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Bacterial endophytes: *Bacillus* spp. from annual crops as potential biological control agents of black pod rot of cacao

Rachel L. Melnick [a], Nina K. Zidack [b], Bryan A. Bailey [c], Siela N. Maximova [d], Mark Guiltinan [d], Paul A. Backman [a,*]

[a] *Department of Plant Pathology, The Pennsylvania State University, 221 Buckhout Laboratory, University Park, PA 16802, USA*
[b] *Department of Plant Science and Plant Pathology, Montana State University, Bozeman, MT 59717, USA*
[c] *USDA/ARS, Sustainable Perennial Crops Laboratory, BARC-West, Beltsville, MD 20705, USA*
[d] *Department of Horticulture, The Pennsylvania State University, University Park, PA 16802, USA*

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Abstract

Diseases are the most important factors limiting production of *Theobroma cacao* in South America. Because of high disease pressure and environmental concerns, biological control is a pertinent area of research for cacao disease management. In this work, we evaluated the ability of four *Bacillus* spp. isolated from vegetable crops, for their ability to colonize *T. cacao* seedlings and reduce the severity of black pod rot (*Phytophthora capsici*). Of the *Bacillus* spp. tested, application of *B. cereus* isolates BT8 (from tomato) or BP24 (from potato) together with the polysilicon surfactant Silwet L-77 (0.24% vol/vol) resulted in long-term (>68 days) stable colonization of cacao leaves. Further investigation revealed that foliar colonization by BT8 and BP24 was primarily epiphytic, with endophytic populations typically representing 5–15% of total foliar bacteria. Significant reductions of disease severity ($P \leqslant 0.05$) on cacao leaf disks challenged with *P. capsici* were recorded from after day 26, and through 68 days following colonization with BT8. No bacterial colonists were observed in or on leaves that developed after bacteria application, suggesting that the bacteria were not capable of systemic movement through vascular tissues. These newly developed, non-colonized leaves from colonized plants exhibited disease suppression, which supports a probable disease suppression mechanism of induced systemic resistance for the BT8 isolate.

Keywords: *Bacillus*; Biological control; Induced systemic resistance; *Phytophthora capsici*; Pathogen; *Theobroma cacao*

1. Introduction

The intimate association of bacterial endophytes with plants offers a unique opportunity for their potential application in plant protection and biological control. Bacteria living inside plant tissues form associations ranging from pathogenic to symbiotic. In a review by Lodewyckx et al. (2002), 81 different bacterial species were reported to form endophytic associations with plants. Multitudes of bacteria reside in leaves, stems, roots, seeds, and fruits of plants that are seemingly neutral in terms of plant health (Surette et al., 2003). However, several studies have also suggested that many endophytic associations are not neutral at all, but are beneficial to plants (Barka et al., 2002; Bailey et al., 2006).

Endophytes offer a wide range of benefits to plants such as promoting growth (Barka et al., 2002; Kang et al., 2007), reducing disease severity (Coombs et al., 2004; Kloepper et al., 2004; Senthilkumar et al., 2007), inducing plant defense mechanisms (Bargabus et al., 2002; Mishra et al., 2006; Bakker et al., 2007), producing anti-herbivory products (Scott, 2001; Sullivan et al., 2007), biologically fixing nitrogen (Stoltzfus et al., 1997; Martínez et al., 2003; Jha and Kumar, 2007), and increasing plant mineral uptake (Malinowski et al., 2000). The beneficial effects that endophytes can confer on plants have made the study of

* Corresponding author. Fax: +1 814 863 7217.
E-mail address: pbackman@psu.edu (P.A. Backman).

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plant-endophyte associations an important research topic for scientists investigating biological control of diseases in annual, biennial, and perennial crops (Bargabus et al., 2004; Kloepper et al., 2004).

According to Backman et al. (1997), the effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance. For example, *Pseudomonas* sp. strain PsJN, an onion endophyte, inhibited *Botrytis cinerea* Pers. and promoted vine growth in colonized grapevines, demonstrating that divergent hosts could be colonized (Barka et al., 2002). Colonization of multiple hosts has been observed with other species of endophytes and plants. For example: *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 reduced *Cucumber Mosaic Virus* in tomatoes and cucumbers (Raupach et al., 1996) as well as anthracnose and Fusarium wilt in cucumber (Liu et al., 1995). Jetiyanon (1994) established that cabbage colonized by endophytes in the greenhouse had season-long reduced black rot in the field due to induction of defense mechanisms. Non-treated cabbage plants reached the economic threshold (symptoms of systemic disease) approximately 33 days after inoculation with *Xanthomonas campestris* pv. *campestris* while the disease progressed slower in plants treated with log 9.0 CFU/ml of either a low virulence isolate of *Xanthomonas campestris* pv. *campestris* or the non-compatible pathogen *Xanthomonas campestris* pv. *malvacearum*. Cabbage colonized by endophytes did not reach the economic threshold for the disease until approximately 50 days after inoculation, which coincided with harvest maturity. These previously reported successes in utilizing bacterial endophytes as biological control agents have led us to investigate the concept of colonizing cacao foliage with bacterial endophytes from other plant species to evaluate their potential as biological control agents (BCAs) to manage diseases.

Theobroma cacao L. is an economically important tree for the many tropical countries where it is grown and for the many chocolate manufacturing countries that process beans into a range of confectionary products. Cacao production in South America is mainly limited by diseases such as black pod rot, caused by three *Phytophthora* spp., frosty pod, caused by *Moniliophthora roreri*, and witches broom, caused by *Moniliophthora perniciosa*, which cause large yield losses throughout the region. Typically, pesticide application costs are beyond the economic means of small-scale growers. Severe disease problems in South America and the large numbers of consumers and farmers interested in either ecologically based pest management or organic farming have led to an increased interest in biological control options for management of cacao diseases.

