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ENDOPHYTIC *BACILLUS* SPP. OF *THEOBROMA CACAO*: ECOLOGY AND

POTENTIAL FOR BIOLOGICAL CONTROL OF CACAO DISEASES

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ABSTRACT

Yield of *Theobroma cacao*, the chocolate tree, is drastically limited by disease. In South America, there are three key diseases: black pod, caused by *Phytophthora* spp.; frosty pod, caused by *Moniliophthora roreri*; and witches' broom, caused by *Moniliophthora perniciosa*. Although chemical control options exist, farmers typically only use cultural disease management such as phytosanitary pruning. Agrochemical use can be problematic in the developing countries where cacao is grown due to large risks to human health and the environment. There has been increased interest in the use of biological control for management of cacao diseases due to problems associated with pesticide use as well as consumer desire for organic chocolate. Fungal species have been the main focus for research on biological control of cacao diseases, while cacao-associated bacteria have been nearly ignored. Previous research demonstrated that a *Bacillus* sp. from tomato was capable of endophytically colonizing cacao foliage and reduced disease severity of *Phytophthora capsici* in detached leaf assays. The previous success of research utilizing bacterial endophytes lead to an investigation on endospore-forming bacterial endophytes associated with cacao tissue.

The present research focused on obtaining and screening endospore-forming cacao bacterial endophytes for their ability to suppress diseases. Approximately 70 isolates were obtained and screened for characteristics of elite biological control agents, such as chitinase production, ability to colonize clean plants, and disease suppression. From these isolates, four were tested in a two-year field study on biological control of witches' broom disease at INIAP in Pichilingue, Ecuador. In this trial, application of *Bacillus pumilus* isolate ET consistently reduced disease severity of witches' broom, when bacteria were applied three times a year and phytosanitary pruning was conducted at the end of each season. Along with this study, it was determined that applied bacterial endophytes persisted in cacao foliage throughout the dry season (6 month period) when applied at the season onset. Bacterial endophytes persisted in the rainy season, but needed to be re-applied midway through the six month season to maintain adequate population levels. In addition to research on suppression of witches' broom, these four bacterial isolates were tested for their ability to suppress cacao pod disease and cherelle wilt in two cacao genotypes: 'Nacional' and 'CCN51'. *Bacillus pumilus* ET reduced cherelle wilt in both genotypes, indicating the presence of a biological component associated with what was once thought to be a physiological condition. None of the four tested isolates reduced diseases on cacao pods, but application of *B. pumilus* ET increased the overall number of healthy pods in the

first two months of the four month experiment. These successful field results indicate the positive potential for the use of native endophytic bacteria to manage cacao diseases.

Further research was conducted to determine the modes of action of the biological control agents as well as whether endophytic bacteria could successfully be combined with endophytic *Trichoderma* spp. currently being tested as biological control agents of cacao diseases. Antibiosis studies indicated that *B. pumilus* ET was antagonistic to *P. capsici* and *M. roreri*, but not *M. perniciosa* in plate antagonism studies. Research was conducted using quantitative real time PCR (Q-PCR) to determine whether colonization of endophytic *Bacillus* spp. activated the expression of cacao expressed sequence tags (ESTs) related to defense. Research indicated that colonization of cacao seedlings with bacterial endophytes activated the expression of cacao ESTs related to plant defense, such as chitinases, pathogenesis-related proteins, and lipoxygenase. Another mode of action that was tested was to determine whether application of endophytic species displaced native endophytes, which were likely neutral in terms of plant health. Analysis of microbial communities utilizing automated ribosomal intergenic spacer analysis (ARISA) indicated that application of potential biological control agents did not impact the community of native *Bacillus* spp. or the overall bacterial community during the sampled periods. Overall, the suppression of disease caused by *B. pumilus* ET was likely due to a combination of chitinase production, induced resistance, and potentially antibiosis. Additional research demonstrated that bacterial endophytes could successfully exist with endophytic *Trichoderma* spp. when applied either to seeds or foliage. *Bacillus* spp. persisted in roots (when applied to seeds only), stems, and foliage, while *Trichoderma* spp. persisted in roots and stems. These species were capable of co-existing in the same niche, therefore could likely be used together in the field. In conclusion, the results reported here indicate that *B. pumilus* ET would likely make an excellent biological control agent, due to its multiple modes of action and ability to be combined with endophytic *Trichoderma* spp.

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Chapter 1

Review of Literature and Research Objectives

Theobroma cacao

Theobroma cacao L. is a tropical understory tree of the *Malvaceae* family (Judd et al., 2002). Cacao is of great economic significance, as its 'beans' are processed into chocolate products. Cacao is the third most important exported commodity in the world, after sugar and coffee, with 3,9247,700 tons produced in 2005 (Donald, 2004) . Despite its agronomic significance, 70% of cacao is grown by small-scale farmers (Donald, 2004). The crop's center of origin lies in Ecuador/Peru, but cacao is grown around the world between the Tropic of Cancer and the Tropic of Capricorn (Bartley, 2005; Cheesman, 1944). Wood and Lass (1987) state that there are three major groups of cacao: Criollo, Forastero, and Trinitario. The Criollo group consists of the first domesticated cacao, used by the Olmec and Mayans. Criollo trees produce the finest chocolate, but are difficult to grow because of high susceptibility to disease and low yield. Forastero trees were selected and grown by Spanish farmers in South America for their high yield and increased resistance to disease. Trinitario trees are natural hybrids of Criollo and Forastero types with higher yield and higher resistance to disease than Criollo trees.

Cacao has a dimorphic branching system, with a shoot growth periodicity (Cook, 1916; Greathouse and Laetsch, 1969; Greathouse et al., 1971). According to Greathouse and Laetsch (1969), cacao seedlings have a main orthotropic shoot. After approximately one year of growth, shoot dimorphism is initiated by abortion of the apical meristem and formation of a pseudowhorl of three to five branches, commonly known as a jorquette. The pseudowhorl is comprised of plagiotropic branches. Leaf arrangement is dependent on shoot arrangement. According to Wood and Lass (1987) cacao leaves on orthotropic shoots have an alternate arrangement, while leaves on a plagiotropic branch have a spiral whorl arrangement. Overall, cacao leaves have a simple blade with a pinnate venation and stomata located solely on the lower surface (Abo-Hamed et al., 1983). Young leaves are red or light green (depending on genotype), flexuous, and

nearly translucent. As they mature, leave expand, become dark green, and lignify. Additionally, stomata mature and the cuticle thickens as leaves reach maturity (Abo-Hamed et al., 1983). These characteristics cause mature leaves to be more resistant to insects and pathogens than young leaves. Although cacao leaves have an interesting structure, cacao flowers and fruit (Fig 1) are of greater economic importance to the exported commodity.

Cacao has small cauliflorous flowers, arising from flower cushions on old wood of trunks and branches (Fig 1-1).

Figure 1-1: Cacao floral cushions bearing flowers on old wood branch of a tree in Valencia, Ecuador.

Perfect flowers are attached to floral cushions by a long, thin pedicel (Wood and Lass, 1987). Nearly 90% of flowers abscise within 32 hours of anthesis, unless successful pollination occurs (Aneja et al., 1999). According to Wood and Lass (1987), flowers are pollinated by flying midges, thrips, or aphids. Fruit, commonly known as pods, form after successful pollination (Fig 1-2A). Eighty percent of immature pods, known as cherelles, wilt before maturation, because of physiological thinning or association with uncharacterized pathogens.

Figure 1-2: A) Healthy 'Nacional' cacao trees bearing fruit, known as pods B) Ms. Melnick holding opened mature cacao pod bearing beans covered in mucilage.

Pods that are not lost to cherelle wilt take five to six months to reach maturity. Mature pods typically contain thirty to forty seeds embedded in white mucilage (Fig 1-2B). Upon maturity, the mucilage-covered seed are fermented for 2 to 3 days to remove this coating. The fermentation method is very depends on the area in which cacao is grown. After fermentation, the seeds are dried and roasted to produce cocoa and chocolate products.

In some areas of South America, cacao is still grown in the traditional *cabrucagem* agroecosystem in which cacao is planted under trees retained from the rainforest (Johns, 1999; Schroth et al., 2000). The *cabrucagem* system not only maintains valued botanical biodiversity, but also provides habitat for insects, primates, bats, and birds (Faria et al., 2006; Johns, 1999; Rice and Greenberg, 2000). In an alternate planting system, farmers intercrop specific shade trees that provide additional agricultural products such as fruit and timber. Several studies have shown that crops intercropped with cacao had higher net returns than cacao alone, due to the additional profit of the other crops (Mathes, 1986; Osei-Bonsu et al., 2002). The key to economic returns in cacao production is to maintain a large number of healthy pods until maturation. The aggressiveness and pervasiveness of cacao diseases makes this a difficult challenge.

Diseases of Cacao

Introduction

Diseases are the most important factors limiting cacao production throughout the world. Pathogenic organisms include viruses, fungi, and fungal-like organisms. Three viruses attack cacao: Cacao yellow mosaic tymovirus (CYMV), Cacao necrosis nepovirus (CNV), and Cacao swollen shoot bandavirus (CSSV). Cacao viruses have a limited distribution, occurring only in Africa. CYMV is limited to Sierra Leone and is characterized by yellow areas on infected leaves (Frison et al., 1999). CNV is a serotype of tomato black ring virus found in Nigeria and Ghana (Owusu, 1971). CNV infected plants have translucent necrotic spots along the main veins of the leaves as well as terminal die-back of the shoots (Frison et al., 1999). Of all viruses, CSSV has the widest distribution, occurring throughout Western Africa, and causes the greatest yield loss. The symptoms of CSSV are swelling of fan branches, chupons, and roots; red vein banding followed by chlorosis on flush leaves; and a roughly fifty percent reduction in bean weight (Hagen, 1993; Ollennu and Owusu, 2002). Infected trees contain fewer pods and infected pods produce fewer beans (Uhde et al., 1993). In addition to decreasing yield, CSSV kills a susceptible tree in three to four years (Hagen et al., 1994; Uhde et al., 1993). CSSV is spread through movement of infected vegetative material, seed transmission (Quainoo et al., 2008), and is vectored by at least fourteen mealybug species (Bigger, 1981; Hanna et al., 1952; Jeger and Thresh, 1993). Although CSSV is highly destructive, it is a minor disease compared to the yield losses caused by fungi and stramenopiles.

Ceratocystis Wilt

In Latin America, infections of cacao by *Ceratocystis* are commonly called "mal de machete", as the use of a machete on infected trees is one way to spread inoculum (Malaguti, 1952). "Mal de machete" is also known as Ceratocystis wilt. Ceratocystis wilt is caused by *C. cacaofunesta*, which has been described as a distinct species from the former classification of *C. fimbriata* (Baker et al., 2003; Engelbrecht and Harrington, 2005). According to Wood and Lass (1987), Ceratocystis wilt likely first developed in Ecuador, but has been less studied due to the

devastation caused by witches' broom and frosty pod (see following). Ceratocystis wilt has been reported in Central America, Latin America, the West Indies, and Asia. According to Engelbrecht et al. (2007), the symptoms of the disease are wilting of leaves and branches, caused by a canker that girdles the vascular system. Cankers on infected branches and tree trunks can be characterized by their discolored brown wood and fragrant odor. In other *Ceratocystis* pathosystems, wounds produced by pruning or harvesting attract insects that inadvertently transport spores to the wounded site. Ceratocystis wilt in cacao is almost always associated with Ambrosia beetles (*Xyleborus* spp.), which infest infected trees (Saunders, 1965). Upon gallery excavation, beetles deposit frass containing conidia and aleuroconidia, which can then be wind or rain dispersed to wounds on other trees (Engelbrecht et al., 2007; Iton, 1960; Iton and Conway, 1961). Although it is not a key pathogen, cacao clone 'CCN-51', which has recently been planted throughout Ecuador, is highly susceptible to this disease (Suárez, 2007). There is no way to save a tree with a trunk canker and management of diseased branches is problematic (Wood and Lass, 1987). The best management strategy is to plant tolerant trees in areas with high infection rates and ensure that clonally propagated material is disease free (Engelbrecht et al., 2007).

Frosty Pod

Frosty pod; also known as watery pod rot, moniliasis, or Quevedo disease; is caused by the hemibiotrophic basidiomycete *Moniliophthora roreri* (Desrosiers and Suárez, 1974). Frosty pod rot was first reported in Quevedo, Ecuador in 1914 by James Rorer (Rorer, 1918). It has since spread to Colombia (Evans, 1981), Costa Rica (Enríquez et al., 1982) , Peru (Krauss and Soberanis, 2001a), and through Central America into Mexico (Phillips-Mora et al., 2006a; Phillips-Mora et al., 2006b). Although the disease is abundant in Western South America, frosty pod has not yet spread across the Amazon to Brazil. Frosty pod has severe economic consequences, as yield losses of up to 100% can occur in highly susceptible fields (Leach et al., 2002). Frosty pod is one of the most unique of the cacao diseases, as the fungus solely infects pods. Infection starts through direct penetration of meiospores germ tubes through natural openings (Desrosiers and Suárez, 1974). Typical early symptoms are spots of mature coloration on immature pods (Wood and Lass, 1987). As the disease progresses, compact creamy white mycelium bearing meiospores cover the pod, resulting in a frosty appearance (Fig 1-3A). The

fungus can penetrate deep into the pod (Fig 1-3B), where the fungus partially or completely destroys beans (Wood and Lass, 1987).

Figure 1-3: A) Frosty pod lesion on a mature cacao pod infected with *Moniliophthora roreri* B) Rotten beans of immature pod due to early infection with *M. roreri*

Evans (1981) estimated that infected pods are covered with roughly 44 million spores per cm^2 or approximately 7×10^8 spores per pod. Although *M. roreri* is a basidiomycete, there is no known basidiocarp stage, yet the pod is covered with meiospores arising from meiotic division, not conidia (Evans et al., 2003).

Frosty pod rot is difficult to manage, because symptoms are rarely noticed until after sporulation of the lesions. At least partial control of frosty pod can be achieved through high and low volume spraying of sulfur, copper, or mancozeb, but chemical means are often too expensive for small-scale farmers (Desrosiers and Suárez, 1974). The most effective frosty pod management methods are removal of infected pods and planting of resistant cultivars. Breeding for resistant varieties is difficult, as the disease only infects pods; therefore, breeders must wait four to five years to screen trees for resistance. There is also increased interest in biological control options for management of this disease through the use of mycoparasitic fungi (Bateman, 2004; Hidalgo et al., 2003; Krauss and Soberanis, 2001b; Krauss and Soberanis, 2002).

Witches' broom

Witches' broom is caused by the hemibiotrophic basidiomycete *Moniliophthora perniciosa* (formerly *Crinipellis perniciosa*) (Aime and Phillips-Mora, 2005). Witches' broom

was first described by Ferreira in 1785 (Silva, 1987). It was found in Suriname in 1895, and then rapidly spread throughout South America (Wheeler and Mepsted, 1988). From its description by Stahl in 1915 until the 1970's, extensive deforestation and exploration of the Amazon basin spread the disease across the Andes, likely by migrating farmers (Griffith and Hedger, 1994). The disease is now present in nearly all regions of South America, where it is a major threat to cacao production (Ceita et al., 2007; Griffith et al., 2003).

The disease cycle of *M. perniciosa* has two distinct phases: biotrophic and necrotrophic. Infection begins in the biotrophic phase when basidiospores, produced by small mushrooms, infect meristematic tissue through direct penetration (Muse et al., 1996). Primary or monokaryotic (1N) mycelium invades the plants and has restricted intercellular growth in the cortical region (Griffith and Hedger, 1994; Griffith et al., 2003; Penman et al., 2000). Eventually, by a mechanism that is not fully understood, the mycelium triggers plant cell responses to incite hypertrophy and hyperplasia, causing characteristic green brooms (Isaac et al., 1992). Symptoms of the biotrophic phase are stem swelling (Fig 1-4A), proliferation of branching, and conversion of flower cushions to vegetative brooms. Isaac et al. (1992) discovered that hypertrophy is accompanied by a loss of the boundaries between host vascular tissue.

Figure 1-4: A) Swelling of infected young cacao stem caused by primary mycelium of *Moniliophthora perniciosa* known as a green broom B) Necrosis of infected cacao stem, known as a dry broom, caused by secondary mycelium of *M. perniciosa*

The second stage of the disease cycle is the necrotrophic stage. This stage is caused by dikaryotic (N+N) or secondary mycelium. *M. perniciosa* is primarily homothallic, therefore is does not need another individual to form the dikaryon (Casselton and Olesnicky, 1998). There is an association of dikaryotic hyphae with tissue death, commonly called dry brooms (Fig 1-4B). Researchers have yet to determine whether the conversion to the dikaryon initiates plant cell necrosis or whether the plant cell necrosis initiates the fungus to undergo plasmogamy to form the dikaryon. The conversion to the necrotrophic stage could be related to production of necrosis and ethylene-producing proteins by the monokaryotic hyphae (Garcia et al., 2007). Necrotic tissue remains attached to the tree, since no abscission layer is formed (Meinhardt et al., 2008). The dry brooms lead to yield loss through destruction of photosynthetic tissue. The pathogen is spread when basidiospores are released from a basidiocarp. In the field, basidiocarps are primarily produced during the wet season, as they require high humidity for formation (Meinhardt et al., 2006).

Witches' broom symptoms are not limited to infection of branches. Infection of flower cushions can cause single, simple, and compound floral brooms, as well as conversion of flower cushions to vegetative tissue (Fig 1-5A) (Isaac et al., 1992).

Figure 1-5: A) Conversion of cacao floral cushion tissue to vegetative tissue due to infection with *Moniliophthora perniciosa* B) Arrow indicates premature abortion of pod formation (commonly known as cherimoya) due to infection with *M. perniciosa*. The remaining two pods are uninfected

Early infection of pods causes the fruit to distort and dry out to form what is called cherimoya (Fig 1-5B), or strawberry fruit (Wheeler and Mepsted, 1988). Larger, mature pods can also be affected, causing the beans to cease to mature or to rot within the pod. Pod infections can result in upwards of ninety percent yield loss, while infections of other tissues greatly damage future yields (Andebrhan et al., 1999).

Researchers throughout South America have worked to develop viable disease management options for witches' broom. Management strategies are similar to those used for frosty pod. Fungicide applications can effectively suppress witches' broom, but are not cost effective for small-scale farmers (Rudgard et al., 1990). Phytosanitation is the best option for disease management in existing plantings (Soberanis et al., 1999). It is suggested that farmers keep the cacao canopy low, so that it is easier to scout and remove infected materials which are often missed in the tops of tall trees (Wood and Lass, 1987). Extensive research has been conducted on resistant varieties of cacao, due to ease of screening seedlings over pods (Holliday, 1955; Purdy et al., 1983; Purdy et al., 1997; Surujdeo-Maharaj et al., 2003). Breeding combined with marker assisted selection, such as QTL (quantitative trait loci) mapping technologies, have lead to discovery of clones tolerant to witches broom, such as 'Scavina 6' (Faleiro et al., 2006; Monteiro and Silva, 1998; Morera et al., 1991; Schnell et al., 2007).

Black Pod

Black pod rot is the most devastating of all cacao diseases, as it is ubiquitous to all cacao growing regions. The disease was first reported as cacao canker in Java in 1924 (Hartley and Rands, 1924). Black pod, also known as Phytophthora rot, is caused by four different species in the Stramenopile genus *Phytophthora*: *P. capsici*, *P. citrophthora*, *P. megakarya*, and *P. palmivora* (Guest, 2007). Three of these species occur in cacao growing regions throughout the world, while *P. megakarya* is restricted to Africa. *P. megakarya* is the most aggressive species, causing 60-100% yield loss compared to the 15-30% losses attributed to *P. palmivora* (Nyassé et al., 2002; Tahi et al., 2006).

The characteristic symptom of black pod is chocolate brown necrotic lesions on infected pods which usually are accompanied by seed rot (Fig 1-6A) (Opeke and Gorenz, 1974).

Figure 1-6: A) Mature cacao pod infected with black pod rot, caused by a *Phytophthora* spp. B) *Phytophthora* canker of a cacao trunk with the origin of the canker arising from the peduncle of an infected pod

Some infected pods become covered in a thin layer of white mycelium bearing sporangia three to five days after infection (Waterhouse, 1974). A trained eye can readily distinguish black pod from frosty pod in the field due to the diaphanous white appearance of mycelium bearing sporangia compared to the thick creamy white mycelium of frosty pod. Another distinguishing feature of black pod infected pods is a seaweed smell (Iwaro et al., 1997). One of the biggest issues with black pod is the ubiquitous supply of inoculum. Under high humidity, infected pods can bear up to 4 million spores (Gregory and Maddison, 1981). Additionally, mummified pods left in the tree can provide inoculum for up to three years (Dennis and Konam, 1994). Although pod infections are the most common symptoms, black rot less commonly causes trunk cankers (Fig 1-6B). Trunk cankers are characterized by discoloration of bark and a characteristic reddish-brown lesion upon removal of the bark, which differs from the brown cankers of Ceratocystis wilt (Okey et al., 1996). Trunk cankers reduce yield by destroying flower cushions, reducing tree vigor, and occasionally girdling the tree (Guest, 2007; Okey et al., 1996).

Scientists throughout the world are currently evaluating viable disease management options for black pod rot. Copper and metalaxyl-based fungicides are short-term remedies to disease management, but are not always economical when the price of cocoa is low (Adejumo, 2005). Combining fertilization with fungicides greatly increases yields over a three year period (Opoku et al., 2004). Additionally, cultural practices, particularly phytosanitary pruning, can also

reduce disease (Ndoumbe-Nkeng et al., 2004; Soberanis et al., 1999). Reducing intercanopy shade through pruning of shade trees also decreases disease by reducing the duration of free moisture available to the pathogen on pod surfaces (Adejumo, 2005). One of the most effective control methods has been planting of resistant varieties (Butler et al., 1998; Lopez-Baez et al., 1996; Monteiro and Silva, 1998). As with frosty pod, screening for pod resistance to black rot is difficult, as it takes four to five years for a tree to produce pods after planting (Tahi et al., 2006). Researchers have developed methods using leaf disks, twigs, and roots to screen for resistant planting materials (Nyassé et al., 2002; Nyassé et al., 1995; Tahi et al., 2006; Tahi et al., 2007; Tahi et al., 2000). Leaf disk assays have been shown to correlate with field resistance, while providing a shortened time for breeding programs (Nyassé et al., 2002; Tahi et al., 2006; Tahi et al., 2007; Tahi et al., 2000). One the most active areas of research for disease control of black pod, as well as frosty pod and witches' broom, has been biological control.

Biological Control

Modes of Action

In the narrowest sense, biological control is the use of beneficial organisms to reduce plant diseases and pests. The term "biological control" is quite a misnomer, as the microbes only suppress disease and are not capable of complete control. Early work on biological control was conducted in the 1950's by Russian scientist who pioneered "bacterial fertilizers" to increase plant growth (Backman et al., 1998). In 1955, Dunleavy illustrated that *Bacillus subtilis* suppressed damping-off of sugar beets. This initial research stimulated further investigation into the development of biological control agents (BCAs).

There are several modes of action for BCAs: mycoparasitism, niche displacement, antagonism, and induced systemic resistance. Mycoparasitism is the ability of an organism to parasitize a fungal pathogen. Disease management through use of a mycoparasite occurs when the introduced organisms reduce the pathogen population through direct attack. *Trichoderma* spp. are common mycoparasites found in nature and are often used as BCAs. *Trichoderma* spp. attack fungi through the combined action of cell-wall degrading enzymes, such as chitinase and glucanase, and direct penetration (Harman et al., 2004). *Trichoderma* spp. have been effective

for management of both soil-borne and foliar fungal pathogens (Bailey et al., 2006; de Souza et al., 2006; Grondona et al., 1997; Rojo et al., 2007).

Niche displacement occurs when a BCA competes with a pathogen for a shared habitat. Several commercial BCAs operate primarily through the mechanism of niche displacement. One of the most studied systems is BlightBan A506 (*Pseudomonas fluorescens strain* A506), which effectively manages fireblight in pome fruits (Greenbook, 2005). *P. fluorescens* A506 colonizes relatively sterile apple blossoms immediately following spray application, and prevents *E. amylovora* from reaching the necessary population levels for quorum sensing (Wilson et al., 1992). BlightBan A506 is also effective in preventing frost damage, as colonization with the BCA excludes ice nucleating bacteria from the system. BlightBan A506 is registered to protect almond, cherry, pome fruits, potato, strawberry, and tomato from frost damage (Stockwell and Stack, 2007). *P. fluorescens* A506 is capable of colonizing a range of crops and protecting against multiple maladies, which has resulted in its continued commercialization.

Another direct mode of action used by BCAs is antagonism. Antagonism occurs when the BCA makes the environment inhospitable for pathogens through the production of enzymes, volatiles, etc. Antagonists can impair growth, germination, or other functions of the pathogen as well as interfere with infection processes. Antagonism is frequently studied, as preliminary screening can be conducted on agar media in an *in vitro* inhibition assay (McSpadden and Fravel, 2002). Antagonistic compounds diffuse through the agar and cause a zone of inhibition around the target pathogen. *Bacillus* spp., a commonly commercialized BCA genus, are known to produce a range of antibiotics (Gupta and Utkhede, 1986; Stein, 2005; Toharisman et al., 2005). One commercial BCA that utilizes antibiosis is NoGall. NoGall, consisting of *Agrobacterium radiobacter* K1026, protects roots from crown gall by producing the bacteriocin agrosin 84, which effectively kills the pathogenic bacterium, *Agrobacterium tumefaciens* (Reader et al., 2005). Treating seeds, graft junctions, and roots with NoGall effectively manages the disease. Many of these antimicrobial compounds have non-specific modes of action, and therefore are effective against multiple pathogens.

Biological Control Utilizing Induced Resistance

Plant disease occurs when pathogens defeat or are not recognized by host defenses, or more precisely do not elicit a timely defense response from the plant (Zehnder et al., 2001). The

activation of plant defense mechanisms is based upon induction of one or more biochemical defense pathways. Some defense pathways are well studied, such as those activated by fungal pathogens, while others are just beginning to be understood, such as the pathway activated by rhizobacteria. The jasmonate and ethylene pathways are induced by wounding and insect attack (van Wees et al., 2000). Systemic acquired resistance (SAR) is a salicylic acid-dependent pathway induced by fungal pathogens, beneficial fungal endophytes, and effector proteins (such as *hrp*) of the type three secretion system of pathogenic bacteria (Pieterse and van Loon, 1999; Strobel et al., 1996). Induced systemic resistance (ISR), a salicylic acid-independent pathway, is induced in response to avirulent pathogens and by some nonpathogenic rhizobacteria (Pieterse and van Loon, 1999). Induced resistance is often investigated in biological control research in the rhizosphere.

Colonization of the rhizosphere with beneficial bacteria has suppressed a range of soil borne pathogens (Baker et al., 1986; Cook et al., 1995; Gupta and Utkhede, 1986; Kuć, 2001; Wilson and Backman, 1999; Xu and Gross, 1986). Although this is typically a localized response, the production of a systemic signal has been shown to reduce foliar pathogens, even though colonization is limited to the roots (Cartieaux et al., 2003; Heil and Bostock, 2002; Kloepper et al., 2004; Zehnder et al., 2001). A reduction in a range of foliar diseases caused by viruses (Murphy et al., 2000; Raupach et al., 1996), fungi (Liu et al., 1995; Zhang et al., 2002), and bacteria (Anith et al., 2004; Liu, 1993) have been reported when roots have been colonized with beneficial microbes. Most research on ISR in relation to biocontrol has focused on rhizobacteria, but a range of fungal and bacterial endophytes of above ground plant tissues are also known to induce systemic resistance to a wide range of pathogens (Bailey et al., 2006; Kishore and Podile, 2005; Kloepper et al., 2004; Kuć, 2001; Liu et al., 2001).

The activation of plant defense mechanisms has been an increasing area for biological control research. This includes the use of biological agents and chemical elicitors to activate plant defenses. Although production of defense products may seem like a costly physiological process, this may not always be true. Not all biological agents and chemicals elicitors cause a large change in gene expression. Some agents prime the plant for later protection against pathogens. Priming is the enhanced ability of a plant to activate defense mechanisms upon infection with a pathogen (Kohler et al., 2002). In other words, a primed plant has a more rapid and stronger induction of plant defense genes upon pathogen infection compared to a non-primed plant. Priming reduces disease while not requiring a large production of defense proteins in the absence of the pathogen (van Hulten et al., 2006).

The knowledge of the molecular and biochemical modes of action of BCAs has led to the identification and development of BCAs. An important aspect of biological control is the understanding that BCAs do not simply utilize one mode of action. Many BCAs utilize multiple modes of action. Arras (1996) concluded that *Candida famata* reduced green mold of orange through niche exclusion, mycoparasitism, and induction of defense mechanisms. The rhizobacteria found in Kodiak suppress diseases through niche exclusion and antibiosis (Brannen and Kenney, 1997). The ability to utilize multiple modes of action should allow for enhanced suppression of plant diseases. With successes in disease management in a broad range of pathosystems, biological control is an increasing area of research for the management of cacao diseases.

Biological Control of Cacao Diseases

Cacao disease and pest management practices have changed significantly in the past twenty years. Affecting these have been several emerging issues that impacted cacao farming such as: 1) increased interest in environmentally safe farming, 2) development of pesticide resistant pathogens, 3) pesticide-exposure related illnesses, 4) consumer interest in ecologically based or organic farming, and 5) the cost of pesticides for small-scale farmers. These issues have lead to increased interest in biological control options for disease management. Although chemical controls can manage disease, they are not cost effective for smallholder farmers who grow 70% of the worlds cocoa (Donald, 2004). Furthermore, chemicals are problematic in an agroforestry system, as they can readily contaminate water supplies, can have negative ecological effects, and can endanger human health. Agricultural workers from developing countries are at high-risk for pesticide-related illness due to improper handling of pesticides, lack of literacy, and lack of protective equipment for pesticide applications (Paumgartten et al., 1998). Biological control by itself or as part of an IPM package offers options which are safer to agricultural workers, environmentally friendly, and are cost effective for small-scale farmers. Additionally, biologically based disease options for the cacao agroforestry system could help maintain biodiversity while increasing cacao yields (Donald, 2004). Recently, The Rainforest Alliance has been providing farmers incentives to plant organic cocoa through a certification program (Rainforest Alliance, 2005).

Initial research into organism associated with cacao has focused on epiphytes. Epiphytes are organisms that live on the surface of plants, such as foliar epiphytes that colonize the phyllosphere. Researchers have investigated cacao epiphytes since the 1950s. Thorold (1952) found epiphytic fungi, lichen, algae, mosses, and vascular plants associated with cacao. More recently, studies have focused on the frequency of microbial epiphytes and their ability to reduce cacao diseases. Epiphytic microbes have been isolated from cacao flowers, pods, and leaves (Hoopen et al., 2003; Krauss and Soberanis, 2001b). Some of the more commonly occurring epiphytes are *Clonostachys* spp., *Trichoderma* spp., and *Fusarium* spp., most of which are mycoparasites (Holmes et al., 2004; Hoopen et al., 2003; Samuels et al., 2000). Isolation and reapplication of these epiphytes effectively reduced witches' broom, frosty pod, or black rot in field trials (Deberdt et al., 2008; Hidalgo et al., 2003; Krauss and Soberanis, 2001b; Krauss and Soberanis W., 2002; Tondje et al., 2007). In addition to these studies, *Trichoderma stromaticum* has been formulated into a product, known as TrichoVab, by Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), the Brazilian institute for cacao research. *T. stromaticum* is a native mycoparasite of *M. perniciosa* (Samuels et al., 2000). TrichoVab was provided to Brazilian farmers to help manage witches' broom, but more research could be conducted, as a range of *Trichoderma* spp. with varying affinities for formulation were discovered in cacao farms throughout the Bahia state of Brazil (de Souza et al., 2006).

In addition to epiphytes, more recent research has focused on endophytes. A study by Arnold et al. (2003) demonstrated that 100% of sampled mature cacao leaves were colonized by fungal endophytes. Colonizing leaves with a cocktail of seven of these endophytes decreased levels of *Phytophthora* infection in a pathogenicity challenge. According to a report by Herre et al. (2007) new cacao leaves are initially free of fungal endophytes, but become colonized by as they mature. After maturation, there is an overall decrease in microbial diversity within a leaf, but all leaves remained colonized. Application of one cacao endophyte, *Colletotrichum gloeosporioides*, reduced black rot severity in field experiments as well as reduced sporulation of pods infected with *M. roreri*, although it did not impact frosty pod incidence during the experimental period (Mejía et al., 2008). Many of the cacao fungal endophytes act as mycoparasites as well as produce antimicrobial compounds that can inhibit pathogen growth (Bailey et al., 2008). Additionally, some endophytic strains can activate plant defense mechanisms. Bailey et al. (2006) investigated endophytic isolates with varying levels of antibiosis and mycoparasitism against *M. roreri*. Macroarray and quantitative-PCR studies demonstrated that colonization positively impacted cacao gene expression as well as the genes

expressed by the fungi. Gene induction patterns seen in the plant-microbe associations were specific to the *Trichoderma* isolates colonizing the seedlings. Several labs are currently investigating the effects of cacao endophytes on gene expression. Although most research has focused on fungal endophytes, the research proposed here will focus on endospore-forming bacterial endophytes.

After the plethora of research using fungal species as BCAs for cacao diseases, researchers decided to investigate the potential of bacteria for biological control. Melnick et al. (2008) demonstrated that *Bacillus cereus* isolate BT8 was capable of long-term colonization of cacao foliage and reduction of *Phytophthora capsici* in a detached leaf assay. The continued disease pressure in a tropical perennial system leads to unique issues in the development of BCAs. BCAs for perennial crops must be capable of long term colonization of treated plant parts and durable protection of susceptible tissues. The BCAs must be robust enough to withstand environmental stresses as well as outcompete the multitude of natural organisms it may encounter. *Bacillus* spp. are excellent candidates for BCAs in tropical perennial crops, such as cacao, because they are known to effectively reduce disease in a range of cropping systems, work in the soil or foliage depending on the isolate used, and environmental tolerance provides for stable formulations which can easily withstand the high heat and humidity found in the tropics. Preliminary research presented in this thesis will show the potential of *Bacillus* spp. in colonizing cacao leaves and suppressing cacao diseases.

Research Objectives

Nearly 30% of the potential annual cacao crop is lost to diseases (Hebbar, 2007). The tropical environment in which cacao is grown causes numerous opportunities for stem, pod, and foliar infections from pathogens. These diseases provide a significant threat to crop sustainability for the smallholder farmers who grow most of the world's beans. A large portion of cacao is grown in developing countries where there is a high risk of pesticide exposure and related illnesses of workers and their families due to lack of protective equipment and the proximity of living spaces to both pesticide storage and use (Paumgartten et al., 1998). For these reasons, it is essential to develop sustainable methods to manage cacao diseases. Biological control utilizing microbes that are native to cacao can offer disease management options with reduced risk to cacao farmers. Previous research by Melnick et al. (2008) illustrated that endophytic *Bacillus*

cereus isolate BT8 from tomato was capable of endophytically colonizing cacao. Colonization with this isolate resulted in not only suppression of disease on colonized leaves, but also on leaves that emerged after initial inoculation and were not colonized by this isolate. This indicated the potential of native cacao endospore-forming bacteria to suppress cacao diseases in treated leaves and the durability for long term colonization as well as protection of newly emerged susceptible tissues that lacked bacterial colonists. Due to this success, we hypothesize that native *Bacillus* spp. from cacao would be capable of acting as biological control agents of cacao diseases.

The first objective of this research was to obtain and screen native endospore-forming endophytic bacteria from cacao trees escaping disease in fields in Ecuador. The bacteria were screened to determine whether they possess the desired qualities of a BCA, such as chitinase production, antagonism to cacao pathogens, identified to species, and the ability to establish long term colonization, to determine which isolates would be tested in the field.

The second objective of this research was to determine whether potential biological control agents had the ability to suppress cacao diseases. Field trials were conducted to determine whether the *Bacillus* spp. were capable of suppressing witches' broom, caused by *Moniliophthora perniciosa*, frosty pod, caused by *Moniliophthora roreri*, black rot, caused by *Phytophthora* spp., and cherelle wilt, which is reported to be a physiological thinning mechanism.

The third objective of this research was to determine whether endophytic colonization of cacao seedlings impacted cacao gene expression. Quantitative real time PCR was utilized to determine the impact of colonization on expression of cacao ESTs putatively related to pathogen defense.

The fourth objective of this research was to determine how applications of endophytic *Bacillus* spp. impacted native microbial communities associated with cacao foliage and pods. Automated ribosomal intergenic spacer analysis (ARISA) was used to determine whether application of endophytes changed the abundance and diversity of species present in the native cacao microbial communities.

The overall goal of this research is to provide sustainable options for disease management in cacao, which are safe for both farmers and the environment. In developing these biological products, we hope that we can gain a better understanding of induced resistance in cacao and how addition of a biological control agent impacts microbial ecology of plant associated microbes.

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Chapter 2

Isolation and Screening of Endophytic Endospore-Forming Bacteria from *Theobroma cacao*

Introduction

Endophytes form an intimate association with their plant host that can beneficially impact plant health. Endophytes are ubiquitous to most plant species and have been detected in a range of tropical plants such as coffee (Santamaria and Bayman, 2005; Vega et al., 2005), Atlantic forest timber species (Lambais et al., 2006), banana (Hidalgo et al., 2003; Vu et al., 2006), and cacao (Arnold et al., 2003). Most endophytes are likely neutral in terms of plant health; while some endophytic microbes improve plant health. Endophytes have been shown to promote growth (Taghavi et al., 2009), reduce herbivory (Koh and Hik, 2007), and suppress disease (El-Tarabily et al., 2009). A broad range of research projects have reported introductions of endophytes into plants, with most of these examining endophytic fungi to reduce disease. Within these studies, there has been focus on the use of fungal endophytes to manage cacao diseases.

Tropical leaves are home to a diverse array of fungal endophytes, being inhabited by fungal species which are not known to occur in other biomes (Arnold and Lutzoni, 2007), leading one to believe that cacao should be host to a diverse group of endophytic species. Herre et al. (2007) found that there is a diverse collection of fungal endophytes colonizing cacao leaves and that overall fungal diversity decreases with leaf age. Researchers have tested these fungal endophytes as potential biological control agents (BCAs) for cacao diseases. 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 *Phytophthora* spp. than endophyte-free leaves. Further work from the Smithsonian lab showed that applications of fungal endophytes in the field reduced the incidence of black pod rot and sporulation of *Moniliophthora roreri* on frosty pod lesions, but these treatments did not reduce frosty pod incidence (Mejía et al., 2008). Additional research has demonstrated that endophytic colonization of cacao with *Trichoderma* spp. activates the expression of cacao ESTs putatively

related to defense genes (Bailey et al., 2006). The prevalence and success of fungal endophytes have led to investigations on the presence and ability of bacterial endophytes to act as potential BCAs for cacao diseases. Bacterial endophytes have also been shown to reduce diseases in a wide range of annual crops, but there are limited studies demonstrating that bacterial endophytes are capable of suppressing diseases of perennial crops.

Endophytic bacteria were capable of suppressing Fusarium wilt on cotton in greenhouse studies (Chen et al., 1995). Although these greenhouse studies were successful, the method of endophyte delivery was too difficult to adapt to large-scale studies. Due to the great diversity of microbes inhabiting plants in the tropics, research on bacterial endophytes focuses on tropical crops. Molecular analysis of microbial communities associated with orange revealed that a high frequency of endophytic *Curtobacterium flaccumfaciens* was associated with asymptomatic plants infected with citrus variegated chlorosis (Araújo et al., 2002) and *in vitro* experiments demonstrated that *C. flaccumfaciens* inhibited growth of the pathogen *Xylella fastidiosa* (Lacava et al., 2004). Another crop of important economic significance to tropical countries is oil palm. Both an endophytic *Burkholderia* sp. and a *Pseudomonas* sp. suppressed incidence of basal stem rot (caused by *Ganoderma boninense*) of oil palm for 8-months after inoculation (Sapak et al., 2008). Despite the success of fungal endophytes in suppressing cacao diseases, little work has focused on the presence, abundance, and potential of bacterial endophytes for biocontrol of cacao diseases. Work by Posada and Vega (2005) studying entomopathogenic fungi found bacteria as endophytic colonists of cacao seedlings produced from surface sterilized seeds. These researchers speculated that the bacteria were actually endophytes of the seed. Although this work found bacterial endophytes in cacao, it did not look at cacao growing in a natural setting. Previous work by Melnick et al. (2008) demonstrated an endophytic *Bacillus* spp. from tomato could endophytically colonize cacao leaves for 60+ days and reduce severity of foliar *Phytophthora* disease in detached leaf assays. Endospore-forming bacteria were used because of their environmental stability and ease of formulation into biological control products. This research further demonstrated that bacterial endophytes could be introduced to cacao, but the presence of native bacterial endophytes still remains unknown. Based upon this previous work, the objective of this research was to isolate and screen endospore-forming bacterial endophytes from cacao trees for their ability to act as potential BCAs. The overall goal was to select several isolates of endophytic endospore-forming bacteria which could be tested as potential biological control agents of cacao diseases in field trials.

