; Hanada et al., 2008; Herre et al., 2007; Rubini et al., 2005). Cacao is home to a diverse microbial community that is still only partially characterized.

Isolation of potential BCAs focused on endospore-forming bacteria because of environmental tolerance and long-term storage in commercial biopesticides (Schisler et al., 2004). The secondary selection criterion was the ability of the isolates to produce chitinase. Many environmental *Bacillus* spp. are chitinolytic (Pleban et al., 1997). This trait can play an important role in the natural environment, allowing the bacteria to degrade the chitin of invertebrates and fungi (Sampson and Gooday, 1998; Watanabe et al., 1990). The two main diseases of cacao in the Americas are caused by *M. royeri* and *M. pernicioso*; therefore chitinolytic bacteria could be beneficial in biological control of cacao diseases. Researchers did not screen *Moniliophthora* spp. *in planta*, as *M. royeri* infections are limited to pods (Evans et al., 2003) and infections arising from laboratory inoculation of *M. pernicioso* take a minimum of 3 months to develop and rarely result in 100% disease incidence (Purdy et al., 1997).

Origin of the bacterial isolates had no effect on the ability of the isolates to colonize cacao leaves, inferring that the isolates tested

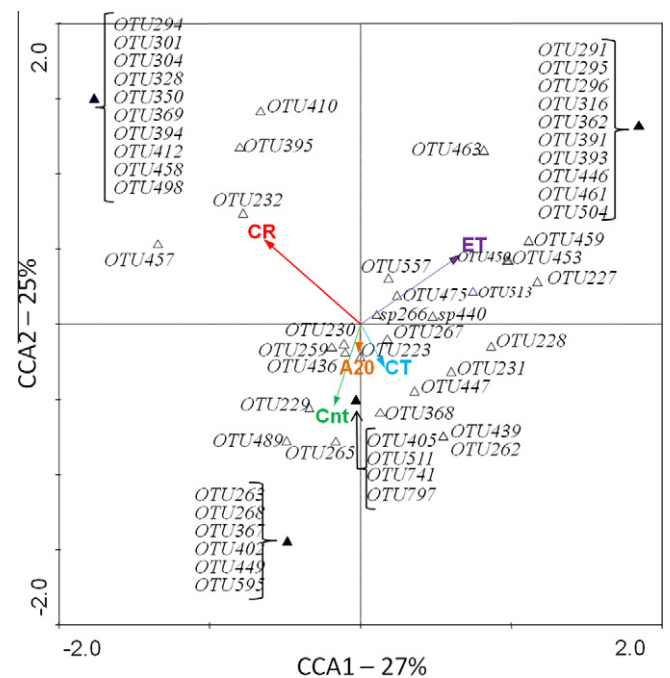


Fig. 5. CCA biplot showing the affect of applying different BCAs on community structure of eubacteria of cacao leaves, as determine by ARISA profile. Bacteria were applied to cacao foliage at 1×10^8 CFU/cm² with 0.2% Silwet. Treatments (indicated by vectors) were untreated control (Cntl), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Microbial OTUs are signified by Δ and OTU## signifies the length of ARISA fragment. The first two axes explain 52% of the relationship between the bacterial community and treatment. This plot indicates that there is no significant difference ($p = 0.1724$) in the community structures of bacteria in leaves receiving treatments with different BCAs.

are not specific to tissue type. Initial internal colonization of leaves occurred through substomatal infiltration caused by Silwet L-77 (Zidack et al., 1992), and then the bacteria survived in the internal environment. Control plants in the chamber studies had lower levels of bacterial colonists (Figs. 1 and 2A), but still had endophytic colonization (Fig. 2B). Endophytic colonization of control leaves was either present before the start of the experiment or was more likely due to phyllosphere bacteria being carried into the plant when the 0.24% Silwet was sprayed into leaves (Zidack et al., 1992). Bacterial endophytes have been isolated from surface sterilized seeds in laboratory studies (Posada and Vega, 2005 and Melnick, unpublished), but no research has looked at isolation from seed immediately after harvest in the field. Further research could focus on whether these bacterial endophytes are vertically or horizontally transmitted, and whether they travel through the plant to colonize tissue that emerges after application. The remaining isolates had similar total and endophytic population levels in internal tissue. Endophytic colonization of cacao leaves by native bacilli varied by approximately $1.0 \log$ CFU/cm² (Fig 2B), while populations of endophytic *B. cereus* from vegetables in cacao leaves varied nearly ≤ 5.5 logs between sampling dates (Melnick et al., 2008) suggesting that the native bacterium may be more adapted to the internal environment of cacao leaves. The low ratio of endospores produced by EET103ht 1.1.1 relative to vegetative cells (Fig 3) suggests that this isolate was neither stressed nor limited by resources in the leaf environment, despite being isolated from a pod.