Previous research on biological control agents for cacao diseases has focused mainly on fungi. Arnold et al. (2003) demonstrated that foliar fungal endophytes reduced leaf damage by *Phytophthora palmivora* (E.J. Butler). Endophytic colonization of cacao seedlings by *Trichoderma* spp. has been reported to activate plant defense cascades in cacao seedlings (Bailey et al., 2006). Additionally, formulations of fungal spores are often environmentally sensitive and have a shorter shelf-life compared to endospore-forming bacteria, as endospores are highly resistant to heat and desiccation (Driks, 2004). In addition to the long-term viability of endospores, endospore-forming bacteria can often be successfully combined with agrochemicals (Jacobsen et al., 2004). Considering this background, endospore-forming bacteria-based BCAs offer a potentially superior alternative to fungal BCAs, due to the resistant nature of the endospore.

Researchers recently, concluded that *Bacillus mycoides* isolate BacJ (Bargabus et al., 2002) and *Bacillus pumilis* isolate 203-7 (Bargabus et al., 2004) suppressed Cercospora leaf spot in sugar beets. Additionally, *Bacillus cereus* isolates BT8 and BP24 have successfully been used to experimentally manage diseases on several crop species including tomato, potato and pecan (Backman, unpublished). We hypothesize that these isolates may also be effective in successfully colonizing and reducing disease severity of black pod rot of cacao. The overall objective of this study was to investigate the ability of *Bacillus* spp. originally isolated from vegetable crops for their ability to colonize cacao foliage and the ability of these isolates to reduce black pod rot severity and to induce host plant resistance.

2. Materials and methods

2.1. Plant material and growth conditions

Pods from open pollinated trees of *T. cacao* var. *comum* for all instances of the variety of cocoa were obtained from the Almirante Centre for Cocoa Studies, Mars Inc. Bahia, Brazil and shipped to the USDA-ARS Sustainable Perennial Crops Lab in Beltsville, MD. Seeds were germinated, planted in soil-less mix, and maintained in a greenhouse. When approximately 20 cm tall, seedlings were transported to The Pennsylvania State University and transplanted into a soil mix consisting of one part potting soil, two parts sand, and two parts Perlite. Plants were maintained in a greenhouse at 60% relative humidity and a photoperiod of 12 h light at 29 ± 3 °C and 12 h dark at 26 ± 3 °C. Ambient light was supplemented with 430W high-pressure sodium lamps, as needed, to obtain 250 μmol/m^{2}s PAR. Automatic retractable shade clothes were used to limit light to a maximum of 1000 μmol/m^{2}s PAR. Plants were drip irrigated several times daily with 1 per 10 strength Hoagland's solution (160 ppm N) to maintain adequate levels of soil moisture and nutrition.

Thirty-six one-year-old trees were used for experiment one, while 45 two-year-old trees were used for experiment two. During the experiment, the emergence of new leaves was recorded. For the purpose of homogenous samples and to reduce statistical variance, leaf development was specified by four different stages of maturity based upon

Table 1
Description of the different developmental leaf stages for cacao leaves; based on Bailey et al., 2005

Leaf stage	Abbrev.	Description
Unexpanded leaves	UE	Leaves less than 1-cm long with limited pigment
Young red leaves	YR	Leaves 1- to 10-cm long, flexuous, and semi-translucent.
Immature green leaves	IG	Leaves 10- to 20-cm long, flexuous, light green
Mature green leaves	MG	Leaves 10- to 20- cm long, rigid, and dark green

morphological differences, as described in Bailey et al., 2005 (Table 1).

2.2. Bacterial isolates

Four different *Bacillus* spp. were evaluated as potential biocontrol agents of cacao against *Phytophthora capsici*: *Bacillus mycoides* isolate BacJ isolated from sugar beet leaves (Bargabus et al., 2002), *Bacillus pumilis* isolate 203-7 from germinating sugar beet seeds (Bargabus et al., 2004), and *Bacillus cereus* isolates BP24 from potato and BT8 from tomato plants sprayed with a colloidal chitin suspension (D. Ploper, Auburn University, unpublished data). All bacterial cultures were stored at −80 °C in Tryptic Soy Broth (Difco) with 20% glycerol.

2.3. Pathogen isolates and inoculum preparation

Phytophthora capsici isolate 73-73 from Ecuador was used for pathogen challenges. This isolate was collected by H. Purdy (Univ. of Florida) from infected cacao pods in the early 1990s, shipped to The Pennsylvania State University, and stored in 10% glycerol in liquid nitrogen. Cultures were removed from storage and routinely cultured on unclarified V8 agar (Lawrence, 1978). Inoculum was prepared from 5-day-old cultures grown on 50 ml unclarified V8 agar in 125 ml Erlenmeyer flasks at 28 °C in an incubator with a 12 h light cycle. Zoospores were obtained following the protocol of Lawrence (1978). Prior to each challenge, a small aliquot of the zoospore suspension was stained with lactophenol cotton blue to stop zoospore movement while the concentration was determined using a hemacytometer (American Optical, Buffalo, NY). Final concentration was adjusted to approximately 5×10^3 zoospores/ml. *P. capsici* 73-73 was periodically reisolated from infected cacao leaves to maintain virulence.

2.4. Plate antagonism assay

An *in vitro* agar plate pairing assay was used to determine if the *Bacillus* spp. were directly antagonistic to *P. capsici*. Two mycelial plugs of *P. capsici* 73-73 were placed 5 cm apart on V8 agar in a 100 mm Petri dish. A streak of a test *Bacillus* sp. was placed between the plugs (2.5 cm from each plug). Control plates consisted of the mycelial plugs without bacteria. Four replicate plates were prepared for each of the four *Bacillus* spp. tested. Plates were incubated at 28 °C, radial growth of *P. capsici* was measured at 1, 2, and 3 days after inoculation and plates were observed daily for development of a zone of inhibition. The experiment was repeated to confirm initial results.