Material and Methods

Isolation of endospore-forming bacterial endophytes from cacao tissue

Potential BCAs were obtained by isolating endophytic endospore-forming bacteria from elite selections of "Nacional" cacao trees that were escaping cacao diseases on the INIAP Estacion Experimental Tropical (INIAP-EET) in Pichilingue, Ecuador. For the purpose of isolating bacterial endophytes, leaves, pods, flower cushions, and branch tissue were collected from these plantings and quickly processed at INIAP-EET. For isolation of endophytic bacteria from leaves, leaf disks were removed, surface sterilized in 20% bleach (NaOCl), and then rinsed three times in sterile distilled water. Sterilized disks were aseptically placed into sterile filter bag with 3 ml of 0.1M potassium phosphate buffer (pH 7.0) and manually triturated using a pestle. Triturated tissue was then placed into a microcentrifuge tube and heated in a water bath at 75°C for 15 min to select for endospore-forming bacteria. Focus was placed on endospore-forming bacteria because of their environmental stability and ease of formulation into biological control products. Once cooled, triturated tissue was plated onto tryptic soy agar (TSA, Difco, Franklin Lakes, NJ) and incubated for 24 - 48 hours at 24°C in the dark. Once individual colonies formed they were streaked onto fresh plates of TSA.

For isolation of bacteria from pods, the pods were sterilized with 20% bleach, and then the exterior surface of the pod was aseptically peeled away to expose the internal husk tissue. Mucilage tissue surrounding seeds was avoided. Sections of tissue just below the husk were removed and placed into sterile 15 ml tubes. For isolation of endophytes from flower cushions and branch tissue, the bark was aseptically removed from tissue to expose the inner wood. Once the internal portions of the tissue were exposed, sections were removed and placed into 15 ml centrifuge tubes. Two ml of 0.1M potassium phosphate buffer was added to all tubes, and then tubes were heated at 75°C for 15 minutes to select for endospore-forming bacteria. Once cooled the tissue was removed from the tube and placed directly onto TSA. TSA plates were incubated for 24-48 hours at 24°C. Once a bacterium began to grow from the tissue, it was isolated and transferred onto fresh TSA. After successful culturing, bacteria were shipped to The Pennsylvania State University, University Park, PA following USDA-APHIS-PPQ guidelines (permit P526P-07261) for further analysis.

Confirmation of endospore production by bacterial endophytes

Confirmation of endospores was conducted by filling 1.7 ml vials with 800 μl of tryptic soy broth (Difco, Franklin Lakes, NJ). The bacteria were inoculated into three vials for each isolate. Tubes were incubated on an incubator shaker at 28°C. After one week, the vials were heated to 75°C for 15 minutes to select for endospore-forming bacteria. Once cooled, 50 μl of the bacterial suspension was plated onto TSA plates in triplicate. The plates were incubated on the lab bench at room temperature (24°C) for 24 hours and observed for bacterial growth. This procedure was repeated to confirm results.

Determining whether isolates are members of the *Bacillus cereus* group clade through use of selective media

To ensure that none of the isolates were *B. cereus*, *B. cereus* HiVeg Agar (HiMedia Laboratories, Mumbai, India) was prepared following manufacturer's directions. Each isolate was spotted onto three replicate plates which were incubated at 28°C for 24-48 hours. *B. cereus* isolates BP24 and BT8 were used as positive controls. The plates were observed for a colorimetric change to yellow, which indicates the presence of *B. cereus* group cells (Mossel et al., 1967). It should be noted that *B. mycoides*, *B. thuringiensis*, and several other species in the *B. cereus* clade will test positive. The experiment was repeated to confirm results.

Determining whether bacterial endophytes possess chitinase activity

A desirable quality for a BCA is the ability to degrade fungal cell walls through the production of chitinolytic enzymes (Kobayashi et al., 2002). A colloidal chitin agar was made by using partial acid hydrolysis of high molecular weight cross-linked chitin to make a chitin nutrient agar (CNA) supplemented with 0.4% chitin (Kokalis-Burelle et al., 1992). Each isolate was streaked onto three CNA plates. Plates were incubated in the dark at 28°C for 7-10 days. Plates were observed daily for clearing around individual colonies, indicating bacterial chitinase production. *B. cereus* isolates BP24 and BT8, known chitinase producers, were used for positive controls. The experiment was repeated to confirm results.

Determining whether bacterial endophytes are directly antagonistic to cacao pathogens

An *in vitro* plate pairing assay was used to determine whether cacao bacterial endophytes were antagonistic to cacao pathogens. The casual agents of black pod rot, *Phytophthora capsici* isolate 73-73 (H. Purdy, Ecuador); frosty pod, *Moniliophthora roreri* (C. Suárez, Ecuador); and witches' broom *Moniliophthora perniciosa* (C. Suárez, Ecuador) were used in the study. Potato dextrose agar (PDA, Difco, Franklin Lakes, NJ) was used, as both fungal and bacterial species were easily cultivated on this medium. For the assay, two mycelial plugs of the select pathogen were placed 5 cm apart on PDA in a 100 mm Petri dish. A streak of a bacterial isolate was placed perpendicularly between the plugs (2.5 cm from each plug). Control plates consisted of mycelial plugs without bacteria. Three replicate plates were prepared for each isolate combination. Plates were incubated at 28°C and radial growth of the pathogen was measured every 1-3 days, depending on the growth of the pathogen. The measurements were used to calculate the growth rate of the pathogen as mm/day. At the end of the experiment, the final colony diameter perpendicular to the bacterial streak was measured. Data were analyzed by PROC GLM followed by Dunnett's analysis using SAS 9.1 (SAS Institute Inc., Raleigh, NC) to determine whether the suppression of growth was statistically significant (α=0.05). The experiment was repeated to confirm initial results.

Sequencing of regions of the 16S rRNA gene to determine species identification of bacterial endophytes

One day before PCR amplification, isolates were streaked onto fresh TSA plates, so that the bacteria were in the vegetative stage. PCR amplification was performed directly from colonies, so DNA extraction was not necessary. A small colony was removed with a 10 μl pipette tip and placed into a PCR tube containing master mix. PCR master mix was composed of 2 μl 10x PCR buffer with 1.5 mM MgCl, 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) and consisted of a total volume of 20 μl. The universal primers used to amplify the small-subunit ribosomal RNA were 530f (5′-GTGCCAGCMGCCGCGG) and 1392r (5'-ACGGGCGGTGTGTRC) (Lane, 1991). PCR amplification was conducted using an Eppendorf Mastercyler Personal Thermal Cycler (Eppendorf AG, Hamburg, Germany) with the following

cycle: 5 min at 95°C; followed by 35 cycles of 94°C for 15s, 58°C for 15s, 72°C for 15s; and final extension at 72°C for 5 min. PCR products were cleaned using ExoSAP-IT (USB Corp., Santa Clara, CA).

DNA was sequenced using the 530f and 1392r universal primers of 16S rRNA. An ABI Hitachi 3730XL DNA Analyzer (Hitachi Ltd., Tokyo, Japan) was used to sequence the amplicons at the Penn State Genomic Core Facility, University Park, PA. 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) to determine the species of the isolate (Wang et al., 2007). Relatedness of isolates was inferred using the neighbor-joining method (Saitou and Nei, 1987) with MEGA 4.0 (Kumar et al., 2008).

Cacao plant material and growth conditions

Cacao clones of 'ICS1' were obtained via rooted cuttings from greenhouse grown plants following the protocol of Maximova et al. (2005). Once rooted, plants were transplanted into a soil mix (two parts fine sand, two parts Perlite, and one part soil) and 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 lmol/m^{2}s PAR. Automatic retractable shade clothes were used to limit light to a maximum of 1000 lmol/m^{2}s PAR. Plants were drip irrigated with 1/10 strength Hoagland's nutrient solution (160 ppm N) to maintain soil moisture and plant nutrition.

Bacterial inoculum preparation and inoculation of cacao foliage with bacterial endophytes

Once potential BCAs were selected, based upon chitinase and endospore-production, the ability of these isolates to colonize cacao leaves was tested in growth chamber studies. The following 14 isolates were tested: 2506ht 2.1.1, A2046 1.1.1, A2075 5.1.7, CCAT1858 2.1.1, CCAT1858 2.1.2, CCAT1858, 2.1.3, CCAT1858 2.1.6, CUR3 2.1.3, CUR3 3.1.1, EET103ht 2.1.1, EET Mn 30/10, UNAP11.2.3, UVAP 1.2.2, UVAP 1.2.3. Bacterial isolates were grown for 9 days in 500 ml of sterile tryptic soy broth (TSB) in 1.8 L Fernbach flasks at 28°C and 120 rpm

on a rotary incubator shaker (New Brunswick Scientific Model M1024-000, Edison, NJ). After growth, the bacterial suspension was centrifuged for 8 minutes at 6,200g to pellet the bacteria. The bacteria were resuspended in sterile 0.1M potassium phosphate buffer (pH 7.0), concentration of the bacterial suspension was adjusted to 1 x 10^8 CFU/ml, and a low surface tension, polysilicon surfactant (Silwet L-77, G.E. Silicones, Tarrytown, NY) was added to the bacterial suspension at a concentration of 0.24% (v/v). The suspension was sprayed onto leaves of foliage using a hand-held aerosol sprayer (Crown Power Pack, Aerovoe Pacific, Gardenerville, NV) until runoff. Five replicate plants were sprayed per treatment. Once leaves were dried, each plant was covered with a plastic bouquet wrapper to ensure minimal cross-contamination between plants/treatments. Plants were maintained in a randomized block design in a growth chamber (Conviron Model PGR16, Winnipeg, Canada) at 28°C with a 12-h photoperiod at 65% RH and 12-h dark period at 65% RH (see Fig 2-1).
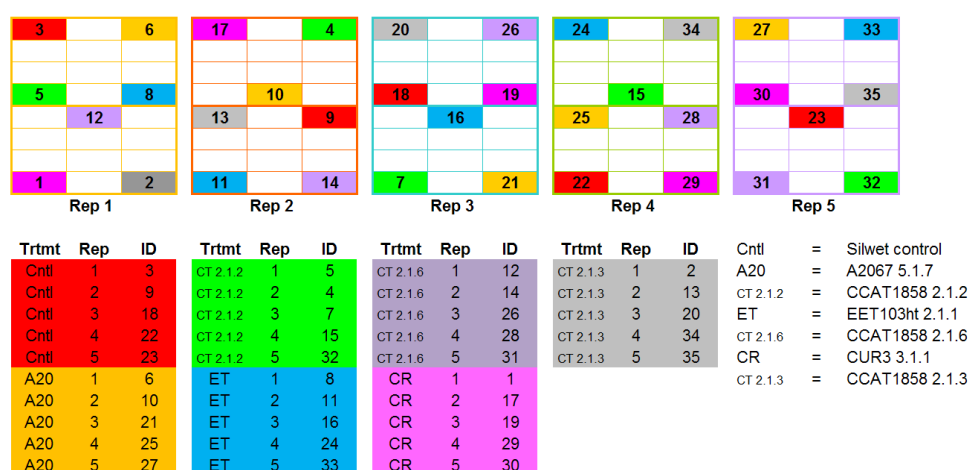
Figure 2-1: Map of growth chamber experimental layout to determine whether bacterial endophytes isolated from cacao tissue were capable of colonizing clean cacao 'ICS1' plants. Plants were sprayed with a log 8.0 CFU/cm^2 bacterial suspension with 0.24% Silwet to run-off, and then placed into bouquet wrappers to prevent cross contamination between plants. Plants were maintained in a randomized block design.

All plants were drip irrigated with 1/10 Hoagland's solution at four-hour intervals. Two separate experiments were conducted to screen all isolates due to space. Isolates with the ability to endophytically colonize cacao tissue at or greater than log 4.0 CFU/cm^2 and with the ability to reduce severity of *P. capsici* in the detached leaf assay were used in a third confirmatory study.

To determine the level of colonization of the bacterial endophytes, four 2.35 cm^2 leaf disks were excised from each leaf in a ''W'' pattern using a cork borer from 3 replicate plants at biweekly intervals. The disks were placed 101 mm x 152 mm stomacher filter bags (SECURE-T

80; Labplas, Sainte-Julie, Quebec) containing 3 ml of sterile 0.1 M phosphate buffer. Samples were agitated with a stomacher blender (Bagmixer 100 MiniMix, Intersciences St. Nom, France) at 100 oscillations per min for 60 sec to remove epiphytic colonists. Fifty μl of the supernatant was plated in triplicate onto TSA using a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA) Plates were incubated at 28°C for 24h, then enumerated using a Spiral Biotech counting grid following manufacturer's instructions. Bacterial populations are reported as CFU/cm^2 of leaf tissue. Endophytic colonization was determined following a similar protocol to epiphytic colonization, except leaf disks were surface sterilized in 10% commercial bleach for 3 minutes, followed by two rinses in sterile distilled water. Disks where then placed in 3 ml of sterile 0.1 M phosphate buffer (pH 7.0) and were triturated as before, but the paddles of the stomacher were set for disruption of the leaf disks. The minimum detectable population level for this methodology was 1.8 log CFU/cm^2.

Detached leaf assay to determine the ability of bacterial endophytes to suppress disease caused by *Phytophthora capsici*

The ability of bacterial endophytes to suppress disease was assessed by challenging leaf disks with *Phytophthora capsici* isolate 73-73 and measuring subsequent disease development. Inoculum was prepared from 5-day-old cultures grown on 50 ml unclarified 20% V8 agar in 125 ml Erlenmeyer flasks at 28°C in an incubator with a 12 h light cycle. Zoospores were obtained in sterile distilled water following the protocol of Lawrence (1978). Prior to each challenge, a small aliquot of the zoospore suspension was stained with lactophenol cotton blue and zoospore concentration was counted using a hemacytometer (American Optical, Buffalo, NY) and adjusted to 5x10^3 zoospores/ml. *P. capsici* 73-73 was periodically re-isolated from infected cacao leaves to maintain virulence.

The detached leaf assay was conducted following the methodology of Melnick et al. 2008. Immature green leaves were detached from plants. Nine cm diameter leaf disks were excised from leaves so that the midrib was in the center of disk and placed abaxial side up on a moist sterile 9-cm Whatman paper in an inverted 100 mm Petri dish. Each leaf disk was inoculated with six 10 μl droplets (approx. 50 zoospores per droplet) of the zoospore suspension. Dishes were sealed with parafilm and maintained in an incubator at 28°C with a 12-h light-dark cycle. Leaves were evaluated every 8–12 h for up to 52 h after inoculation by measuring the

lesion diameter and the percent of necrotic area under the droplet of zoospore suspension (0–400%). Disease was determined over time and was used to calculate area under the disease progress curve (AUDPC) (Shanner and Finney, 1977). Data were statistically analyzed for significance using ANOVA followed by Tukey's HSD using the SAS 9.1 program with a 95% confidence level.

Results

Isolation of endospore-forming bacterial endophytes from *Theobroma cacao*

There were 69 isolates obtained from cacao plant tissue sampled. All isolates are listed in Table 2-1.

Table 2-1: Characterization of endospore-forming bacterial endophytes isolated from cacao trees. The table lists isolate identification, the genotype of the host of origin, the disease the trees were considered to be tolerant to, the host tissue the isolate was derived from, and the date each bacterial endophyte was isolated.

	Isolate ID	Genotype of tree bacteria were from	Reported tolerance of genotype	Plant tissue isolated from	Date tissue obtained
1	103.hm(2)	EET-103	Witches broom	Leaf	10-Mar-06
2	103.hm.2.1.1	EET-103	Witches broom	Branch	10-Mar-06
3	103.ht.2.1.1	EET-103	Witches broom	Branch	10-Mar-06
4	103ht 2.1.2	EET-103	Witches broom	Branch	10-Mar-06
5	2073 2.1.1	Nacional 2073	-	Branch	19-Mar-06
6	2073 2.1.2	Nacional 2073	-	Branch	19-Mar-06
7	2073 2.1.3	Nacional 2073	-	Branch	19-Mar-06
8	2506tallo 1.1.1	Nacional 2506	-	Leaf	Mar-06
9	2506hm 2.1.1	Nacional 2506	-	Branch	Mar-06
10	2506hm2.1.1(2)	Nacional 2506	-	Branch	Mar-06
11	2506ht2.1.1	Nacional 2506	-	Branch	Mar-06
12	A2046 1.1.1	Nacional A2046	-	Pod	19-Mar-06
13	A2076 1.1.1	Nacional A2076	-	Pod	19-Mar-06
14	A2076 3.1.1	Nacional A2076	-	Leaf	19-Mar-06
15	A2076 3.2.1	Nacional A2076	-	Leaf	19-Mar-06
16	A2076 5.1.1	Nacional A2076	-	Floral cushion	19-Mar-06
17	A2076 5.1.2	Nacional A2076	-	Floral cushion	19-Mar-06
18	A2076 5.1.3	Nacional A2076	-	Floral cushion	19-Mar-06
19	A2076 5.1.4	Nacional A2076	-	Floral cushion	19-Mar-06
20	A2076 5.1.5	Nacional A2076	-	Floral cushion	19-Mar-06
21	A2076 5.1.6	Nacional A2076	-	Floral cushion	19-Mar-06
22	A2076 5.1.7	Nacional A2076	-	Floral cushion	19-Mar-06

	Isolate ID	Genotype of tree bacteria were from	Reported tolerance of genotype	Plant tissue isolated from	Date tissue obtained
23	A2076 5.1.8	Nacional A2076	-	Floral cushion	19-Mar-06
24	CCAT1858 1.1.1	ICCO CCAT1858	Frosty pod	Pod	19-Mar-06
25	CCAT1858 2.1.1	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
26	CCAT1858 2.1.2	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
27	CCAT1858 2.1.3	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
28	CCAT1858 2.1.4	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
29	CCAT1858 2.1.5	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
30	CCAT1858 2.1.6	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
31	CCAT1858 2.1.7	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
32	CCAT4688 2.1.1	ICCO CCAT1858	Frosty pod	Branch	19-Mar-06
33	CUR3 1.1.1	CUR3	Witches broom	Pod	19-Mar-06
34	CUR3 1.1.2	CUR3	Witches broom	Pod	19-Mar-06
35	CUR3 2.1.1	CUR3	Witches broom	Branch	19-Mar-06
36	CUR3 2.1.2	CUR3	Witches broom	Branch	19-Mar-06
37	CUR3 2.1.3	CUR3	Witches broom	Branch	19-Mar-06
38	CUR3 2.1.4	CUR3	Witches broom	Branch	19-Mar-06
39	CUR3 2.2.1	CUR3	Witches broom	Branch	19-Mar-06
40	CUR3 2.2.2	CUR3	Witches broom	Branch	19-Mar-06
41	CUR3 3.1.1	CUR3	Witches broom	Leaf	19-Mar-06
42	EB1922 1.1.1	EB1922	Witches broom	Pod	19-Mar-06
43	EB1922 1.1.2	EB1922	Witches broom	Pod	19-Mar-06
44	EB1922 1.1.3	EB1922	Witches broom	Pod	19-Mar-06
45	EB1922 5.1.1	EB1922	Witches broom	Floral cushion	19-Mar-06
46	EB1922 5.1.2	EB1922	Witches broom	Floral cushion	19-Mar-06
47	EET 103Tallo1.1.1	EET-103	Witches broom	Leaf	Oct-05
48	EET103 ht 1.1.1	EET-103	Witches broom	Leaf	Oct-05
49	EET103.ht.2.1.1	EET-103	Witches broom	Branch	Oct-05
50	EET103Mm	EET-103	Witches broom	Pod	Oct-05
51	EET103Mm 30/10	EET-103	Witches broom	Pod	10-Mar-06
52	EET103Tallo 3-10	EET-103	Witches broom	Branch	10-Mar-06
53	ET62 2.1.1	EET-62	Witches broom	Branch	19-Mar-06
54	ET62 2.1.5	EET-62	Witches broom	Branch	19-Mar-06
55	ET62 2.1.6	EET-62	Witches broom	Branch	19-Mar-06
56	ET62 2.1.7	EET-62	Witches broom	Branch	19-Mar-06
57	ET62 5.1.1	EET-62	Witches broom	Floral cushion	19-Mar-06
58	ET62 5.1.3	EET-62	Witches broom	Floral cushion	19-Mar-06

	Isolate ID	Genotype of tree bacteria were from	Reported tolerance of genotype	Plant tissue isolated from	Date tissue obtained
59	L25H64	L25H64	Witches broom	Branch	20-Mar-06
60	L25H64 2.1.3	L25H64	Witches broom	Branch	20-Mar-06
61	SCA 1.1.1	Scavina	Witches' broom	Pod	19-Mar-06
62	SCA(5) 1.1.1	Scavina	Witches' broom	Pod	19-Mar-06
63	SPEC541 1.1.1	SPEC 54/1	Witches' broom	Pod	20-Mar-06
64	UNAP1 1.1.1	UNAP-1	Frosty pod	Pod	19-Mar-06
65	UNAP1 1.2.2	UNAP-1	Frosty pod	Pod	19-Mar-06
66	UNAP1 1.2.3	UNAP-1	Frosty pod	Pod	19-Mar-06
67	UVAP 1.1.1	UVAP	Frosty pod	Pod	19-Mar-06
68	UVAP 1.2.2	UVAP	Frosty pod	Pod	19-Mar-06
69	UVAP 1.2.3	UVAP	Frosty pod	Pod	19-Mar-06

Of the isolates obtained, 14% were from leaves, 17% were from floral cushions, 25% were from pods, and 44% were from branches. All tissues sampled were colonized by endospore-forming bacterial endophytes.

Screening of bacterial endophytes for qualities of elite BCAs

One-hundred percent of the isolates produced endospores during the confirmation study, indicating the success of the isolation technique (Table 2-2). It should be noted that production of endospores and chitinase were used as selection criteria to select for bacteria to conduct further growth chamber analyses to determine the ability of bacteria to colonize trees and reduce disease. Antagonism in *in vitro* plate assays was not used as a selection criterion, but was simply conducted to characterize the isolates.

Table 2-2: Results of screening of bacterial endophytes for elite quality of BCAs such as endospore-production; chitinase production; positive reactions on *B. cereus* agar; antagonism toward the cacao pathogens *Moniliophthora roreri* (MR), *M. perniciosa* (MP), and *Phytophthora capsici* via *in vitro* plant assays; and disease suppression in detached leaf assays. Putative species identifications are reported. \Stars listed under endospore production and chitinase production indicates a positive result. Stars listed under pathogens indicate statistically significant disease suppression at α=0.05. For disease suppression, blank spaces indicate isolates in which disease suppression was not tested, yes is answered if statistically significant disease suppression occurred, and no is answered if disease suppression *in planta* was absent.

ID	Species ID	Endospore Prdtn	Cereus agar	Chitinase Prdtn	MR	MP	PC	Reduce Disease
						Antagonism towards:		
1	*Bacillus subtilis*	*			*	*	*	
2	*Bacillus pumilus or safensis*	*			*	*	*	
3	*Bacillus pumilus or safensis*	*			*	*	*	
4	*Bacillus pumilus*	*						
5	*Bacillus subtilis*	*			*	*	*	
6	*Lysinibacillus fusiformis*	*				*		
7	*Bacillus subtilis or amyloliquefaciens*	*			*	*	*	
8	*Bacillus* sp.	*	*				*	
9	*Bacillus pumilus*	*						
10	*Bacillus cereus*	*			*		*	
11	*Bacillus cereus* or *thuringiensis*	*	*	*	*		*	No
12	*Bacillus cereus*	*	*	*	*			No
13	*Bacillus flexus or megaterium*	*						
14	*Lysinibacillus sphaericus*	*						
15	*Bacillus pumilus or safensis*	*			*	*	*	
16	*Bacillus pumilus*	*			*	*	*	
17	*Lysinibacillus sphaericus*	*						
18	*Lysinibacillus sphaericus*	*						
19	*Bacillus cereus or thuringiensis*	*					*	
20	*Lysinibacillus sphaericus*	*					*	
21	*Bacillus cereus or thuringiensis*	*	*					
22	*Lysinibacillus sphaericus*	*		*	*	*		Yes
23	*Solibacillus silvestris*	*					*	
24	*Bacillus cereus or thuringiensis*	*			*		*	
25	*Bacillus firmus*	*		*			*	Yes
26	*Bacillus cereus* or *thuringiensis*	*		*				Yes
27	*Bacillus cereus* or *thuringiensis*	*	*	*	*			Yes
28	*Paenibacillus* spp.	*						
29	*Bacillus cereus* or *thuringiensis*	*	*		*		*	
30	*Bacillus cereus* or *thuringiensis*	*	*	*	*			Yes
31	*Bacillus cereus*	*	*					
32	*Paenibacillus alvei*	*					*	
33	*Bacillus subtilis*	*			*	*	*	
34	*Bacillus subtilis*	*						
35	*Bacillus pumilus*	*				*	*	
36	*Lysinibacillus sphaericus*	*						
37	*Lysinibacillus fusiformis*	*			*			Yes
38	*Bacillus cereus or thuringiensis*	*		*			*	

ID	Species ID	Endospore Prdtn	Cereus agar	Chitinase Prdtn	Antagonism towards: MR	MP	PC	Reduce Disease
39	*Bacillus safensis or pumilus*	*				*	*	
40	*Solibacillus silvestris*	*				*		
41	*Bacillus subtilis*	*		*		*		Yes
42	*Bacillus pumilus*	*			*	*		
43	*Bacillus megaterium*	*			*			
44	*Bacillus subtilis*	*			*	*	*	
45	*Bacillus flexus*	*						
46	*Bacillus cereus*	*						
48	*Bacillus cereus or thuringiensis*	*	*	*				
49	*Bacillus pumilus*	*		*	*		*	Yes
50	*Bacillus pumilus*	*						
51	*Bacillus* spp.	*		*			*	No
52	*Bacillus mycoides*	*						
53	*Bacillus subtilis or B. amyloliquefaciens*	*			*	*	*	
54	*Bacillus subtilis*	*			*	*	*	
55	*Bacillus subtilis*	*			*	*	*	
56	*Bacillus subtilis or B. amyloliquefaciens*	*			*	*	*	
57	*Bacillus mycoides*	*	*					
58	*Bacillus cereus or thuringiensis*	*	*				*	
59	*Bacillus cereus*	*	*		*		*	
60	*Brevibacillus brevis*	*		*		*		
61	*Bacillus megaterium*	*	*					
62	*Bacillus megaterium*	*	*					
63	*Bacillus cereus or thuringiensis*	*	*		*	*	*	
64	*Bacillus subtilis*	*			*	*	*	
65	*Bacillus pumilus*	*			*		*	
66	*Bacillus cereus or thuringiensis*	*	*	*				No
67	*Paenibacillus sp.*	*						
68	*Bacillus cereus*	*		*				No
69	*Bacillus cereus or thuringiensis*	*		*				No

Sixteen of the isolates (23%) were chitinolytic. There were differential abilities to be antagonistic to the three tested cacao pathogens. Of the isolates tested, 42% were antagonistic to *M*. *roreri*, 33% were antagonistic to *M*. *perniciosa*, and 49% were antagonistic to *P*. *capsici*. Twenty-five percent of isolates inhibited the growth of both *Moniliophthora* spp. Species that inhibited the growth of *M*. *roreri* did not uniformly inhibit the growth of *M*. *perniciosa* and vice-versa, despite the genetic closeness of these pathogens. Of isolates that inhibited *M*. *roreri*, 45% did not inhibit

the growth of *M. perniciosa* while 27% of isolates that inhibited *M. perniciosa* did not inhibit *M. roreri*. Twenty-two percent of isolates inhibited the growth of all three pathogens. After sequencing, it was determined that more isolates tested positive for *B. cereus* through sequencing than those that tested positive on the *B. cereus* selective media. Only chitinolytic isolates that tested negative on *B. cereus* agar were tested for the ability to colonize cacao foliage and suppress *P. capsici*. Several of the endophytes that were not directly inhibitory to *P. capsici* in the plate assays, were inhibitory to disease development *in planta*.

Inoculation of cacao leaves with bacterial endophytes to confirm endophytic ability

All isolates tested were capable of colonizing cacao foliage when sprayed onto plants with 0.24% Silwet L-77, indicating that the isolates were endophytes. The initial colonization data is located in Figure 2-2.
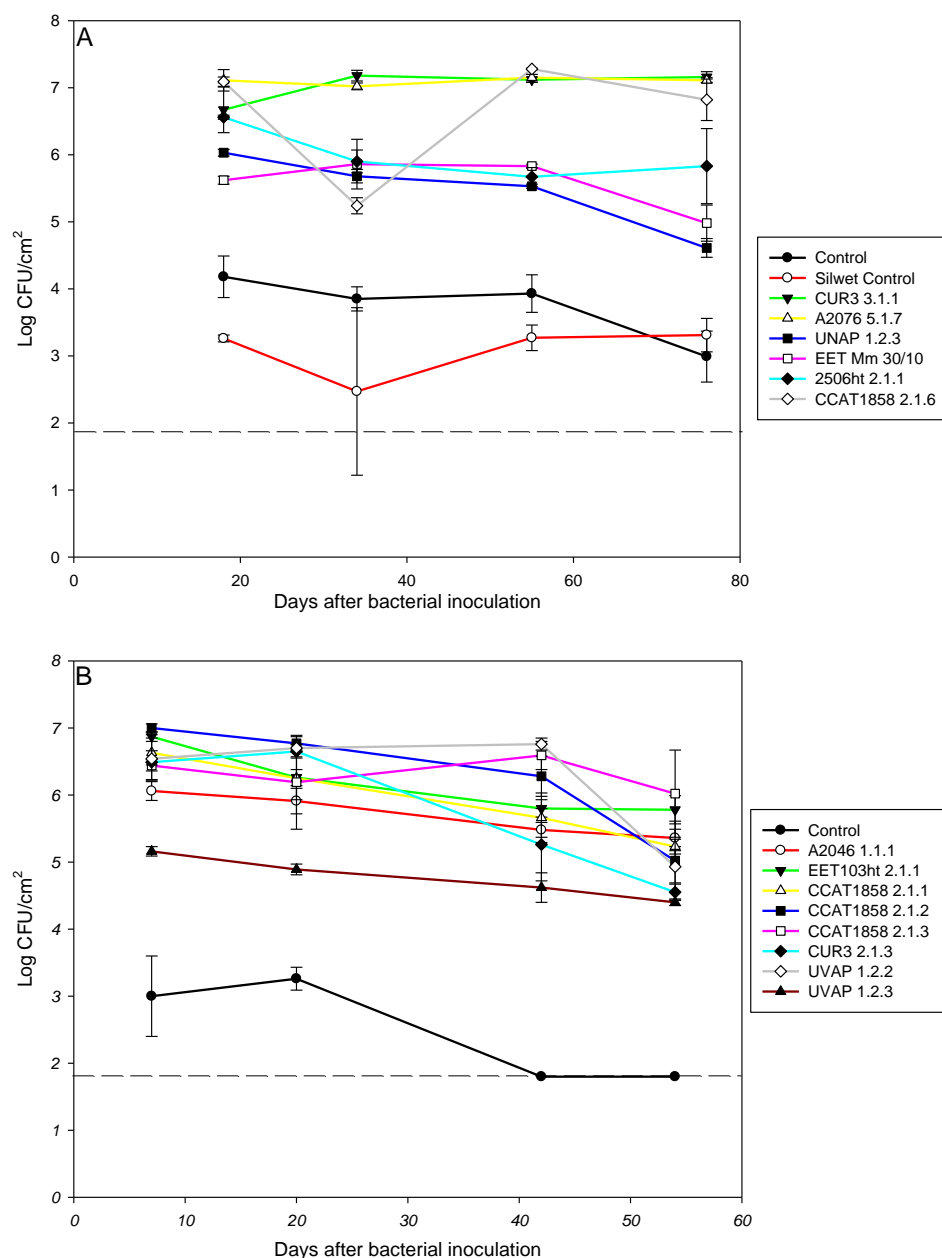
Figure 2-2: Mean bacterial colonization of mature cacao leaves sprayed with either 0.24% Silwet or 0.24% Silwet + log 8.0 CFU/ml bacterial endophyte. Control represents leaves that were never sprayed and Silwet control represents leaves that were sprayed with 0.24% Silwet L-77 in 0.1M potassium phosphate buffer without bacteria. The remaining treatments represent chitinolytic cacao bacterial endophyte applied at log 8.0 CFU/ml with 0.24% Silwet. Bars extending around the means indicate the standard error 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.

Leaves from control plants were colonized by endophytes, but at a lower level than those

receiving bacterial treatments. Most isolates were capable of long-term colonization at relatively

high populations levels (log 6.0-7.5 CFU/cm^2). UVAP 1.2.3 was capable of endophytically colonizing leaves, but only at low population levels compared to other colonists.

Based upon these 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 EETht103 2.1.1) were used in subsequent studies to determine both the microbial ecology of colonization as well as their abilities to suppress disease in detached leaf assays.
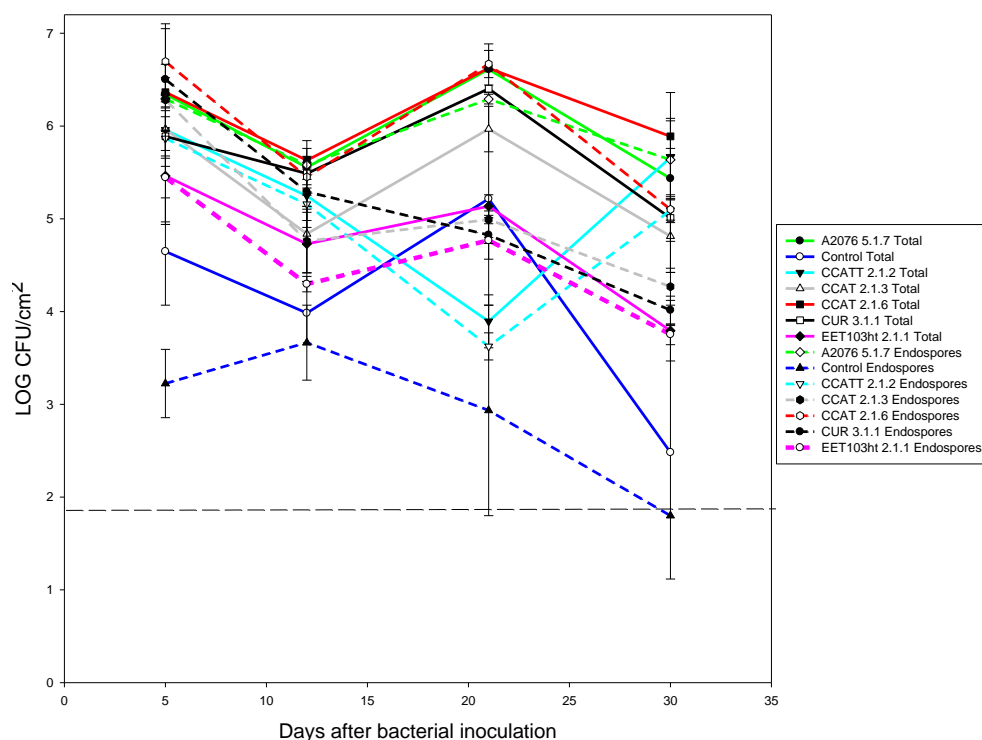
Figure 2–3: Mean epiphytic colonization of mature cacao leaves at 5, 12, 21, and 30 days after inoculation with bacterial endophytes for total colonization (vegetative cells and endospores) and for endospores Total bacterial colonization is represented by solid lines, while populations of endospore-bearing bacteria colonizing the epiphytic and endophytic portions of plants are represented by dashed lines. Application of bacteria to cacao leaves occurred at day 0 when plants where 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 0.1M potassium phosphate buffer. Bars extending around the means indicate the standard error of that mean. The horizontal dashed line indicates the minimum detection level of the experiment.

All isolates were capable of colonizing the phyllosphere of cacao leaves. In terms of total colonization (vegetative cells + endospores), colonization was variable among isolates, varying up to one log CFU/cm^2 (Fig 2-3). Colonization was detected in control plants, with populations at one to two log CFU/cm^2 lower than those in treated plants. Endospore production in control plants was far below that of treated plants at 3 to 4 log CFU/cm^2 lower. Colonization also

occurred in the endophytic portion of leaves, but at lower levels than colonization of the epiphytic areas of leaves (Fig 2-4).
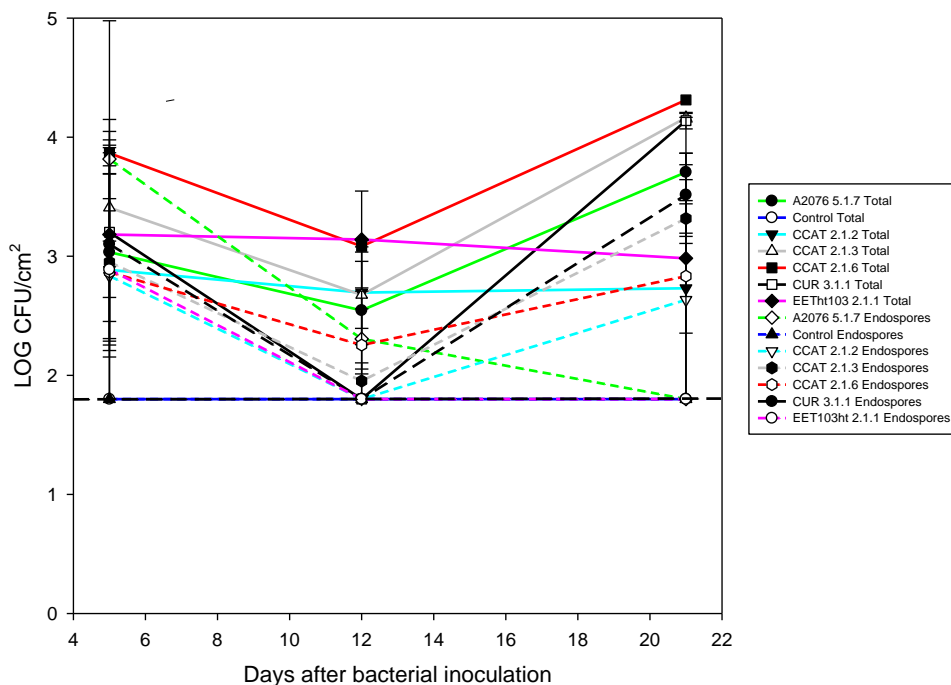
Figure 2-4: Mean endophytic colonization by total bacteria (vegetative cells + endospores) and populations of endospore-bearing bacteria in mature green cacao leaves at 5, 12, and 21 days after inoculation with cacao bacterial endophytes. Total endophytic colonization is represented by solid lines, while endophytic endospores are represented by dashed lines. Inoculation of cacao leaves with bacteria occurred at day 0 when plants where sprayed with 0.24% Silwet L-77 (vol/vol) + log 8.0 CFU/ml bacterial suspension. Bars extending around the means indicate the standard error of that mean. The horizontal dashed line indicates the minimum detection level for the experiment. All data points on this line indicate that the microbial populations were below this detection threshold.

Control plants did not have detectable levels of endophytic bacteria. Populations of endophytic bacteria were approximately two log CFU/cm^2 less than total endophytic colonists (vegetative + endospores). Several isolates had less than the detection threshold levels of endospores in leaves at 12 days after inoculation, but these populations recovered by 21 days. Overall, all isolates tested had long term persistence in the endosphere, and showed the ability to grow (as evidenced by vegetative cells) in cacao leaves.

Identification of bacterial species from the 16S rRNA gene

Successful amplification and sequencing occurred for all isolates. Below is the alignment of amplified 16S sequences to determine the relatedness of the bacterial endophytes to each other and to endospore -forming endophytes from vegetable crops, used in previous studies with cacao (*B. cereus* isolate BP24 and *B. cereus* isolate BT8, Melnick et al. 2008) and apple (A1-1, A1-5, Ae-1, FL-1, and FL-5, Poleatewich, unpublished) (Fig 2-5).

46
59
B. cereus BP24
49
12
66
69
68
31
21
10
76
38
41
19
30
58
B. cereus BT8
91
Apple.FL-5
27
24
11
26
8
52
Bacillus mycoides
71 57

Bacillus **c**

34
5
33
50
54
53
64
65
35
30
44
7
3
99
16
42
15
56
47
48
4
2
9
1
39
55

Bacillus **a**

87
45
25 *Bacillus flexus*
13
43
Apple.Ae-1 *Bacillus megaterium*
26
100 Apple.A1-5
61
62
98
23
100
40 *Solibacillus silvestris*
63
6 *Lysisnibacillus fusiformis*
83
98 37
14
17 *Lysisnibacillus sphaericus*
52 18
82 20
22
36
28 *Paenibacillus* spp.
32 *Paenibacillus alvei*
96
62 67
56
60 *Brevibacillus brevis*
Apple.FL-1
100
51 *Bacillus* spp.

0.07 0.06 0.05 0.04 0.03 0.02 0.01 0.00

Figure 2-5: Relatedness of 69 bacterial endophytes from cacao, two isolates from vegetables (*B. cereus* isolate BP24 and *B. cereus* BT8) and five endospore-forming bacterial endophytes from apple. Neighbor joining analysis was conducted in MEGA 4.0 (Tamura et al., 2007). Species identification was determined by BLAST analysis of sequences using SEQMATCH of RDP 10.14.

Bacillus a is composed of *B. amyloliquefaciens*, *B. pumilus*, *B. safensis*, and *B. subtilis*. Species located in the *Bacillus* c genus are species of the *B. cereus* clade. The presence of BP24 and BT8 in *Bacillus* c indicates the success of the methodology, as both are known to be *B. cereus*.

Discussion

This study represents the first known report of isolation of bacterial endophytes from cacao growing in its natural environment. Bacteria associated with cacao have been isolated from trees, but not as endophytic colonists. Macagnan et al. (2006) isolated endospore-forming bacteria and actinomycetes from the carposphere of cacao pods. The cacao rhizosphere is also home to actinomycetes in the genus *Streptomyces* (Barreto et al., 2008). Despite working with plant associated bacteria, neither of these studies looked at endophytic organisms. Additionally, there is a high diversity of fungal endophytes that are known to inhabit cacao (Bae et al., 2009; Bailey et al., 2008; Hanada et al., 2008; Herre et al., 2007) that the isolated bacteria must co-exist with in cacao tissue.