Since chitinolytic bacilli suppressed *P. capsici* in the detached leaf assay, it is assumed that mechanisms other than chitinase production are involved in disease suppression. The unique nature of the leaf environment could potentially incite antagonism of *P. capsici* by the *Bacillus* spp. Additionally, the native *Bacillus* spp. may

have induced resistance, as *B. cereus* BT8 induced resistance against *P. capsici* in cacao in the detached leaf assay (Melnick et al., 2008). The ability of the bacterial isolates to colonize cacao leaves at high and persistent levels was not always correlated to disease suppression, as 30% of chitinolytic isolates were robust colonists but were not capable of suppressing *P. capsici* in the detached leaf assay. *B. pumilus* EET103ht 1.1.1 had the lowest levels of both total colonization (Figs. 1B and 2A) and endophytic colonization (Fig. 3), yet was capable of suppressing disease in the detached leaf assay. Disease suppression by *B. pumilus* EET103ht 1.1.1 *in planta* likely was related to the ability of the isolate to antagonize *P. capsici* (Table 1) or induce resistance. It should be noted that *Bacillus* EET103 Mm30/10, *B. cereus* UNAP1 1.2.3, and *B. cereus* UVAP 1.2.3 actually increased disease by 40–55% (Table 2), despite, *Bacillus* EET103 Mm30/10 inhibiting the growth of *P. capsici* (Table 1) in the dual plate assay. These results provide further support that use of a dual plate assay in primary selection can eliminate microbes that could suppress disease *in planta* or select for microbes that could potentially enhance disease.

ARISA analysis indicated that there are at least 21 bacilli OTUs inhabiting the sampled cacao leaves, compared to our 15 unique species obtained through the traditional sampling methods we used. It can be assumed that our methodology underestimated the true diversity present in cacao tissue. Sampling revealed only a portion of the eubacteria inhabiting cacao trees, as indicated by the 58 distinct bacterial OTUs amplified during BARISA. BARISA fragment sizes of pure cultures were similar in size to bacilli from Geib et al. (2009), indicating successful amplification via this method. ARISA analysis found no statistically significant differences between the treatments in either eubacteria or bacilli populations, indicating that inundative application of bacteria to the cacao trees did not shift the bacterial communities at three months after application. These results indicate that the bacteria were not likely redistributed at high population levels and that one application of a BCA would not ensure long-term protection of the tree. No inference can be made on the fungal community present in the leaves. Previous lab sampling found that populations of non-native bacteria (Melnick et al., 2008) and native bacteria in leaves (Fig. 1) tend to decrease over time. Populations at three months after colonization had likely naturally declined, while the bacterial community recovered from the disturbance of an inundative application of a single bacterial endophyte. This also indicates that colonization studies from greenhouse grown rooted cuttings are not adequate in estimating colonization in field settings due to exposure to differing conditions and competition with native microbes. Future work on the influence of BCAs on microbial community population dynamics in the field should focus on a collecting a time series to determine (1) how the community shifts at initial application (2) the time in which it takes the native microbial community to recover from the disturbance.

Our ongoing research focuses on screening isolates for their ability to suppress *M. perniciosa* in seedlings and to suppress the development of pod diseases in a field setting. Biological control testing focusing on consortium of several beneficial microbes could be highly beneficial to reducing disease. Endophytic *Trichoderma* spp. from cacao have been shown to provide protection from disease in the field (Krauss et al., 2010), are known to be mycoparasites of *Moniliophthora* spp., and can change the expression of cacao ESTs related to defense during endophytic colonization (Bailey et al., 2006). Combinations of *Trichoderma* spp. and *Bacillus* spp. may result in disease suppression using multiple mechanisms and should be studied.

Overall, this research demonstrated that bacilli are common endophytes of cacao trees and likely play an important role in the native microbial community of cacao trees. The microbial community of cacao is more diverse than previously described, as it is

home to both fungal and bacterial endophytes coexisting in cacao tissues. Additionally, the endophytic microbes likely fluctuate between the epiphytic and endophytic environment, being able to survive in each. The characteristics of some of the bacterial endophytes to act as potential BCAs indicated that these bacteria may act as natural antagonists of pathogenic organisms in the natural environment.

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