2.5. Bacterial inoculum preparation and colonization

Seven days prior to colonization of cacao foliage, the four *Bacillus* spp. were grown in 500 ml of sterile Tryptic Soy Broth in 2.8 L Erlenmeyer flasks. Flasks were incubated for 7 days at 28 °C and 120 rpm on a rotary incubator shaker (New Brunswick Scientific, Edison, NJ). The bacterial suspension was adjusted to 1×10^8 CFU/ml and a low surface tension, non-ionic organosilicone surfactant (Silwet L-77, G.E. Silicones, Tarrytown, NY) was added to a concentration of 0.24%. The bacterial/surfactant suspension was sprayed onto foliage using a hand-held aerosol sprayer (Crown Power Pack, Aerovoe Pacific, Gardenerville, NV) until leaves were wetted. Immediately following spraying, attached leaves were evaluated on a light box to verify infiltration into sub-stomatal cavities (Zidack et al., 1992). Following bacterial application, plants were maintained in a randomized block design in an environmental room (Conviron Model No. BDW120, Winnipeg, Canada) at 28 °C. Lighting was controlled by high intensity discharge lamps at 300 μmol/m^{2}s PAR to comprise a 12-h photoperiod at 55% RH and 12-h dark at 75% RH, with all plants drip irrigated as in the greenhouse (described above).

This study was conducted in two separate experiments. Experiment one consisted of six treatments with six cacao plants per replicate. Treatment one consisted of non-sprayed plants and treatment two was the surfactant control sprayed with a solution of 0.24% Silwet L-77 as previously described. Each of treatments 3–6 included the same solution of 0.24% Silwet L-77 plus 203-7, BacJ, BP24, or BT8 at 1×10^8 CFU/ml.

Experiment two was preformed as a repeat of the successful colonists of experiment one (BP24 and BT8) with a Silwet-sprayed control for a total of 3 treatments, each with 15 cacao plants per treatment. During spraying, plants were placed inside individual plastic tents to prevent cross contamination, and then organized into a randomized complete block design. To prevent excess water in the growth room, the relative humidity was reduced compared to experiment one. The environmental parameters were 12-h light at 65% RH and 12-h dark at 55% RH. Plants were drip irrigated as described above.

2.6. Determination of levels of bacterial colonization

For experiment one, two mature green (MG) leaves were collected per plant from 3 of the 6 replicate plants per treatment (6 leaves per treatment) at 5, 12, 19, 26, 33, 41, 56, 62,

and 70 days post-inoculation (dpi) with *Bacillus* spp. Five 2.35 cm^2 leaf disks were excised from each leaf in a "W" pattern using a size 10 cork borer. The five disks from the individual leaves were placed in a 101 mm × 152 mm stomacher filter bags (SECURE-T 80; Labplas, Sainte-Julie, Quebec) containing 3 ml of sterile 0.1 M phosphate buffer. Samples were triturated at 100 oscillations per min for 60 s with a stomacher blender (Bagmixer 100 MiniMix, Intersciences St. Nom, France). Fifty microliters of the supernatant from each bag was plated in triplicate on yeast extract dextrose agar (YED, Difco, Detroit, MI) using a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA) for a total of six biological replicates per treatment. Plates were incubated at 28 °C for 24 h. Following incubation, bacteria were enumerated using a Spiral Biotech counting grid following manufacturer's instructions. Bacterial populations are reported as CFU/cm^2 of leaf tissue. The minimum detectable population level using these sampling methods is 1.8–2.0 log CFU/cm^2.

For experiment two, four mature green (MG) leaves were collected per plant from five replicate plants per treatment at 5, 11, 18, 25, 33, 46, 54, and 68 dpi. Each of the four individual leaves underwent separate sampling methods to differentiate between total and endophytic populations as well as vegetative cells and endospores. Two leaves per replicate plant were washed for 4 h under running tap water to remove epiphytic colonists, while the remaining two leaves were not washed. For each plant, the following procedure was conducted: to determine the total population of bacterial colonists, leaf disks were excised from a non-washed leaf and processed as described in experiment one. Fifty microliters of the supernatant was plated in triplicate onto YED. The remaining supernatant was heated for 15 min in a 75 °C water bath to select for endospore-forming bacteria in the total population. The heated supernatant was plated onto YED as previously described. For these and all subsequent samples, plates were incubated and subsequent colonies were enumerated as in experiment one.

A washed leaf from each replicate was used to determine the population of endophytic colonists. From this leaf, five leaf disks were excised, as previously described and triturated in the stomacher blender. The 50 μl was plated in triplicate onto YED, while the remaining supernatant was heated to select for endophytic *Bacillus* endospores.

The remaining washed leaf was used to ensure that the washing step removed epiphytic colonists. Five leaf disks were placed into a stomacher bag in 3 ml buffer and aggressively agitated in a stomacher blender. The supernatant was plated in triplicate on YED. The remaining supernatant was not heated, as this was verification of epiphyte removal.

In addition to determining the levels of bacterial colonists on mature leaves, immature green (IG) leaves were sampled to determine the populations of bacterial colonists on leaves used for the detached leaf assay. Immature green leaves were sampled following the methods for mature green leaves. The remaining portions of the leaves were used for the disease challenge assay (below). The leaves were processed following the same protocols utilized for mature green leaves.