Isolations demonstrated that bacterial endophytes are present in a range of cacao tissues, indicating their ubiquitous nature in the cacao environment. Of the isolates obtained, most inhabited branches. This finding is not an indication of bacterial endophytes being the most abundant in branches, but more likely reflects the biases of the isolation methodology. Few flower cushions were removed from the trees, as not to cause extensive damage. Additionally, a large number of leaves were sampled, but fewer bacterial endophytes were isolated from these leaves. As seen in previous work, applied bacterial endophytes were capable of persisting for 68+ days in cacao leaves (Melnick et al., 2008). The adverse nature of the sterilization and sampling techniques likely prevented the isolation of more bacteria from selected leaves. The heat treatment eliminated all bacteria that did not produce endospores, which likely eliminated most bacterial genera as well as endospore-forming bacteria which were growing in a vegetative stage at the time of sampling. The primary selection criteria was endospore formation, as endospore-forming microbes are environmental tolerance, which leads to the ability of a bacterium to tolerate long term storage in a commercial formulation and organic pesticide.

Bacillus spp. form the resistant spore structure in response to resource insufficiency, so simply growing the cultures to exhaustion in liquid medium usually induces spore formation, allowing for easy production of endospores (Driks, 2004). All isolates were endospore producers, so additional selection criteria were needed to choose which isolates to test in the field. Chitinase production combined with negative results on *B. cereus* agar was used to determine which isolates to utilize in further trials on ability of the isolates to colonize young cacao plants and reduce disease in detached leaf assays. *B. cereus* agar was used instead of sequencing, as it was a rapid screening methodology. It was not until after isolates were screened and initial field testing commenced until it was determined that the *B. cereus* agar did not correctly identify all isolates of *B. cereus* in the collection.

Colonization assays with the 14 tested bacteria demonstrated that all isolates were capable of endophytically colonizing cacao leaves, but isolates had a differential ability to colonize leaves. Most isolates were capable of colonizing cacao tissue at log 5.5-7.0 CFU/cm^2, except isolate UVAP 1.2.3, which had consistently low levels of endophytic colonization (~log 2.5 CFU/cm^2). UVAP 1.2.3 was eliminated from future consideration, due to the low levels of colonization. Several isolates, such as EETht103 2.1.1, existed predominately as vegetative cells in the leaf endosphere. Eight of the tested isolates suppressed the severity of *P. capsici* in detached leaf assays: A2064 1.1.1, A2076 5.1.7, CCAT1858 2.1.1, CCAT1858 2.1.2, CCAT1858 2.1.6, CUR3 3.1.1, EET103ht 2.1.1, and UVAP 1.2.2 at the different time points following inoculation. It should be noted that isolates capable of antagonizing cacao pathogens on *in vitro* plate assays did not always suppress *P. capsici* in the detached leaf assay. Specifically, isolates 2506ht 2.1.1 and EET103Mm 30/10 suppressed the growth of *P. capsici in vitro*, but did not suppress disease severity in detached leaves. The ability of the isolates to colonize cacao leaves was not always correlated to disease suppression. Of the chitinolytic isolates tested, only 57% of the colonists suppressed disease. It should be mentioned that UVAP 1.2.3 was not capable of suppressing disease and had low levels of endophytic colonization, indicating that the isolate may not have been able to maintain suitable population levels to suppress disease. Based upon all of these studies, isolates A2076 5.1.7, CCAT1858 2.1.2, CUR3 3.1.1, and EETht103 2.1.1 were used for further field studies on biological control of witches' broom disease and cacao pod maladies.

A diverse range of endospore-forming bacteria were isolated from cacao tissue. Sixty eight percent of all isolates were members of either the *Bacillus a* or *Bacillus c* clades. *B. pumilus* and *B. subtilis* were common inhabitants of cacao tissue. Of the most abundant isolates,

29% were *B. cereus* group cells, indicating that this common soil saprophyte is also a common endophytic colonist of cacao tissue. There were a few less common *Bacillus* spp., such as *B. flexus*, *B. firmus*, and *B. megaterium*. Additionally, 4% of the isolates were *Solibacillus silvestris*. *Lysinibacillus* spp. were relatively common (12% of isolates), while there were only three isolates which where *Paenibacillus* spp. and one isolate from the genus *Brevibacillus*. The diversity of endophytic endospore-forming bacteria isolated from cacao was similar to those isolated from apple (see Figure 2-5), *Cinnamomum longepaniculatum* (Wang et al., 2009), orange (Araújo et al., 2002), and other plants.

According to the Center for Disease Control (2009), *Bacillus cereus* causes two percent of reported foodborne illness in the U.S. annually. Despite the ability of 8 out of 18 serotypes of *B. cereus* to cause human disease (Gilbert and Parry, 1977), this bacterial species is a ubiquitous organism in soil and as an endophyte. *B. cereus* has been detected in branches of sweet orange and tangerine (Araújo et al., 2002), coffee leaves (Shiomi et al., 2006), ginseng roots (Cho et al., 2007), carrots (Surette et al., 2003), and many other crops. Many environmental *B. cereus* isolates are chitinolytic (Chang et al., 2003; Pleban et al., 1997), indicating that chitinase production is common to *B. cereus*. One area of further research that should be conducted is to determine whether endophytic *B. cereus* isolates produce the emetic toxins and enterotoxins capable of causing foodborne illnesses. Since this species has been isolated as an endophyte of plant tissue, including those directly consumed by humans (Surette et al., 2003), it is imperative to conduct further investigations of endophytic strains of *B. cereus* as these isolates could pose potential risks to human health. Additionally, *B. cereus* has been used in a range of biological control studies (Cho et al., 2007; Shiomi et al., 2006); therefore a thorough study needs to be conducted to determine whether utilizing this species is a safe practice for biological control.

A key remaining area of future research with these isolates, which is presented in later chapters, is the screening of the isolates for their abilities to reduce disease in the field. Although laboratory characterization of potential BCAs is useful in identifying potential BCAs, only field testing can verify that these isolates can reduce disease under natural conditions. Further experiments focused on testing whether an elite set of isolates were capable of reducing witches' broom, frosty pod, black pod, and cherelle wilt of cacao. Lastly, culture independent methods could be useful in characterizing the microbial communities associated with cacao. The isolation methodologies used in this chapter solely focused on endospore-forming isolates, but a variety of bacterial genera are likely associated with cacao. Other research has already illustrated that fungal endophytes are associated with cacao (Arnold et al., 2003). Culture independent

technologies, such as T-RFLP, ARISA, and pyrosequencing can be used to detect culturable and noncultarable microbes and may eliminate sample size bias, heat treatment effects, and competition of the microbes for nutrient sources on media. Another benefit to these technologies is the reduction of time and labor in the laboratory compared to isolation and independent sequencing of all cultured isolates. Overall, this research indicates that cacao supports a high diversity of endophytes from multiple kingdoms that could potentially be BCAs of cacao diseases.

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Chapter 3

Biological Control of Witches' Broom of Cacao with Bacterial Endophytes

Introduction

   Theobroma cacao L. is an economically significant crop, as the seeds are processed into a range of cocoa products. A major portion of cacao is produced on small-scale farms throughout tropical regions. Cacao production is difficult, because of several disease problems. A recent estimate states that 30% of the global cacao crop is lost to disease (Hebbar, 2007). One of the most devastating diseases to the South American continent is witches' broom, caused by the agaric basidiomycete *Moniliophthora perniciosa* (Aime and Phillips-Mora, 2005). The disease occurs throughout cacao growing regions in South America, causing between 50 to 90% loss of yield in infected fields (Meinhardt et al., 2008). It is estimated that 200,000 workers lost their jobs in Ilheus, Brazil following the initial outbreak of this disease (Pereira et al., 1996). The disease cycle has two phases characterized by the two vegetative phases of the pathogen, primary and secondary mycelium, as seen in Figure 3-1.
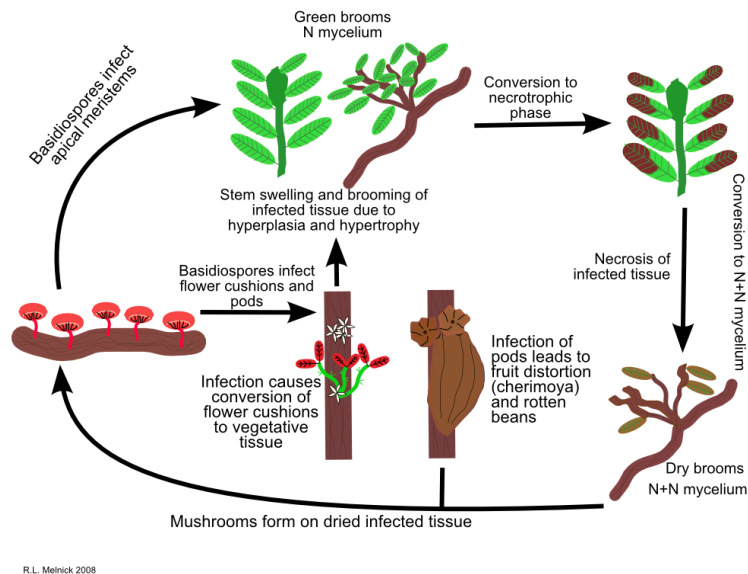
Figure 3-1: Disease cycle of witches' broom of cacao caused by *Moniliophthora perniciosa*, causal agent of witches' broom disease. The disease cycle is intimately linked to the life cycle of the fungus. Symptoms

of the infection are typically observed on infected branches, but pods and flower cushions can also become infected.

The first phase of witches' broom is caused by the primary (1N) mycelium of *M. perniciosa*. Initial infections arise from basidiospores that directly penetrate meristematic tissue. After initiating infection, there is a latent stage, where *M. perniciosa* is often considered to be an obligate biotroph (Wheeler and Suárez, 1993). Primary mycelium produces the most conspicuous symptoms on axiliary and terminal brooms where it causes stem swellings and proliferation of branching, commonly known as green brooms, 2-3 months after infection. Eventually, by a mechanism that is not fully understood, there is a conversion to the necrotrophic stage of the disease cycle, which is associated with the conversion to dikaryotic (N+N) mycelium. Dead brooms remain attached to the tree, since no abscission layer is formed (Meinhardt et al., 2008). In addition to infecting foliage, *M. perniciosa* can also infect floral cushions, causing the conversion to vegetative tissue, as well as infections of developing pods, where it can rot the beans.

Although there has been extensive research on witches' broom, disease management still remains problematic. Phytosanitation remains the best management option (Soberanis et al., 1999). Diseased tissues are pruned from the trees to reduce inoculum. Although this method is quite successful, it is often ignored due to the high labor input (Purdy and Schmidt, 1996). Fungicides can reduce disease by protecting pods and reducing inoculum, but has little impact on pre-existing brooms (Tovar, 1991). A larger problem is that small-holder farmers cannot afford pesticides or application equipment. Cacao varieties with partial resistance are currently being deployed to combat disease, but it is difficult and expensive to switch an entire field of perennial trees to more resistant genotypes. Current issues with disease management combined with the desire for environmentally based farming options have led to research on biological control of cacao diseases.

Much of the initial research on biological control of witches' broom has focused on mycoparasites, such as *Trichoderma* spp. and *Clonostachys rosea* (de Souza et al., 2006; Krauss and Soberanis, 2001a; Krauss and Soberanis, 2002). Although the mycoparasite *Trichoderma stromaticum* was formulated and sold, in the field it was displaced by native isolates from the environment and did not activate the expression of cacao defense genes during endophytic colonization (de Souza et al., 2006; de Souza et al., 2008). While early work on biological control of cacao diseases focused on mycoparasites, recent studies have centered on fungal endophytes. Cacao harbors a range of endophytic fungi some of which suppress cacao diseases

(Arnold et al., 2003; Herre et al., 2007; Rubini et al., 2005). Colonization of cacao with endophytic *Trichoderma* spp. activated cacao plant defense genes, delayed the drought response, and promoted growth (Bae et al., 2009; Bailey et al., 2008; Bailey et al., 2006). These successful biocontrol projects utilizing fungal endophytes led to research centered on endophytic bacteria of cacao. Melnick et al. (2008) demonstrated that endophytic *Bacillus* spp. from vegetable crops colonized cacao foliage and suppressed *Phytophthora capsici* on leaves that emerged after colonization; therefore lacked bacterial colonists. This success led to research on native endospore-forming endophytic bacteria from cacao. Sixty-nine isolates of endophytic endospore-forming bacteria were obtained from cacao trees and screened to select four potential biological control agents (BCAs) (see Chp. 2).

Biological control is intimately linked to microbial ecology. Plants are home to a microbial community that is often not well characterized, since approximately 90 to 99% of microbes cannot be cultured using current methodologies (Curtis et al., 2002; Pace, 1997). Application of BCAs can sometimes alter the native microbial communities associated with the plant host. Application of *Bacillus cereus* UW85 to soybean roots caused dramatic alterations to the bacterial rhizosphere community (Gilbert et al., 1993). Some BCAs do not have a dramatic effect on overall non-pathogenic bacterial communities. Work by Bowers et al. (1996) demonstrated that introduction of a beneficial *Streptomyces* sp. did not change the diversity of the native *Streptomyces* community in the potato rhizosphere, but did decrease the number of *S. scabies* isolates causing scab. Since a high percentage of microbes in the environment cannot be cultured, nucleic acid based methods have been central to the study of microbial ecology. No published works on the microbes associated with cacao have utilized culture-independent methods to characterize the cacao microbial communities, nor have researchers looked at how application of the BCAs impact native microbial communities associated with cacao. One technology that could be utilized is automated ribosomal RNA intergenic spacer analysis (ARISA). ARISA is a molecular fingerprinting technique used to study microbial diversity from environmental samples. PCR is conducted to amplify the rRNA spacer region (Jensen et al., 1993). Automation involves using a DNA analyzer to measure the length of the rRNA spacer and the fluorescence of the fragments generated using fluorescent labeled primers to estimate the diversity and abundance of the microbial community (Fisher and Triplett, 1999). ARISA cannot determine the exact counts or identify the organisms present, but can provide useful estimates of diversity and abundance. Differences occurring between treatments can be used to determine how a specific treatment impacts the overall community structure. The objectives of the research

presented in this chapter are to test the ability of endospore-forming bacterial endophytes from cacao to suppress witches' broom disease caused by *M. perniciosa* and to determine how application of bacterial endophytes impact native endophytic bacterial communities associated with cacao leaves using ARISA.

Materials and Methods

Cacao clonal material and maintenance

Young cacao trees were obtained by grafting of budwood onto rootstock. Budwood was obtained from cacao clones EET-19, 'Nacional' A2126, 'Nacional' A2634, and CCN-51 on Instituto Nacional Autónomo de Investigaciones Agropecuarias Estacion Experimental Tropical (INIAP-EET) in Pichilingue, Ecuador. Budwood was grafted onto rootstock of seedlings from open pollinated IMC67 trees. Additionally, IMC67 seedlings were allowed to develop as a rootstock control. After grafting, plants were maintained under ambient conditions in a greenhouse at INIAP-EET and treated with insecticides as needed to manage aphid and mite problems.

Bacterial isolates and application of potential biological control agents

Four different cacao endophytes were evaluated as potential biocontrol agents of cacao pod diseases: *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET all isolated from cacao trees (see Chp. 2). To prepare the bacterial inoculum, the bacteria were grown in 500 ml of sterile tryptic soy broth (TSB) in a 1L flask. Flasks were incubated for 9 days at 28°C and 120 rpm on a rotary incubator shaker (Thermolyne BIG Bill, Duquesne, IA). The bacteria were allowed to settle to the bottom of the flask overnight and the broth was decanted. Bacteria were resuspended in sterile distilled water and the bacterial concentration was adjusted to approximately 1×10^8 CFU/ml. Bacterial treatments were applied to plants on 23 May 2007. Applications were made using the methods of Melnick et al. (2008). Bacterial suspensions were applied at 1×10^8 CFU/ml with 0.20% Silwet (GE Silicons, Tarrytown, NY) using handheld sprayers. Control treatments consisted of 0.20% Silwet in sterile

distilled water. Applications of bacterial treatments were made to four plants each of each cacao clone for a total of 100 plants. After application, plants were maintained in a greenhouse at INIAP-EET under ambient conditions and watered as needed.

Field experiment for biological control and witches' broom and evaluation of disease severity

Three weeks after initial application of bacterial endophytes (13 Jun 2007), trees were planted into the field in Pichilingue, Ecuador in a nested block design, so that plants within blocks were grouped (or nested) by bacterial treatment to prevent cross-contamination through bacterial redistribution and during future bacterial reapplications (Fig 3-2).

Figure 3-2: Nested block design of the field experiment to evaluate the ability of endophytic bacilli to suppress witches' broom disease of cacao. Small trees were grouped into blocks by bacterial treatment, and then planted under large trees infected with high levels of disease (trunks of large trees represented by dark green blocks). Yellow blocks indicate areas that had high levels of sunlight, due to lack of coverage by the upper canopy. Individual trees are labeled by cacao clone (A2126, A2634, CCN-51, EET-19, and IMC67) and then by the bacterial treatment applied, as follows: Silwet control (CNTL), and isolates *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Bacteria were applied to plants maintained in the greenhouse May 2007 and plants were planted into the field in June 2007. Bacteria were reapplied to plants in the field in Jan 2008, Mar 2008, May 2008, Jan

2009, and Apr 2009. Phytosanitary pruning to remove infected tissues occurred before the reapplication in Dec 2008, May 2008, and Jan 2009.

Small trees were planted in the field under large cacao trees with high levels of witches' broom infections. The infected larger trees not only provided shade to the smaller trees, but also provided basidiospores to initiate natural infections. Plants were rated throughout the experiment to measure disease severity by counting the number and types of brooms (apical, lateral, terminal) as well as cankers, then converting the information into the following disease severity scale:

Table 3–1: Disease severity scale for rating witches' broom severity on small trees.

Severity Rating	Disease Symptoms
0	No disease present
1	Infection of one lateral or axillary shoot
2	Infection of 2 lateral or axillary shoots or 1 terminal shoot
3	Infection of a lateral/axillary and terminal shoot Infection of 2 terminal shoots Infection of terminal shoot with extensive stem swelling Stem canker indicating systemic infection
4	Infection of 2 lateral/axillary shoots with infection of terminal shoots Infection of 3+ terminal shoots Stem canker with 1 brooms
5	Presence of dry brooms on the plant 5+ branches with brooms Stem canker with 2+ brooms
6	Plant killed due to disease

Disease was measured twice during the 2007 dry season (25 Sept & 21 Nov). Plants were pruned at the onset of the 2008 rainy season (5 Jan 08) to remove infected tissue. The bacteria were reapplied to the plants in the field, as previously stated, on 7 Jan 08. Disease severity was evaluated monthly throughout the rainy seasons (7 Feb, 7 Mar, 21 Apr, and 21 May). Near the end of the rainy season plants were pruned to remove infected tissue and bacterial treatments were reapplied (30 May). During the 2008 dry season, disease was evaluated twice (21 Sep and 3 Dec). At the end of the dry season, plants were pruned to remove infected tissue and bacterial treatments were reapplied (7 Jan 09). During the 2009 rainy season, disease was evaluated on 21 Apr, 28 May, and 25 Jun. The timeline for the application of the bacteria and phytosanitary pruning can be found in Figure 3-3. Each date was analyzed separately as well as by determining

area under the disease progress curve (AUDPC) for the rainy season data (Shanner and Finney, 1977). Statistical significance was determined through PROC MIXED followed by Tukey's analysis ($\alpha=0.05$) using SAS 9.1 (SAS Institute Inc., Raleigh, NC).
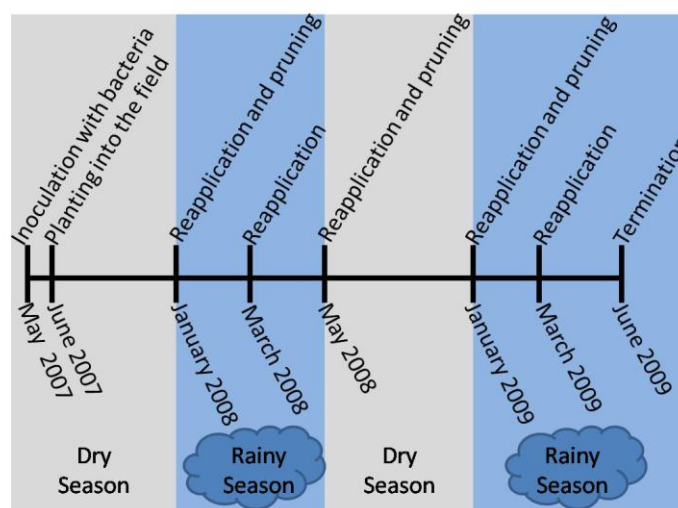
Figure 3-3: Timeline for the application of bacterial endophytes and phytosanitary pruning of brooms in the two year field study on biological control of witches' broom of cacao using endospore-forming bacterial endophytes.

Determination of endophytic colonization of leaves by *Bacillus* spp.

Colonization of cacao leaves was determined immediately after initial application in May 2007, at the end of the dry season in November 2007, mid-way through the rainy season in March 2008, and at the end of the rainy season in May 2008. To determine endophytic colonization, mature cacao leaves (that had been directly sprayed) were removed from the plants at each of the above dates. Leaves were brought back to the lab and four 1.7 cm diameter leaf disks were cut from leaves using a hole punch. Leaf disks were surface sterilized in 20% commercial bleach for 3 minutes followed by two rinses in sterile distilled water. Leaf disks were then placed in a stomacher filter bag (SECURE-T 80; Labplas, Sainte-Julie, Quebec) with 3 ml of sterile 0.1M potassium phosphate buffer (pH 7.0). Disks were manually triturated using a mortar, and then 50 µl of supernatant was plated onto tryptic soy agar (TSA). Plates were incubated in the dark at 24°C for 24-48 hours. At 24 hours after inoculation with bacteria, plates were rated for the presence or absence of bacterial colonies, while plates were enumerated at remaining times to determine the level of bacterial colonization. Endophytic colonization was reported as CFU/cm^2.

Determining the affect of application of BCAs on the microbial communities of cacao leaves

Cacao trees and application of BCAs

An experiment was conducted during the 2008 rainy season to determine whether application of the potential BCAs changed the microbial communities naturally associated with cacao leaves. Fifteen large 'Nacional' cacao trees with no visible disease were selected for the experiment. The trees were roughly 25 years old and were randomly distributed throughout the small planting (1-2 hectares). Trees of similar size and structure were chosen with non-treated trees separating all treated trees. The small trees used in the biological control experiment were not used for analysis of the microbial community, as the presence of the pathogen might have altered the microbial communities; therefore be impossible to differentiate the affect of the pathogen from those of the BCAs. Treatments were the same bacterial treatments and formulations as the biological control experiment. Bacteria were grown and prepared as previously stated. Branches in the north, east, south, and west cardinal direction were tagged and sprayed with bacterial solution on the same day small trees were sprayed during the 2008 rainy season (Jan and March). Three replicate trees were sprayed with each treatment. Branches were evaluated monthly for disease.

Sampling leaves for use in automated ribosomal intergenic spacer analysis (ARISA)

Before reapplication of bacteria in March and at the end of May, leaves were sampled for ARISA analysis to determine the impact applications of BCAs have on the bacterial community of cacao leaves. For each tree, two leaves were removed from each of the four sprayed branches and combined to represent sampling from one tree. Plugs were removed from collected leaves, surface sterilized as previously stated, and then placed into RNALater (Applied Biosystems, Foster City, CA) to preserve the tissue for nucleic acid extraction. Tissues were shipped in RNALater to Penn State for later analysis.

***DNA extraction and ARISA analysis to determine if application of potential BCAs altered the
microbial community of the cacao leaf.***

DNA extraction from cacao leaves followed the protocol of Michiels et al.(2003). DNA
was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).
Analysis was conducted to determine how the BCAs impacted the community of related species
(gram-positive endospore-forming bacteria) as well as and total bacteria in cacao leaves. To
determine the community structure of bacilli (*Bacillus* and closely related genera such as
Paenibacillus), a nested PCR was conducted following the protocol of Garbeva et al. (2003).
Primer set BacF 5'-GGGAAACCGGGGCTAATACCGGAT (Garbeva et al., 2003) and R1378,
5'-CGGTGTGTACAAGGCCCGGGAACG (Heuer and Smalla, 1997) were used for the first
PCR reaction and primer set labeled F968-HEX /R1378 (Heuer et al., 1997) were used for the
second reaction. PCR was conducted 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 25μl. The reactions for each PCR step were conducted following Garbeva et
al. (2003). For the PCR reaction to determine the structure of the total bacterial community, the
primers ITSF (5′-GTCGTAACAAGGTAGCCGTA) and labeled ITSReub-HEX (5'-
GCCAAGGCATCCACC) (Cardinale et al., 2004) were used. PCR was conducted using the
previously described reaction mix. The reaction conditions were 95°C for 3 min; 28 cycles of
95°C for 30 sec, 58°C for 1 min, 72°C for 1.5 min; with a final extension of 72°C for 10 min.
Verification of all PCR reactions was conducted through gel electrophoresis and PCR products
were stored at -20°C.

The length and relative abundance of fluorescent labeled fragments of the16S-23S rDNA
IGS region were determine by analyzing 1 μl of fluorescent labeled PCR product on an ABI
Hitachi 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) with an internal ROX
size standard at the Penn State Core Genomic Facility, University Park, PA. Fluorograms were
analyzed using Genemapper® Fragment Analysis Software v 4.0 to record peak length and
quantify relative abundance of each peak. Data from fragments are reported as operational
taxonomic units (OTUs).

Multivariate analysis of ARISA profiles via ordination analysis

ARISA profiles were analyzed using unconstrained ordination analysis through conical correspondence analysis (CCA) in CANOCO version 4.5 (Leps and Smilauer, 2003; ter Braak and Smilauer, 2002). For the profiles of both bacilli and total bacteria, analyses determined that profile data were unimodal via conical correspondence analysis (having a beta diversity > 4) (Leps and Smilauer, 2003). Fragment relative abundance was used for the data input and analyzed using Hill's scale. Data were visualized by creating biplots of unconstrained axes which best explained the data using CanoDraw (ter Braak and Smilauer, 2002). The microbial composition of leaves with different bacterial treatments was measured with Monte Carlo simulations with 999 iterations. Analysis for bacilli specific primers utilized 29 species, while analysis for general bacterial primers consisted of 58 unique species.

Results

Dry Season 2007: May 2007-December 2007

Application of bacterial endophytes with 0.2% Silwet L-77 resulted in successful endophytic colonization of all leaves tested. Some of the control plants had fewer total bacterial colonists (log 4.0 CFU/cm^2) compared to the larger populations colonizing treated leaves, but this was dependant on the cacao clone. Not all control leaves had detectable levels of bacterial colonization (detection threshold was 1.8×10^2 CFU/cm^2). Measurements at the end of the 2007 dry season indicate that endophytic colonists persisted for at least six months after initial application, despite that no reapplication occurred throughout the dry season (Fig 3-4).
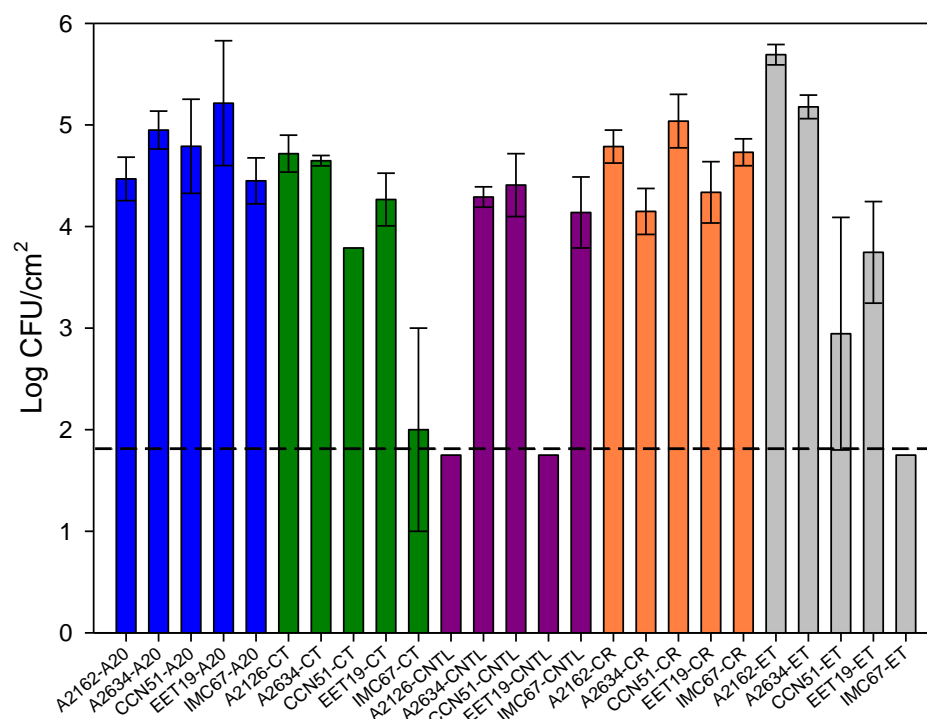
Figure 3-4: Mean endophytic colonization of cacao leaves during Nov of the 2007 dry season. Leaves were sprayed with log 8.0 CFU/cm^2 bacilli + 0.20% Silwet six months prior to determining colonization. Plants were maintained in a greenhouse for three weeks, and then planted into the field. Cacao clones (A2126, A2634, CCN-51, EET-19, IMC67) where inoculated with the following bacterial treatments: Silwet control (CNTL), and isolates *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. The dashed line indicates the detection threshold of log 1.8 CFU/cm^2. Bars extending from the means represent standard errors of that mean.

Colonization was relatively persistent across the different cacao clones for *L. sphaericus* A20 and *B. subtilis* CR. Colonization by *B. cereus* CT and *B. pumilus* ET was not persistent across the clones, as colonization did not persist to detectable levels in IMC67 plants. Since the means were of colonization of leaves from all replicates, it is likely that no detectable colonization existed. Control plants were colonized by bacteria, indicating that they became naturally colonized by bacteria under field conditions that differed in morphology from the introduced species.

Overall disease incidence was low throughout the 2007 dry season. There was very little disease in September and only slightly more in November, as reported in Table 3-2.

Table 3-2: Average disease severity rating for bacterial treatment of the cacao clones for the dry season of 2007 (May 07– Dec 07). Disease was evaluated in Sep and Nov on a 0 to 6 severity scale (Table 3-1). Bacterial treatment means followed by the same lower case letter within columns and cacao variety followed by the same capital letter indicate treatments that are not significantly different using Tukey's HSD (α=0.05).

| | Average disease severity by bacterial treatments | | | | | | | | | |
| | Control | | A20 | | CT | | CR | | ET | |
Clone	Sep	Nov	Sep	Nov	Sep	Nov	Sep	Nov	Sep	Nov
A2126 A	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
A2634 A	0 a	0 a	0.5 a	0.8 a	0 a	0 a	0 a	0 a	0 a	0 a
CCN-51A	0 a	0 a	0 a	0 a	0 a	0 a	0.3 a	0.3 a	0 a	0.5 a
EET-19 B	2.0 a	3.0 a	0.5 a	1.8 a	0 a	0 a	0.5 a	0.5 a	0.3 a	0.3 a
IMC-67 A	0.5 a	0.7 a	0.3 a	0.5 a	0 a	0 a	0 a	0 a	0 a	0 a

During the 2007 dry season, only cacao clone significantly affected disease severity, due to very low disease incidence. Susceptible clone EET-19 had significantly higher levels of disease than the remaining four clones. There was no significant treatment x clone interaction. Although treatment did not affect disease severity, no plants inoculated with bacterial isolate *B. cereus* CT where infected by witches' broom and plants inoculated with CR and ET follow a trend of reduced disease, as seen in Figure 3-5.
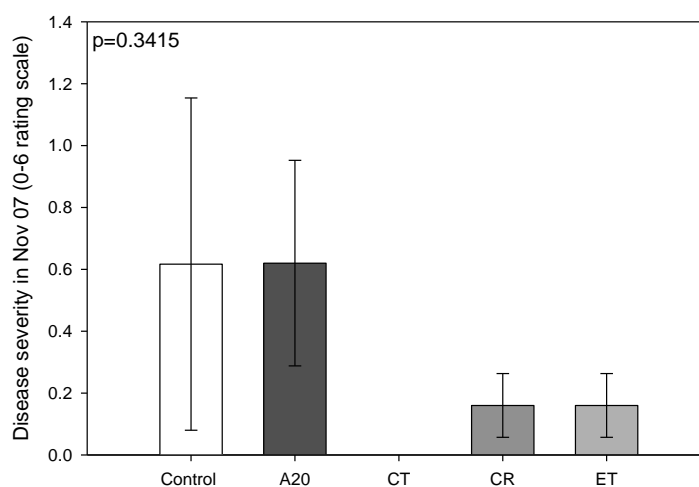
Figure 3-5: Mean disease severity (percentage of diseased plants) of witches' brooms in Nov of the 2007 dry season (May 07-Dec 07) by treatment. Plants were maintained in a greenhouse for three weeks, and then planted into the field. Plants were inoculated with the following bacterial treatments: Silwet control (CNTL), and isolates *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Bars extending from the means represent standard errors of that mean.

The single application of *B. cereus* CT in May resulted in complete disease suppression throughout the six month dry season.

Rainy Season 2008: January 2008 – May 2008

  Colonization was highly variable among the four replicate plants, as seen by the large error bars. Colonization of treated plants was between log 3.0 and 4.5 CFU/cm^2 in March. Colonization persisted between log 4.0 and 5.0 CFU/cm^2 through May 2008 (Fig 3-6). Leaves of control plants had relatively high levels of indigenous bacterial colonists (~ log 3.0 CFU/cm^2 in March and log 4.0 CFU/cm^2 in May), but these were predominantly gram-negative bacteria, not the gram-positive, spore formers applied. Additionally, colony morphology of bacteria from control plates differed greatly from bacteria from treated plants.
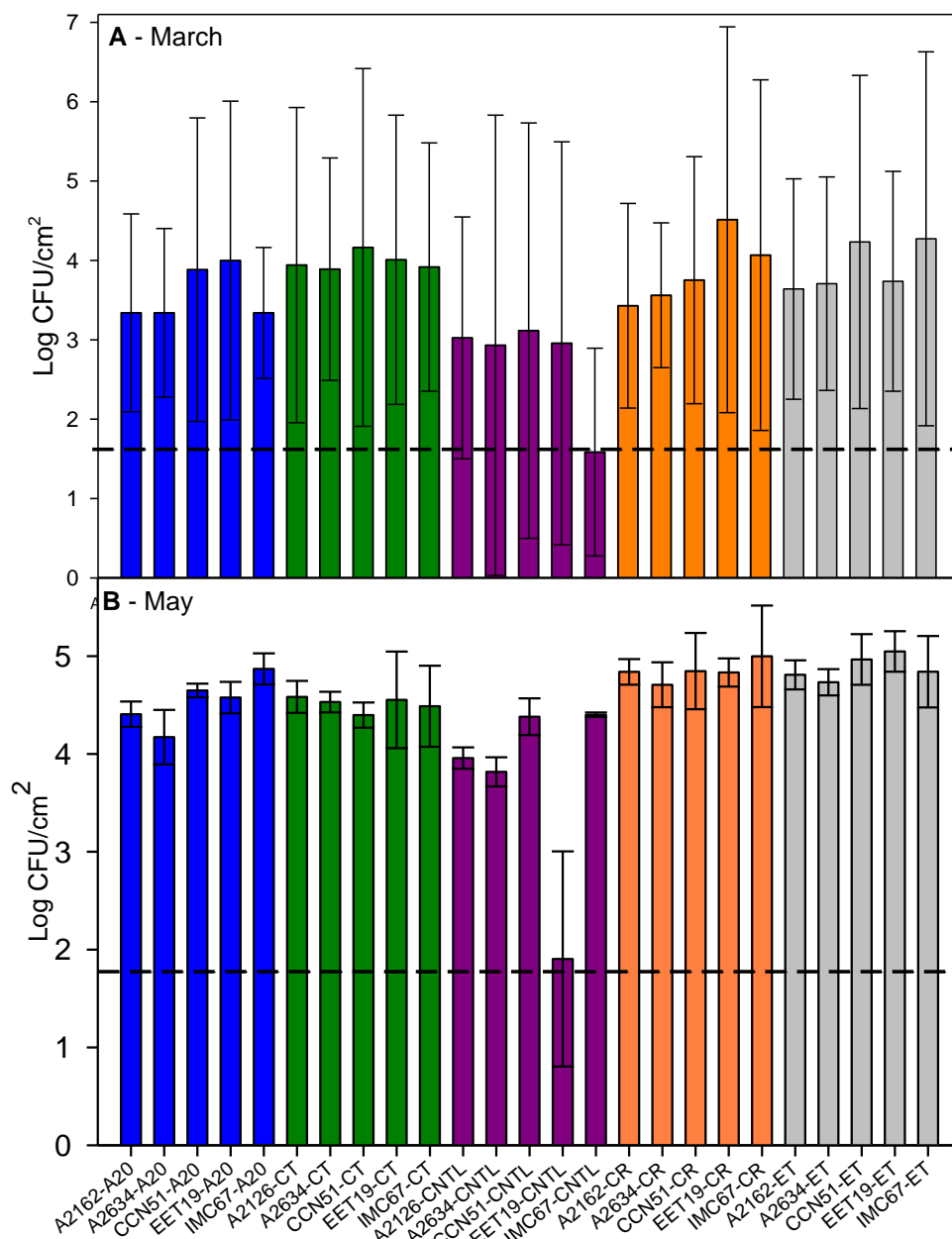
Figure 3-6: Mean endophytic colonization of cacao leaves during the 2008 rainy season **A** represents colonization in March 2008 before reapplication while **B** represents colonization in May 2008. Leaves were sprayed with log 8.0 CFU/cm^2 *Bacillus* spp. + 0.20% Silwet in Jan 08 and Mar 08. Cacao clones (A2126, A2634, CCN-51, EET-19, and IMC67) where inoculated with the following bacterial treatments: Silwet control (CNTL), and isolates *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Note that although control plants had high levels of bacterial colonist, these colonists were mainly gram-negative bacteria. Dashed line indicates the detection threshold of log 1.8 CFU/cm^2. Bars extending from the means represent standard errors of that mean.

Colonization throughout the rainy season was relatively uniform both between treatments and cacao clones. Only untreated control plants of susceptible 'EET-19' had low levels of bacterial colonists, just above the minimum detection level. Colonization was more persistent throughout the rainy season than in the previous dry season, regardless of bacterial isolate or cacao clone. Throughout the course of the 2008 rainy season, clone had no significant effect on overall disease severity (Fig. 3-7).
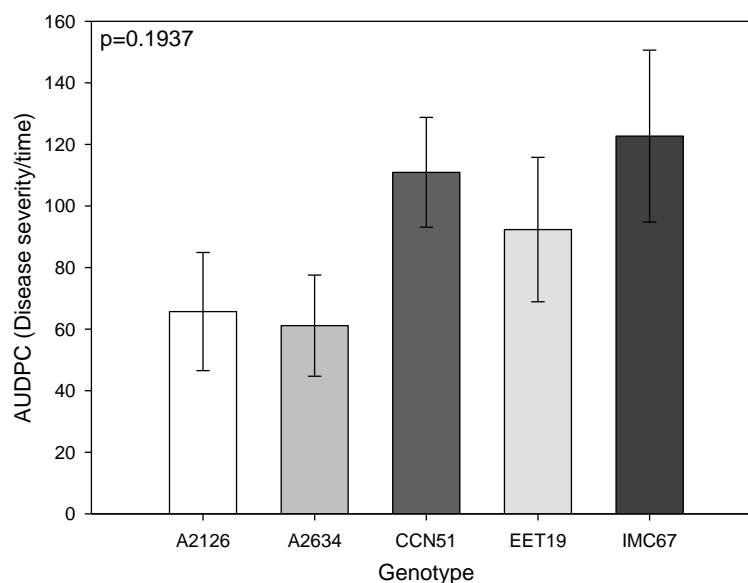
Figure 3-7: Mean area under the disease progress curve for witches' broom severity throughout the course of the 2008 rainy season (Jan 08-May08) by clone. Cacao clones were A2126, A2634, CCN-51, EET-19, and IMC67. AUDPC is based on the disease severity in Table 3-2. Bars extending from the means represent standard errors of that mean.

Although clone did not significantly affect disease during the rainy season, treatment of plants with bacterial endophytes resulted in statistically significant witches' broom suppression from non-treated control plants. *B. pumilus* ET reduced disease severity at each individual rating date (Fig 3-8) when data were combined from all cacao clones and also throughout the rainy season, as indicated by the area under the disease progress curve (AUDPC) (Fig 3-9)There was no significant clone x treatment interaction.
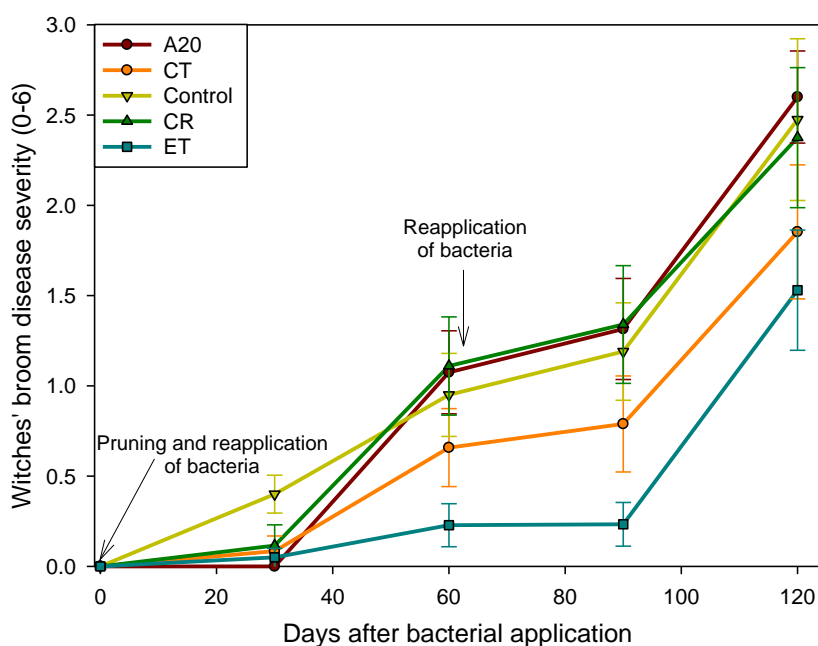
Figure 3-8: Mean witches' broom disease severity by days after bacterial application for bacterial treatments averaged across all cacao clones and measured on a 0-6 severity scale throughout the 2008 rainy season (Jan 08-May 08). Phytosanitation of the trees and initial bacterial applications occurred at day 0, with reapplication of bacteria occurring at day 62. Bacteria were applied at log 8.0 CFU/cm^2 with 0.20% Silwet on both dates. Treatments consisted of: *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Control plants were sprayed with 0.2% Silwet in sterile distilled water. Bars extending from the means represent standard errors of that mean.

Colonization of cacao seedlings with *B. pumilus* ET resulted in statistically significant suppression of witches' broom disease severity throughout the dry season. This was especially noted between days 35 and 60 after application of bacteria, as plants treated with *B. pumilus* ET displayed a delayed onset of disease from plants sprayed with other treatments and the control. Reapplication at 62 days continued the reduced rate of disease development in plants treated with *B. pumilus* ET until roughly 90 days after initial reapplication, but did not stop the increase in disease seen on plants of all treatments observed 120 days after initial reapplication. Although there was an increase in disease at the end of the rainy season, final disease severity in plants treated with *B. pumilus* ET remained lower than plants treated with the Silwet control. In addition to suppressing disease at early dates, analysis of AUDPC showed that *B. pumilus* ET suppressed witches' broom throughout the entire season (Fig 3-9).
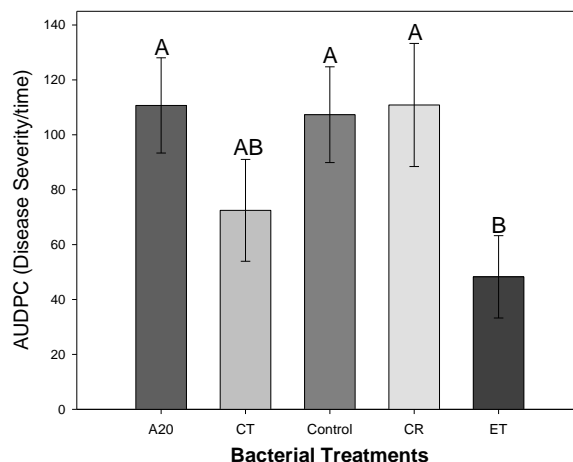
Figure 3-9: Mean area under the disease progress curve for witches' broom severity throughout the course of the 2008 rainy season (Jan 08-May08) by bacterial treatment. Treatments consisted of: *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Control plants were sprayed with 0.2% Silwet in sterile distilled water. AUDPC is based on the disease severity ratings in Table 3-1. Bars extending from the means represent standard errors of that mean. Differing letters above the means indicate statistically significant difference based upon Tukey analysis ($\alpha=0.05$).

Plant colonized with *B. pumilus* ET had significantly reduced disease (p=0.045) when compared to Silwet controls, *L. sphaericus* A20, and *B. subtilis* CR. Plant colonized with *B. cereus* CT had reduced levels of diseases, but not at statistically significant levels. It is interesting to note that despite high levels of endophytic colonization, both *L. sphaericus* A20 and *B. subtilis* CR did not reduce disease. Additionally, despite natural colonization by bacterial endophytes, control plants had relatively high levels of disease

Multivariate analysis of ARISA profiles via ordination analysis

Unimodal ordination analysis by CCA determine that none of the treatments had a significant effect on the microbial composition of bacilli in leaves collected in either March (p=0.0662) or May (p=0.0776) (Fig 3-10).
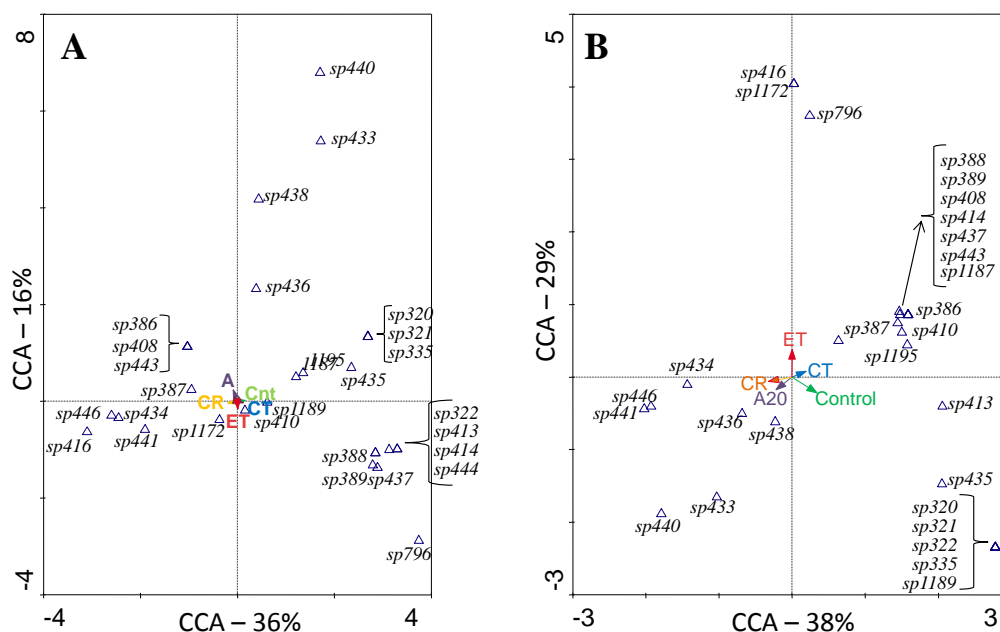
Figure 3-10: CCA biplots showing how application of potential biological control agents of cacao diseases in Jan 08 and March 08 impacted bacilli OTUs in cacao leaves in A) March 08 (before reapplication) and B) May 08. Bacteria were applied to cacao foliage at $1x10^8$ CFU/cm^2 with 0.2% Silwet. Treatments were untreated control (Cnt), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Microbial OTUs are signified by △ and Sp## signified the species type from length of ARISA fragment. Location of species within the biplots indicates the relative influence of the OTUs in defining the species types.

In terms of bacilli, 28 unique bacterial OTUs (as determined by fragment length) were present in leaves, with 16S-23S intergenic spacer region varying from 320 – 1195 bp. The applied potential BCAs had no impact on the diversity of bacteria endophytically colonizing cacao leaves at three months after colonization. For the March data, the first-axis of the biplot explains 36% of the variance in the data, while the second-axis explains 16% of the variance. The variance does not separate bacterial treatments, as all treatment have values of nearly zero on both axes. For the May data, the first-axis explains 38% of the variance on the samples, while the second-axis explains 29% of the data. Communities of leaves treated with CT and A20 share similar OTUs as seen by the overlapping samples with negative values on both axes. For all these differences, none were statistically significant at α=0.05.

Unimodal ordination analysis by CCA infers that treatment of leaves with *B. pumilus* ET impacted the total bacterial community in cacao leaves in March (Fig 3-11).
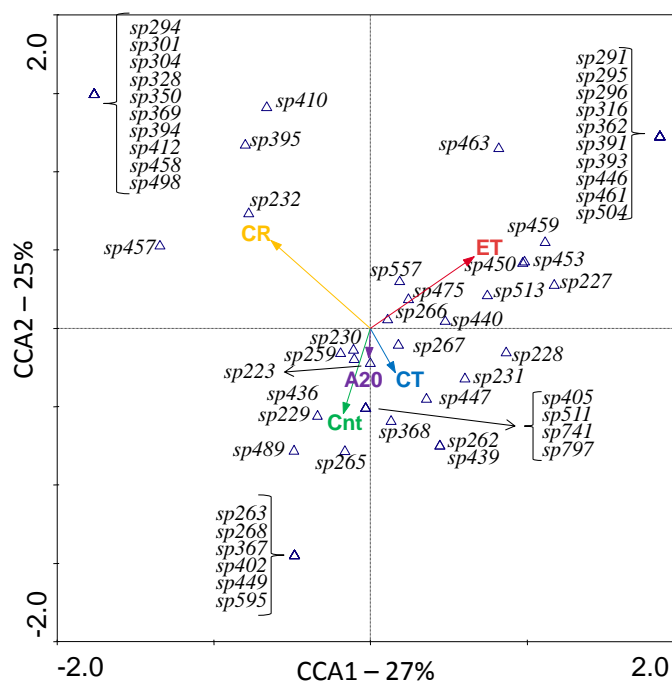
Figure 3-11: CCA biplots showing how application of potential biological control agents of cacao diseases in Jan 08 impacted microbial OTUs in cacao leaves in March 08. Bacteria were applied to cacao foliage at $1 \times 10^8 CFU/cm^2$ with 0.2% Silwet. Treatments were untreated control (Cnt), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Microbial OTUs are signified by △ and Sp## signified the species type from length of ARISA fragment. Location of species within the biplot indicates the relative influence of the OTUs in defining the species types.

In terms of total bacteria, 58 unique bacterial OTUs (as determined by fragment length) were present in all treated leaves, with 16S-23S intergenic spacer region varying from 223 – 797 bp. The first-axis of the biplot explains 27% of the variance in the data, while the second-axis explains 25% of the variance. Overall, there is no statistically significant difference (p=0.1724) in the microbial communities as determined by the Monte Carlo test. Communities of leaves treated with CT and A20 share similar OTUs as seen by the overlapping samples with negative values on the second-axis, and close location on the first axis. Additionally, there is no significant variance in the OTUs between control leaves and leaves treated with A20, indicating that this BCA had little impact on microbial communities. Communities in leaves treated with CR were varied from leaves treated with CT and varied on the second axis from control leaves.

Dry Season 2008: May 2008-December 2008

As in the previous dry season, there were low levels of disease and there were no
statistical separations between bacterial treatments, as seen in Table 3-3. Additionally, cacao
clone had no significant affect on disease severity.

Table 3-3: Average disease severity ratings of bacterial treatments by cacao clone for the dry season of
2008 (May – Dec). Disease was rated in Sept and Nov on a 0 to 6 severity scale (Table 3-2). Bacterial
treatment means followed by the same lower case letter within columns and cacao variety followed by the
same capital letter indicate treatments that are not significant different according to Tukey's HSD (α=0.05).

| | Average disease severity by bacterial treatments | | | | | | | | | |
| | A20 | | CT | | Control | | CR | | ET | |
Clone	**Sep**	**Nov**	**Sep**	**Nov**	**Sep**	**Nov**	**Sep**	**Nov**	**Sep**	**Nov**
A2126 [A]	0 a	0.5 a	0 a	0.3 a	0	1.8 a	0 a	1.0 a	0 a	1.5 a
A2634 [A]	0 a	0 a	0 a	0.8 a	0	0.3 a	0 a	0 a	0 a	1.0 a
CCN-51[A]	0 a	0.3 a	0 a	0.5 a	0.5 a	0.8 a	0 a	0.5 a	0.3 a	0.8 a
EET-19 [A]	0 a	1.5 a	0 a	1.5 a	0	2.3 a	0 a	1.0 a	0 a	1.0 a
IMC67 [A]	0 a	0.3 a	0 a	0.0 a	0	1.0 a	0 a	0.3 a	0 a	0.8 a

The only plant and treatment that had relatively high levels of disease were 'EET-19' control
plants. Although it had the highest disease for the season, it was not statistically significant. The
disease incidence by treatment was slightly higher in the 2008 dry season (Fig 3-12) as compared
to the 2007 dry season (Figure 3-5), but overall disease severity remained low.
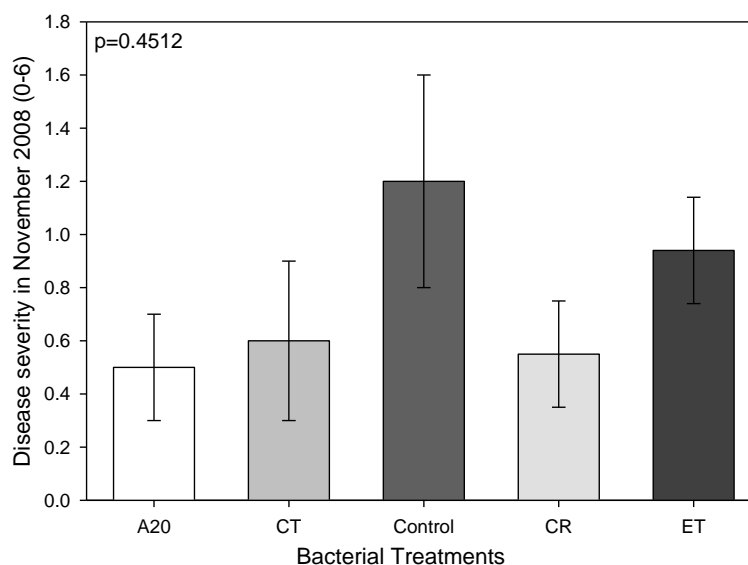
Figure 3-12: Mean witches' broom disease severity throughout in Nov of the 2008 dry season (May 08-Dec 08). Leaves were sprayed with log 8.0 CFU/cm^2 bacilli+ 0.20% Silwet six months prior to this determination. Plants were maintained in a greenhouse for three weeks, and then planted into the field prior to the 2007 rainy season Bacterial treatments were Silwet control (CNTL), and isolates *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Bars extending from the means represent standard errors of that mean.

No treatment or cacao clone reduced disease during the 2008 dry season. This result is similar to the results in the 2007 dry season, where bacterial treatments did not impact disease development, due to low overall disease severity.

Rainy Season 2009: January 2009 – June 2009

Due to a communication issue with our collaborators, disease ratings were not taken monthly through the course of the 2009 rainy season, as they were in 2008. Because of this problem, disease evaluations are missing for Feb and Mar, but were taken in Apr, May, and June. Throughout the course of the 2009 rainy season, clone had a significant effect on disease (Fig. 3-13). 'Nacional' clone A2634 had higher overall disease severity (p<0.0002) than all other clones.
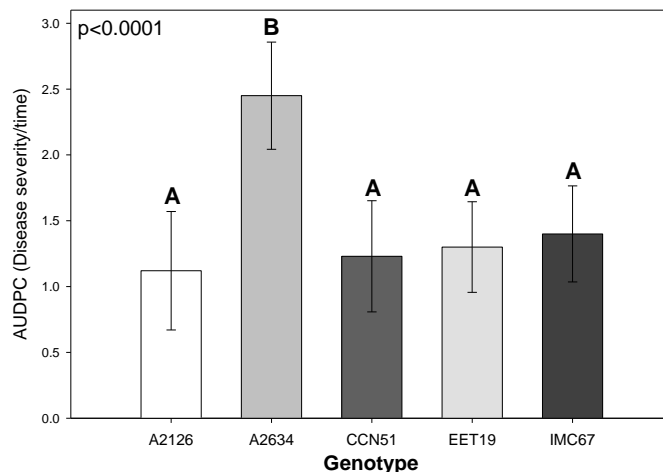
Figure 3-13: Mean area under the disease progress curve for witches' broom severity throughout the course of the 2008 rainy season (Jan 08-May08) by clone. Cacao clones were A2126, A2634, CCN-51, EET-19, and IMC67. AUDPC is based on the disease severity in Table 3-2. Bars extending from the means represent standard errors of that mean. Differing letters above the means indicate statistically significant difference based upon Tukey analysis (α=0.05).

This was different than the previous rainy season, when no clones were significantly different.

Applications of the bacteria in Jan 2009 and Apr 2009 did not result in disease suppression throughout the course of the rainy season, as shown in Figure 3-14. The 2009 rainy season was one of the driest on records, so overall disease severity at the end of the season was less than the previous rainy season.
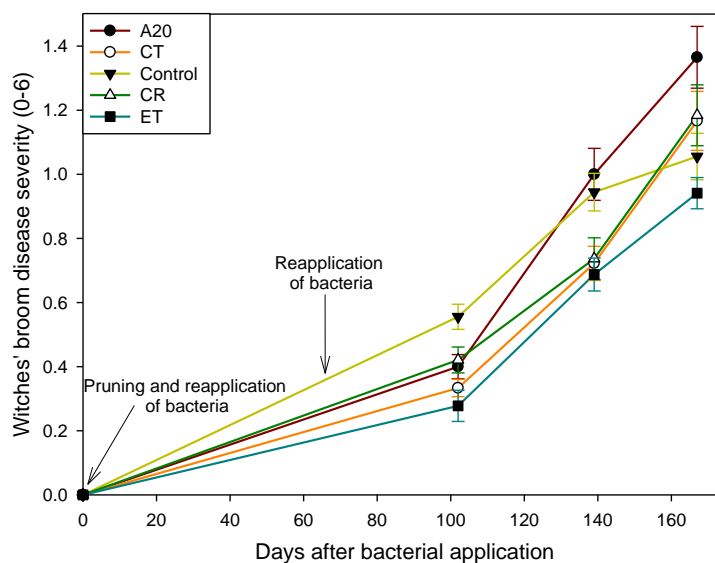
Figure 3-14: Mean area under the disease progress curve for witches' broom severity throughout the course of the 2009 rainy season (Jan - Jun) by bacterial treatment. AUDPC is based on the disease severity in table 3-2. Phytosanitation of the trees and initial bacterial applications occurred at day 0, with reapplication of

bacteria occurring at day 63. Bacteria were applied at log 8.0 CFU/cm^2 with 0.20% Silwet on both dates. Treatments were *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET, and control. Bars extending from the means represent standard errors of that mean.

Application of bacteria to plants did not significantly suppress disease at any of the individually dates. Application of bacteria also did not significantly suppress disease, based upon statistical analysis (p=0.4849) of the AUDPC from Apr through Jun (Fig 3-15). Application of *B. pumilus* ET reduced disease throughout the 2009 rainy season, just not at significant levels.
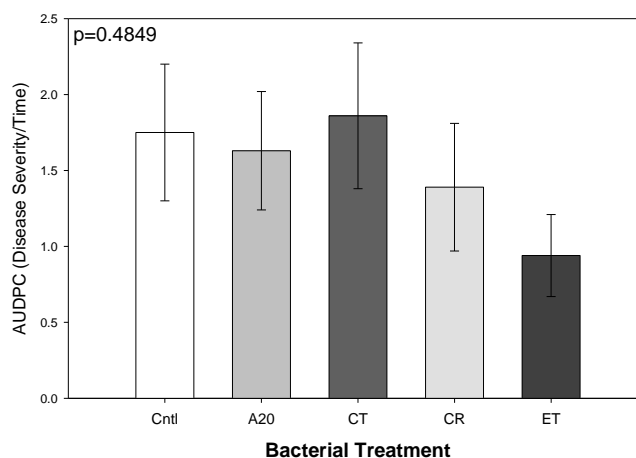
Figure 3-15: Mean witches' broom disease severity throughout the 2009 rainy season by bacterial treatment. Phytosanitation and bacterial application occurred on 7 Jan 09 and reapplication of bacteria occurred on 1 Apr. *Bacillus* spp. were sprayed onto cacao foliage at log 8.0 CFU/cm^2 +0.20% Silwet. Control plants were sprayed with 0.2% Silwet. Treatments were *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET, and control. Bars extending from the means represent standard errors of that mean.

The lack of statistically significant disease suppression during the rainy season may not be a result of the inability of the bacteria to suppress diseases, but rather a combination of reduced disease due to the dry environment and missing ratings in Feb and Mar. As seen in Figure 3-15, even though it is not significant, plants treated with *B. pumilus* ET had lesser levels of disease at individual dates as well as when looking at the AUDPC than control plants. Overall, *B. pumilus* ET reduced witches' broom disease severity when applied 3 times annually and combined with phytosanitary pruning. Disease was suppressed for the entire 2007/2008 dry/rainy season cycle.

Discussion

Application of bacterial isolates following the methodology of Melnick et al. (2008) resulted in endophytic colonization of cacao leaves grown under natural field conditions. Leaves remained colonized throughout the 2007 dry season, with only one application, verifying the ability of the applied bacterial isolates to persist in the natural cacao environment. Colonization was robust, as introduced bacterial colonists endophytically persisted in cacao leaves six months after application, despite the fact that there were no reapplications throughout the dry season. Colonization endured through the rainy season, as bacterial colonists continued in leaves for the 3-months between applications. Colonization was comparable between clones sprayed with the same treatment. This result differed from the dry season were, as noted for IMC-67 plants sprayed with isolates CT and ET, some clone/treatment combinations had low colonization. This is likely due to a combination of the differing environmental conditions as well as the midseason reapplication of the bacterial endophytes. The colonization data indicate that the introduced bacterial endophytes persist in the cacao environment with three applications per year.

Throughout the experiment, disease severity by clone differed greatly between the dry and rainy seasons. During the 2007 rainy season, EET-19 had statistically higher disease than other clones (Table 3-2). During both the 2008 rainy and dry season (Jan 08-Dec 08), clone had no significant effect on witches' broom disease, while during the 2009 rainy season, 'Nacional' clone A2634 had significantly higher disease than other clones. Based upon the variability between seasons, it is not possible to draw an overall conclusion upon resistance of clones or to make recommendations to farmers. It appears that the clones were heavily influenced by the differing environments during the seasons, opposed to the overall disease resistance or susceptibility. The plants in the experiments also flushed (a development of new leaves) and formed jorquettes at different times, which influenced disease development. *M. perniciosa* infection is intimately linked to the production of new tissue, as basidiospores infect actively growing apical meristems (Meinhardt et al., 2008). Throughout the two year experiment, there was no interaction between clones and bacterial treatments, indicating that clone had no influence on the ability of the potential BCAs to reduce disease. Cacao fields in Ecuador, and many other cacao growing regions, tend of have great genetic diversity, including the planting of seeds resulting from open-pollination (Lerceteau et al., 1997). Cacao is genetically diverse, so the ability of *B. pumilus* ET to reduce disease for all clones tested should readily allow for adoption of this potential BCA for large-scale use.

Research indicates the potential of *B. pumilus* ET as a BCA for witches' broom of cacao. Although bacterial treatment did not cause statistically significant disease suppression during the 2007 dry season, no disease developed on plants colonized with *B. cereus* CT and plants colonized with *B. pumilus* ET and *B. subtilis* CR had low disease severities. This initial early reduction could have beneficial implications for cacao nurseries, as it could lead to protection of young plants, which could potentially increase the number of overall plants being produced for future planting by farmers. The first year of data indicates applications of *B. pumilus* ET twice through the rainy season were effective in reducing witches' broom disease severity. This disease management, via spray application and pruning, is reduced compared to the monthly or biweekly spray schedules used in previous research on biological control of cacao diseases (Deberdt et al., 2008; Krauss and Soberanis, 2002; Mejía et al., 2008). Application of *B. pumilus* ET combined with pruning could readily be used by low input cacao farmers, as a moderate increase in input could potentially have large impacts on yield. Another benefit to utilizing *Bacillus* spp. is that large commercial cacao plantations could incorporate this potential BCA to their pre-existing management strategies. *B. pumilus* ET could be used alone or in an alternating spray schedule with commercial fungicides to manage the disease. An additional advantage to the use of *Bacillus* spp. over other microbes, is that endospore-forming bacteria tank-mixed with agrochemicals (Edgecomb and Manker, 2007), which would allow plantations to simply add it to current routine with little increase in overall labor.

Work was also conducted to elucidate the mode of action utilized by *B. pumilus* ET to reduce witches' broom disease. Previous work with *in vitro* plate assays demonstrated that ET did not inhibit the growth of *M. perniciosa* under laboratory conditions (see Chp. 2). This result demonstrates that ET may not be antagonistic to the pathogen under the tested conditions. ARISA was conducted to determine whether application of the bacterial endophytes impacted the endophytic microbial communities of cacao leaves. Application of *Bacillus* spp. did not impact the community of *Bacillus* spp. associated with the cacao leaves. Work with potatoes demonstrated that introduction of suppressive strains of *Streptomyces* spp. did not impact the diversity of the native nonpathogenic *Streptomyces* communities in the soil, but suppressed populations of *S. scabies,* causal agent of potato scab (Bowers et al., 1996). Application of potential BCAs did not have a significant impact on the overall bacterial communities present in cacao leaves (at either $\alpha=0.05$ or $\alpha=0.10$). ARISA data on bacilli confirm the colonization data of leaves collected through the 2008 rainy season, as populations of endophytic endospore-forming bacteria isolated from untreated control leaves were at similar levels to treated leaves.

Some microbial communities change in response to short-term disturbance, but will revert to preexisting structure after the disturbance dissipates. Populations of leech associated bacteria fluctuated several hours after feeding, but the change was short-term as the abundance of *Aeromonas* sp. decreased after four days .(Kikuchi and Graf, 2007). It is possible that sampling of the leaves at three months after inoculation with BCAs was too removed from the disturbing activity to detect changes to the microbial communities. It should also be noted that nucleic-acid based methodologies have their own flaws. One issue is with estimates of abundance, is that more abundant species will be preferentially amplified during PCR. An additional issue is that diversity measurements are impacted by the molecular probes used, as species that better anneal with the primers will be preferentially amplified (Reysenbach et al., 1992). Copy number of the rRNA gene can also create bias in the PCR amplification, which makes measuring true abundance of species nearly impossible (Farrelly et al., 1995). Also, DNA based techniques do not differentiate between actively growing cells and dead or inactive cells in the environment; therefore an RNA based technology give a more accurate picture of microbes that are actively inhabiting the niches of an environment . Despite these issues, the ARISA analysis provides a better picture of the bacterial community of the cacao leaves as well as further explains the detected endophytic colonization of untreated control leaves. Although ET did not alter the endophytic community of the cacao leaves during the time measured, molecular analyses were not conducted to determine how ET impacted *M. perniciosa* within the plant. It is possible that ET could have reduced the growth of the pathogen within the plant, through either direct or indirect means.

For small-scale farmers, fungicides are not often part of integrated pest management both because of the cost of the chemical and the labor involved with their use. For successful adoption of a BCA in the developing countries where cacao is grown it should be 1) effective at reducing disease 2) should have reasonably few applications throughout the growing season and 3) should be easy to use and store. *B. pumilus* ET fits all these categories. It effectively suppressed witches' broom, with three applications and one pruning. In terms of adoption into a growing situation, *B. pumilus* ET would likely be ideal to use with four applications a year (an additional application in the rainy season) and two phytosanitary prunings, one at the end of each season. Farmers in Ecuador who already follow INIAP's recommendation to prune at the end of each season will have no increase in pruning when implementing this potential BCA. The largest hurdle to adoption of BCAs would be the initial investment of spray equipment. Mist blowers, although effective in cacao plantations, will likely not be adapted by small-scale famers (Pereira

et al., 1996). Backpack sprayers are the best option in terms of cost and ease of use, but are difficult to ensure full coverage of a large tree (Bateman, 2004). The additional benefit to *B. pumilus* ET is endospores production. Formulations of endospore-forming bacteria have long shelf lives and are environmentally resistant to the heat and humidity of the tropics (Driks, 2004). Farmers could simply store the product at room temperature. Also, a cottage industry could be developed for production, so that ET could be supplied to farmers at a reduced cost. Such systems have been successfully developed at the Fundación ProINPA in Bolivia

The endophytic nature of *B. pumilus* ET could also increase the benefits of this BCA, when compared to previously tested microbes, through the activation of plant defense mechanisms. *Trichoderma stromaticum* was commercially formulated and distributed by the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLEC) of Brazil as product named as Trichovab (de Souza et al., 2006; Sanogo et al., 2002). *T. stromaticum* is a mycoparasite initially isolated from a dry broom infected with *M. perniciosa* (Samuels et al., 2000). Although this species is an effective mycoparasite of *M. perniciosa*, it was only capable of limited endophytic colonization of cacao seedlings and did not activate the expression of cacao ESTs related to defense (de Souza et al., 2008). Other work demonstrated that mycoparasitic *Trichoderma* spp. and *Clonostachys rosea* strains could reduce disease in bioassays, but had no impact on witches' broom in the field (Krauss and Soberanis, 2001b). To the contrary, endophytic *Trichoderma* spp. were shown not only to endophytically colonize seedlings, but also to promote growth and delay the onset of the negative physiological impacts of drought, (Bae et al., 2009), as well as activate the expression of cacao ESTs related to defense (Bailey et al., 2006). Further research presented in Chp. 5 will discuss the ability of the tested bacterial endophytes to impact the expression of cacao ESTs related to defense.

Several areas should be investigated in continuing research with *B. pumilus* ET including improved timing of bacterial applications to better suppress plant disease. Further research should focus on gaining a better understanding of the spatial and temporal changes in the cacao microbial communities resulting from application of BCAs. Both the culture-dependent and culture-independent methodologies used in this study would have been more instructive if sampling were done immediately after application and at a biweekly or monthly interval thereafter. These results would better explain fluctuations in the microbial communities due to application and better illustrate the impact applying potential BCAs has on native bacterial communities. Time course sampling of cacao leaves by Herre et al. (2007) for fungal endophytes demonstrated that cacao leaves lack culturable fungal endophytes at the onset of leaf

development, but become colonized as the leaf expands. In older mature cacao leaves, the diversity of fungal endophytes decreases. More intensive sampling can better elucidate the interaction between cacao and its bacterial endophytes, as this previous study showed that cacao endophytes can fluctuate through time. Further research should focus on reapplication at monthly timing intervals and potentially biweekly between March and April when inoculum load is high. After determining an effective application schedule, large-scale studies need to be conducted to determine how the *B. pumilus* ET performs in field situations. Future large scale trials should focus both on disease suppression as well as whether application of *B pumilus* ET has a beneficial impact on cacao yield, as infection of cacao tissue of *M. perniciosa* can result in loss of 50 to 90% of cacao yield (Meinhardt et al., 2008). Although there are still some unanswered questions about utilizing endophytic bacteria to suppress witches' broom disease, this research shows the potential for endophytic *B. pumilus* ET as a biological control agent of witches' broom diseases.

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Chapter 4

Biological Control of Cacao Pod Diseases with Bacterial Endophytes

Introduction

Throughout cacao growing regions, only a small portion of potential pods reach maturity. Most pods are lost early in development to cherelle wilt or later to pests and diseases. In an unmanaged plantation in Costa Rica, only 18% of pods remained healthy at the end of the growing season, due to losses from disease and cherelle wilt (Porras and Gonzalez, 1984). Cherelle wilt is currently thought to result from a natural physiological thinning mechanism of the tree, causing a 19 to 92% loss of potential pods (Pyke, 1933). Naundorf and Vellamil (1949) summarized that excessive rain and temperature extremes exacerbate cherelle wilt and that fruit borne on floral cushions bearing few flowers are less likely to be affected. Pods on the main trunk are less susceptible to wilt than those on branches (Uthaiah and Sulladmath, 1981). Research on the etiology of cherelle wilt indicates that phytohormones may be involved. Wilted pods have lower levels of cytokinins than healthy pods, and no cytokinins are detected in pods after the end of the susceptibility period to cherelle wilt (Uthaiah and Sullabmath, 1980). The process of wilt initiation remains unknown, but once initiated, pod death is a product of xylem occlusion by a mucilage-like substance (Nichols, 1961). Although researchers agree that wilting is impacted by phytohormones, studies suggest that biotic factors, such as weak pathogens, may also be involved (Nichols, 1965).

Additional pods are lost to the diseases black pod, frosty pod, and witches' broom. Black pod is caused by several *Phytophthora* spp. and symptoms begin as dark necrotic lesions on the pods (Guest, 2007). Aggressive species can cover the entire pod and rot beans within a few days. Black pod is the most devastating of all cacao diseases because of its worldwide distribution. Losses to black pod range from 20 to 30% of global cacao yield (Ndoumbe-Nkeng et al., 2004). Frosty pod is caused by the agaric basidiomycete *Moniliophthora roreri* and is the only cacao disease that exclusively infects pods. Visible symptoms of frosty pod are premature ripening, deformed pod tissue, and necrotic lesions which rapidly become covered with mycelium bearing meiospores (2N spores). Infected tissue is quickly liquefied, resulting in loss of the seeds

(Phillips-Mora and Wilkinson, 2007). Frosty pod is a rapidly expanding problem, as the disease has spread from the western portions of South America, to all cacao growing regions in Central America. Despite the extensive geographic distribution, it has not crossed the Andes to the cacao production regions of Brazil.

Witches' broom is caused by the sister pathogen of frosty pod, *Moniliophthora perniciosa*. Although foliar symptoms of the disease are the most conspicuous, the fungus can also infect pods. Infection of young pods and flower cushions can lead to abortion of pod development, resulting in young distorted pods commonly known as 'cherimoya'. Infection of older pods leads to fruit distortion and bean rot (Meinhardt et al., 2008). Current management options for pod diseases are removal of infected pods to reduce inoculum (Krauss and Soberanis, 2001a) and planting of tolerant cultivars. However, there are no truly resistant cacao cultivars for any of these diseases. 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 the aforementioned reasons; there has been increased research on biological control as a sustainable disease management option.

Several researchers have investigated biological control options as alternatives to fungicides to manage pod diseases. Research in Africa has focused on using *Trichoderma* spp. to reduce black pod. *Trichoderma asperellum* strain PR11 reduced incidence of black rot caused by *Phytophthora megakarya* in field trials, but it did not perform as well as the contact copper hydroxide fungicide Kocide (Deberdt et al., 2008). In a study in Peru, applications of mycoparasitic strains of *Clonostachys rosea* or *Trichoderma* spp., as singular species and in combination, reduced incidence of frosty pod (Krauss and Soberanis, 2001b). In other studies, applications of mycoparasitic *C. rosea* reduced sporulation of *M. roreri*, but did not reduce frosty pod incidence (Hidalgo et al., 2003). Additionally, *C. rosea* reduced sporulation of frosty pod lesion, despite not reducing disease incidence. Research has also been conducted on fungal endophytes. Mejía et al. (2008) demonstrated an endophytic isolate of *Colletotrichum gloeosporioides* reduced incidence of black pod rot in the field, but other endophytes tested did not reduce disease. Although there has been great success with fungal species reducing disease, research on bacteria as potential biological control agents (BCAs) of cacao diseases has been limited.

Bacteria have been used to manage several diseases in perennial tree crops. Applications of *Bacillus subtilis* reduced avocado black spot, cause by *Pseudocercospora purpurea*, to levels similar to chemical fungicides (Korsten et al., 1997). *Bacillus* spp. were also shown to reduce

brown rot of peach (Altindag et al., 2006), apple replant disease (Utkhede and Smith, 1992) and mango anthracnose (Senghor et al., 2007). Melnick et al. (2008) found that an endophytic *Bacillus* sp. from tomatoes could colonize cacao foliage and suppress *P. capsici* in detached leaf assays. Endospore-forming bacteria are resistant to environmental stresses and can readily be formulated into a product with a long shelf-life that often can be mixed with agrochemicals. Based upon previous successes with endospore-forming bacterial endophytes, the objective of this research was to investigate the potential for biological control of cacao pod diseases (cherelle wilt, black pod, frosty pod, and witches' broom) using native endophytic *Bacillus* spp. Success in reaching this objective would offer additional disease management strategies to small-holder farmers offering the potential to fit into sustainable integrated management practices.

Materials and Methods

Cacao trees and pods

Experiments were conducted at two sites in Ecuador: the INIAP Estación Experimental Tropical (INIAP-EET) in Pichilingue, Ecuador and the Rio Lindo commercial farm in Pichilingue, Ecuador. The Rio Lindo farm was located approximately 4 km from INIAP-EET. Research at the INIAP-EET was conducted on ~20 year old 'Nacional' trees that were offspring of crosses between cacao 'EET95' x 'Silecia 1'. Six-hundred 'Nacional' flowers at INIAP-EET were hand pollinated over a period of three days in mid-December 2008. Hand pollination was conducted because of a previous history of low pod set in these trees. Upon pollination, a plastic bag was placed over pods to protect them from the environment and pests. Research at the Rio Lindo site was conducted on a 20 ha section of a 150 hectare monoculture farm of 'CCN-51' trees produced from rooted cuttings of genetically similar trees. Trees were estimated to be 6-years old. Flowers were open pollinated and a plastic bag was secured around pods that were estimated to be no more than two-weeks old. Six-hundred pods were bagged as before.

Bacterial isolates and application of potential biological control agents

Four different bacilli were evaluated as potential biocontrol agents of cacao pod diseases: *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET, all isolated from cacao trees. These isolates were previously shown to have elite qualities for biological control agents such as chitinase production and disease suppression (see Chp. 2). The four isolates were grown in 500 ml of sterile tryptic soy broth (TSB) contained in 1L flasks. Flasks were incubated for 9 days at 28°C and 120 rpm on a rotary incubator shaker (Thermolyne BIGBill, Barnstead International, Dubuque, IA). The bacteria were allowed to settle to the bottom of the flask and the broth was descanted. Bacteria were resuspended in sterile distilled water and the bacterial concentrations were adjusted to 1 x 10^8 CFU/ml. Immediately before application, Silwet L-77 a polysilicon surfactant (G.E. Silicones, Tarrytown, NY) was added to bacterial suspensions at 0.20% (v/v). Control treatments consisted of 0.20% Silwet in sterile distilled water. Five minutes before application of the potential BCAs, plastic bags covering the pods were removed. Only symptomless pods were chosen for the experiment. Bacterial suspensions were sprayed onto pods using a hand-held pump aerosol sprayer until droplet run-off. Bacteria were applied in mid-January at both sites. At application time, national pods were four weeks old and 'CCN-51' pods were estimated to be six weeks old. Each of the five treatments was applied to pods on the same tree, so that trees were individual experimental units. For trees with large pod populations, more than one replication of the five treatments was applied to each tree. Trees were chosen based on availability of pods and were randomly distributed amongst field sites (see Fig 4-1 and Fig 4-2). The 'Nacional' experiment had 78 replicates per treatment, while the 'CCN-51' experiment had 82 replicates per treatment.
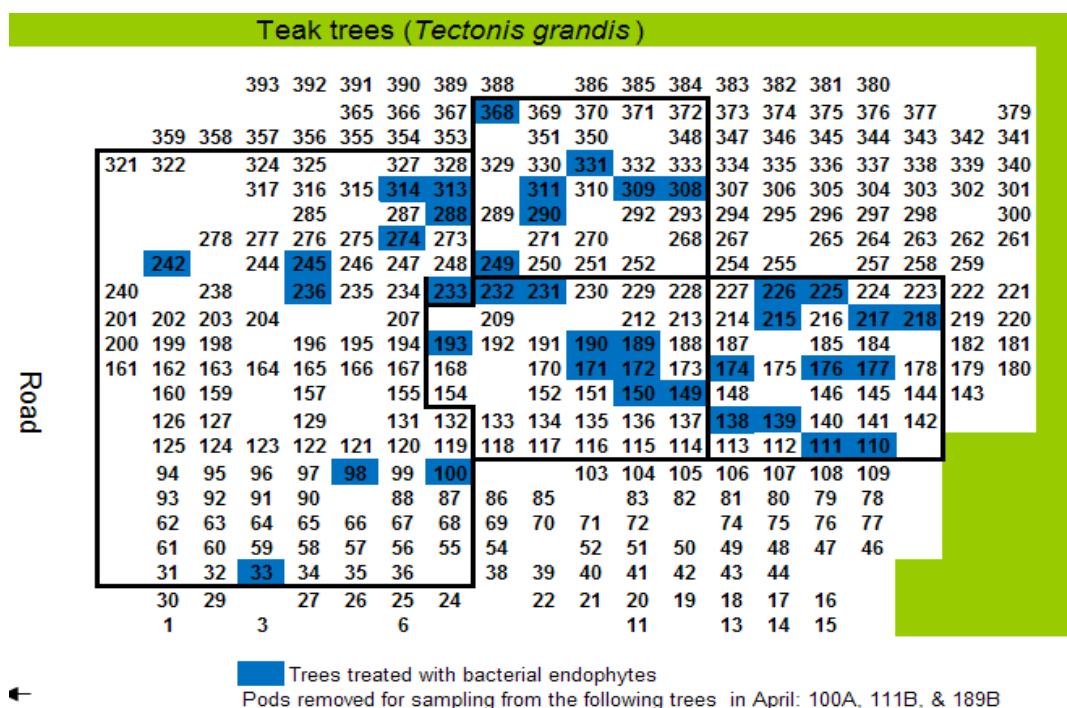
Figure 4-1: Map of 'Nacional' cacao trees treated with bacterial endophytes at INIAP-EET in Pichilingue, Ecuador. Four-week old pods obtained through hand pollination were sprayed to run-off with a 1x 10^8 CFU/ml suspension of bacteria with 0.2% Silwet (v/v). All five treatments were applied to pods on a single tree, allowing for individual trees to be a block in the experiment. Blue squares indicate trees that were treated with the bacterial endophytes and white backed numbers indicate trees that were not treated with bacteria. Trees were in rows with a 3 meter by 3 meter spacing. The black bars indicate the grouping of trees based on similar disease pressure for later statistical analysis.
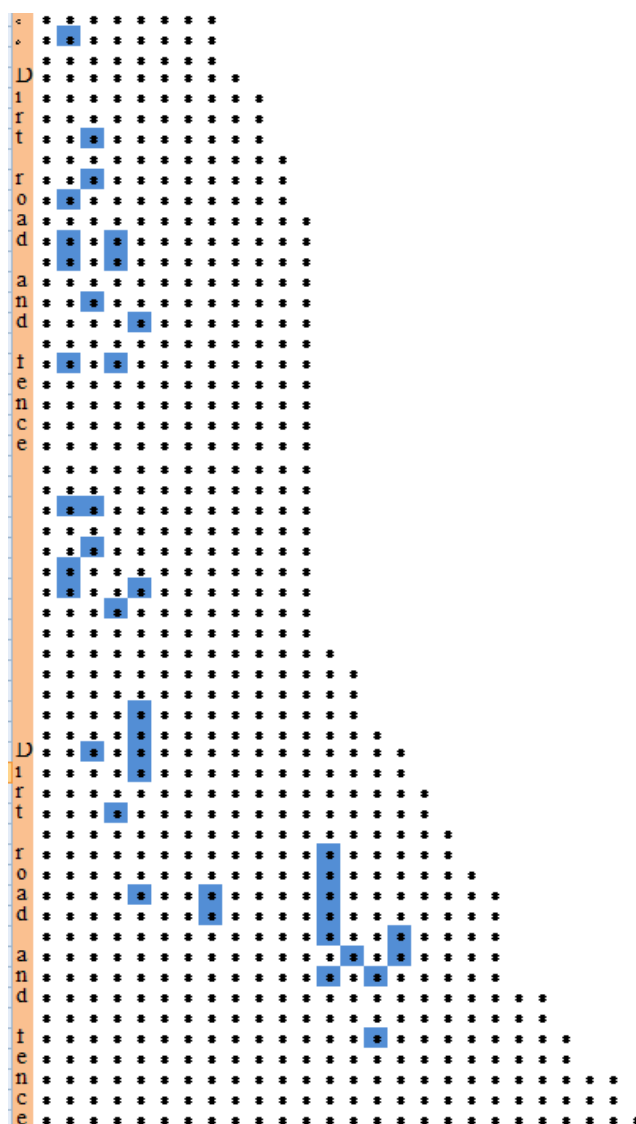
Figure 4-2: Map of 'CCN-51' trees treated with bacterial endophytes at the Rio Lindo commercial farm in Pichilingue, Ecuador. Dots with a blue background indicate trees in which bacteria were applied to the pods. Six-week old open pollinated pods were sprayed to run-off with a 1x 10^8 CFU/ml suspension of bacteria with 0.20% Silwet (v/v). All five treatments were applied to pods on a single tree, allowing for individual trees to be a block in the experiment. Trees were in rows with a 2 meter by 2 meter spacing.