2.7. Detached leaf disk assay to test for disease suppression

The ability of *Bacillus* spp. to suppress disease was assessed by challenging leaf disks with *P. capsici* zoospores and measuring subsequent disease development. Immature green (IG) leaves were detached from plants at 6, 12, 19, 26, 32, 45, 56, and 68 dpi, then separated and washed as previously described. Leaf disks with a 9-cm diameter were excised from leaves so that the midrib was in the center of disk (Bailey et al., 2005) and placed adaxial side up on a moist sterile 9-cm Whatman paper in an inverted Petri dish. Each leaf disk was inoculated with six isolated drops (10 μl each) of a zoospore suspension at a concentration of 5×10^3 zoospore per ml or 50 zoospores per 10 μl drop. Petri dishes were sealed with Parafilm and incubated at 28 °C with a 12-h light-dark cycle. Leaves were rated every 8–12 h for approximately 52 h after inoculation by evaluating lesion diameter, percent necrosis under the droplet of zoospore suspension (0–400%), and disease severity based on the following subjective scale:

0 - no disease apparent
1 - one to three water soaked small spots approximately 0.5–1.0 mm (<10% necrotic area under the water droplet (AUWD))
2 - several larger water soaked or necrotic spots ⩾10% to <30% necrotic AUWD
3 - larger necrotic areas ⩾30% to <60% necrotic AUWD
4 - area under the water droplet ⩾60% to <100% necrotic AUWD
5 - area under the water droplet ⩾100% necrotic to <200% (2 times the AUWD)
6 - necrotic area ⩾200% to <300% of the AUWD
7 - necrotic area ⩾300% to <400% AUWD
8 - necrotic area ⩾400% of the AUWD

If appropriate, intermediate ratings were recorded for all lesions evaluated. Disease progress curves were created for all measurements over time, and then the area under the disease progress curve (AUDPC) was calculated (Shanner and Finney, 1977). Data were statistically analyzed for significance using ANOVA analysis followed by a Tukey test using the SAS program (SAS Institute Inc., Raleigh, NC). A 95% confidence level was used for all analyses, so that $P \leqslant 0.05$ were considered to be statistically significant.

3. Results

3.1. Plate antagonism assay

There were no statistically significant differences ($P \leqslant 0.05$) in radial growth when *P. capsici is* grown alone,

or when grown in close proximity to any of the four *Bacillus* spp. treatments.

3.2. Colonization of cacao leaves with Bacillus spp.

Immediately after spray application and for a period of 10 min thereafter, cacao leaves receiving Silwet L-77; or any of the bacterial suspensions containing Silwet L-77, had water soaked areas scattered throughout, indicating sub-stomatal infiltration of the spray solution. One day after initial colonization, small necrotic lesions formed at the leaf margins of some young red (YR) leaves on plants treated with the Silwet L-77 formulation applied alone or in combination with bacteria. The damaged leaves readily developed into mature green (MG) leaves that were slightly misshapen at the margins.

The initial experiment (Fig. 1A) demonstrated that cacao leaves were successfully colonized by 203-7, BT8, BacJ, as well as the BP24 mixture. BP24 was found to have two distinct colony types, and was therefore called a mixture. The unsprayed control and surfactant control (Silwet L-77) plants had limited colonization (near the