After application of bacteria, pods were maintained uncovered to allow for natural pathogen infection to develop. A typical insecticide regime was conducted at the commercial Rio Lindo farm throughout the duration of the experiment, while no chemicals were applied at the INIAP site. These differences allowed for experimentation at both a typical large scale commercial farm utilizing 'CCN-51' trees and a low input 'Nacional' farm more characteristic of small scale farmers in Ecuador.

Determination of pod colonization by bacterial endophytes

Twenty-four hours after inoculation and after an overnight rain event, four pods per treatment were collected from the trial at the INIAP site to determine the success of endophytic colonization. The length and width of each pod was used to estimate pod surface area. To determine epiphytic colonization, the small pods were placed into 2 ml of sterile distilled water in a sterile 15 ml tube. The tube was heated to 75°C for 15 minutes to select for endospores. Once cooled, the tube was vigorously shaken for 30 sec and 50 μl of supernatant was plated in duplicate onto tryptic soy agar (TSA) plates. To determine whether endophytic colonization occurred, pods were removed from the tubes and surface sterilized in 20% commercial bleach for 5 minutes followed by two rinses in sterile distilled water. Pods were aseptically cut into sections which were placed onto TSA and incubated in the dark for 24-48 hours, after which, plates were observed for bacterial growth.

Pods were also collected at both sites at 3 months after bacterization and tested for levels of endophytic colonization, to determine the longevity of the bacteria in the endosphere. Three pods were harvested per treatment at each site and washed with soap and water. Once dried, the outer surface of the pod was aseptically removed to expose internal tissue. Sections (1 cm x 1cm) were removed from pods (mucilage and seeds were avoided), heated to 75°C for 15 minutes to select for endospores, and then placed onto TSA. The level of epiphytic colonization was not determined, as it was difficult to sample nearly mature pods. Plates were incubated on the dark at 24°Cfor 3 days and evaluated for the presence of bacterial growth from pod sections.

Evaluation of cherelle wilt and pod diseases

Pods were rated monthly for presence/absence of cherelle wilt, black pod, frosty pod, and witches' broom. 'CCN-51' pods matured and were harvested at 4 months after bacterization, terminating the experiments, while 'Nacional' pods reached maturity at 5 months. These data were used to calculate disease incidence over time. Area under the disease progress curve was calculated as disease incidence over time (Shanner and Finney, 1977). Data were analyzed at each sampling data as well as utilizing AUDPC using PROC GLM followed by Tukey's and Dunnett's analyses (α=0.05) for disease incidence using SAS 9.1 (SAS Institute Inc., Raleigh, NC).

Results

Colonization of cacao pods with bacterial endophytes

Pods treated with endophytic bacteria developed normally with no apparent negative impact of treatments on pod development. Application of bacterial endophytes with Silwet resulted in colonization in both the epiphytic (Fig. 4-3) and endophytic environments (Table 4-1).
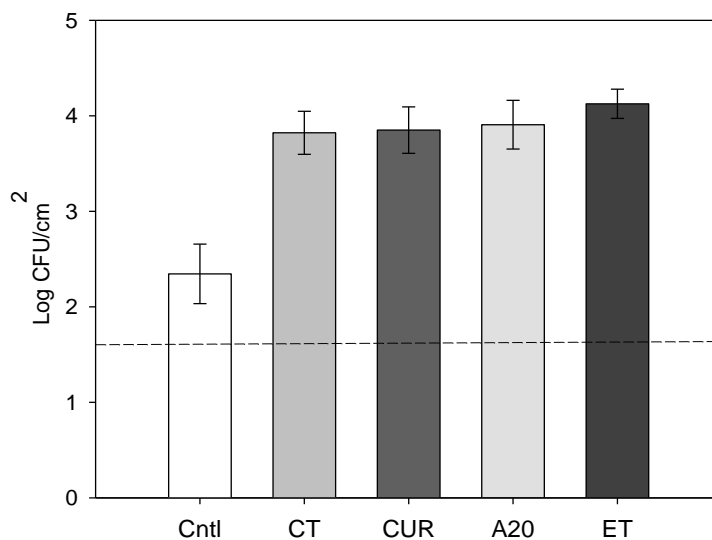
Figure 4-3: Mean initial epiphytic colonization of immature 'Nacional' cacao pods 24 hrs after treatment when sprayed with either 0.20% Silwet control or 0.20% Silwet + log 8.0 bacteria (for the 4 bacterial treatments). Bacterial treatments consisted of *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Control pods had low levels of naturally occurring endospore-forming epiphytes. The dotted line indicates the minimum detectable level for the bacteria (log 1.8 CFU/cm^2). Bars extending from means indicated the standard error of that mean.

One hundred percent of the pods treated with bacteria were endophytically colonized with endospore-forming bacteria (Table 4-1).

Table 4-1: Mean percentage of sampled pods with endophytic endospore-forming bacterial colonists. Treatments were Control, *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. % Jan indicates the percentage of pods with endophytic colonization 24 hours after inoculation with bacteria, while % Apr indicates the percentage of pods with any heat stable endophytic colonists tested 3 months after inoculation.

Treatment	% Jan	% Apr
Control	0	33.33
CT	100	100
CR	100	66.67
A20	100	66.67
ET	100	33.33

Endospore-forming bacteria were not detected in the untreated pods 24 hours after application, but were present 3 months after application. For sampling at three months after inoculation, 6 one cm^2 pod pieces were excised from each test pod and placed into 2 plates (with three sections per plate). Bacteria did not grow out of every pod piece on the media. Epiphytic colonization was not tested at 3 months after inoculation with bacteria due to the difficulty in sampling large mature pods.

Effect of biological control agents on cherelle wilt and pods diseases

Pods on 'Nacional' trees at the INIAP plot were not infected with black pod rot, while none of the pods on the 'CCN-51' trees were infected by witches' broom. For 'Nacional' pods, *B. pumilus* ET significantly increased the number of healthy pods in March when compared to control treatments (p=0.0262) (Fig 4-4A) using Dunnett's analysis, but not in later months. Although not at statistically significant levels, there was a trend throughout the experiments that *B. pumilus* ET treated pods had the highest incidence of healthy pods.
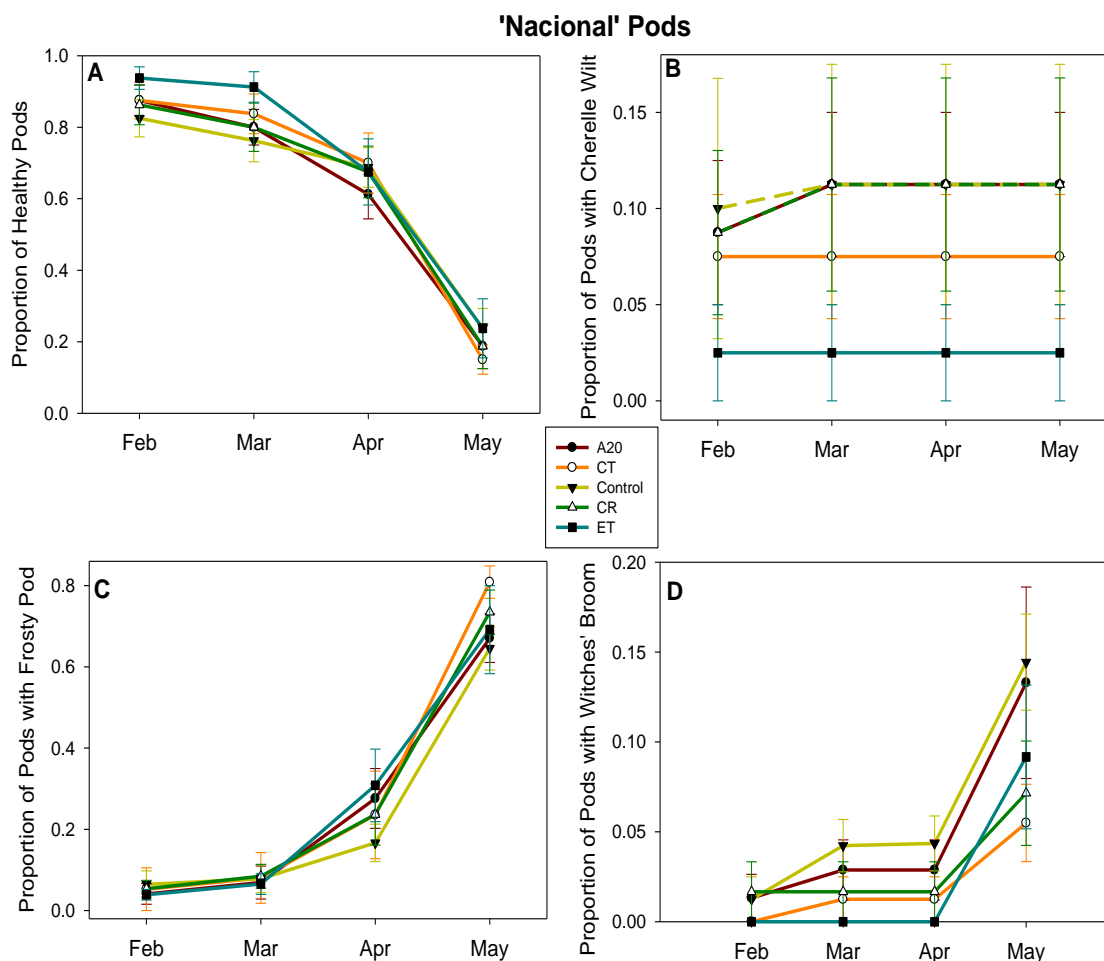
Figure 4-4: Mean proportion of A) healthy pods, B) cherelle wilt, C) frosty pod, and D) witches' broom out of 78 'Nacional' pods treated with each bacterial treatment at INIAP-EET. Control pods were sprayed with 0.20% Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU/cm^2 solution of bacterial isolate + 0.20% Silwet L-77. Treatments were control, *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Pods were sprayed in January and were evaluated monthly for the presence of cherelle wilt, frosty pod, and witches' broom. Proportions were calculated within each block over the time course of the experiment. Bars extending from means indicated the standard error of that mean.

No isolate reduced cherelle wilt one month after application, but *B. pumilus* ET reduced cherelle wilt in March (p=0.044) as determined through Dunnett's analysis (Fig 4-4B). Cherelle wilt did not develop further after the March rating, as all pods were physiologically resistant to further wilt. No bacterial isolate reduced frosty pod (Fig 4-4C) or witches' broom (Fig 4-4D)

In terms of disease development throughout the four months of the experiment, none of the treatments significantly reduced disease or increased the proportion of healthy pods

throughout the season, as determined by AUDPCs of the individual diseases or the overall numbers of healthy pods through the season (Fig 4-5).
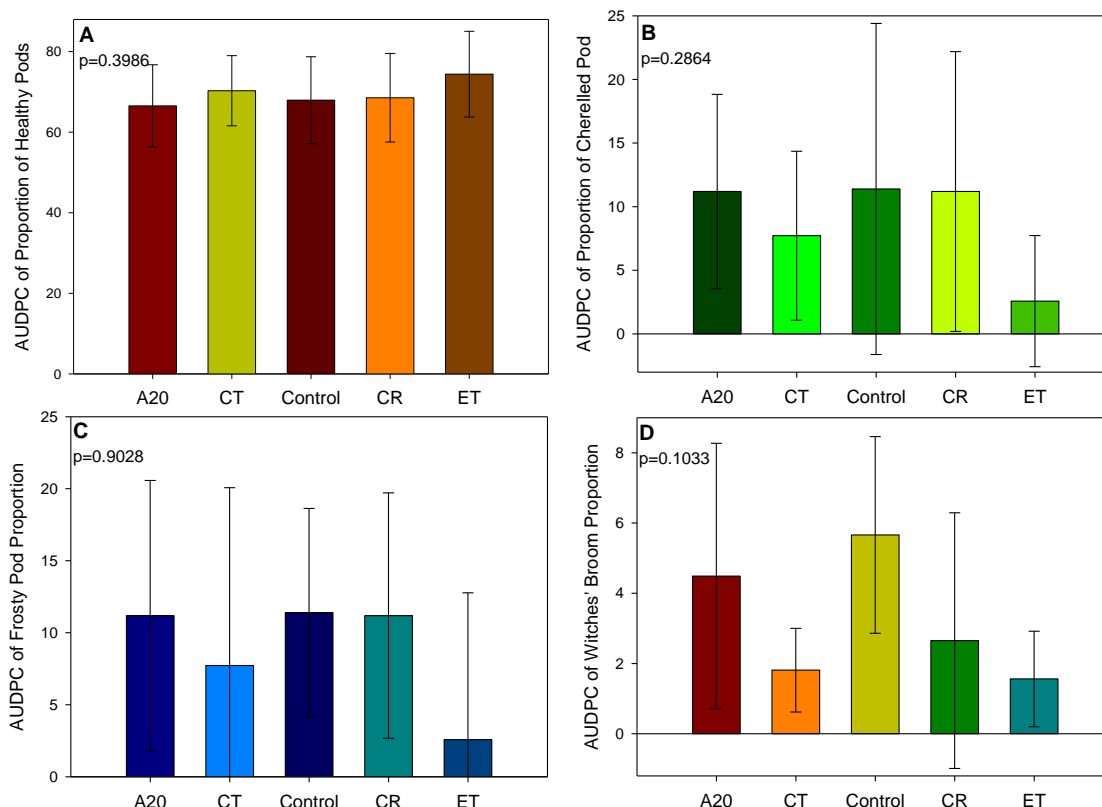
Figure 4-5: Mean AUDPC of incidences of A) healthy pods, B) cherelle wilt, C) frosty pod, and D) witches' broom out of 78 'Nacional' pods per treatment blocked by location at the INIAP-EET. Control pods were sprayed with 0.20% Silwet L-77, while the remaining treatments were sprayed with log 8.0 $CFU.cm^2$ solution of bacterial isolate + 0.20% Silwet L-77. Treatments were control, *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Pods were sprayed in January and evaluated monthly in February, March, April, and May for the presence of cherelle wilt, frosty pod, and witches' broom. Bars extending from means indicated the standard error of that mean.

Cherelle wilt and witches' broom both had low overall incidence in the trial. Application of *B. pumilus* ET resulted in a 32% reduction in witches' broom when compared to control, but not at a statistically significant level. Even with an 83% reduction in cherelle wilt by application of *B. pumilus* ET, the high levels of variability in the control treatment precluded a significant effect.

For 'CCN-51' pods, *B. pumilus* ET significantly decreased cherelle wilt throughout the study when compared to both control pods and pods treated with *L. sphaericus* A20 (Fig 4-6B). Although *B. pumilus* ET reduced cherelle wilt, there was no statistically significant increase in the

number of healthy pods throughout the season (Fig 4-6A). Application of the bacteria did not significantly decrease incidence of black pod rot (Fig 4-6C) or frosty pod (Fig 4-6D), but there was low overall incidences of these diseases.
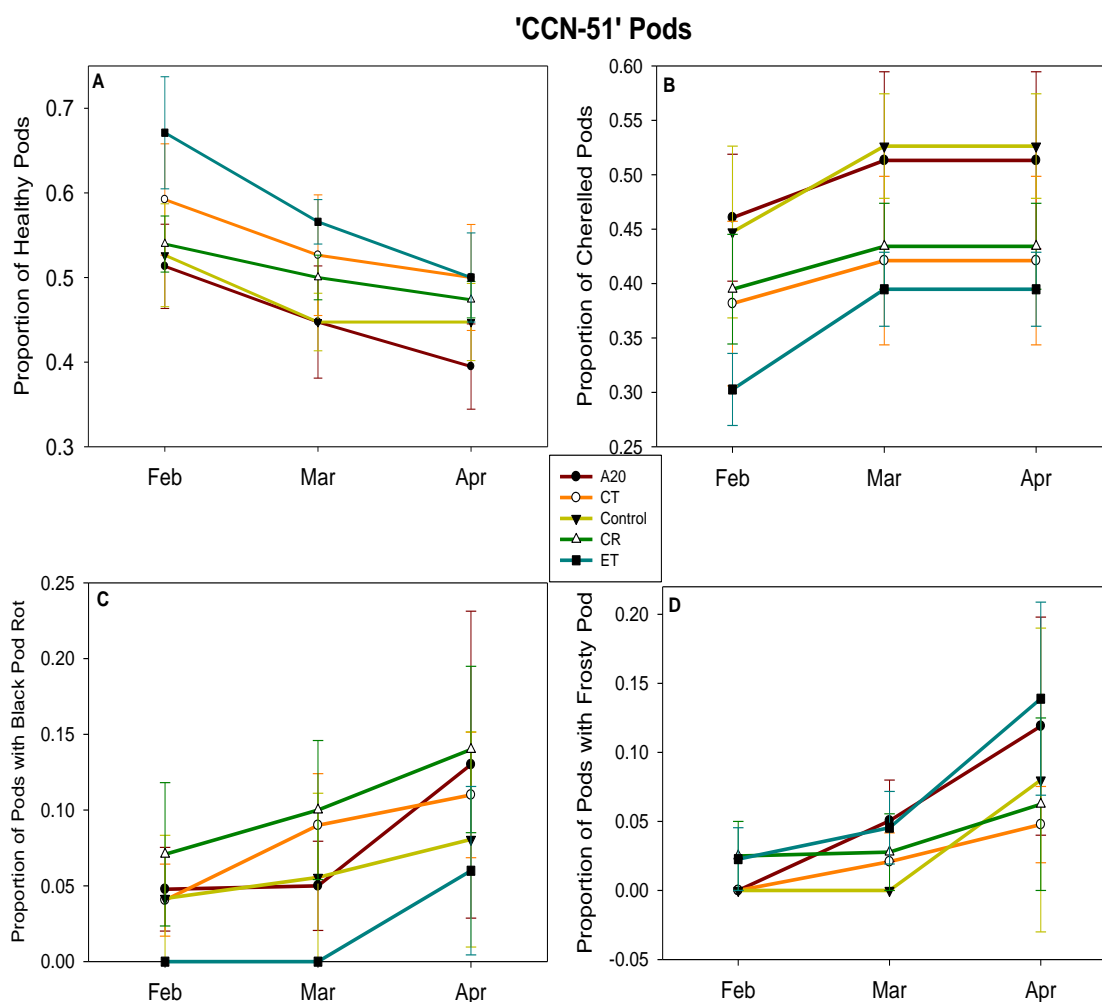
Figure 4-6: Mean proportions of 82 'CCN-51' treated pods per treatment with A) healthy pods, B) cherelle wilt, C) black pod, and D) frosty pod at the Rio Lindo commercial farm. Control pods were sprayed with 0.20% Silwet L-77, while the remaining treatments were sprayed with log 8.0 $CFU.cm^2$ solution of bacterial isolate + 0.20% Silwet L-77. Treatments were control, *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Pods were sprayed in January and evaluated monthly for the presence of cherelle wilt, frosty pod, and witches' broom. Proportions were calculated by block over the time course of the experiment. Bars extending from means indicate the standard error of that mean.

None of the bacterial treatments significantly reduced the AUDPC of black pod (Fig 4-7C) or frosty pod (Fig 4-7D), but application of *B. pumilus* ET decreased the total amount of cherelle

wilt in the field through the season (Fig 4-7B) when compared to both control and *L. sphaericus* A20.
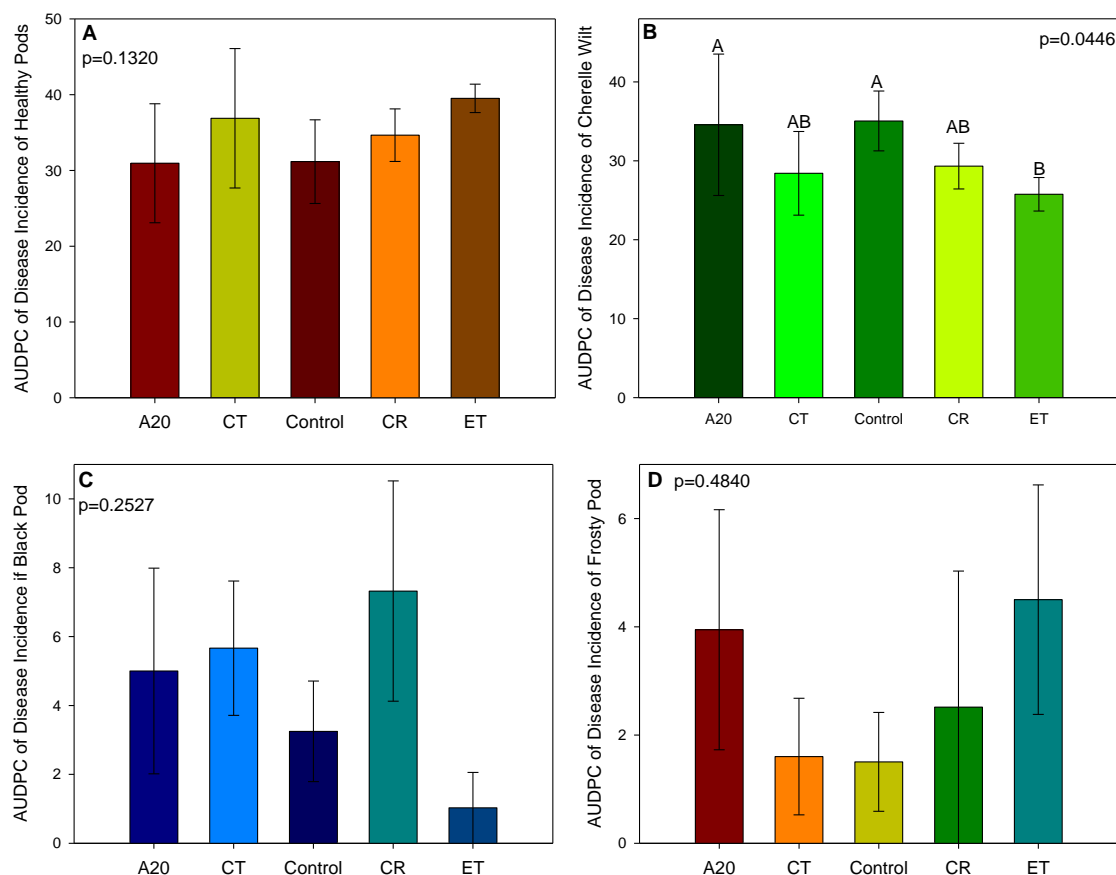
Figure 4-7: Mean AUDPCs for incidence of A) healthy pods, B) cherelle wilt, C) black pod, and D) witches' broom out of 82 'CCN-51' pods per treatment blocked by tree location at the Rio Lindo farm. Control pods were sprayed with 0.20% Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU/cm^2 solutions of *Bacillus* isolate + 0.20% Silwet L-77. Treatments were control, *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET. Pods were sprayed in January and evaluated monthly in February, March, April, and May for the presence of cherelle wilt, frosty pod, and witches' broom. Bars extending from means indicate the standard error of that mean.

In both 'Nacional' and 'CCN-51' plots, bacterial treatments had significant effects on cherelle wilt, but no significant impact on development of any of the other disease development. There was a trend between sites as *B. pumilus* ET always had the least amount of cherelle wilt and the highest proportion of healthy pods.

Discussion

Twenty-four hours after application of bacteria the applied endophytes successfully colonized pods in both the epiphytic and endophytic environments. These results indicate that active growth on the plant surfaces could be a second location of biological control to complement the mechanisms endophytes use to suppress diseases. It also indicates that endophytes are not limited to existing solely in the internal portion of tissue, but occupy other niches. There were no detectable endophytic colonists in control pods immediately after application however; there were low populations of endospore-forming epiphytes colonizing the pod surface. These endospores probably represent both a small amount of drift from nearby treatments and native epiphytes growing on the surface. Despite initially lacking endospore-forming endophytes, there were increases in endospore-forming bacterial endophytes in control pods 3-months after inoculation with bacteria. Applied bacteria colonizing both the epiphytic and endophytic environments of the pod supports our previous research on vegetable endophytes as bacterial colonists of cacao. In that study, foliar applications of *Bacillus* spp. resulted in continuous colonization of both epiphytic and endophytic niches of cacao leaves throughout the 7 week sampling period (Melnick et al., 2008).

When pods were sampled 3-months after treatment, endophytic colonization studies revealed that applied bacterial colonists did not inhabit every pod piece plated. Since pods sampled in January were very small (<4 cm in length) it was possible to plate the entire pod in sections. For larger pods, the six 1 cm^2 sections used to represent the endophytic environment of a rapidly expanding pod could have excluded endospore-forming bacterial endophytes that were present in the pods. The patchy nature of endophytic colonization in older pods was likely due to a combination of factors such as pod expansion and competition with other endophytic microbes present in these same tissues. Fungal endophytes are also known to inhabit cacao pods (Crozier et al., 2006; Evans et al., 2003; Mejía et al., 2008); therefore introduced bacterial endophytes must either co-exist or compete with natural inhabitants.

This is the first report in which application of a BCA suppressed cherelle wilt. Studies of fungal mycoparasites by Krauss and Soberanis (2001b) in Peru and Deberdt et al. (2008) in Africa indicated that biological control agents had no impact on cherelle wilt in field trials. In 'Nacional' trees, *B. pumilus* ET significantly reduced cherelle wilt for two months after application, while cherelle wilt was reduced at both one and two months after application to 'CCN-51' pods. Despite the differences in genotype, environment, and field management

strategies, reduction of cherelle wilt followed the same trend at both sites. Pods treated with *B. pumilus* ET had the least amount of cherelle wilt when compared to pods treated with *L. sphaericus* A20 or control pods.

The impact on cherelle wilt is significant, as reducing the number of pods lost to cherelle wilt could potentially increase the number of healthy pods thus potentially increasing yield. 'CCN-51' pods lost to cherelle wilt were reduced from 52% in the control to 38% in the ET treated pods. Since this is the single largest effect for any of the "diseases", it points to the potential for large yield increases if these pods can be protected to maturity. The suppression of cherelle wilt supports the early hypothesis of Nichols (1965) that there is a biological component to cherelle wilt. Despite several research projects, Koch's postulates have never been conducted to determine the cause of the wilt; therefore it is not possible to fully speculate on how *B. pumilus* ET reduced wilt. Since *Bacillus* spp. are known to produce both hormones (Idris et al., 2007) and antibiotics (Ongena and Jacques, 2008) and stimulate host defenses (van Wees et al., 2008), these results do little to clarify the roles of a disease component versus physiological thinning that have both been indicated as mechanisms cherelle wilt. Further work should be conducted on the etiology of cherelle wilt and the microbes associated with floral cushions and young pods.

One consistent observation on disease management in both sites was that one application of bacterial endophytes was not enough to reduce disease from early pod set to harvest. Cherelle wilt was reduced throughout the first two months after application, but the bacterial protection did not persist to increase the number of healthy pods that survived to harvest. A similar lack of reduction of frosty pod occurred when the fungal endophytes *Colletotrichum gloeosporioides* and *Clonostachys rosea* were applied to cacao trees monthly (Mejía et al., 2008). Other researchers have had more success with using fungal BCAs to manage pod diseases. Krauss and Soberanis (2001b) demonstrated that mixtures of mycoparasites reduced frosty pod when applied at monthly intervals. Deberdt et al. (2008) reduced incidence of black pod through biweekly applications of mycoparasitic *Trichoderma asperellum* PR11. In nearly all previous research, potential BCAs were reapplied every 2 to 4 weeks. The increasing numbers of pods resulting from suppression of cherelle wilt introduced a complicating issue in this experiment, as it also increased the number of susceptible pods on the trees available to become infected at later dates. The increase in susceptible pods clearly requires that additional measures be taken to protect the increased number of healthy pods throughout the course of the experiment.

Data on pod diseases indicate that there were effects on witches' broom (on 'Nacional') and on black pod rot (on 'CCN-51') because application of *B. pumilus* ET inhibited development

of these diseases at early dates, but not at statistically significant levels. 'Nacional' pods treated with *B. pumilus* ET had no witches' broom for the first three months of the experiment, while the disease developed on all other treatments. Also, 'CCN-51' pods treated with ET had no black pod for the first two months. No treatment effected frosty pod in either of the experiments. Reapplication did not occur throughout the experiment and the patchy colonization present in mature pods could indicate that there were not enough bacterial colonists present to reduce disease that developed on the pods surviving wilt. It could also be that the bacteria did not grow at the same rate as the quickly expanding pod to uniformly colonize large pods. Studies by Herre et al. (2007) illustrated that cacao leaves are initially free of culturable fungal endophytes, but eventually 100% of the internal leaf tissue is colonized by fungi during development. Even within a small 16 mm x 16 mm region of the leaf, there is a patchy nature to the colonization by fungi when looking at individual species. It is likely that the pods also become inhabited by endophytes as they mature and expand, which could partially explain the patchy nature of endospore-forming colonists in this experiment. The bacteria must both colonize the rapidly expanding pod and compete with other bacterial and fungal endophytes colonizing the pod tissue. These results point to the need for additionally application of BCAs in further research.

Further research should focus on beginning BCA applications at flower onset and continuing with repeated bacterial applications until pod maturity. Application of bacilli to reduce pod diseases should be conducted with reapplication of BCAs in both biweekly and monthly application strategies during the rainy season to determine whether these alternative spray regimens will increase the efficacy of the BCA. It is possible that additional applications of endophytic bacilli would allow higher levels of the beneficial bacterial in the epiphytic and in the endophytic portions of the pod to result in enhanced disease suppression. An additional area of research should be combining successful *B. pumilus* ET with beneficial mycoparasites or fungal endophytes through co-colonization. Plants consist of complex microbial communities, containing both fungal and bacterial endophytes. Due to the complexity of the natural microbial community, applications of a combination of microbes may be more successful at displacing neutral endophytes or plant pathogens, which could result in overall improved disease suppression. An ideal combination of microbes would likely persist in the environment and have the ability to reduce the multiple diseases that infect cacao trees.

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Chapter 5

Effect of Endophytic Colonization of Cacao Seedlings by *Bacillus* spp. on Cacao Defense Cascades

Introduction

Theobroma cacao L., the chocolate tree, is grown by small-holder farmers throughout the tropics. The main threat to cacao production worldwide is incredible yield lost to diseases. The key diseases for cacao grown in South America are black pod (caused by *Phytophthora* spp.), frosty pod (caused by *Moniliophthora roreri*), and witches' broom (caused by *M. perniciosa*). Throughout the world, approximately 1 million tons of cacao beans are lost to pest and pathogens annually, making disease management an important issue in sustainable cacao production (Hebbar, 2007). Fungicides have been shown to be effective in disease management, but are cost prohibitive for small-holder farmers (Bateman et al., 2005; Opoku et al., 2004). Additionally, there has been increased interest in both integrative pest management options for cacao as well as organic cacao production, leading to research on biological control of cacao diseases (Slingerland and Gonzalez, 2006).

Cacao is host to a diverse range of microbes, several of which have been investigated for their ability to suppress cacao diseases (Mejía et al., 2008; Rubini et al., 2005). Most current research on biological control of cacao diseases has focused on fungal species. Mycoparasitic *Trichoderma stromaticum* has been used to suppress foliar witches' broom disease (Bastos, 1988; Samuels et al., 2000). Endophytic *Trichoderma martiale* strain ALF 247 reduced disease severity of black pod in field trials (Hanada et al., 2009). Researchers in Brazil identified epiphytic actinomycetes and endospore-forming bacteria from cacao pod surfaces and actinomycetes from the cacao rhizosphere (Barreto et al., 2008; Macagnan et al., 2006). Several of these isolates were capable of inhibiting the germination of *Phytophthora palmivora* and *M. perniciosa* spores and fungal growth in a laboratory settings but no isolate suppressed *P. palmivora* in field experiments.

One mechanism of disease reduction reported for biological control agents (BCAs) is to induce host resistance to pathogens, in which the BCA activates the expression of defense related genes to protect the plant from diseases. Plant disease occurs when pathogens defeat host

defenses, or alternatively, are not recognized by the host. In the latter case, it would be more precise to say that successful pathogens do not elicit a timely defense response from the plant (Zehnder et al., 2001). Biochemical plant defenses result from induction of one or more pathways. The jasmonate and ethylene pathways are initiated by wounding and insect attack (van Wees et al., 2000). Systemic acquired resistance (SAR) is a salicylic acid-dependent pathway, induced by fungi (pathogenic and beneficial) and effector proteins (such as *hrp*) of the type III secretion system of pathogenic bacteria (Pieterse and van Loon, 1999; Strobel et al., 1996). Induced systemic resistance (ISR), a salicylic acid-independent pathway, is induced in response to avirulent pathogens and by some nonpathogenic microbes (Pieterse and van Loon, 1999). Induced resistance is often investigated in biological control research. BCAs can activate these pathways as well as genes within these pathways to result in reduce disease development over time (Conn et al., 2008). Although production of defense products may seem like a costly physiological process, this may not always be the case because some agents prime the plant for later protection against pathogens. Priming is the enhanced ability of a plant to activate defense mechanisms upon infection with a pathogen (Kohler et al., 2002). Priming reduces disease while not requiring a large production of defense proteins in the absence of the pathogen (van Hulten et al., 2006).

Cacao BCAs have been shown to impact plant defense products in cacao. Colonization of cacao seedlings with *Streptomyces griseus* isolate Ac26 resulted in accumulation of peroxidases and polyphenol-oxidases in cacao leaves, indicating that the isolate likely induced resistance (Macagnan et al., 2008). Bailey et al. (2006) demonstrated that colonization of cacao seedlings by endophytic *Trichoderma* spp. induced the expression of cacao expressed sequence tags (ESTs) related to defense, but the expression pattern was highly dependent on the isolate of *Trichoderma* colonizing the cacao seedlings. The benefits of induced resistance research combined with previous success at induction of resistance by other cacao BCAs has lead to the objective of determining whether endophytic colonization of cacao seedlings by endophytic *Bacillus* spp. activates the expression of defense related genes in cacao. These endophytes are naturally associated with cacao (see Chp. 2) and have been shown to significantly reduce both witches' broom disease (see Chp. 3) and cherelle wilt (see Chp. 4) in field trials. An additional objective was to determine whether endophytic *Bacillus* spp. utilized priming as modes of action for the repression of cacao diseases.

Materials and Methods

Cacao seedlings source and growth

Pods were removed from an open pollinated Pound7 cacao tree at the CATIE research station in Turrialba, Costa Rica and shipped to the Sustainable Perennial Crops Lab of USDA-ARS in Beltsville MD. Seeds were removed from the pods, surface sterilized in 20% bleach for five minutes, and then rinsed three times in sterile distilled water. Sterilized seeds were plated three to a plate onto 1.5 % water agar. Plates were sealed with parafilm and incubated at room temperature on the lab bench until seed germination.

Bacterial isolates and sources

Four different bacterial endophytes were evaluated as potential biocontrol agents of cacao pod diseases: *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Bacillus pumilus* ET, all isolated from cacao trees in Ecuador (see Chp. 2). These isolates were previously tested for their ability to reduce cacao diseases (see Chp. 3 & 4). Cultures were maintained on tryptic soy agar (TSA) and maintained in long -term storage in tryptic soy broth (TSB) with 20% glycerol under liquid nitrogen.

Phytophthora capsici isolate and sources

Phytophthora capsici isolate 73-73 was obtained by H. Purdy in Ecuador in the early 1990s, shipped to The Pennsylvania State University, and stored in 20% glycerol in liquid nitrogen. Cultures were removed from storage and cultured on unclarified V8 agar made with 20% V8 (Lawrence, 1978).

Preparation of bacteria and inoculation of cacao seedlings with *Bacillus* spp.

One-week old seedlings grown in petri dishes

The bacterial isolates were inoculated into 500 ml of sterile TSB in 1.8 L Fernbach flasks. The flasks were maintained on a rotary incubator shaker (New Brunswick Scientific, Edison, NJ) at 28°C and 150 rpm and grown for nine days to incite endospore production. The bacterial suspension was centrifuged for 8 minutes at 6,200g to pellet the bacteria. The bacterial pellets were resuspended in sterile pH 7.0, 0.1M potassium phosphate buffer. The concentration of the bacterial suspension was determined at OD_{600} using a spectrophotometer (Bausch & Lomb Co., Rochester, NY) then adjusted to 1 x 10^6 CFU/ml. Inoculum concentration was verified by dilution plating onto TSA.

The following treatments where applied to germinated cacao seeds: control, *L. sphaericus* A20, *B. cereus* CT, *B. subtilis* CR, and *B. pumilus* ET. For the control plants, seeds were placed in sterile 0.1M potassium phosphate buffer for five minutes then allowed to dry under a laminar flow hood for five minutes. For bacterial treatment, seeds were placed in the bacterial suspension for five minutes then allowed to dry under a laminar flow hood for five minutes. After drying, seeds were placed onto three pieces of 9.0 cm Whatman paper wetted with sterile distilled water in a petri dish. Plates were sealed with parafilm and incubated on the lab bench at room temperature (24°C). Each treatment consisted of 14 seeds, with two seeds per plate to comprise seven replicates for the five treatments.

Cacao seedlings grown in magenta boxes

Further experiments were conducted on whole seedlings utilizing the two isolates that performed best in the field (*B. cereus* CT and *B. pumilus* ET) to determine how they impacted gene expression at different developmental stages. Seeds were received and inoculated with bacteria as previously described. In preparation for planting, double magenta boxes (20 cm high and 6.5 cm square, Magenta Corp., Chicago, IL) with four 0.5 cm diameter holes in bottom box were filled with 80g of sterile soilless mix (2:2:1; sand:perlite:Promix) (Promix Growing Media, Premier Horticulture, Quakertown, PA) and the holes were taped closed. Treated seeds were

aseptically placed 3 cm deep in the soil and the soilless mix was wetted with 20 ml of sterile distilled water. There were 36 replicate plants for each treatment. Boxes were maintained in a controlled environment chamber (model M-2, EGC Corp., Chagrin Halls, OH) at 25°C, 60%±5% relative humidity, and a 12h light/12h dark photoperiod. Irradiance in the chamber was 50 μmol/m^{2}s PAR. Plants were maintained throughout the course of the experiment, and nine replicates per treatment were harvested at specific time points. Eleven days after planting, the top box and tape were removed. Seedlings were watered every other day and once weekly with commercial Scotts fertilizer (All Purpose, Scotts Miracle-Gro Corp., Maryville, OH) for the rest of the experiment to maintain adequate soil moisture and plant nutrition. Four collection periods occurred throughout the course of the experiment: time 1, time 2, and time 3, and time 3+priming. Each collection point consisted of 9 replicate plants per treatment. Time points and collected materials are listed below in Table 5-1:

Table 5-1**:** Time series for collection of tissue samples to determine colonization, challenge with *P. capsici*, and preserve tissue for quantitative real time PCR (QPCR) analysis.