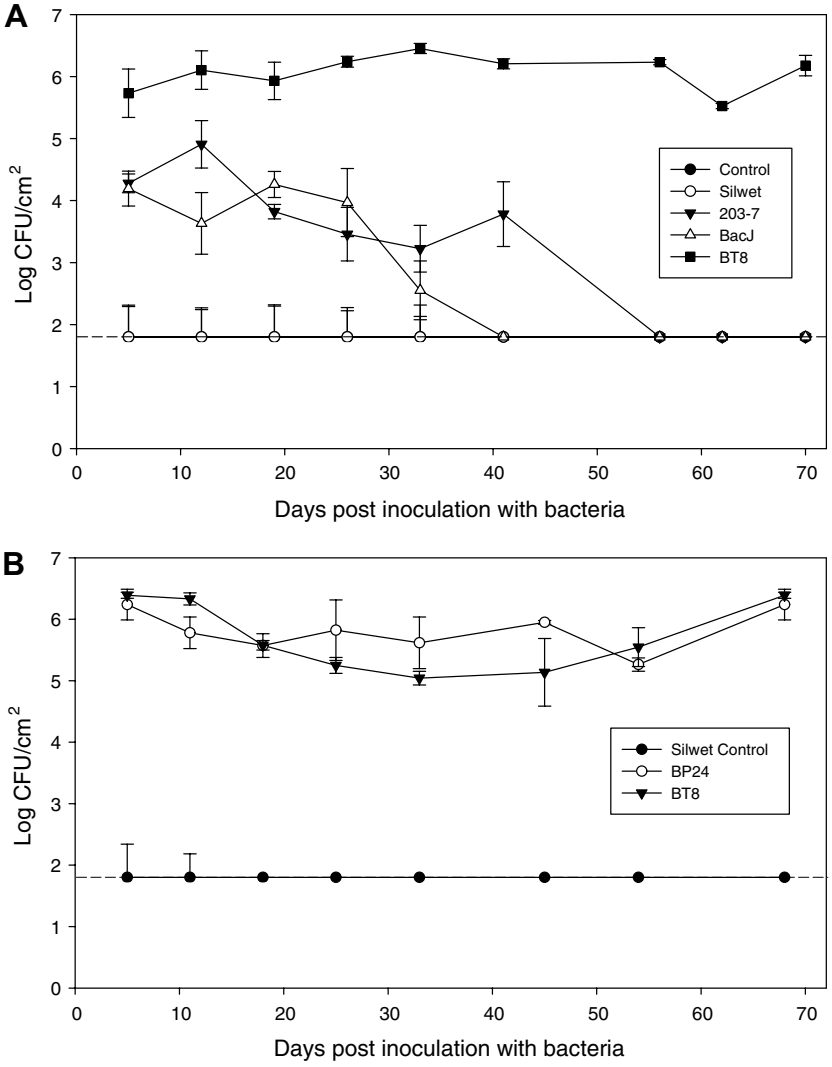

Fig. 1. (A) Mean total bacterial colonization of mature green cacao leaves sprayed with either 0.24% Silwet or 0.24% Silwet + log 8.0 bacteria (experiment one). The unsprayed control represents leaves that were never sprayed and Silwet control represents leaves that were sprayed with 0.24% Silwet L-77 + 0.1 M potassium phosphate buffer without bacteria. BacJ is a *B. mycoides* isolate, 203-7 is *B. mojavensis* isolate, and BT8 is a *B. cereus* isolate. Leaves from untreated control and Silwet L-77 treated plants had low levels of colonization from in-chamber drift of BT8 during initial colonization. Initial colonization of cacao occurred at day 0 with colonization measured 5, 12, 19, 26, 33, 41, 56, 62, and 70 days after initial colonization. Bars extending from the means represent standard errors of that mean. (B) Mean total bacterial colonization of mature green cacao leaves sprayed with either 0.24% Silwet or 0.24% (Silwet control) Silwet + log 8.0 bacteria (experiment two). Silwet control represents leaves sprayed with the Silwet L-77 formulation without bacteria, BT8 represents a *B. cereus* isolate, and BP24 represents a *B. cereus* isolate. Colonization levels were assessed on 5, 11, 18, 25, 33, 46, 54, and 68 days after initial colonization. Leaves on Silwet control plants had low levels of bacterial colonists due to cross-contamination from treated plants while in the randomized block design. BP24 and BT8 remained colonized throughout the duration the experiment. Bars extending from the means represent the standard error of that mean. The dashed line indicates the minimum detection level of the experiment. All data points on this line indicate that the microbial populations were below detection levels.

minimal threshold of detection), likely due to aerosolized bacteria solution drifting in the environmental chamber during spraying. Bacterial colonists observed on control treatments were phenotypically identical to BT8. The treatment containing BacJ provided colonization that rapidly declined and was undetectable after 32 dpi, while 203-7 declined more slowly persisting on cacao leaves for 58 days (Fig. 1A). BT8 colonized cacao leaves throughout the duration of the experiment (70 days). Colonization studies showed that populations persisted in sprayed mature leaves at high levels with little variability within treatments, fluctuating between log 5.5 and log 6.5, respectively (Fig. 1A). Data from BP24 is not included because the original spray inoculum was contaminated. Although contaminated in the first experiment, it was a long-term colonist. A non-contaminated isolate was used in the second experiment.

A second experiment was conducted with BP24 and BT8 to provide more details on the distribution of bacteria (epiphyte vs. endophyte) in colonized cacao leaves and the status of the bacterial colonists (vegetative cells vs. endospores). In this experiment, we again observed short-term epiphytic colonization of control plants (Fig. 1B). This colonization was likely due to bacterial transfer from treated to nearby untreated leaves within a very humid growth room while in the randomized block design. Colonization for both treatments of bacteria-treated plants lasted the duration of the experiment (68 days), but remained restricted to leaves that were originally sprayed with test bacteria (Fig. 1B). As in the initial experiment, total colonization of leaves treated with BP24 and BT8 persisted with little variability, fluctuating around log 6.0 and log 4.8, respectively (Fig. 1B). Although Silwet L-77 facilitated sub-stomatal infiltration to allow for endophytic colonization, colonization persisted in both epiphytic and endophytic environments (Fig. 2). For both bacteria, colonization of foliage was predominantly epiphytic (approx. 90%). Here, we observed that while levels of epiphytic colonists remained relatively stable, endophytic colonization was less stable, fluctuating from log 2.5 to log 5.5 CFU/cm^2 (Fig. 2) for both isolates. At 25 dpi, BP24 endophytic colonization was lower than our detection threshold, but by the next sampling and thereafter, there was population resurgence.

In addition to variations in spatial locations of bacterial colonization, there were also fluctuations in the relative levels of vegetative cells and endospores within the foliar environment (Fig. 3). Numbers of vegetative cells and endospores of BT8, differed by no more than one log. Although leaves colonized with BT8 had endospores throughout the experiment, there were no detectable levels of BP24 endospores from 18 dpi through the termination of the experiment (70 dpi).

Colonization of YR or IG leaves that were directly sprayed with either isolate, were detected at low levels from 0 to 19 dpi. There was no bacterial colonization of newly developed IG leaves at 26, 32, 45, 56, and 68 dpi. In these later observations, the leaves did not exist when the seedlings were sprayed, and there was no apparent movement of the bacteria from leaves that were sprayed to leaves that developed after colonization (data not presented).

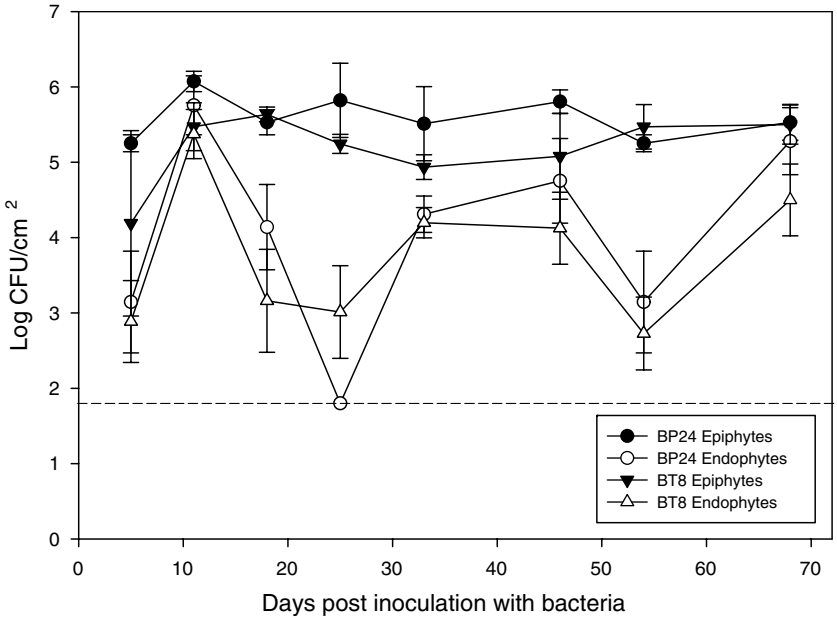

Fig. 2. Mean epiphytic and endophytic colonization of mature green cacao leaves (Fig. 1B) at 5, 11, 18, 25, 33, 46, 54, and 68 days after colonization by *B. cereus* isolates BT8 and BP24. Initial colonization of cacao leaves occurred at day 0 when plants where sprayed with 0.24% Silwet L-77 (vol/vol) + log 8 bacterial suspension. Bars extending from the means represent the standard error of that mean. The dashed line indicates the minimum detection level of the experiment. All data points on this line indicate that the microbial populations were below detection levels.