Time	Days after inoculation with bacteria	Tissue sampled for colonization	Tissue challenged with *P. capsici*	Tissue collected for QPCR
Time 1	11 days	Entire seedling including roots	-	Entire seedling
Time 2	18 days	Immature green leaf and stem	Immature green leaf	Immature leaf and stem
Time 3	32 days	Mature green leaf and stem	-	Mature leaf and stem
Time 3 + priming	32 days	-	Entire plant	Mature leaf challenged with *P. capsici*

Time 1 consisted of 11-day old entire seedlings from unopened magenta boxes. Time 2 consisted 18-day old seedlings at the onset of leaf flush, one week after the top magenta box was removed. Time 3 consisted of seedlings sampled 32 days after planting, at the onset of primary leaf maturation. At time 3+priming, an additional 9 replicates of 32-day old seedlings were challenged with *P. capsici* zoospores and incubated for one day.

Determination of the presence and levels of bacterial colonization in treated cacao seedlings

One-week old seedlings grown in petri dishes

After one week of growth, two 1 cm stem sections were collected from Petri dish grown seedlings. Sections were surface sterilized in 20% commercial bleach for three minutes followed by three rinses in sterile Milli-Q water. The sections were plated onto TSA and placed into a 28°C incubator for 48 hours. Plates were observed for growth of bacteria from the cut ends of stems. They were also compared to the phenotypic characteristics of the applied bacterium.

Cacao seedlings grown in magenta boxes

Cacao seedlings grown in magenta boxes were sampled over four time period (Table 5-1) to determine colonization, challenge tissue with *P. capsici*, and preserve samples for QPCR analysis. At time 1 (11 days after inoculation with bacteria, and before opening the magenta boxes) two 1 cm stem sections were removed from the seedlings above the cotyledon and two below the cotyledon near the soil line. Sections were surface sterilized as previously stated and then two pieces (one from below and one from above the cotyledon) were placed onto TSA and cornmeal dextrose agar (CMDA) respectively. CMDA was used to determine whether fungal species were present in cacao tissue, while TSA was used to determine whether bacterial species were present.

At time point two, samples were collected after boxes were opened (18 days after planting); therefore were not from a sterile environment. Stem section sampling consisted of removal of two 1 cm sections from immediately above the soil level and two 1 cm sections from near the top of the stem. Sections were surface sterilized and plated as previously stated and Colonization of immature green leaves was assessed by removing five disks from a leaf in a "W" pattern using a 15.5 mm diameter cork borer. All disks were surface sterilized following the same protocol used for stems. One complete disk was placed onto CMDA and another was placed onto TSA. The remaining three surface sterilized disks were placed into a stomacher filter bag (Filtra-Bag, Labplas Inc., Québec, Canada) with 3 ml of sterile pH 7.0, 0.1 M potassium phosphate buffer. Disks were triturated and 50 μl were plated in duplicate onto TSA plates. All plates were incubated at 28°C and observed for bacteria growth and phenotype. For time point 3

(32 days after planting), sampling of stems occurred as at time 2 and sampling for mature green leaves occurred exactly as indicated for immature leaves at time 2. Samples were not taken from time 3+priming for colonization as plants were an extra nine replicates of time 3, therefore colonization was inferred by sampling of time 3.

Challenge of leaves with *P. capsici* to determine whether bacterial colonization suppresses disease

Challenge of immature leaves from cacao seedlings grown in magenta boxes at time 2 (18-days after inoculation)

Immature green leaves were used for the detached leaf challenge. One immature leaf was detached from the plant and the lid of a 100 mm Petri dish was used to cut disks so that the midrib was the center of the leaf disk. Disks were placed abaxial side up on a moist 10 cm Whatman 9 cm filter paper. Zoospores of *P. capsici* 73-73 were obtained following the method of Lawrence (1978) from five day old cultures. Zoospore concentration was determined using a hemacytometer and adjusted to approximately 5×10^3 zoospores/ml. Six 10 μl drops of the zoospore suspension were placed on the leaf, three on each side of the midrib. Petri dishes were sealed with parafilm incubated at room temperature (25°C) under fluorescent lights (alternating 12 hour light/dark cycle). Lesions were assessed every eight to twelve hours by measuring lesion diameters and the percentage of necrosis under the drop for 52 hours.

Challenge of entire cacao seedlings grown in magenta boxes at time three + priming (32 days after inoculation)

Zoospores, obtained as previously stated, were sprayed onto 32-day old seedlings using a handheld pump aerosol sprayer (Nalge Nunc International Corp., Rochester, NY) so that all leaf tissue was wetted. Plants were covered with a plastic bag and maintained in the hood for 24 hours (Fig 5-1).

Figure 5-1: Cacao seedlings inoculated with *Bacillus* sp. through seed treatment. Treatments were control, *Bacillus cereus* CT, and *Bacillus pumilus* ET. Plants were challenged with *Phytophthora capsici* zoospores ($5x10^3$ CFU/ml) through spray application onto leaves at 32-days after inoculation with bacteria. Seedlings were bagged and incubated for 24-hours to allow time for symptoms of the pathogen to develop.

Disease severity was assessed 24 hours after inoculation with *P. capsici*. To assess disease severity, a 1.8 cm x 1.8 cm glass cover slip was place on three areas of the leaf in a "V" pattern. The number of lesions under the cover slip was counted at each spot and the average number of lesion per/cm^2 was determined from this rating. The length and width of the leaf was measured and then the number of lesions were counted on half of the leaf and used to estimate the number of lesions per leaf area.

Preservation of cacao tissues for determination of expression of cacao ESTs

For seedlings grown in Petri dishes, entire seedlings were collected at one week after inoculation with bacterial endophytes. Samples consisted of the two seedlings per treatment contained in one Petri dish for a total of 7 replicates per treatment. Upon harvesting the seedlings, the cotyledons were removed. Sampling of seedlings grown in magenta boxes occurred at time 1, 2, 3, and time 3 + pathogen (see Table 5-1). Each time point consisted of 9 replicate plants for each of the three treatments. Time one samples consisted of entire seedlings.

Seedlings were removed from the boxes, the cotyledons were removed, and soil was rinsed from the roots. Two samples were taken at time 2: the stem and one immature green leaf from the initial flush. To reduce the variability, leaves and cotyledons were removed from the seedlings before collection. Time 3 consisted of seedlings sampled 32-days after planting. Two samples were taken from each plant: a mature green leaf and the stem with cotyledons and leaves removed. Priming samples consisted of mature leaves only.

For all times and tissue types, samples were flash frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted and treated with DNAseI following the methodology of Bailey et al. (2005). RNA concentration was determine by measuring the OD_{260} and OD_{280} using a spectrophotometer. cDNA synthesis and QPCR was conducted as described in Bae et al. (2005). Two μg of RNA was used to synthesize first-strand cDNA using a SuperScript[TM] III First-Strand Synthesis System for RT-PCR kit (Invitrogen Corporation, Carlsbad, CA). Synthesized cDNA was diluted 10-fold for use in Q-PCR. QPCR analysis was conducted with the primers listed in Table 5-2 using a Mx3005P QPCR system (Stratagene, La Jolle, CA) and Brilliant[®] SYBR[®] Green QPCR Master Mix (Stratagene, La Jolla CA). Reactions consisted to 12.5 μl 2x Brilliant[®] SYBR[®] Green QPCR Master Mix, 5 μl of 10-fold diluted cDNA, 5 μl combined primer set (2.5 μl forward and 2.5 μl reverse), 0.375 μl diluted ROX reference dye (final concentration = 300nm), and 2.125 μl DEPC treated water. Primers used for QPCR are listed in Table 5-2. The following thermal profile was used: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec; and finally 95°C for 1 min, 55°C for 3 min, and 95°C for 30 s.

Table 5-2**:** Primers of cacao ESTs related to defense used in QPCR analysis.

Putative gene function (EST designation)	Acession No.	Sequence (5' to 3')	Anticipated size (bp)	EST Citation
Actin (*TcACT*)	CF973918	F: CAGACTTTGAGTTCACTTGACACAG / R: AGTGTCTGGATTGGAGGATCTATCT	200	Verica et al. 2004
Lipoxygenase (*TcLOX*)	CF973956	F: ACATACTCAGTCACCCATTGTTTG / R: TTGGCCAAGTATTATCTAGAGGTTG	218	Verica et al. 2004
MAP kinase 3 (*TcMAPK3*)	CF974368	F: TGAATAGAAAGCCTCTATTTCCTGG / R: GAGATCAATAGCCAATGGATGAAC	204	Verica et al. 2004
MAP kinase kinase 4 (*TcMKK4*)	CF974565	F: CAAGCTCCATATATCTCCAAGGTAA / R: CTTGCCTTACTTACATGCCCATA	239	Verica et al. 2004
Protein-related protein 1 (TcPR1)		F: CACTCGGCCTCCACGTTG / R: CCAGAGGGCGATTTCAAGC		M.J. Guiltinan, Penn State
Protein-related protein 4 (TcPR4)		F: ACGAATAATGGCTGGGTCATACC / R: GCCATTCCAAGGGCTTATCA		M.J. Guiltinan, Penn State
Protein-related protein 6 (TcPR6)		F: TTTCTCGGAGGTCGGTTGTC / R: CACAAATGTAAGCGCAGAGCC		M.J. Guiltinan, Penn State
Protein-related protein 10 (TcPR10)		F: TGCATTCGTAGAAACCTTGTGC / R: GTGAATGTAGGGACACATTTTGCAGA		M.J. Guiltinan, Penn State
Osmotin-like (TcOsmotin-like)	AY766059	F: ATTAGATGCACGGCAGATATCATAG / R: CAGAACACAACCCTGTAGTTAGTCC	242	A.R. Ramos, E.S. Gander, L.H. Marcellino; EMBRAPA
Cytochrome P450 (C.P450-ICS)	EH057657	F: CTCAGGCGACACCCTCCA / R: GTCCACCACAACCCCATC		G.A.L. Lealm Uni. de Sao Paulo
ACC Synthase (TcACC)	CF974124	F: CTCATATCAGCCCAACAGTAGAAAC / R: TTACTCTTTCAATGAGGAGGTTCTG	243	Verica et al. 2004
Protein phosphatase 2C (TcPP2C)	CF972808	F: ACAGCTTCTTGATTGCTTAGGTG / R: TACAGGTTTCCTAGATCTATCGGTG	215	Verica et al. 2004
Nonexpresspr of PR gene 1 (TcNPR1)	CF974051	F: CTTGATTTATCAAACCATCCTCCTC / R: AAGAAATTTCCTGAAGAAGAGACTCC	219	Verica et al. 2004
Chitinase 1 (TcChi1)		F: CAAAGCCTTCTTGCCACGAT / R: TGCCGCCTGGTCGCTA		M.J. Guiltinan, Penn State
Chitinase 7 (TcChi7)		F:TCCCACTACCTGTATTACCTTGACC / R: TTAGCTCGTCATGTGGCCC		M.J. Guiltinan, Penn State

At the end of the amplification reaction, a dissociation curve was run to ensure that only the gene of interest was amplified during the PCR reaction. The constituently expressed cacao ACTIN gene (CF973918) was used as an expression control. The data were analyzed as described by Pfaffl (2001).

Results

Young cacao seedlings produced in petri dishes

All stems treated with bacteria were positive for bacterial endophytes and had the same phenotypic identity of the applied organism, while stems from control plants lacked endophytic colonists. Colonization of seedlings with bacteria, regardless of isolate, appears to cause a down regulation (as compared to the non-colonized control) of the following ESTs: osmotin-like, ACC synthase, and chitinase 7 (Chi7) (Fig. 5-2). The expression of ACC synthase is highly variable in control seedlings. All isolates, except A20, repressed the expression of protein phosphatase 2C.
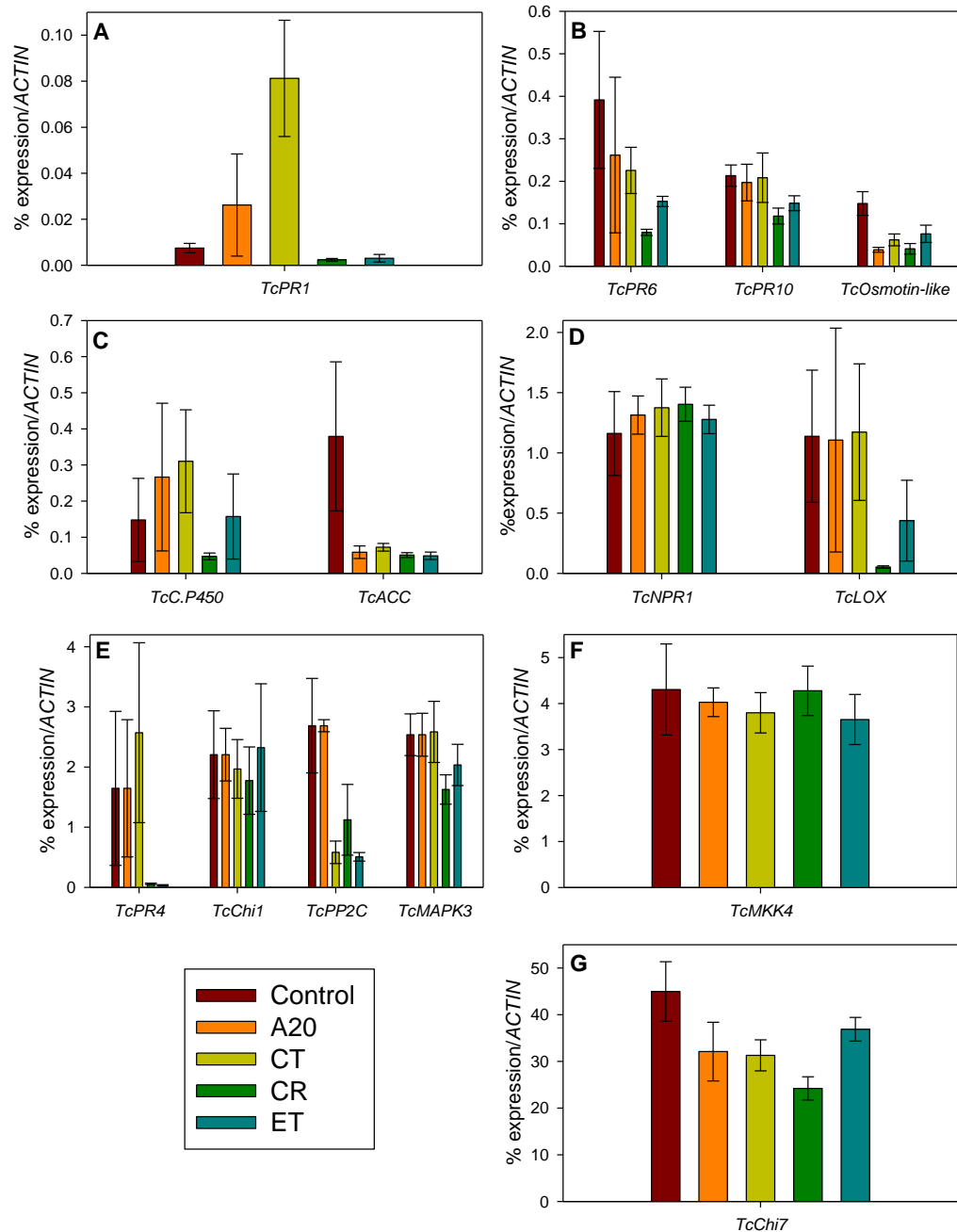
Figure 5-2: Mean expression of cacao ESTs putatively involved in cacao defense from seedlings grown on wetted Whatman paper one week after inoculation of germinated seeds with cacao bacterial endophytes. Mean relative mRNA of TcPR1, TcPR6. TcPR10, *TcACC*, Tc*PP2C*, *TcOsmotin-like*, Tc*C.P450, TcAcc, TcNPR1, TcLOX, TcPR4, TcChi1, TcPP2C, TcMAPK3, TcMKK4, and TcChi7* of 7 replicate seedlings calculated with respect to *TcACTIN* and presented as percentage of *ACTIN*. Treatments were control,

Lysinibacillus sphaericus A20, *Bacillus subtilis* CR, *Bacillus cereus* CT, and *Bacillus pumilus* ET. Bars extending around the means indicate the standard error of that mean.

Colonization by CR and ET repressed pathogenesis-related protein 1 (PR1), pathogenesis-related protein 4 (PR4), pathogenesis-related protein 6 (PR6), and pathogenesis-related protein 10 (PR10), while colonization with CT caused high expression levels of PR1. Colonization with CR also repressed expression of cytochrome P450, lipoxygenase (LOX) (Fig 5-2C), and mitogen-activated protein kinase 3 (MAPK3) (Fig 5-2E). None of the bacteria affected the expression of nonexpressor of pathogenesis-related protein 1 (NPR1), chitinase 1 (Chi1), or mitogen-activated protein kinase kinase 4 (MKK4).

Colonization of cacao seedlings inoculated with bacterial endophytes and grown in magenta boxes

Bacterial colonists were found in all treated stems at all time points, indicating the ability of colonists to persist in rapidly growing tissues. Colonization by fungi was not detected on CMDA. Gram-positive bacterial colonists were found in all treated stems, indicating the success of endophytic colonization due to seed treatment. No endophytic colonists were detected in immature leaves after inoculation with *Bacillus* spp. It should be noted that the aggressive sterilization process damaged the sensitive young tissue if the immature leaves.

Bacillus spp. were capable of colonizing mature green leaves of cacao seedlings following seed treatment (Fig. 5-3).
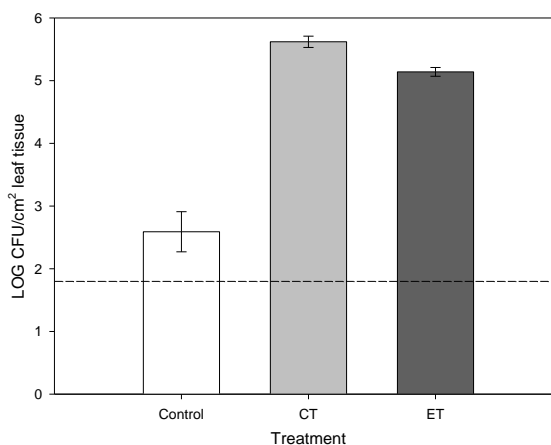
Figure 5-3: Mean endophytic bacterial colonists of mature cacao leaves on seedlings grown from seeds treated with endophytic *Bacillus* spp. Control seeds were dipped in sterile 0.1M potassium phosphate buffer, while seed inoculated with *Bacillus pumilus* ET and *Bacillus subtilis* CT were treated with 1×10^6

CFU/ml bacteria in phosphate buffer. Colonization of leaves was determined 32 days after inoculation with bacteria and planting. Bars extending around the means indicate the standard error of that mean. The dashed line indicates the minimum detection level for the experiment at log 1.8 CFU/cm^2.

There were low levels of bacterial colonists in the stems and leaves of control plants (3.0 log CFU/cm^2 less than treated), but these bacteria were gram negative, therefore not related to the introduced bacteria. Treatment of seed with bacterial endophytes resulted in colonization of mature leaves at 3.5 log CFU/cm^2 above that of the native colonists found in control plants. Additionally, only gram positive bacteria were detected in treated plants, and only gram negative bacteria were detected in untreated control plants. There was only a half log CFU/cm^2 difference in levels of colonization between the two bacterial isolates (CT and ET).

Impact of bacterial colonization on disease development of *P. capsici* on cacao leaves

Challenge of immature green leaves with zoospore droplets at time 2 (18 days after inoculation)

Colonization of cacao leaves with both CT and ET did not reduce *P. capsici* disease severity in detached leaf assays (Fig. 5-4).
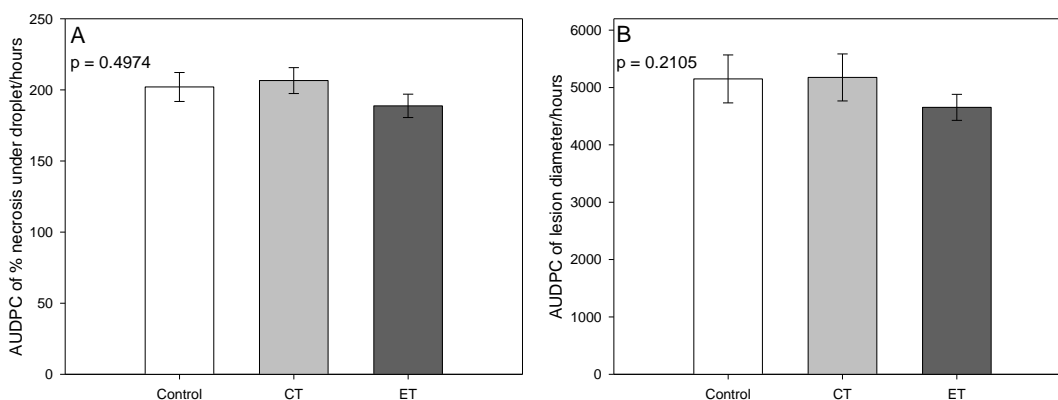
Figure 5–4: Mean area under the disease progress curve of disease severity due to *Phytophthora capsici* 18-days after planting of *Bacillus* treated seedlings. Leaves were removed from seedlings and challenged with 50 zoospores/μl of *Phytophthora capsici* by placing 10 μl droplets on the leaves. Disease severity was rated every 8-12 hours for 55 hours then used to determine the AUDPC. Bars extending around the means indicate the standard error of that mean.

Challenge of seedlings with zoospores at time 3 (32 days after inoculation) to determine impact of endophytic colonization on expression of cacao ESTs after pathogen infection

Plants colonized by *B. cereus* CT had reduced the number of *P. capsici* lesions per cm^2 leaf tissue (Fig. 5-5).
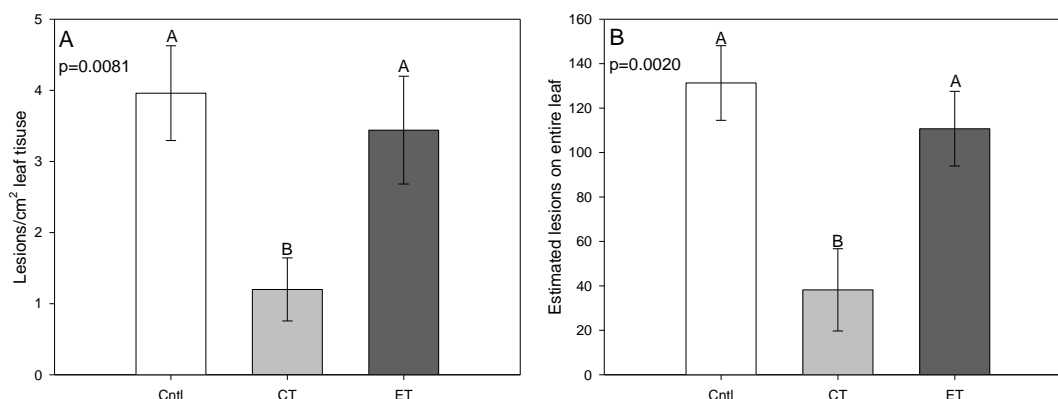
Figure 5–5: Mean *Phytophthora capsici* disease severity as A) lesions/cm^2 leaf tissue and B) lesions covering the leaf that were sprayed with a solution of a $5x10^3$ zoospores/ml solution of *P. capsici* 32-days after planting *Bacillus* treated cacao seeds. Seedlings were bagged and incubated for 24-hours. Bars extending around the means indicate the standard error of that mean. Differences in letters above the bar indicate significant differences at α=0.05 via Tukey analysis.

Impact of colonization of bacterial endophytes on expression of cacao ESTs

Colonization of cacao seedlings with *B. cereus* CT increased expression of the osmotin-like EST in stems at 32 days after colonization (Fig 5-6A) and also mature leaves, but only after inoculation with *P. capsici*. Immature leaves were collected at time 2, while mature leaves were collected at time 3, as well as challenged with *P. capsici* (time 3 + priming). Leaves were not present at time one, therefore were not collected. Colonization did not impact expression of ACC synthase, protein phosphatase 2C or cytochrome P.450 in either leaves or stems.
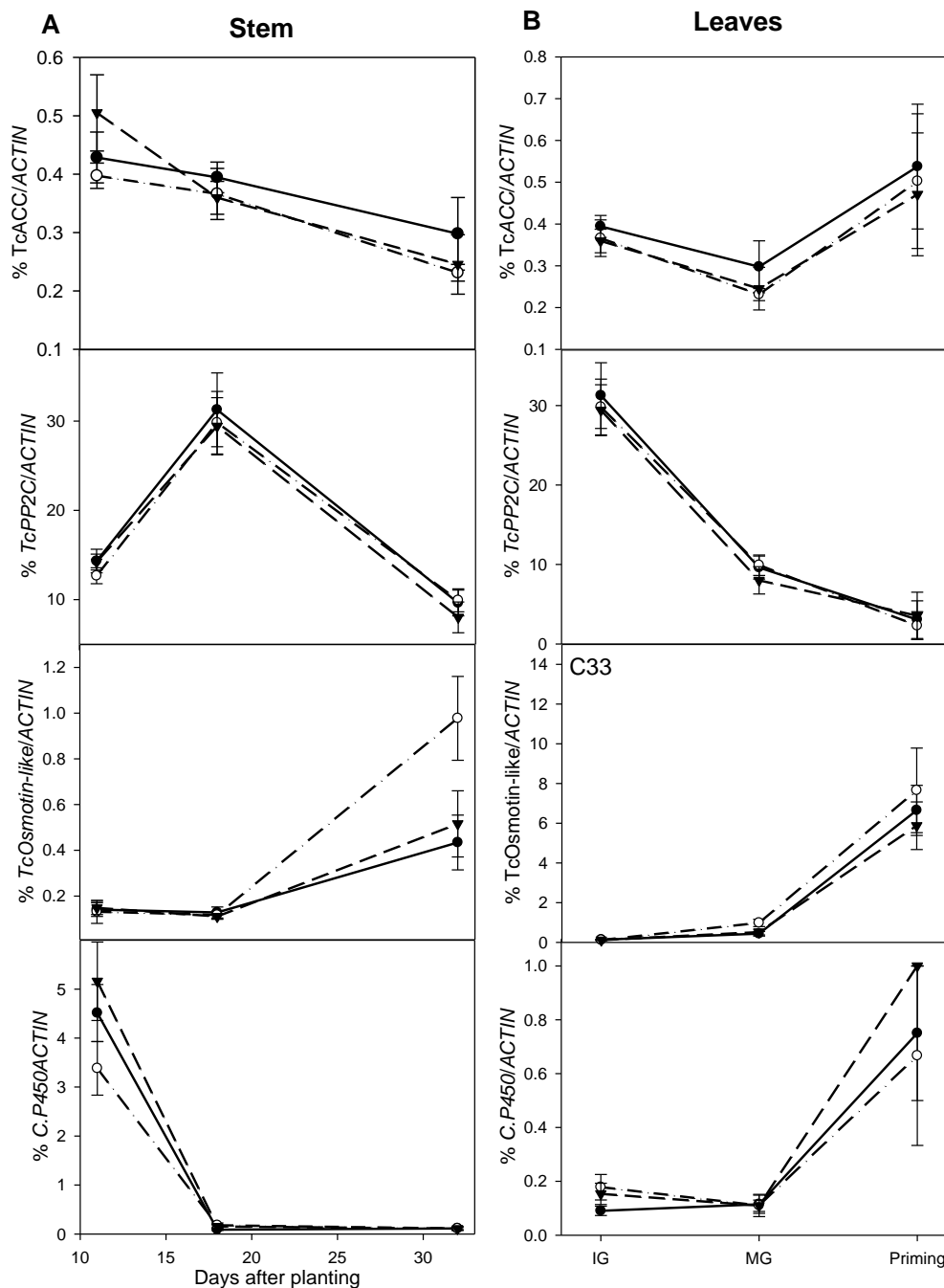
Figure 5-6: Mean expression of cacao ESTs putatively involved in cacao defense pathways in (A) stems and (B) leaves of 11, 18, and 32 days old cacao seedlings grown from seeds treated with endophytic *Bacillus* spp. IG leaves are from 18 day old seedlings, MG represents mature green leaves from 32 day old seedlings and priming were MG leaves at 32 days inoculated with *Phytophthora capsici* zoospores. Mean relative mRNA of *TcACC*, *TcPP2C*, *TcOsmotin-like*, and *Tc*C.*P450* of 9 replicate seedlings calculated with respect to cacao actin and presented as percentage of *ACTIN*. Control (●) plants were treated with sterile phosphate buffer, CT (O) plants were treated with *Bacillus cereus* CT, while ET (▼) plants were treated with *Bacillus pumilus* ET. Bars extending around the means indicate the standard error of that mean.

There were no differences between treatments for the expression of Chi1, Chi7, LOX, or MAPK3 (Fig. 5-7).
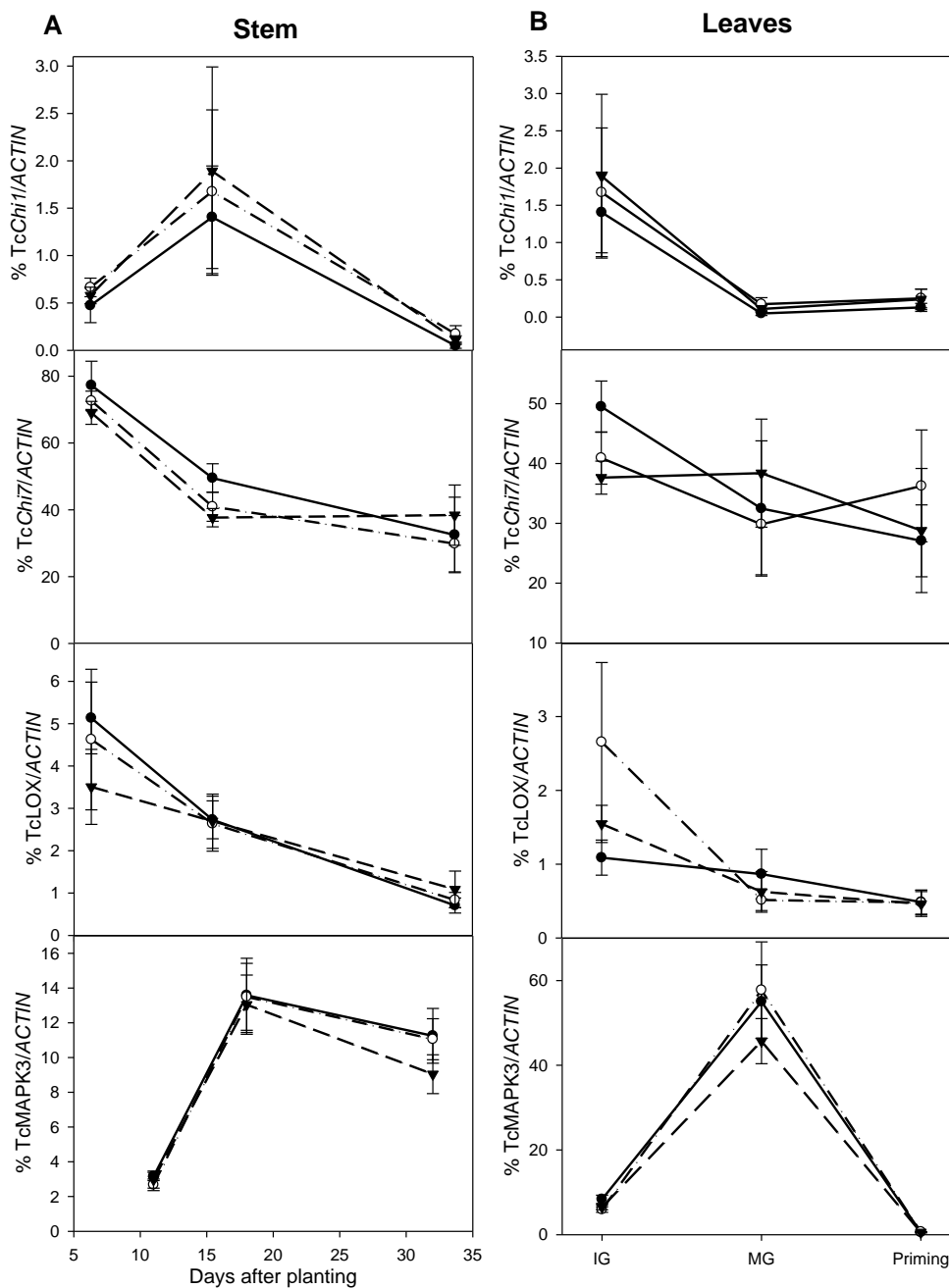
Figure 5-7: Mean expression of cacao ESTs putatively involved in cacao defense pathways in (A) stems and (B) leaves of 11, 18, and 32 days old cacao seedlings grown from seeds treated with endophytic *Bacillus* spp. IG leaves are from 18 day old seedlings, MG represents mature green leaves from 32 day old seedlings and priming were MG leaves at 32 days inoculated with *Phytophthora capsici* zoospores. Mean relative mRNA of *TcChi1*, *TcChi7*, *TcLOX*, and *TcMAPK3* of 9 replicate seedlings calculated with respect to cacao actin and presented as percentage of *ACTIN*. Control (●) plants were treated with sterile

phosphate buffer, CT (○) plants were treated with *Bacillus cereus* CT, while ET (▼) plants were treated with *Bacillus pumilus* ET. Bars extending around the means indicate the standard error of that mean.

        B. pumilus ET repressed expression of MKK4 in stems at all time points (Fig 5-8A), while both CT and ET activated the expression of MKK4 in mature green leaves. Colonization with the *Bacillus* spp. did not impact the expression of NPR1, PR1, or PR4 in stems or leaves (Fig. 5-8).
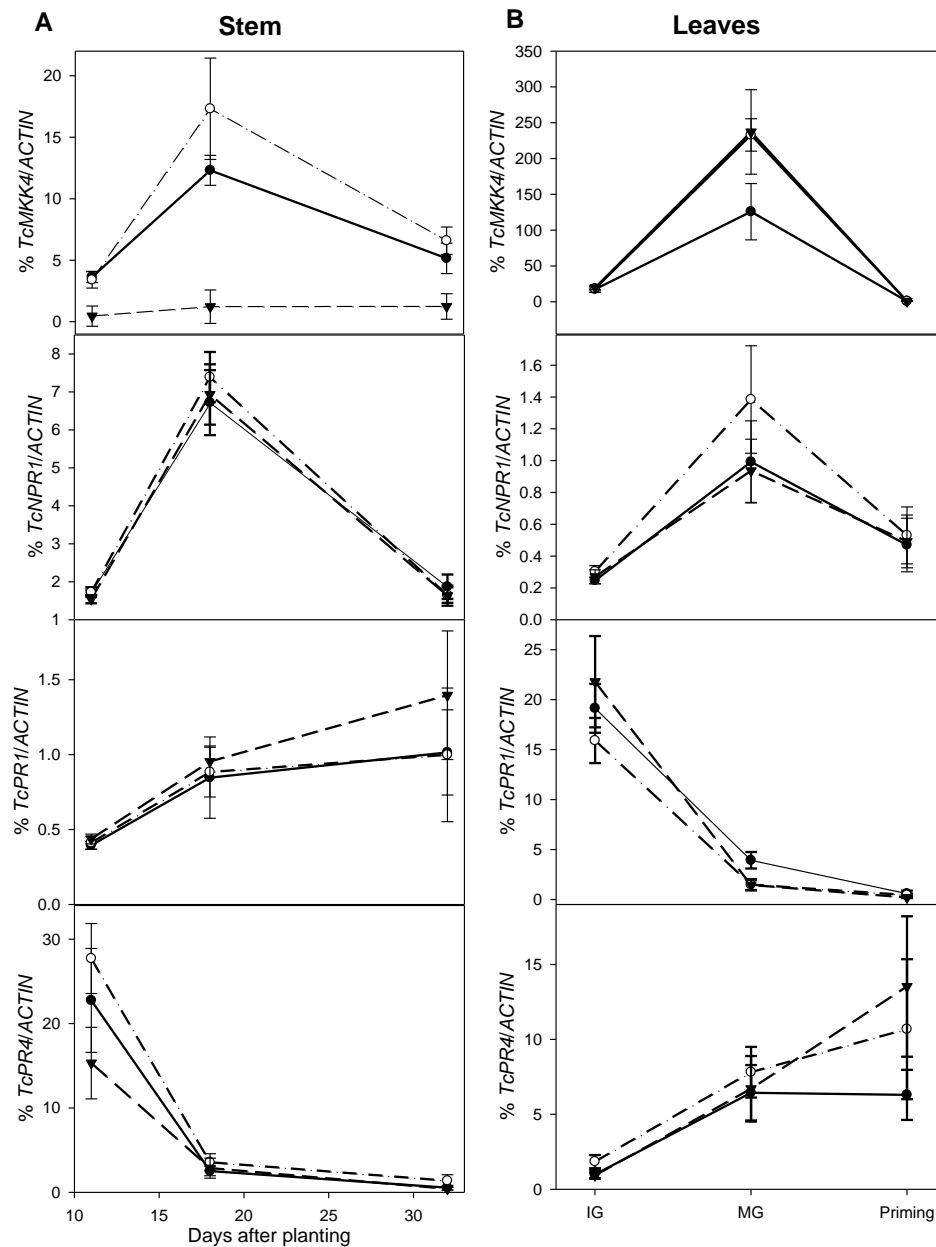
Figure 5-8: Mean expression of cacao ESTs putatively involved in cacao defense pathways in (A) stems and (B) leaves of 11, 18, and 32 days old cacao seedlings grown from seeds treated with endophytic

Bacillus spp. IG leaves are from 18 day old seedlings, MG represents mature green leaves from 32 day old seedlings and priming were MG leaves at 32 days inoculated with *Phytophthora capsici* zoospores. Mean relative mRNA of *TcMKK4*, *TcNPR1*, *TcPR1*, and *TcPR4* of 9 replicate seedlings calculated with respect to cacao actin and presented as percentage of *ACTIN*. Control (●) plants were treated with sterile phosphate buffer, CT (○) plants were treated with *Bacillus cereus* CT, while ET (▼) plants were treated with *Bacillus pumilus* ET. Bars extending around the means indicate the standard error of that mean.

Colonization by CT reduced the expression of PR6 at 11-days after inoculation, but increased expression at 18 days after inoculation (Fig 5-9). Additionally, ET repressed the expression of PR10 in 18-day old stems, while CT increased the expression in 32-day old stems.
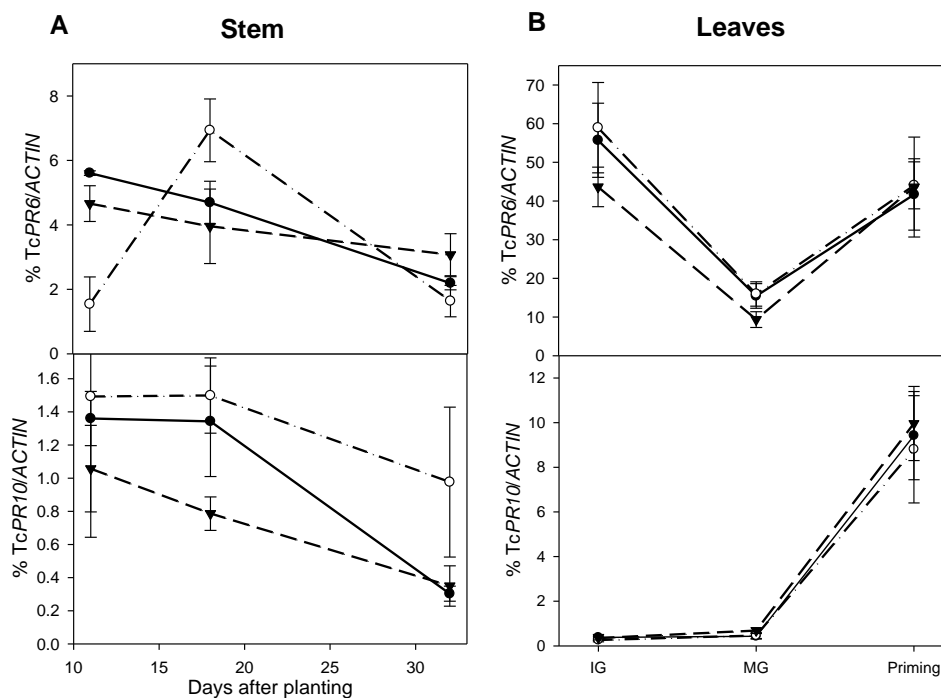
Figure 5-9: Mean expression of cacao ESTs putatively involved in cacao defense pathways in (A) stems and (B) leaves of 11, 18, and 32 days old cacao seedlings grown from seeds treated with endophytic *Bacillus* spp. IG leaves are from 18 day old seedlings, MG represents mature green leaves from 32 day old seedlings and priming were MG leaves at 32 days inoculated with *Phytophthora capsici* zoospores. Mean relative mRNA of *TcPr6*, *TcPR10* of 9 replicate seedlings calculated with respect to cacao actin and presented as percentage of *ACTIN*. Control (●) plants were treated with sterile phosphate buffer, CT (○) plants were treated with *Bacillus cereus* CT, while ET (▼) plants were treated with *Bacillus pumilus* ET. Bars extending around the means indicate the standard error of that mean.

Discussion

Successful colonization of the seedlings resulted from seed treatment with each of the tested bacterial endophytes. Colonization occurred in stems and mature leaves. However, endophytic colonists were not detected in immature leaves at time 2 (18 days after inoculation),

but were present in mature leaves at time 3 (32 days after inoculation). The lack of endophytic colonists in immature leaves was likely from the high concentration of bleach used in the sterilization process affecting the fragile immature leaves and any endophytes they might contain. Immature leaves are very flexible and sensitive and were damaged in the sterilization process both by the bleach and the handling of the leaf disks. The fact that mature leaves were all endophytically colonized by the *Bacillus* spp. further indicates that immature leaves were likely colonized, as it is unlikely that the *Bacillus* spp. would move from the stems to leaves that were fully developed. It is most likely that the bacteria colonized the leaves at the onset of development, which was found to occur in other unpublished experiments (R.L. Melnick, M.D. Strem, and B.A. Bailey; unpublished). Roots were not sampled for colonization in this experiment, but previous work has demonstrated that 100% of roots from seedlings grown from *Bacillus* treated seed were endophytically colonized (Melnick, Strem, and Bailey, 2008, unpublished). This is the first report of bacterial endophytes colonizing cacao seedlings through seed inoculation. Prior work with endophytic *Trichoderma* spp. indicated that cacao seedlings could be colonized through treatment of seeds (Bailey et al., 2008). Previous findings indicated that endophytic *B*. *cereus* isolate BT8 from tomato colonized cacao foliage that it was applied to, but did not grow to colonize tissue that emerged after inoculation of cacao with the isolate (Melnick et al., 2008). It is not possible to say whether the ability of the bacteria to colonize newly emergent tissue is due to the differing isolates, different application strategies, plant age, or the use of bacterial endophytes that are native to cacao.

An additional finding is the presence of endophytic bacteria colonizing untreated control plants, despite all seeds being surface sterilized and planted into a sterile production system. These bacteria were morphologically different than the applied *Bacillus* spp. and were gram negative. This colonization was not detected in plants grown from *Bacillus* treated seeds, indicating that the seed endophytes could not compete with the introduced isolates. Although not well studied, bacterial endophytes have been previously detected in the seeds of perennial trees such as Norway spruce (Cankar et al., 2005) and Western white pine (Ganley and Newcombe, 2006). A previous experiment on fungal endophytes of cacao detected bacterial endophytes colonizing plants grown from surface sterilized seeds (Posada and Vega, 2005). It is likely that colonization of control plants resulted from naturally occurring seed endophytes that lacked the ability to compete with the applied *Bacillus* spp., as the gram negative bacterium was never detected in plants inoculate with the endophytes. These bacteria could potentially play an

important role in the fermentative step of cacao processing, as microbes are essential to the process (Camu et al., 2007).

Colonization of seedlings with *Bacillus* spp. did not reduce *P. capsici* lesion expansion on detached immature leaves nor did the bacteria impact expression of defense ESTs in immature leaves. The work of Melnick et al. (2008) utilizing *B. cereus* BT8 from tomato demonstrated that endophytic colonization of leaves did not suppress *P. capsici* until 30 days after initial colonization and disease suppression continued at each sampled time point after. It is likely that the bacteria did not protect the plants from *P. capsici* in these rapidly developing leaves. In mature leaves, the presence of *B. cereus* CT significantly reduced the number of lesions covering leaves that were challenged with *P. capsici*. *B. pumilus* ET did not reduce the number of lesions. Immature leaves were not challenged at this date (32 days after inoculation), as they were not present. Mature leaves were not used for the detached leaf assay, as lesions develop after challenge with *P. capsici* zoospores do not expand as they do on immature leaves (Melnick, 2006). Despite this problem, spraying zoospores onto mature leaves resulted in small necrotic lesions. Although all lesions were relatively the same size, we could readily count the number of lesions and determine differences in numbers of lesions as reflective of relative resistance of the leaf. This whole plant methodology is a new technique that works well for seedlings and could allow for better analysis of disease suppression, as it does not rely on the presence of the transient immature leaf stage. Additionally, it does not require leaves be removed from the plant and wounded during the cutting of leaf disks. This new methodology seems to be more robust than previously tested methods using detached leaves (Melnick et al., 2008; Nyassé et al., 2002; Tahi et al., 2007).

Bacterial treatment of seedlings produced in Petri dishes had a unique impact on the expression of the tested ESTs. All isolates repressed the expression of osmotin-like EST, ACC synthase, and Chi7 in seedlings. It should be noted that expression of ACC synthase was highly variable in control seedlings, likely due to the early developmental age of the plants. It should also be noted that colonization with CR resulted in repression of ESTs related to defense against plant pathogens (PR4, MAPK3, and Chi7) as well as the jasmonic acid pathway (LOX) (Wasternack and Parthier, 1997), while the remaining four isolates did not cause similar repression. The overall repression of ACC synthase indicates that the ethylene pathway is not likely involved in early interactions between cacao and the tested bacterial endophytes (Morgan and Drew, 2006). Further, the lack of activation of PR1 (except by CT), Chi1, PR4, PR6, and PR10 indicates that these bacilli are not likely activating the SAR defense pathway typically

initiated by infection with fungal pathogens (Metraux et al., 1990). None of the ESTs tested were upregulated in seedlings grown in petri dishes at 7 days after inoculation with the bacteria, except PR1 upregulation due to colonization with isolate CT.

Colonization by *Bacillus* spp. did not impact expression of ACC synthase, Chi1, cytochrome P450, LOX, MAPK3, NPR1, PR4, and protein phosphatase 2C at any date in stems, leaves, or after challenge with *P. capsici*. Although the expression of these ESTs was not impacted by the presence of the bacteria, they were influenced both by cacao tissue type as well as the developmental stage of the seedlings. Expression of ACC synthase, Chi7, and LOX steadily decreased over time in stems, while expression of PR1 steadily increased as seedlings developed. There was a marked decrease in expression of Chi1, protein phosphatase 2C, PR1, and PR6, as leaves developed from immature to mature leaves while there was an increase in expression of MAPK3, MKK4, PR4, and NPR1.

In terms of the effect of endophytes on the expression of cacao ESTs, *B. cereus* CT increased the expression of osmotin-like proteins in stems of 32 day old seedlings, but not in leaves. The expression of PR6 was repressed by the presence of *B. cereus* CT in the stems of 11-day old seedlings, but activated expression in stems one week later. Additionally, *B. cereus* CT increased the expression of PR10 in the stems of 32-day old seedlings, but expression between replicates was highly variable. The observation that *B. pumilus* ET reduced the expression of MKK4 in 11, 18, and 32-day old stems, but did not impact MKK4 expression in leaves indicating that ET most impacted expression of these genes in stems. ET also increased the expression of PR4 in leaves challenged with *P. capsici* when compared to control plants. *B. pumilus* ET reduced the expression of PR10 in immature leaves. Both *Bacillus* spp. had increased levels of MKK4 in MG leaves at 32 days after colonization. The variation in gene expression between the isolates indicates that the various *Bacillus* spp. had differential impacts on the expression of cacao ESTs related to defense. These isolates are not likely involved in priming, as NPR1, which is essential for priming was not induced by the presence of the bacteria (Conrath et al., 2006). Work with plant growth promoting rhizobacteria demonstrated that these nonpathogenic plant-associated microbes induced a salicylic acid-independent (ISR) pathways that was linked to the ethylene and jasmonic acid pathways through *etr1* and *jar1*, respectively (Pieterse et al., 2000). EST expression patterns of the cacao bacteria indicate that they are not likely utilizing this ISR pathway.

As mature leaves collected at time 3 and mature leaves used in priming were the same age, we can compare the differences in expression by these two treatments to determine how *P.*

capsici impacts expression of defense ESTs in cacao at 24 hours after challenge. Infection by zoospores caused an increase in the expression of ACC synthase, cytochrome P450, PR6, PR10, and osmotin-like EST, in mature leaves, while decreasing the expression of MAPK3, MKK4, and NPR1. Previous research indicates that tomatoes infected with *P. infestans* had increased levels of ACC synthase and ethylene in response to the pathogen (Spanu and Boller, 1989), which is similar to the response found in cacao. Infection of potato with *P. infestans* increased the expression of cytochrome P450 and pathogenesis related proteins, which are all related to cell defense (Beyer et al., 2001). Habanero peppers infected with *P. capsici* had increased levels of MAPK transcription, but infection had no impact on NPR1 (Nakazawa-Ueji et al., 2009). Cacao appears to have a different response to *P. capsici* in terms of the expression of MAPK and NPR1 than habanero pepper. In cacao, *P. capsici* infection caused did not change the expression of Chi1, LOX, PR1, and protein phosphatase 2C. All ESTs considered, *P. capsici* appears to be activating a salicylic acid-dependent response as well as ethylene response due to the transcription of ACC synthase and pathogenesis related proteins (Van Loon and Van Strien, 1999), but further ESTs should be tested to confirm this result.

Future research should look at the nature of cacao seed endophytes and focus on confirming their presence and characterizing the microbes colonizing seeds. It is possible that these endophytes may play a role during the fermentative step of the cocoa beans processing. Additionally, they could play a role in defense against pathogens, although these isolates could not compete with the bacterial endophytes introduced to the seed. While seed-borne endophytes have been reported for several fungal endophytes colonizing grasses (Schardl et al., 2004), there is only a limited amount of literature on the presence of seed endophytes in perennial tree species. Future research on the ability of bacterial endophytes to induce resistance should focus on looking at a larger set of genes, to determine whether the colonization impacts ESTs that were not tested in this analysis. The selected set of ESTs was screened as candidate ESTs for defense response, based upon previous experiments. The bacterial endophytes do not appear to have a large impact on expression of cacao ESTs as has colonization with endophytic *Trichoderma* spp. (Bailey et al., 2006). Additionally, suppression of witches' broom by *B. pumilus* ET in the field resulted from application of the bacterial isolate to the cacao foliage (see Chp. 3). Work to elucidate the differences in expression between seed colonization and foliar application of the *Bacillus* spp. could indicate which application methodology could best reduce disease as well as the potential to use both application strategies to best suppress disease. In conclusion, there is a wide range of research yet to be conducted in the area of induced resistance in cacao that could

not only better the understanding of biological control, but also how the plant responds to infection from the unique set of pathogens that infect plants in South America.

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Chapter 6

Co-colonization of cacao seedlings with *Trichoderma* spp. and *Bacillus* spp.

Introduction

Cacao (*Theobroma cacao* L.) is a tropical perennial tree grown for the production of cocoa and chocolate products. Cacao faces major losses due to pest and diseases. *Moniliophthora perniciosa*, causal agent of witches' broom and *Moniliophthora roreri*, causal agent of frosty pod, have drastically reduced cacao bean production in Latin America. In many areas of South America, cacao is grown by small-scale low input farmers whose only real management options are tolerant trees and phytosanitation. One problem with the use of resistant cultivars is that farmers cannot readily replace preexisting plantings with new trees, due to the perennial nature of cacao. Phytosanitation is highly effective in reducing disease, but can require high labor input and also requires farmers to regularly scout trees (Soberanis et al., 1999). Small-scale farmers typically do not use modern agrochemicals, as their use is cost prohibitive. Due to the inadequacy of available management practices as well as a general need for more sustainable cacao production, much current research focuses on developing biological control options for the management of cacao diseases.

Tropical plants are considered to be important harbors of endophytes, supporting a high abundance and diversity of both common and rare species (Arnold and Lutzoni, 2007). This high microbial diversity appears to be true for cacao, as it is host to a diverse range of microbes, several of which have been investigated for their ability to suppress cacao diseases (Mejía et al., 2008; Rubini et al., 2005). Most recent research on biological control of cacao diseases has focused on fungal species. One genus that has been seen to have a close relationship with *Theobroma* species has been *Trichoderma* (Evans et al., 2003; Samuels et al., 2006). Epiphytic *Trichoderma stromaticum* has been used to suppress foliar witches' broom disease, through acting as a mycoparasite (Bastos, 1988; Samuels et al., 2000). Krauss and Soberanis (2001) found that mycoparasitic *Trichoderma* spp. reduced growth of both witches' broom and frosty pod in bioassays. Although *Trichoderma* spp. are most commonly found in soil, a unique subset of *Trichoderma* spp. survive as endophytes within cacao trees (Evans et al., 2003; Hanada et al.,

2008; Holmes et al., 2004). A diverse collection of endophytic *Trichoderma* spp. are currently being investigated for the ability to suppress witches' broom, frosty pod, and black pod of cacao (Bailey et al., 2008; Tondje et al., 2007). These isolates have been shown to be antagonistic to cacao pathogens as well as being capable of endophytically colonizing cacao stems and roots (Bailey et al., 2008), indicating their potential for biocontrol of cacao diseases.

Another active area of research has been the utilization of cacao bacterial endophytes to suppress witches' broom (see Chp. 3) and cacao pod diseases (see Chp. 4). Endophytic *Bacillus pumilus* ET reduced incidence of witches' broom and cherelle wilt, a physiological thinning mechanism with a likely biological component. Although most research on biological control has focused on introducing a single species to suppress disease, combinations of biological control agents to enhance suppression of a targeted disease or to expand the range of diseases controlled have been an increasing area of research. Combining different species of plant growth promoting rhizobacteria decreased the number of tomato and pepper plants infected with *Fusarium* and *Rhizoctonia* (Domenech et al., 2006). Application of the yeast *Pichia guilermondii* with the bacterium *Bacillus mycoides* resulted in increased control of *Botrytis* disease on strawberries because of the additive effects of the different mechanisms used by the two organisms (Guetsky et al., 2002). Although some researchers have tested combinations of fungal species to manage cacao diseases (Krauss and Soberanis, 2001), no research has been reported on combinations of fungal and bacterial species to reduce cacao diseases.

The objective of the research presented in this chapter is to test the ability of endophytic *Trichoderma* spp. and *Bacillus* spp. from cacao to colonize cacao tissues and whether combinations of these endophytes can coexist in cacao seedlings. Seedlings were also challenged with *Phytophthora* spp. to determine whether the colonization of plants with both fungal and bacterial endophytes had an increased benefit on disease suppression. An additional objective was to determine whether combinations of two successful endophytes (*Bacillus pumilus* ET and *Trichoderma hamatum* 219b) might activate multiple defense genes to have an additive benefit on induced resistance against pathogens.

Material and Methods

Bacterial isolates: source and growth

Endophytic *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *Bacillus subtilis* CR and *Bacillus pumilus* ET were isolated from "Nacional" cacao trees largely escaping disease on the INIAP Estacion Experimental Tropical (INIAP-EET) in Pichilingue, Ecuador (see Chp. 2). Bacteria were shipped from INIAP-EET to The Pennsylvania State University. All cultures were stored in tryptic soy broth (TSB, MO BIO Laboratories, Inc., Carlsbard, CA) with 20% glycerol in liquid nitrogen.

Trichoderma isolates: source and growth

Trichoderma caribbaeum var. *aequatoriale* DIS320c was isolated as a trunk endophyte from *Theobroma gileri* in Vicente Maldonado, Pichincha Province, Ecuador (Evans et al., 2003) and was classified by Samuels et al. (2006). *Trichoderma koningiopsis* G.J.S. 01-07 was isolated from cacao pods infected with the frosty pod pathogen *M. roreri* that were lying on leaf litter in Ecuador (Samuels et al., 2006). *Trichoderma hamatum* 219b was isolated from *Theobroma gileri* pods in Ecuador (Evans et al., 2003). Cultures were maintained on corn meal dextrose agar (CMDA) (Difco, Franklin Lakes, NJ). All cultures are stored with 20% glycerol in liquid nitrogen.

Phytophthora isolates: source and growth

Phytophthora citrophthora was obtained from infected cacao pods in Brazil. *Phytophthora megakarya* was obtained from infected cacao pods in Ghana. *P. capsici* was isolated by H. Purdy from infected cacao tissue in Pichilingue, Ecuador. Cultures were maintained and grown on V8 agar (20% V8) and stored in 20% glycerol in liquid nitrogen.

Antagonism study between *Bacillus* spp. and *Trichoderma* spp.

An *in vitro* agar plate pairing assay was used to determine if the *Bacillus* spp. were directly antagonistic to *Trichoderma* spp. Two mycelial plugs of the tested *Trichoderma* spp. were placed 5 cm apart on tryptic soy agar (TSA, MO BIO Laboratories, Inc., Carlsbard, CA) in a 100 mm Petri dish. A streak of *Bacillus* sp. was placed between the plugs (2.5 cm from each plug). Control plates consisted of the mycelial plugs without bacteria and bacteria without *Trichoderma* sp. Three replicate plates were prepared for each combination between the *Bacillus* spp. and *Trichoderma* spp. tested. Plates were incubated at room temperature (24°C), radial growth of the *Trichoderma* spp. was measured, and fungal and bacterial colonies were observed for the development of a zone of inhibition.

Germination of *Trichoderma* conidia is essential for foliar application and subsequent colonization of cacao tissue; therefore it was necessary to determine whether the bacterial isolates affected germination. Bacterial suspensions were adjusted to 1×10^{8} CFU/ml in 0.1M potassium phosphate buffer. Conidia were collected by flooding cultures grown on CMDA with sterile distilled water and the concentration was adjusted to approximately 3.5×10^{6} CFU/ml in sterile distilled water. Solutions were mixed at a 1:1 ratio (500 µl each) in the following mixtures in triplicate: phosphate buffer/water (B.Cntl/T.Cntl), B.Cntl/320c, B.Cntl/01-07, B.Cntl/219b, A20/01-07, CT/01-07, CR/01-07, A20/320c, and CT/320c, CR/320c, ET/219b. Solutions were incubated for 24 hours at 25°C, and then where placed onto a glass slide. Total spores and germinated spores were counted to determine effects of bacteria on germination.

Production of cacao seedlings and growth in soilless-medium

Pods were removed from open pollinated *T. cacao* "comum" trees (Lower Amazon Amelonado type) from the Almirante Cacau, Inc. farm in Itabuna, Bahia, Brazil. These pods were used as the seed source for the first two experiments. For the experiment on cacao gene expression (experiment 3), pods were removed from open pollinated *T. cacao* Pound7 trees on the CATIE research station in Turrialba, Costa Rica. Pound 7 pods were used because "comum" pods were not available during this experimental period. All pods were shipped to the Sustainable and Perennial Crop Lab (SPCL) of USDA-ARS. Seeds were removed from the pods and surface sterilized in 20% commercial bleach for 5 minutes, then rinsed three times in sterile

distilled water. Seeds were then plated three per plate onto 1.5% water agar to allow for germination and were incubated at room temperature (25°C) on the lab bench. Double magenta boxes (20 cm high and 6.5 cm square, Magenta Corp., Chicago, IL) with four 0.5 cm diameter holes bored through the bottom box were filled with 80g of sterile soilless mix (2:2:1; sand:perlite:Promix, Premier Horticulture, Quakertown, PA), the holes were taped closed, and the closed boxes were autoclaved for 40 minutes.

Inoculation of cacao seedlings with *Trichoderma* spp.

The soilless mix in magenta boxes was inoculated with the *Trichoderma* sp. by placing two 2 mm plugs of the fungus onto the soil with 25 ml of sterile distilled water. Soil from the control treatment was inoculated solely with 25 ml sterilized distilled water. Boxes were closed and incubated at 25°C for five days. After five days, the surface sterilized germinated seeds were planted into *Trichoderma* inoculated soil (3-days after inoculation). Seedlings were grown in a controlled environment chamber (Model M-2, EGC Corp., Chagrin Halls, OH) at 25°C, 60%$\pm$5% relative humidity, and a 12h light/12h dark photoperiod. Irradiance in the chamber was 50 μmol $m^{-2}s^{-1}$ PAR. Approximately twenty days after planting (at the onset of leaf development) the top box and tape covering the drain holes were removed. Seedlings were watered to maintain adequate soil moisture and then fertilized once a with Scotts Miracle-Gro fertilizer (All Purpose Plant Food, The Scotts Miracle-Gro Company, Marysville, OH) once a week. Nine replications were carried out for each time/treatment combination.

Preparation of *Bacillus* spp. and inoculation of cacao foliage of seedlings pre-colonized with *Trichoderma* spp.: Experiment One

The three bacterial isolates were grown in 500 ml TSB in 1.8L Fernbach flasks. The flasks were incubated on a rotary incubator shaker (New Brunswick Scientific, Edison, NJ) at 28°C and 150 rpm and grown for 9 days (to incite endospore production). The bacterial solution was centrifuged for 8 minutes at 6,200g to pellet the bacteria. The bacterial pellets were resuspended in sterile 0.1M potassium phosphate buffer (pH 7.0). The concentration of the bacterial suspension was determined at OD_{600} using a spectrophotometer (Bausch & Lomb Co.,

Rochester, NY) then adjusted to 1 x 10^8 CFU/ml. The polysilicon surfactant, Silwet L-77, was added to the adjusted bacterial suspension so that it was 0.24% of the total concentration (vol/vol). The control treatment consisted of 0.1M potassium phosphate buffer with 0.24% Silwet.

The following bacterial treatments where applied to one month old seedlings precolonized with *Trichoderma*: Control, A20, CR, and CT to allow for the following treatment combinations bacterial control/*Trichoderma* control (B.Cntl/T.Cntl), A20/T.Cntl, CR/T.Cntl, CT/T.Cntl, B.Cntl /320c, B.Cntl /01-07, A20/320c, CR/320c, CT/320c, A20/01-07, CR/01-07, and CT/01-07. Bacterial treatments were applied by spraying the suspensions seedlings using a handheld pump aerosol sprayer (Nalge Nunc International Corp., Rochester, NY), as see in Figure 6-1.

Sterile soil inoculated with *Trichoderma* sp.

Plant germinated seed into *Trichoderma* colonized soil

Spray bacterial solution onto first flush. Sampled after second flush.
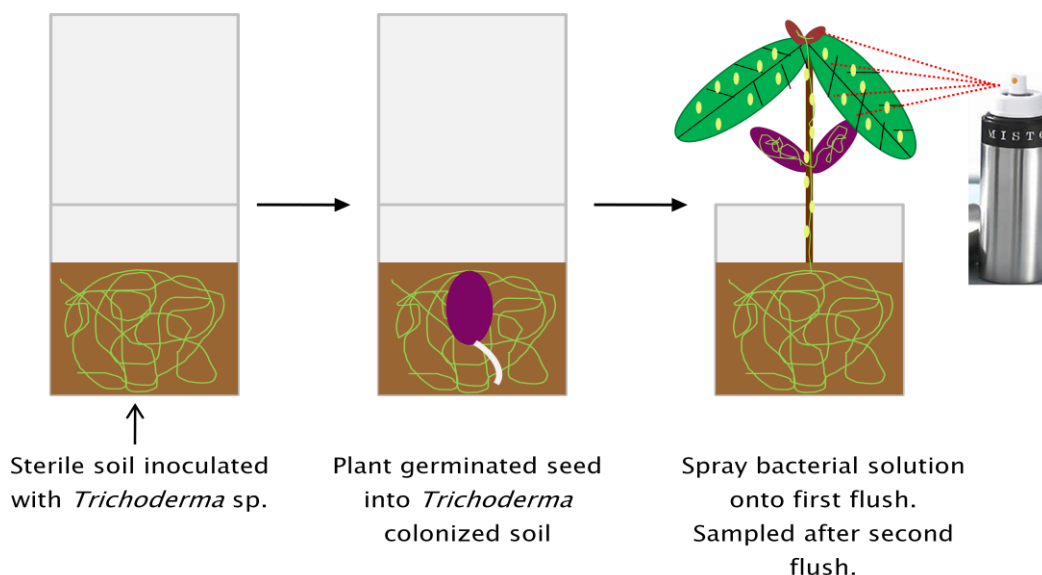
Figure 6-1: Methodology used in experiment one to test the ability of *Trichoderma* spp. and *Bacillus* spp. to co-colonize cacao seedlings. Germinated seedlings were planted into soilless mix in double magenta boxes three days after inoculation with *Trichoderma* spp. *Trichoderma* treatments were *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Twenty days after planting the magenta boxes were opened and at 29 days after planting, bacterial endophytes were sprayed onto the seedlings at $1x10^8$ CFU/ml with 0.24% Silwet using an aerosol sprayer. Bacterial treatments were bacterial control (B.Cntl), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, and *B. subtilis* CR.

Leaves were allowed to dry and plants were returned to the growth chamber. Plants were maintained in a randomized block design in the growth chamber. Seedlings were then watered to maintain adequate soil moisture and fertilized with Scotts Miracle-Gro fertilizer once weekly.

Inoculation of cacao seeds with bacterial endophytes and planting into *Trichoderma* inoculated soil: Experiment Two

A second experiment was conducted to determine if inoculating cacao with bacilli through seed treatment would result in endophytic colonization and whether this colonization could persist in the presence of *Trichoderma* spp. Soil and magenta boxes were prepared, *Trichoderma* was inoculated onto the soil, and seeds were sterilized and germinated as in experiment one. Two days after seed germination, defined as radical emergence, seeds were inoculated with the following treatments: 0.1M phosphate buffer (B.Cntl), A20/T.Cntl, CR/T.Cntl, CT/T.Cntl, B.Cntl /320c, B.Cntl /01-07, A20/320c, CR/320c, CT/320c, A20/01-07, CT/01-07, and CT/01-07. The bacteria were prepared as in experiment one, expect the concentration was adjusted to $1x10^6$ CFU/ml. Silwet L-77 was not added as it allows for substomatal infiltration during foliar application. Seeds were removed from the plates two days after surface sterilization and placed into the bacterial suspension. Controls for the bacterial seed treatments consisted of sterile 0.1M phosphate buffer. The suspension was periodically agitated for five minutes, seeds were removed and allowed to dry, and then seeds were directly planted into *Trichoderma* colonized soil (Fig 6-2).

| Sterile soil inoculated with *Trichoderma* sp. | Seeds dipped in 10^6 bacterial suspension | Plant bacterial treated seeds into *Trichoderma* colonized soil | Sample seedling |
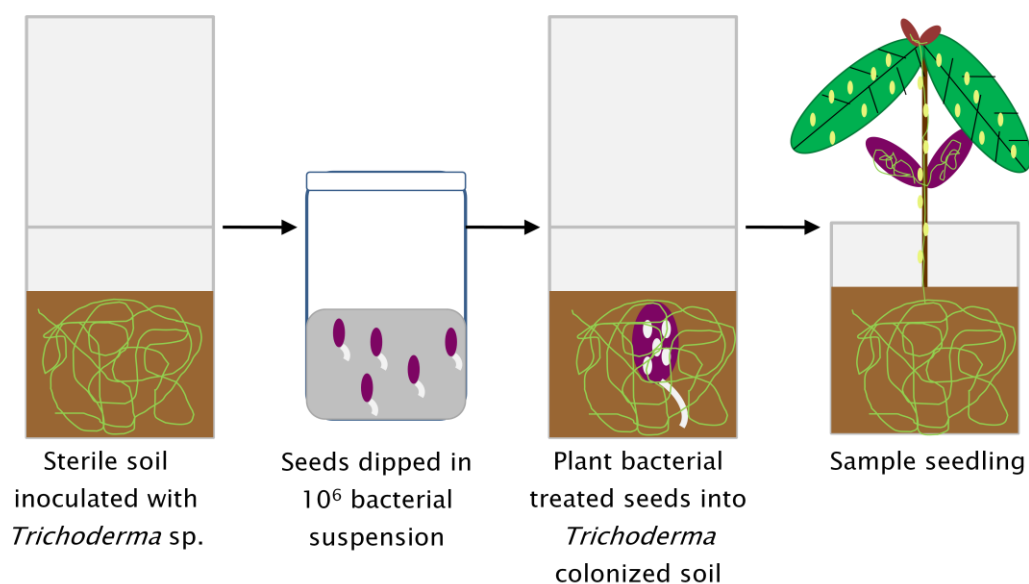
Figure 6-2: Methodology used in experiment two to test the ability of *Trichoderma* spp. and *Bacillus* spp. to co-colonize cacao seedlings. Two days after seed surface sterilization, seeds were treated with bacterial endophytes by placing the seeds into the $1x10^6$ CFU/ml bacterial suspension for 5 minutes. Bacterial treatments were bacterial control (B.Cntl), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, and *B. subtilis* CR. Immediately after treatment with bacilli, seed were planted into soil three days after inoculation with *Trichoderma* spp. *Trichoderma* treated were: *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07).

Inoculation of Pound7 cacao seedlings with *Trichoderma* spp. and *Bacillus* spp. to determine impacts on gene expression: Experiment Three

A third experiment was conducted to determine whether endophytic colonization of cacao with both fungal and bacterial endophytes impacted the expression of cacao ESTs related to defense. For this experiment, *B*. *pumilus* isolate ET was used as the bacterial endophyte, due to its ability to reduce disease in field trials (see Chp. 3 and 4) and *T*. *hamatum* 219b due to its success in activating defense cascades (Bailey et al., 2006). These endophytes were applied following the protocol stated in experiment one (planting of sterile cacao seeds into *Trichoderma* inoculated soil followed by foliar inoculation of *B. pumilus* one month after planting). Plants were maintained in opened magenta boxes in the growth chamber until advent of the second leaf flush (29 days after inoculation with bacteria), then sampled as described below.

Determination of colonization of cacao seedlings by *Trichoderma* and *Bacillus*

Plants were removed from the growth chamber and sampled to confirm colonization by fungal and bacterial endophytes. In experiment one and three, plants were sampled one month after foliar inoculation with bacteria. In experiment two, plants were sampled twenty days after bacterial inoculation of cacao seeds. To determine whether the microbes colonized the stem, eight 1 cm stem sections were removed from the seedlings, four from above and four below the cotyledons. The stem sections were surface sterilized in 20% bleach for 3 minutes, followed by three rinses in sterile distilled water. Four sections (2 from above and 2 from below the cotyledon) were plated onto TSA to determine bacterial colonization and the remaining four sections were plated onto CMDA to determine colonization by *Trichoderma* spp. To determine colonization of roots in experiment two, roots were removed from the soil and washed in water. Eight 1 cm stem sections were removed from roots and were surface sterilized in 20% bleach for 3 minutes, followed by three rinses in sterile distilled water. Four sections were plated onto TSA to determine bacterial colonization and the remaining four sections were plated onto CMDA to determine colonization by *Trichoderma* spp.

Colonization of leaves was assessed by removal of five 15.5 mm leaf disks from one mature leaf per replicate in a 'W' pattern. Disks were surface sterilized following the same

protocol as the stems. Two disks were placed onto CMDA to determine colonization by *Trichoderma*. To determine the level of endophytic bacterial colonization of leaves, the remaining three surface sterilized disks were placed into stomacher filter bags (Filtra-Bag, Labplas Inc., Québec, Canada) with 3 ml of sterile 0.1 M potassium phosphate buffer. Disks were triturated and 50 μl of supernatant were plated in duplicate onto TSA plates. Colonization of roots was assessed by removing the plant from the soil and washing soil from the roots. Six 2 mm root sections were removed from different areas of the root systems. Sections were surface sterilized as with other tissue. Three sections were plated onto CMDA and three sections were planted into TSA. All plates were incubated at 28°C for up to five days and observed for signs of fungal and bacterial growth.

Challenge of immature green cacao leaves with *Phytophthora capsici* to determine the ability of endophytes to suppress cacao disease

Immature green leaves were used for a detached leaf challenge to determine whether endophytes suppressed disease. Leaves were removed from the second leaf flush at 46 days after planting for experiment one, from the first leaf flush at 22 days after planting for experiment two, and from the second leaf flush at 50 days after planting during experiment three. For the challenge, two leaves were detached from the plant and 100 mm diameter disks were cut out of the leaf so that the midrib was the center of the leaf disk. The disks were placed abaxial side up atop a moist 10 cm Whatman filter paper a 100 mm Petri dish. Zoospores were obtained following the method of Lawrence (1978). Zoospore concentration was determined using a hemacytometer and adjusted to 5×10^3 zoospores/ml in sterile distilled water. For experiment one and two, one leaf was challenge with *P. megakarya* and the other leaf was challenged with *P. citrophthora*. *P. capsici* was used for experiment three, due to the low virulence of the other two *Phytophthora* isolates. Six 10 μl drops of the zoospore suspension were placed on each leaf disk, three on each side of the midrib near the center of the leaf blade. Petri dishes were sealed with parafilm and incubated at room temperature (25°C) under fluorescent lights (12 h light/dark cycle). Lesions were assessed every eight to twelve hours by measuring lesion diameter and the percentage of necrosis under the droplet (0-400%) until 52 hours after challenge.

Preservation of cacao tissue for RNA extraction and analysis of expression of cacao ESTs by QPCR due to colonization by endophytes

Mature leaves were preserved at the same time as sampling for colonization during experiment three. Mature leaves were removed from each plant and flash frozen in liquid nitrogen, and stored in -80°C. Total RNA was extracted and treated with DNAseI following (Bailey et al., 2005). RNA concentration was determine by measuring the OD_{260} and OD_{280} using a spectrophotometer. cDNA synthesis and QPCR was conducted as described in (Bae et al., 2005). Two μg of RNA was used to synthesize first-strand cDNA using a SuperScript^{TM} III First-Strand Synthesis System for RT-PCR kit (Invitrogen Corporation, Carlsbad, CA). Synthesized cDNA was diluted 10-fold for use in quantitative real time PCR (Q-PCR) analysis. QPCR analysis was conducted with the primer sets listed in Table 6-1 using a Mx3005P QPCR system (Stratagene, La Jolle, CA) and Brilliant$^\circledR$ SYBR$^\circledR$ Green QPCR Master Mix (Stratagene, La Jolle, CA). Each reaction was composed of 12.5 μl 2x Brilliant$^\circledR$ SYBR$^\circledR$ Green QPCR Master Mix, 5 μl of 10-fold diluted cDNA, 5 μl combined primer (2.5 μl forward and 2.5 μl reverse), 0.375 μl diluted ROX reference dye (final concentration = 300nm), and 2.125 μl DEPC treated water. The following thermal profile was used: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec; and finally 95°C for 1 min, 55°C for 3 min, and 95°C for 30 s.

Table 6-1**:** Primers of cacao ESTs related to defense used in QPCR analysis.

Putative gene function (EST designation)	Accession No.	Sequence (5' to 3')	Anticipated size (bp)
Actin (*TcACT*)	CF973918	F: CAGACTTTGAGTTCACTTGACACAG	200
		R: AGTGTCTGGATTGGAGGATCTATCT	
Lipoxygenase (*TcLOX*)	CF973956	F: ACATACTCAGTCACCCATTGTTTG	218
		R: TTGGCCAAGTATTATCTAGAGGTTG	
Mitogen-activated protein kinase 3 (*TcMAPK3)*	CF974368	F: TGAATAGAAAGCCTCTATTTCCTGG	204
		R: GAGATCAATAGCCAATGGATGAAC	
Mitogen-activated protein kinase kinase 4 (*TcMKK4)*	CF974565	F: CAAGCTCCATATATCTCCAAGGTAA	239
		R: CTTGCCTTACTTACATGCCCATA	
Pathogenesis-related protein 1 (TcPR1)		F: CACTCGGCCTCCACGTTG	
		R: CCAGAGGGCGATTTCAAGC	
Pathogenesis-related protein 4 (TcPR4)		F: ACGAATAATGGCTGGGTCATACC	
		R: GCCATTCCAAGGGCTTATCA	
Pathogenesis-related protein 6 (TcPR6)		F: TTTCTCGGAGGTCGGTTGTC	
		R: CACAAATGTAAGCGCAGAGCC	
Pathogenesis-related protein 10 (TcPR10)		F: TGCATTCGTAGAAACCTTGTGC	
		R: GTGAATGTAGGAGACATTTTGCAGA	
Osmotin-like (TcOsmotin-like)	AY766059	F: ATTAGATGCACGGCAGATATCATAG	242
		R: CAGAACACAACCCTGTAGTTAGTCC	
Cytochrome P450 (C.P450-ICS)	CAB214EST018	F: CTCAGGCGACACCCTCCA	
		R: GTCCACACCAACCCCATC	
ACC Synthase (TcACC)	CF974124	F: CTCATATCAGCCCAACAGTAGAAAC	243
		R: TTACTCTTTCAATGAGGAGGTTCTG	
Protein phosphatase 2C (TcPP2C)	CF972808	F: ACAGCTTCTTGATTGCTTAGGTG	215
		R: TACAGGTTTCCTAGATCTATCGGTG	
Nonexpressor of PR1 (TcNPR1)	CF974051	F: CTTGATTTATCAAACCATCCTCCTC	219
		R: AAGAATTTCCTGAAGAAGAGACTCC	
Chitinase 1 (TcChi1)		F: CAAAGCCTTCTTGCCACGAT	
		R: TGCCGCCTGGTCGCTA	
Chitinase 7 (TcChi7)		F:TCCCACTACCTGTATTACCTTGACC	
		R: TTAGCTCGTCATGTGGCCC	

At the end of the amplification reaction, a dissociation curve was run to ensure that only the genes of interest were amplified during the PCR reactions. The constitutively expressed ACTIN gene (CF973918) was used as an expression control. The data were analyzed as described by (Bae et al., 2005; Pfaffl, 2001).

Results

Preliminary tests to determine whether fungal and bacterial endophytes were antagonistic to each other

None of the bacteria tested were found to inhibit the growth of any of the *Trichoderma* spp. and none of the *Trichoderma* spp. inhibited the growth of bacterial colonies in *in vitro* plate assays (data not presented). When in mixed culture, none of the bacteria inhibited the germination of *Trichoderma* conidia as seen in Table 6-2.

Table 6-2: Mean percentage *Trichoderma* conidia that germinated when combined with bacterial isolates. Treatments are: bacterial control (B.Cntl), *Lysinibacillus sphaericus* A20, *Bacillus cereus* CT, *B. subtilis* CR, *B. pumilus* ET, *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), *T. koningiopsis* G.J.S. 01-07 (01-07), and *T. hamatum* 219b. Data are presented as percent of total conidia that germinated after 24 hours.

Treatment	% Germination
B.Cntl/T.Cntl	No spores
B.Cntl/320c	98.3
B.Cntl/01-07	91.7
A20/320c	100
CR/320c	96.9
CT/320c	95.6
A20/01-07	100
CR/01-07	100
CT/01-07	100
ET/219b	100

Experiment one: Inoculation of cacao of foliage with bacterial endophytes from seedlings colonization with *Trichoderma* spp

Microbial colonization of roots, stems, and leaves

Planting cacao seedlings into soil inoculated with *Trichoderma* spp. resulted in endophytic colonized of stems and roots. None of the *Trichoderma* spp. colonized cacao leaves in any of the experiments, regardless of whether plants were treated with bacterial endophytes or

not. Bacterial endophytes colonized stems and leaves, but not roots through foliar application. Fungal and bacterial endophytes were found to coexist in stems. In experiment one, *Bacillus* spp. were abundant in leaves both as total colonists (persisting in the epiphytic and endophytic leaf portions) (Fig 6-3A) and as endophytes (Fig 6-3B).
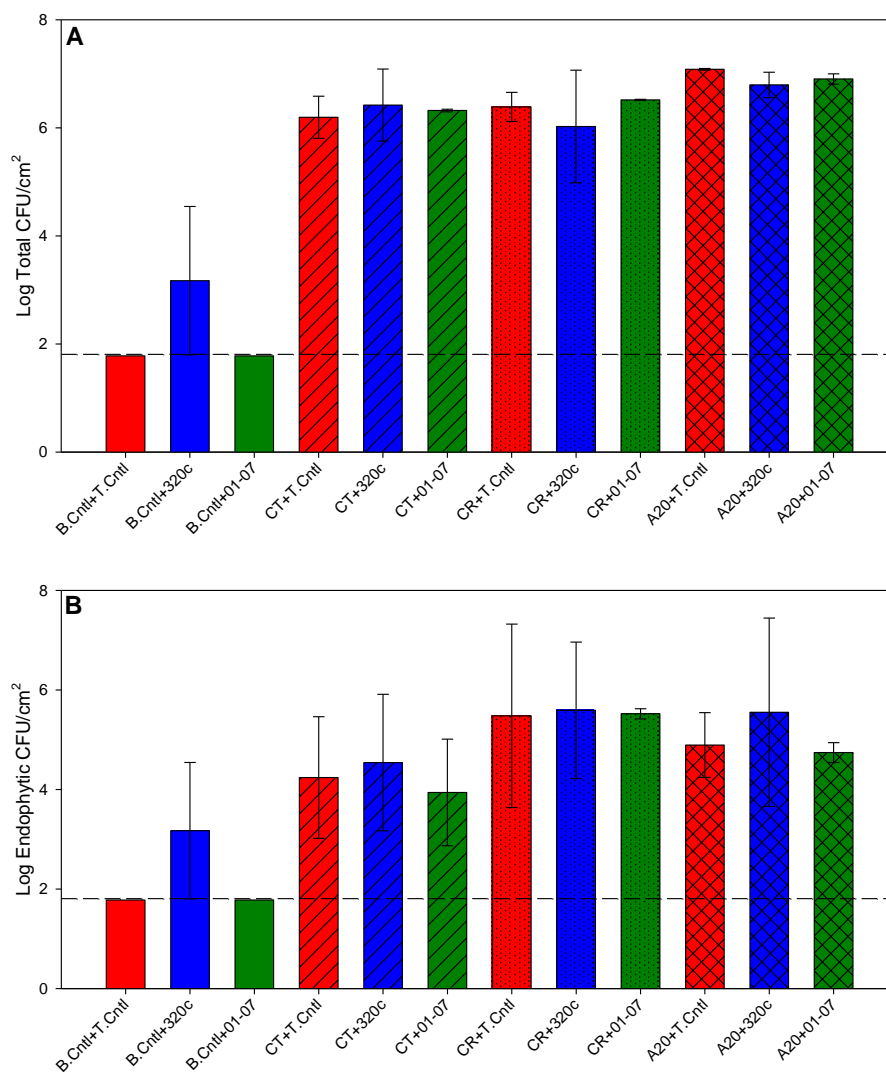
Figure 6-3: Mean bacterial colonization of cacao leaves from experiment one. Mean A) total colonists (epiphytes and endophytes) and B) endophytic colonists of mature green cacao leaves on seedlings grown in *Trichoderma* inoculated soil, and then inoculated with endophytic *Bacillus* spp. by spraying log 8.0 CFU/ml bacteria with 0.24% Silwet L-77 (vol/vol). Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Bacterial colonization was determined 46 days after planting of the seeds (29 days after inoculation with bacteria). Bars extending around the means indicate the standard error of that mean. The dashed line indicates the minimum detection level of the experiment (1.8 log

CFU/cm^2 leaf tissue); bars below this line indicate population levels in leaves that were too low to be detected.