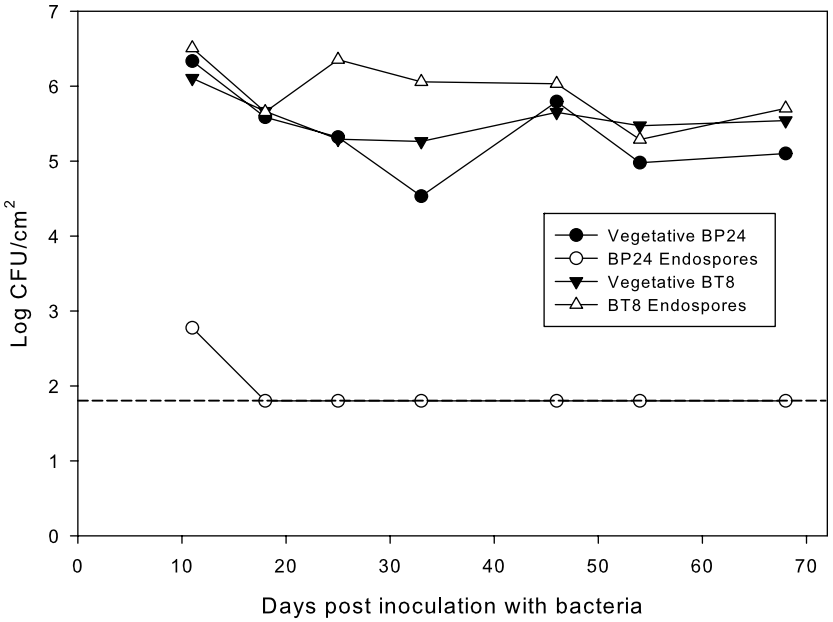

Fig. 3. Levels of vegetative cells and endospores in mature cacao leaves treated with either *Bacillus cereus* BP24 or BT8 and sampled at days 5, 11, 18, 25, 33, 46, 54, and 68 days after colonization. Initial colonization of cacao leaves occurred at day 0 when plants where sprayed with 0.24% Silwet L-77 (vol/vol) + log 8.0 bacterial suspension. Populations of vegetative cells were developed by determining total bacteria by direct plating of leaf triturate, and subtracting that value the number of endospores developed by heat treating the same sample for 15 min at 75 °C. The dashed line indicates the minimum detection level of the experiment. All data points on this line indicate that the microbial populations were below detection levels.

3.3. Disease suppression in detached leaf disk assays

When leaf disks from YG leaves were excised from *Bacillus*-treated and control (Silwet or untreated) cacao plants and inoculated with a *P. capsici* zoospore suspension there were no significant effects ($P \leqslant 0.05$) detected between control treatments and the BP24 treated plants at any date. (Fig. 4). There were also no significant differences ($P \leqslant 0.05$) in *P. capsici* disease severity on YG leaves harvested from plants treated with BT8 bacteria from 5 to 26 dpi. However, plants treated with BT8 had significantly lower disease severities than either the control or BP24 treated plants ($P \leqslant 0.05$) at 32, 45, 52, and 68 dpi on newly emerged, non-colonized IG leaves (Figs. 4 and 5).

4. Discussion

4.1. Plate antagonism assay

There was no antagonism detected in dual plate assays. These results indicate that the test *Bacillus* spp. probably did not directly antagonize or produce antibiotics against *P. capsici* in this assay.

4.2. Colonization of cacao leaves with Bacillus spp.

These experiments demonstrate that *Bacillus* spp. from annual crops are capable of long-term colonization of cacao foliage. The Silwet-L77 surfactant used to apply the bacteria did not significantly affect plants, although the slight necrosis at the margin of some young leaves was probably due to the use of higher Silwet levels than have been used in other systems (0.01–0.2%) (van Wees et al., 2000; Jetiyanon, 1994). The limited colonization seen on Silwet control plants was likely due to bacteria dripping on foliage, despite the plastic tents used during bacterial application. We also demonstrated that the different *Bacillus* strains had differential ability to colonize cacao leaves. BT8 and the BP24 were able to establish long-term colonization, while colonization by BacJ and 203-7 was recorded for shorter durations and with differing rates of decline.

Overall, total foliar colonization in BT8 and BP24 treated plants remained relatively stable, despite the fluctuating ratios of epiphytes to endophytes and of vegetative cells to endospores. Although the distribution of an endophyte colonizing leaves has not been fully investigated, researchers have examined endophytic community structure. Herre et al. (2007) indicated that colonization of cacao by fungal endophytes was not homogeneous. Additionally, fluctuations of endophytic communities commonly occur in nature in response to seasons (Mocali et al., 2003), temperature (Ju et al., 2006), and the presence or absence of pathogens and subsequent defense products (Mengoni et al., 2003).

4.3. Bacterial endophytes suppress disease severity of P. capsici in a leaf disk assay

Detached leaf disk assays provided opportunity to screen large numbers of test plants in laboratory conditions in significantly shorter time (2–3 days) due to rapid development of disease in our model system. Similarly, a cacao

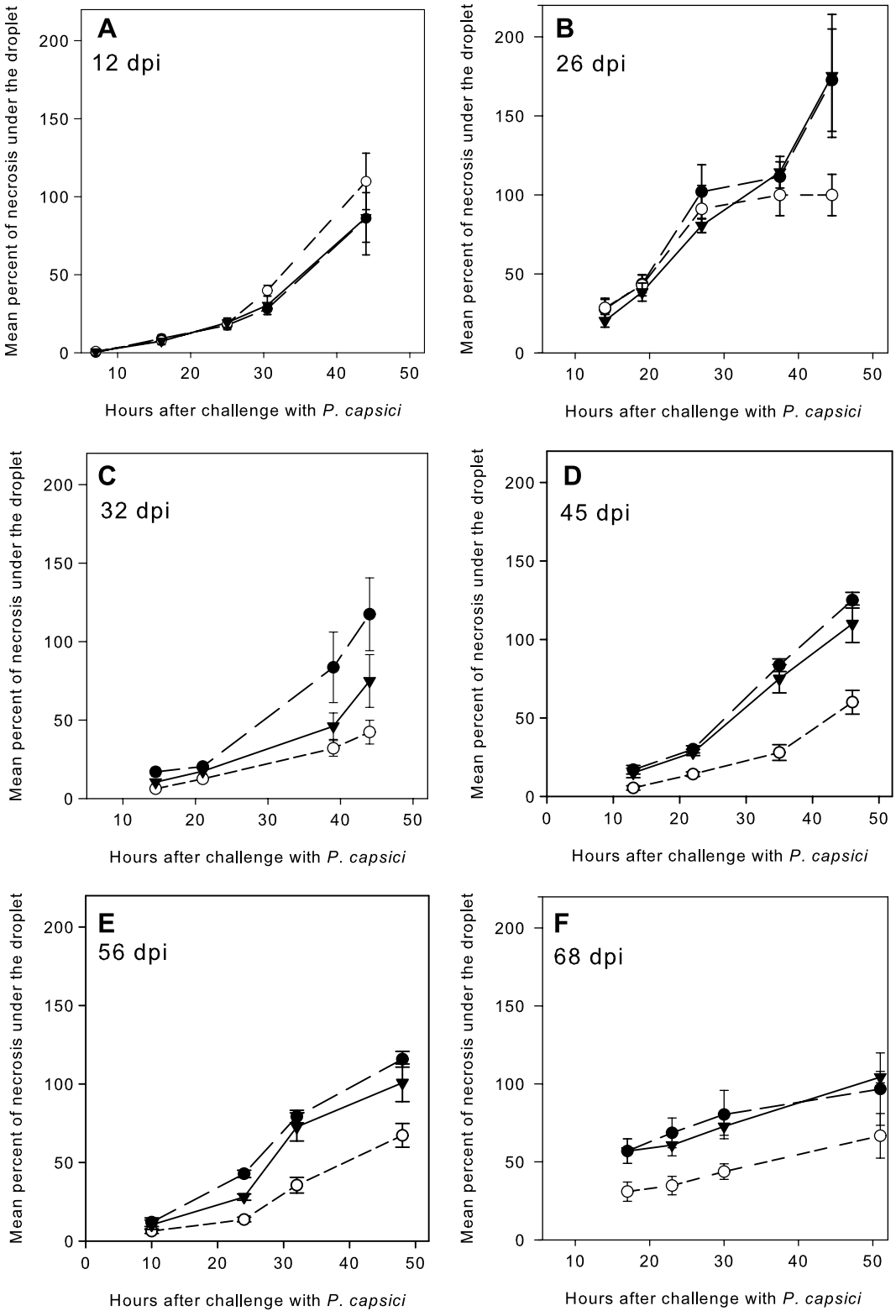

Fig. 4. Disease severity, measured as mean percent necrosis under the zoospore droplet, on young green leaves challenged with droplets of *P capsici* zoospores (50/10 μl drop) during the course of experiment on leaves from Silwet (●), BT8 (○), and BP24 (▼) treated plants. Each time point represents a distinct set of leaves that were detached from the plant and used for the pathogen challenge. IG leaves challenged at 12 days after inoculation (dpi) were present during bacterial application, therefore were colonized with bacteria. IG leaves challenged at 12, 25, 32, 45, 56, and 68 dpi developed after bacterial application and lacked bacterial colonists. Bars extending from means represent the standard error of that mean. (A) IG leaves challenged 12 dpi (B) IG leaves 25 dpi (C) IG leaves challenged 32 dpi (D) IG leaves challenged 45 dpi (E) IG leaves challenged 56 dpi (F) IG leaves challenged 68 dpi. BT8 reduced lesion development in comparison to both the Silwet control and BP-24 during all sampling dates except 12 dpi.

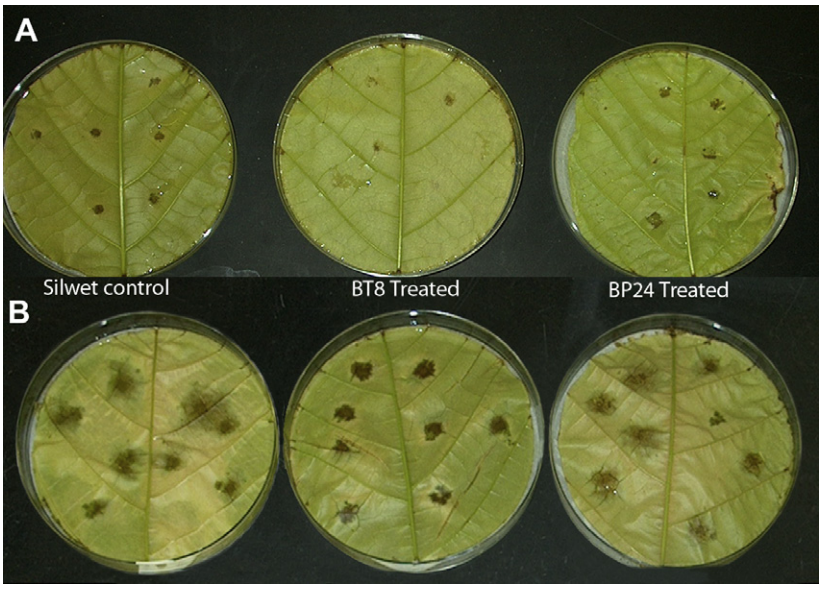

Fig. 5. Leaf discs (YG stage) challenged with 10 μl suspension of *P. capsici* zoospores (50 per droplet) 45 days after cacao plant colonization with either *Bacillus cereus* BP24 or BT8 applied with Silwet surfactant (0.24% Silwet in 0.1 M phosphate buffer). The third treatment is a Silwet control without bacteria. Leaf disks are (A) 21.5 h after challenge with *P. capsici* and (B) 43 h after challenge. BT8 treated plants had significantly lower levels of disease severity ($P \leqslant 0.05$) from the Silwet control and the plants treated with BP24 at both time intervals. Leaves used in these bioassays were formed after the colonization step and remained non-colonized throughout the term of this experiment.

leaf disk assay was developed to aid in screening for genotypes resistant to *Phytophthora* spp. and that have demonstrated to correlate to field resistance (Tahi et al., 2006). Applying this methodology, we observed that although both BP24 and BT8 species colonized cacao leaves, only BT8 suppressed *P. capsici* lesion expansion. Neither isolates protected leaves that were directly sprayed, even though populations were high both on and in the leaf tissue.

Phytophthora suppression by BT8 only occurred in non-colonized leaves that had developed after bacterial applications were made and persisted in the older leaves of the BT8 colonized plants. These results strongly suggest that the colonized plants are exhibiting systemically induced disease resistance in the immature non-colonized leaves with a signal likely originating from the colonized mature leaves. An unexpected result of the leaf disk assay was that disease suppression induced by BT8 was not observed until >26 dpi. This is different from previous reports on other pathosystems were endophytes were shown to induce disease suppression more rapidly (Wilhelm et al., 1998; Barka et al., 2002). Additionally, colonization with bacterial endophytes in annual crop systems has resulted in the accumulation of plant defense products beginning at 7–17 days after colonization (Jetiyanon, 1994; Bargabus et al., 2002). It is also of interest to note that at 11 and 19 days after inoculation, disease suppression was not detected on IG leaves that contained high levels of *Bacillus* spp. This strongly suggests that there is no direct effect (e.g. antagonism) involved in the disease suppression recorded 32–68 days after colonization.

The concept of induced resistance can be further seen by the presence of endospores. The endospores present in BP24 colonized leaves at 6 and 11 dpi were likely present in the bacterial suspension during application or possibly were formed due to short-term activation of defense products by Silwet L-77, since Silwet is reported to induce short-term production of PR proteins in cabbage (Jetiyanon, 1994). The lack of endospores suggests that BP24 colonized cacao leaves with high population levels, but likely did so without activating plant defenses since it did not reduce disease. When total endospore populations of BT8 were high, levels of endophytic BT8 colonists were low (more were epiphytic). Additionally, levels of endophytic BT8 endospores were high when plant resistance to *P. capsici* was high (Figs. 2 and 3). Endospores are produced by *Bacillus* spp. when vegetative cells are stressed (Driks, 2004). Therefore, it is possible that endophytic colonization by BT8 induces plant defense products, which in turn stresses the colonizing *Bacillus* causing it to produce endospores. The results presented in this manuscript demonstrate that some *Bacillus* spp. from vegetable crops were capable of long-term colonization of cacao leaves and subsequent disease reduction. These results are consistent with other experiments where endophytes from other hosts colonized new hosts' tissues and subsequently reduced disease (Barka et al., 2002; Anith et al., 2004; Compant et al., 2005). These data offer further support for the potential of endophytic microbes in management of cacao diseases suggested by the works of Arnold et al. (2003) and Bailey et al. (2006). Additionally, this research provides evidence that endophytes from diverse kingdoms

are able to colonize cacao leaves and potentially could be a viable option for biological control of cacao diseases.

4.4. Potential of *Bacillus* spp. as biological control agents for cacao diseases

Overall, *Bacillus* spp. isolates BT8 and BP24 were effective long-term colonists of cacao leaf tissue, but only BT8 reduced disease severity in a detached leaf assay, which demonstrates that not all endophytic colonists can suppress disease. Disease suppression was sustainable (>68–70 days) after a single application, but reapplication would likely be necessary in a cacao planting due to its perennial non-deciduous nature that supports a typical leaf life of about one year. Based on previous experience, application of *Bacillus* spp. may have the advantage of longer time periods between applications compared to chemical control methods (Adejumo, 2005), which would result in a disease management system that requires less labor. This could be a great advantage considering that labor costs are a primary concern limiting the utilization of agronomic practices for disease management in cacao. BT8 and BP24 were not isolated from cacao or the tropical regions where cacao is grown and this could present potential problems of regulatory and environmental nature. Thus, natural cacao endospore-forming endophytes may be better suited for long-term colonization of cacao and could activate plant defense mechanisms more successfully than BT8. Our ongoing research is focused on screening a collection of endospore-forming endophytic bacteria from cacao leaves, pods, flower cushions, and branches (Melnick, unpublished). The application of endospore-forming bacteria native to the region could reduce the regulatory and environmental concerns associated with use of non-native microbes.

A better understanding of the molecular aspects of microbe-induced disease suppression would facilitate the development of successful biocontrol strategy. This study demonstrated that *Bacillus* spp. from vegetable crops were not directly antagonistic to *P. capsici* 73-73 in the plate antagonism assay; therefore, disease suppression most likely occurred through other mechanisms. Experiments should be conducted to determine actual nature and time course of the defense response. An understanding of the defense mechanisms in cacao after endophyte colonization would contribute to optimal application of biological control by providing insights into the possibility of broad spectrum disease suppression. Broad spectrum resistance would be beneficial in cacao growing regions where trees are often under simultaneous pressure from multiple diseases including witches' broom, frosty pod, and black pod rot.

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