There was no detectable bacterial colonization in the bacterial control plants (B.Cntl) except for plants that were colonized by 320c. Further investigation revealed that 320c mycelium was inhabited by a gram negative bacterium. Leaves colonized with 320c and an applied bacterial endophyte lacked the gram negative bacterial contaminant. In terms of bacterial colonization of foliage in the presence of fungal endophytes, all of the *Bacillus* spp. readily colonized the cacao leaves. The presence of *Trichoderma* spp. did not impact colonization of *Bacillus* spp., as population levels of the bacilli were similar between non-inoculated *Trichoderma* controls and *Trichoderma* treated seedlings.

Disease suppression due to colonization with cacao endophytes

Very low levels of disease developed on all leaves challenged with *P. megakarya* (Fig 6-4).
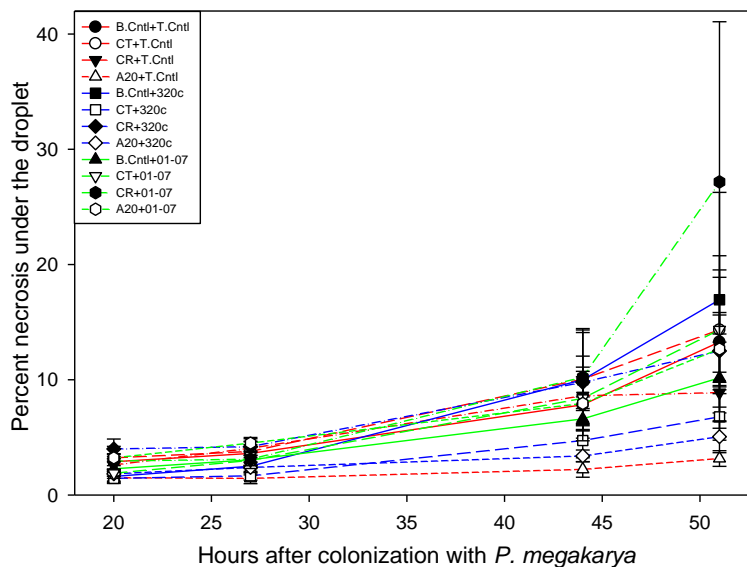
Figure 6-4**:** Mean percent necrosis under the 10 μl droplet of zoospores in experiment one. Cacao seeds were planted into soil inoculated with *Trichoderma* spp., then *Bacillus* spp. were sprayed at log 8.0 CFU/ml onto foliage with 0.24% Silwet L-77 29 days after planting. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl) *T. caribbaeum* var. *aequatoriale* DIS320c, and *T. koningiopsis* G.J.S. 01-07.   Immature leaves were detached from the plant then challenged with 50 zoospores/μl of *P. megakarya*. Bars extending around the means indicate the standard error of that mean.

Colonization of plants with A20 by itself or in combination with 320c appears to reduce *P. megakarya* development, especially at later observation times. When analyzed individually, no *Trichoderma* spp. or *Bacillus* spp had a significant impact on disease development when compared to the untreated controls (Fig 6-5). There was no significant interaction between the bacterial and *Trichoderma* treatments.
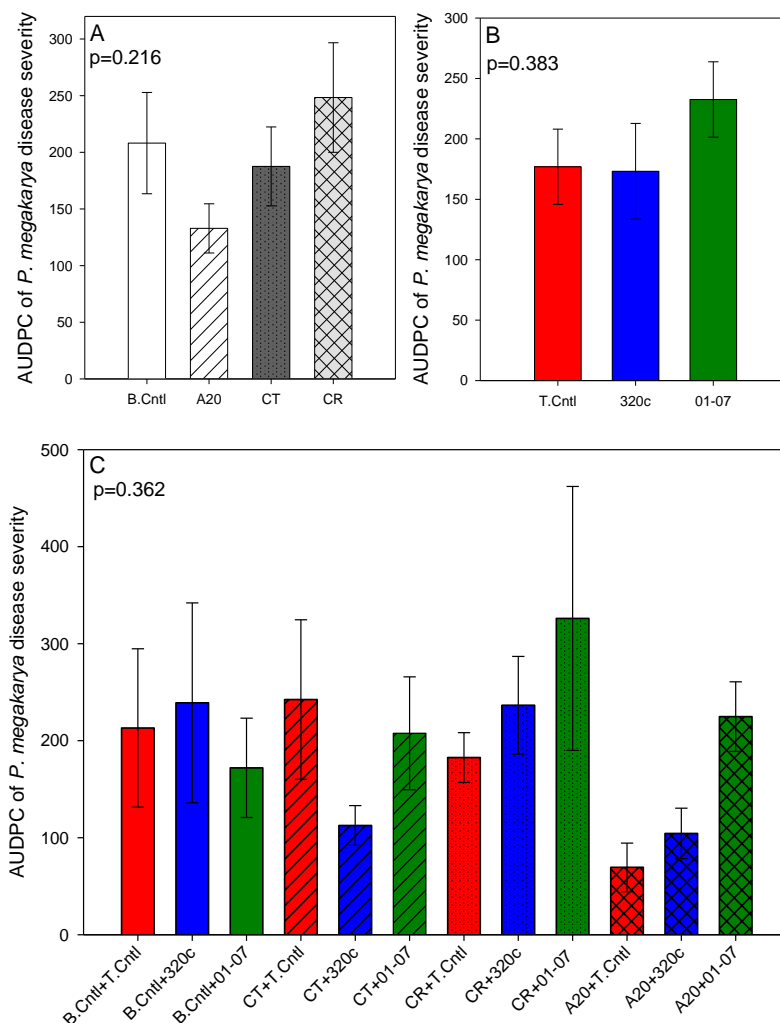
Figure 6-5: Area under the disease progress curve (AUDPC) for disease development of *Phytophthora megakarya* over time in experiment one for A) Bacillus treated, B) *Trichoderma* spp. treated, and C) Bacillus spp. + *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Cacao seeds were planted into soil inoculated with *Trichoderma* spp., and then cacao foliage was sprayed with 8.0 log CFU/ml bacterial suspension with 0.24% Silwet L-77 29 days after planting. Immature leaves were detached from the plant then challenged with 50 zoospores/10μl of *P. megakarya*. Bars extending around the means indicate the standard error of that mean.

Disease developed differently depending on whether *P. citrophthora* or *P. megakarya* was used as the challenge organism. There was a general trend that seedlings without *Trichoderma* colonists tended to have less disease than seedlings colonized with *T. koningiopsis* (Fig 6-6).
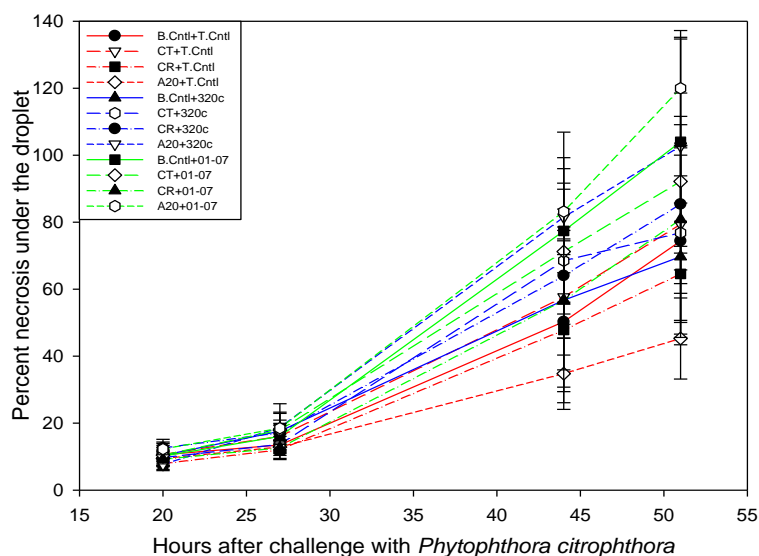
Figure 6-6: Mean percent necrosis under the 10 µl droplet of zoospores in experiment one. Cacao seeds were planted into soil inoculated with *Trichoderma* spp., then *Bacillus* spp. were sprayed at log 8.0 CFU/ml onto foliage with 0.24% Silwet L-77 29 days after planting. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl) *T. caribbaeum* var. *aequatoriale* DIS320c, and *T. koningiopsis* G.J.S. 01-07. Immature leaves were detached from the plant then challenged with 50 zoospores/µl of *Phytophthora citrophthora*. Bars extending around the means indicate the standard error of that mean.

In terms of AUDPC, colonization with bacterial or fungal endophytes had no significant impact on disease development either separately or in combination (Fig 6-7).

Figure 6-7: Area under the disease progress curve (AUDPC) for disease development of *Phytophthora citrophthora* over time in experiment one for A) Bacillus treated, B) *Trichoderma* spp. treated, and C) Bacillus spp. + *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Cacao seeds were planted into soil inoculated with *Trichoderma* spp., and then cacao foliage was sprayed with a bacterial suspension 8.0 log CFU/ with 0.24% Silwet L-77 29 days after planting. Immature leaves were detached from the plant then challenged with 50 zoospores/10μl of *P. citrophthora*. Bars extending around the means indicate the standard error of that mean.

Experiment Two: Planting cacao seeds inoculated with bacterial endophytes into soil colonized with *Trichoderma* spp.

Microbial colonization of roots, stems, and leaves

Populations of bacteria were not enumerated in leaves, but colonization was measured similar to fungi and reported as presence or absence of endophytic colonization. The morphology of the isolated bacteria was the same as applied bacteria. Planting cacao seeds into *Trichoderma* colonized soil resulted in colonization of roots and stems, but not foliage. Seed treatment with *Bacillus* spp. resulted in colonization of cacao roots, stems, and foliage. *Trichoderma* spp. and bacterial endophytes coexisted in roots and stems of inoculated seedlings.

Disease suppression due to colonization with cacao endophytes

None of the isolates of the bacteria or *Trichoderma*, whether applied individually or in combination, were capable of reducing disease severity of *P. megakarya* at any of the sampled times (Fig. 6-8).
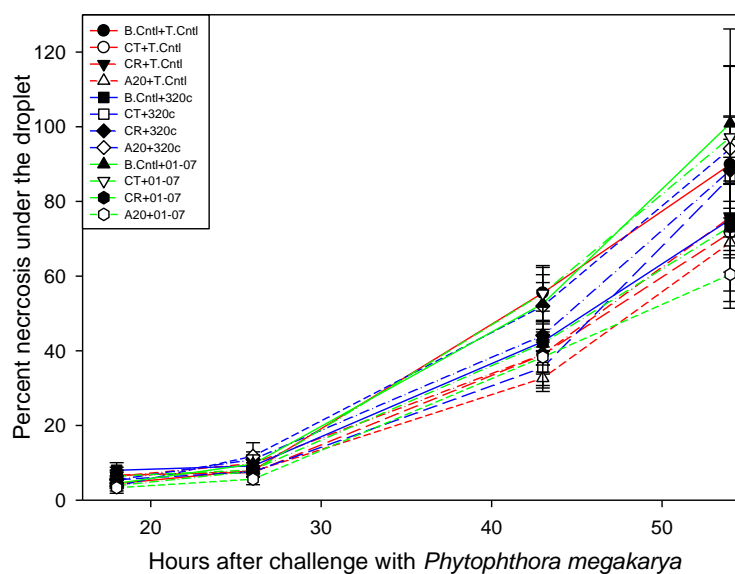
Figure 6-8: Mean percent necrosis under the 10 µl droplet of *Phytophthora megakarya* zoospores in experiment two 20 days after inoculation of seeds with bacteria. Cacao seeds were treated with log 6.0

CFU/ml bacterial and were then planted into soil inoculated with *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl) *T. caribbaeum* var. *aequatoriale* DIS320c, and *T. koningiopsis* G.J.S. 01-07. Immature leaves were detached from the plant, and then challenged with 50 zoospores/µl of *P. megakarya*. Bars extending around the means indicate the standard error of that mean.

Neither bacterial nor fungal endophytes reduced total disease severity of *P. megakarya* (Fig 6-9).
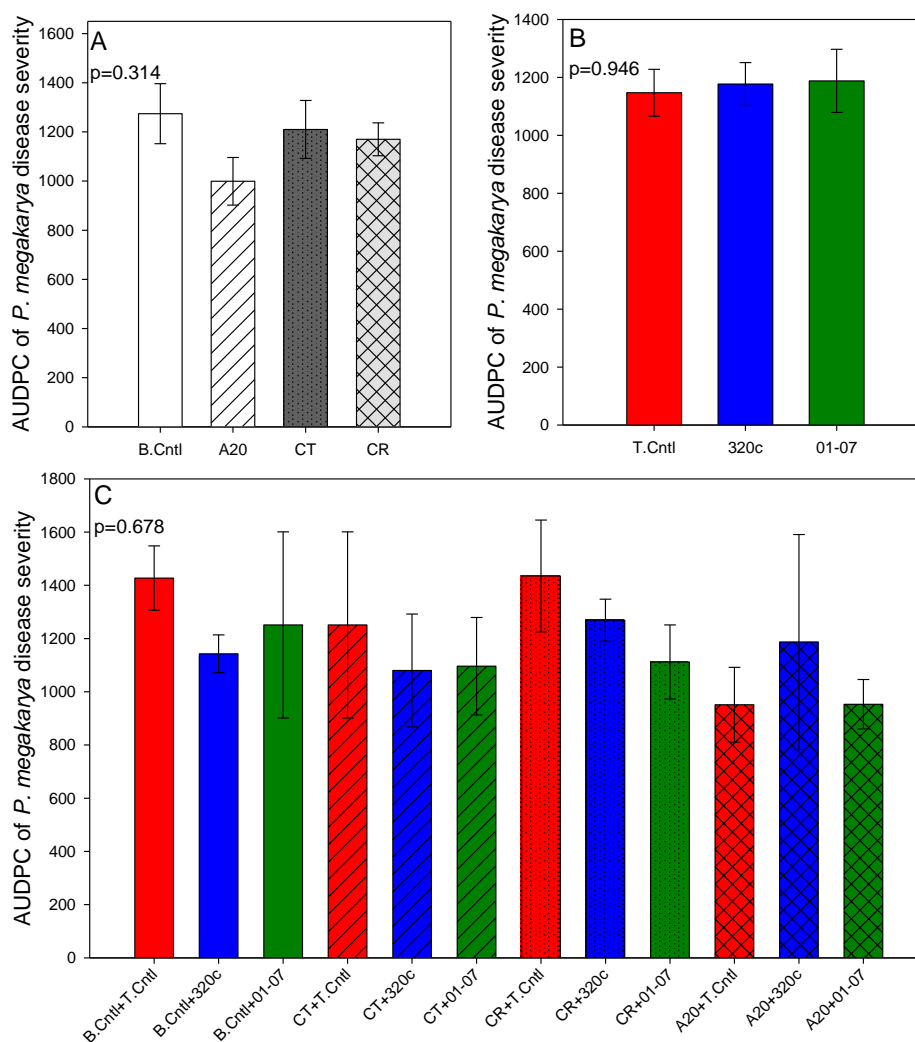
Figure 6-9: Area under the disease progress curve (AUDPC) for disease development of *Phytophthora megakarya* over time in experiment two 20 days after inoculation of seeds with bacteria for A) Bacillus treated, B) *Trichoderma* spp. treated, and C) Bacillus spp. + *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Cacao seeds were treated with log 6.0 CFU/ml bacterial suspension and then planted into soil inoculated with *Trichoderma* spp. Immature leaves were detached

from the plant then challenged with 50 zoospores/10µl of *P. megakarya*. Bars extending around the means indicate the standard error of that mean.

Colonization of cacao seedlings with A20 in combination with *Trichoderma* 01-07 resulted in low levels of disease near the end of the disease challenge (Fig 6-10).
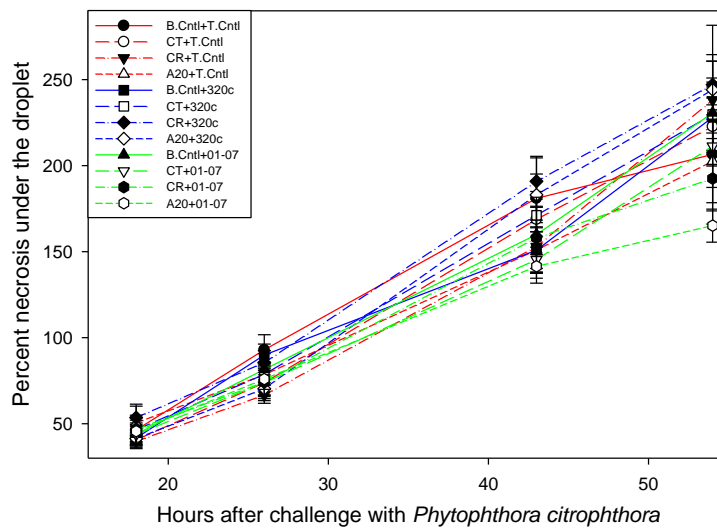
Figure 6-10: Mean percent necrosis under the 10 µl droplet of *Phytophthora citrophthora* zoospores in experiment two 20 days after inoculation of seeds with bacteria. Cacao seeds were treated with log 6.0 CFU/ml bacterial and were then planted into soil inoculated with *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl) *T. caribbaeum* var. *aequatoriale* DIS320c, and *T. koningiopsis* G.J.S. 01-07. Immature leaves were detached from the plant, and then challenged with 50 zoospores/µl of *P. citrophthora*. Bars extending around the means indicate the standard error of that mean.

None of the treatments, either bacterial or fungal, reduced overall disease severity of *P. citrophthora* (Fig 6-11). Also the additive effect of combining fungal and bacterial endophytes did not significantly suppress severity of *P. citrophthora*.
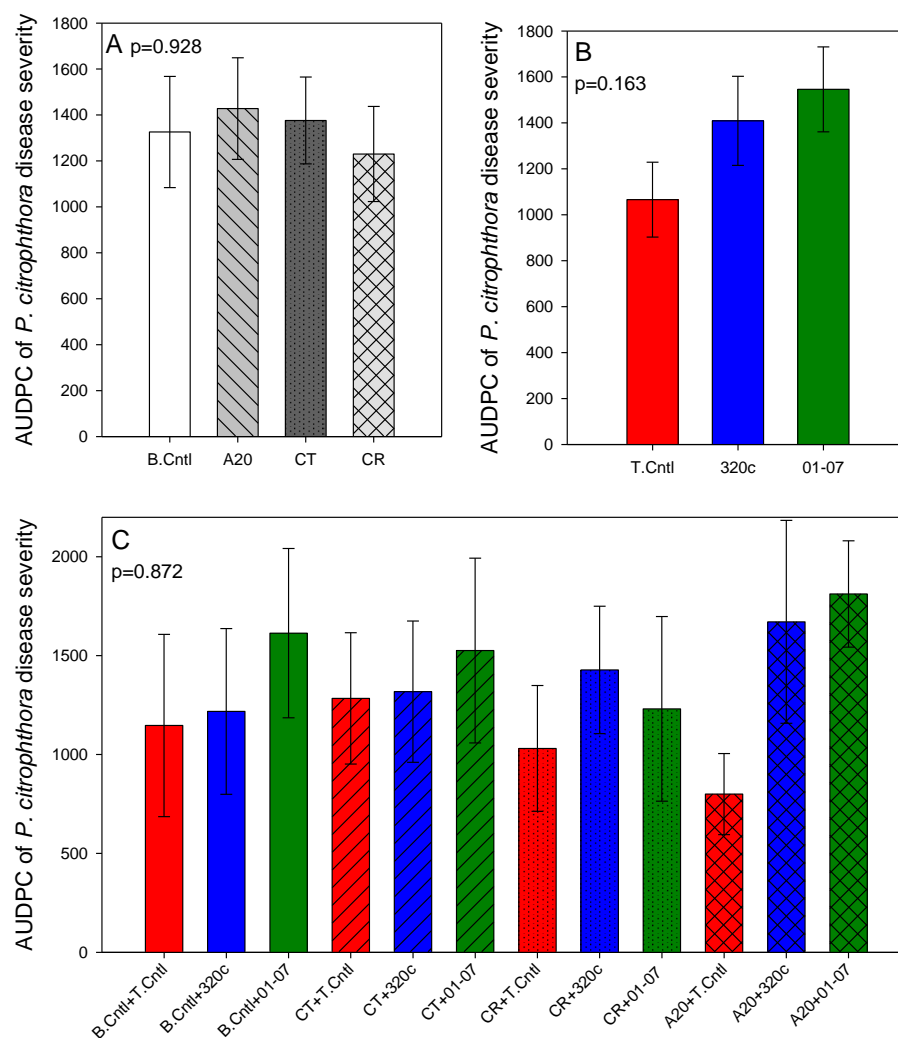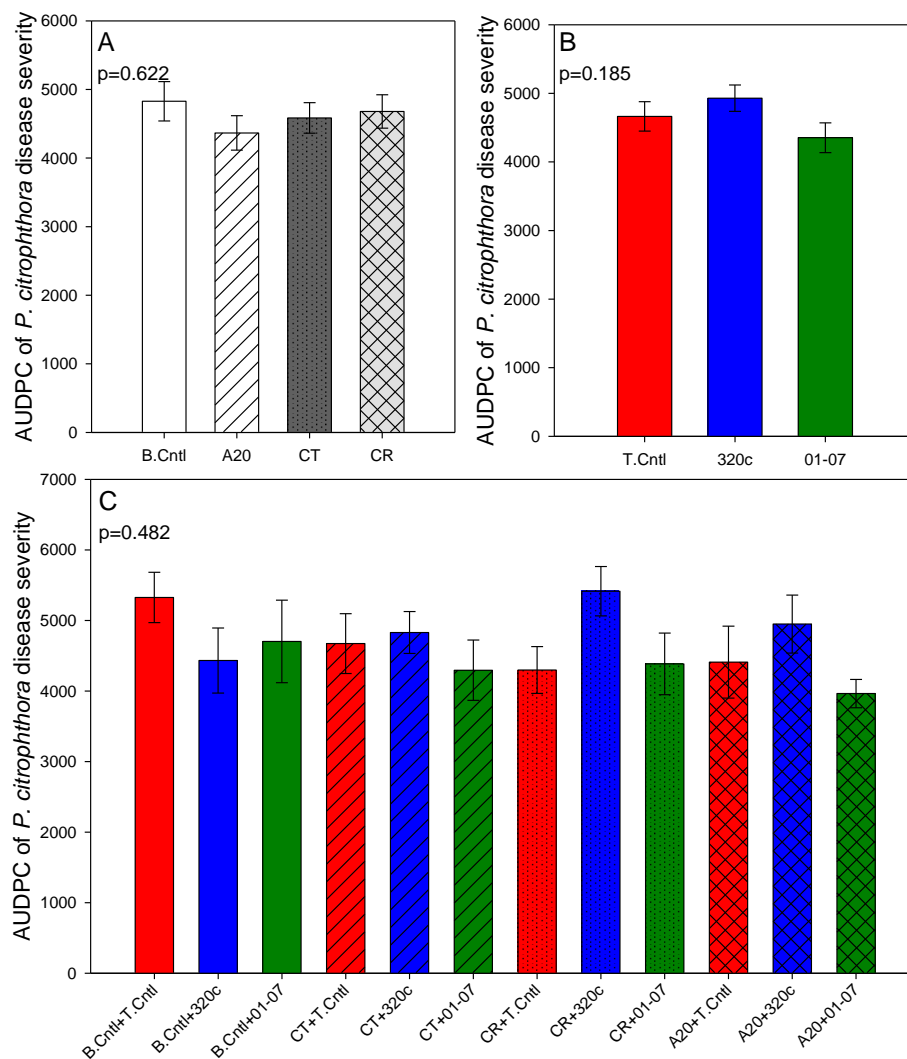
Figure 6-11: Area under the disease progress curve (AUDPC) for disease development of *Phytophthora citrophthora* over time in experiment two 20 days after inoculation of seeds with bacteria for A) Bacillus treated, B) *Trichoderma* spp. treated, and C) Bacillus spp. + *Trichoderma* spp. Bacterial treatments were as follows: B.Cntl (Silwet control), *Bacillus cereus* CT, *Bacillus subtilis* CR, and *Lysinibacillus sphaericus* A20. *Trichoderma* treatments are *Trichoderma* control (T.Cntl), *T. caribbaeum* var. *aequatoriale* DIS320c (320c), and *T. koningiopsis* G.J.S. 01-07 (01-07). Cacao seeds were treated with log 6.0 CFU/ml bacterial suspension and then planted into soil inoculated with *Trichoderma* spp. Immature leaves were detached from the plant then challenged with 50 zoospores/10μl of *P. citrophthora*. Bars extending around the means indicate the standard error of that mean.

Experiment three: Inoculation of cacao foliage with *B. pumilus* ET and root inoculation of *T. hamatum* 219b spp.

Microbial colonization of roots, stems, and leaves

The presence or absence of endophytic colonization by *B. pumilus* ET and *T. hamatum* 219b of stems and leaves are reported in Table 6-3. ET and 219b coexisted in the endophytic environment of roots and stems, while neither were detected colonizing immature leaves. All replicates had the same results.

Table 6-3: The presence of endophytic colonization of stems, leaves, and roots by *T. hamatum* 219b and *B. pumilus* ET.

Bacteria	Fungus	Fungi Stem	Bacteria Stem	Fungi Leaf	Bacteria Leaf	Fungi Root	Bacteria Root
B.Cntl	T.Cntl	no	no	no	no	no	no
ET	T.Cntl	no	yes	no	no	no	yes
B.Cntl	219b	yes	yes	no	no	yes	no
ET	219b	yes	yes	no	no	yes	yes

Sampling was conducted to enumerate the populations of ET colonizing immature cacao leaves, but bacteria were not isolated. This was likely due to high levels of bleach used in the sterilization process, as leaves appeared to be infiltrated by the bleach.

Disease suppression due to colonization with cacao endophytes

Colonization of plants with endophytes did not significantly suppress disease when challenged with *P. capsici* (see Fig 6-11), but there is a general trend that colonization with *B. pumilus* ET either by itself or in combination with *T hamatum* 219b reduced disease when compared to untreated control plants (Fig 6-12).
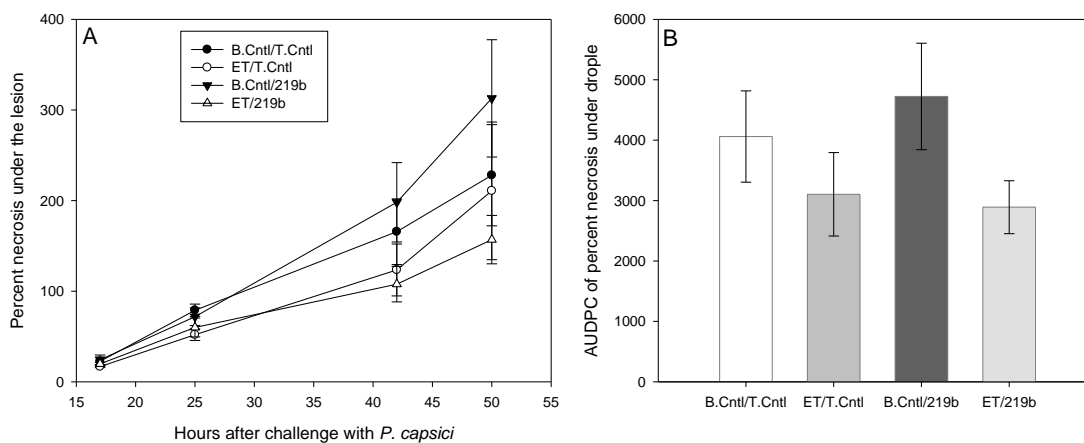
Figure 6-12: A) Mean disease severity of immature cacao leaves challenged with *Phytophthora capsici* zoospores (50 zoospores/10μl) in experiment three 50 days after planting of cacao seeds. Graph A) represents the percentage of necrosis under the 10 μl droplet when rated every 8-12 hours and B) is the AUDPC of graph A. Sterilized cacao seeds were planted into soil inoculated with *T. hamatum* 219b were inoculated with 8.0 log CFU/ml *B. pumilus* ET with 0.24% Silwet L-77 through spray application one month after planting. Immature leaves were detached from the plant then challenged with 50 zoospores/μl of *P. capsici.* Bars extending around the means indicate the standard error of that mean.

Although colonization did not cause significant disease suppression it did impact the expression of some of the tested cacao ESTs related to defense (Fig 6-13).
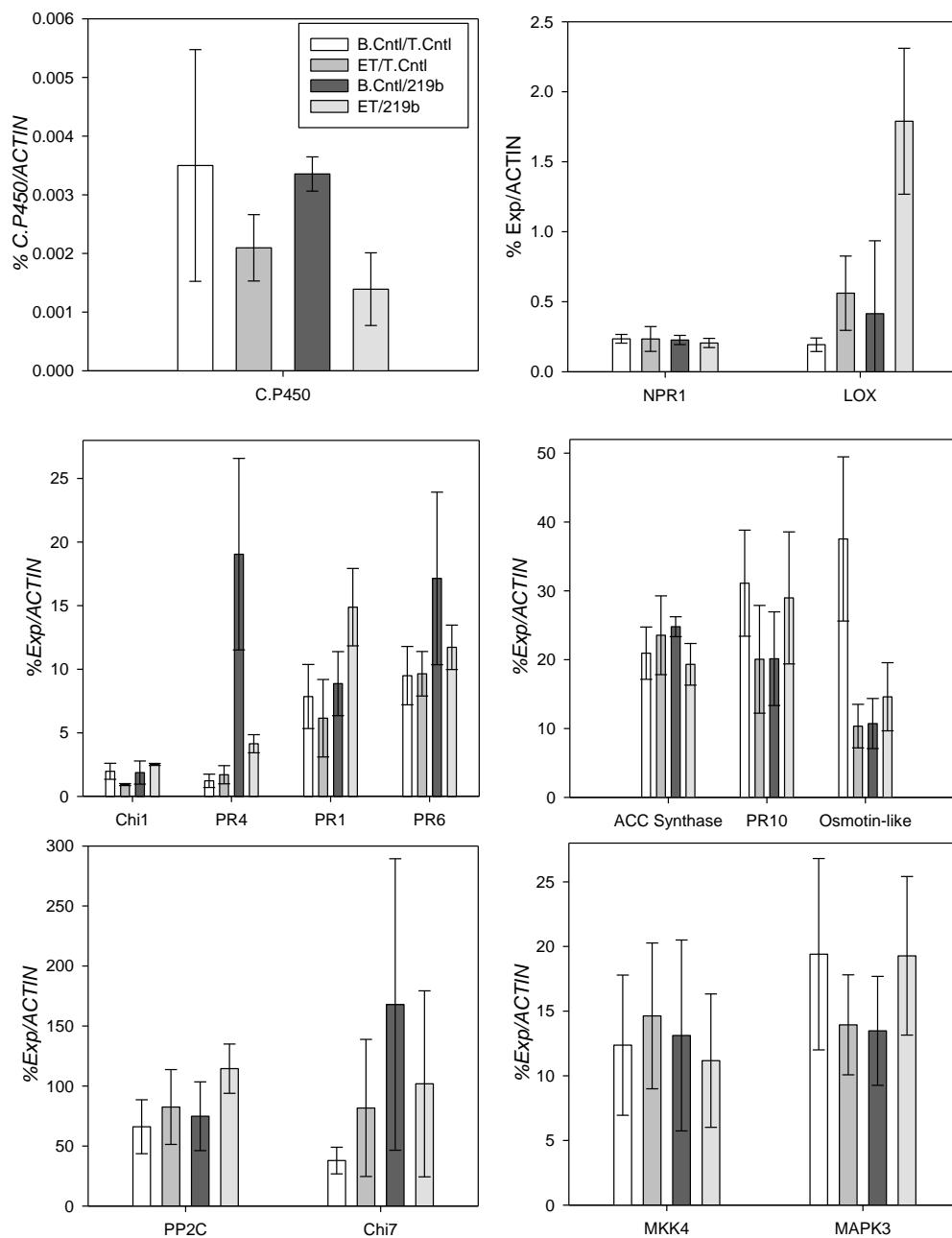
Figure 6-13: Mean expression of cacao ESTs putatively involved in cacao defense pathways in leaves of two-month old cacao seedlings grown from seeds planted into soil inoculated with *T. hamatum* 219b and treated with foliar application of 8.0 log CFU/ml *Bacillus pumilus* ET with 0.24% Silwet L77 one month after planting. Treatments are endophyte free (B.Cntl/T.Cntl), ET/*Trichoderma* control (ET/T.Cntl), bacterial control/219b (B.Cntl/219b), and treated with both ET and 219b (ET/219b). Mean relative expression of mRNA of *TcC.P450*, *TcNPR1*, *TcLOX*, *TcChi1*, *TcPR4, TcPR1, TcPR6, TcACC Synthase*, *TcPR10, TcOsmotin-like, TcPP2C*, *TcChi7*, *TcMKK4*, and *TcMAPK3* is reported for the 5 replicate seedlings calculated with respect to cacao actin and presented as percentage expression of the EST compared to *ACTIN*. Bars extending around the means indicate the standard error of that mean.

Colonization of cacao seedlings with both ET and 219b increased the expression of the LOX, which did not occur when singular isolates were introduced. *T. hamatum* 219b increased expression of PR4 when colonizing seedlings by itself, but not when combined with *B. pumilus* ET. All endophytes decreased the expression of the osmotin-like EST. The expression of the other cacao ESTs were not impacted by the colonization by the endophytes, either by themselves or when combined.

Discussion

The colonization data from all three experiments indicates that both bacterial and fungal endophytes were capable of co-inhabiting cacao tissue. All bacterial endophytes colonized cacao leaves and stems both when applied to seeds and when sprayed onto foliage with 0.24% Silwet. In the stems and roots, regardless of the isolates used, the bacterial endophytes coexisted with fungal endophytes except when bacteria were applied to foliage as they did not colonize roots. *Trichoderma* spp. were not capable of colonizing leaves with or without the presence of the bacteria, therefore the lack of colonization was either due to the capabilities of the fungus or to the application methodology. These results indicate that bacterial and fungal endophytes can coexist in cacao trees and that there is potential to develop a biological control formulation for cacao diseases that is a combination of beneficial bacterial and fungal species. The species tested probably compete for resources such as space and nutrients when inhabiting cacao tissue, but would likely survive together as a part of a larger microbial community inhabiting cacao trees. Previous work has suggested that applying combinations of microbial agents to plants can result in better overall disease suppression through either synergistic or additive effects (Guetsky et al., 2002; Yu et al., 2006). Formulations of the multiple microbes might better suppress disease in the field through utilizing multiple modes of action as well as more efficiently displacing pathogenic microbes from the community.

The presence of the gram negative bacteria associated with *T. caribbaeum* var. *aequatoriale* DIS320c was an interesting finding. Colonies grown on CMDA did not have the typical "wet" appearance, indicative of a bacterial contamination, as CMDA is not conducive to bacterial growth. It was not until the fungus was grown on TSA, after the establishment of the experiment, that the bacterial contamination was recognized. This bacterium was capable of endophytically colonizing the cacao tissue inoculated at 320c, but work was not done to elucidate

whether it did so through inhabiting the hyphae of the fungus or whether the bacteria persisted in the tissue by itself. It should be noted that the bacterium contaminating 320c was not detected when plants were inoculated with the bacterial endophytes, either through spray application or seed treatment. The lack of the gram negative bacteria in the presence of the introduced bacteria indicate that the bacterial contaminate of the hyphae could not persist and actively grown in the presence of the native cacao bacteria. This could be due to the inability of the gram negative bacterium to compete with the bacterial endophytes or due to an antagonistic reaction to the introduced species.

The introduction of the beneficial microbes never significantly suppressed the development of *Phytophthora* lesions in detached leaf assays, regardless of the *Phytophthora* spp. used. These results could be due to the controlled environment that was required to follow APHIS quarantine restrictions. It is possible, as seen in Chp. 5, that challenging entire treated plants with zoospores could have resulted in disease suppression, even when it was absent in detached leaves. However, this methodology is difficult to accomplish, due to the destructive sampling necessary for determining endophytic colonization, the limited numbers of plants, and space available in growth chambers. Additionally, detached leaves are wounded and not indicative of a leaf found in nature. The difficulty in screening biological control agents of cacao diseases is the obstacle of developing assays for pathogen infection. Work with *Phytophthora* spp. has focused on root, twigs, branches, and detached leaves (Nyassé et al., 2002; Tahi et al., 2006; Tahi et al., 2000). This methodology is artificial, but does not require a large amount of pods and can be done using young seedlings in the period before flowering and fruit onset. The artificial nature of the bioassay used in these experiments, although the best assay under the conditions and regulations, could have confounded any beneficial suppression of the pathogen that the endophytes induced.

In terms of induction of cacao ESTs related to plant defense, neither ET nor 219b had a significant effect on expression of the tested ESTs in cacao seedlings. Seedlings colonized with 219b had increased expression of PR4, but this was not seen when colonized with both ET and 219b. Colonization with both ET and 219b increased the expression of LOX. This increase was likely due to an additive effect of the isolates, as neither ET nor 219b increased expression of LOX when colonizing seedlings alone. Additionally, colonization of seedlings with any isolate reduced the expression of the osmotin-like EST, suggesting that endophytic colonization by the tested isolates may reduce the expression of this antifungal protein (Yun et al., 1998). Overall, the lack of impact on levels of gene expression could have resulted in the lack of disease

suppression due to colonization with the tested microbes. If the endophytes are not inducing resistance, they may not have been able to suppress this disease. Beyond gene expression, the lack of disease suppression may also have been due to the pathogen used. *T. hamatum* metabolites were seen to suppress the growth of *M. roreri* in plates and the isolate appeared to be an effective mycoparasite of *M. roreri* in *in vitro* assays (Bailey et al., 2008), but this isolate was not tested against *Phytophthora* spp. Additionally, all bacterial isolates tested were antagonistic to *P. capsici* in *in vitro* assays in Chapter 2, but this did not translate to significant disease suppression in this experiment. All tested bacterial isolates suppressed disease development of *P. capsici* lesions on detached cacao 'ICS1' leaves in previous studies (see Chp. 2), but these experiments were conducted with more mature plants grown a non-sterile environment. The lack of suppression in the current studies readily could have been due to experimental differences, the differing plant genotypes, and/or differing plant age, amongst other variations.

Future work should focus on testing a wider variety of microbial combinations to find the right mixture to reduce disease. There are a range of endophytes which have been shown to reduce disease (Krauss and Soberanis, 2001; Mejía et al., 2008) and working to combine these isolate could be highly beneficial to disease management. Additionally, future work should focus on field testing of microbial combinations. Due to the nature of the diseases caused by *Moniliophthora* spp., field tests are better to screen isolates than laboratory testing. Since *M. roreri* only infects pods it is not possible to test biological control agents against this disease in seedlings or even young trees. *M. perniciosa* infections can be obtained in controlled settings (Purdy et al., 1997), but these inoculations rarely result in 100% disease incidence. Witches' broom symptoms also take several weeks (~12) to develop, which makes screening a large number of isolates difficult due to the large requirements of both space and time. With both of these diseases, small-scale studies, like those in Chp. 3 and Chp. 4, would be the best way to test the effectiveness of the microbial combination. Overall, further research is needed on the combinations of microbes, as research presented in this chapter indicates that fungal and bacterial endophytes could successfully be introduced to cacao plants and coexists in cacao tissues.

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Appendix A

Melnick, R.L., Solis, K.S., Suárez, C., and Backman, P.A. 2008. Field evaluation of endophytic endospore-forming bacteria with cacao successions for management of witches' broom of cacao during the dry season, 2007. Plant Disease Management Reports 2:V136.

This trial was conducted using clones of four varieties of cacao at the Tropical Experimental Research Station of INIAP in Pichilingue, Ecuador. The four varieties included the: susceptible clone (EET-19), moderately resistant clones (A2162 and A2634) and resistant clone (CCN-51). On 23 May, five bacterial treatments consisting of endophytic endospore-forming bacteria previously isolated from trees on the INIAP station were applied to six month old greenhouse grown plants. The bacterial treatments applied to each variety were: 1) no bacteria, Silwet control; 2) A2076 5.1.7; 3) CCAT1858 2.1.2; 4) CUR3 3.1.1; and 5) EETht103 2.1.1. Bacterial suspensions were applied at log 8.0 CFU/ml in water with 0.20% Silwet L-77 using hand-held aerosol sprayers. On 13 Jun, all inoculated plants were placed in the field three weeks after treatment in a randomized nested block design with four replicates. To best standardize natural infection, potted trees were placed under larger trees with high levels of witches' broom infection. Witches' broom incidence was determined on 25 Sep and 21 Nov. Mean separation was conducted using Tukey's honestly significant difference test (HSD) (α=0.05).

Cacao variety had a significant effect on disease severity, with reduced disease observed on susceptible and resistant cacao successions. There were no significant differences in disease severity between varieties treated with bacterial endophytes and the Silwet treated control plants at either rating date. Visual observations suggest bacterial treatments did not decrease disease, due to low levels of disease incidence at the trial site during the dry season. However, none of the plants colonized by endospore-forming bacterium CCAT1858 2.1.2 were diseased in any of the cacao lines, indicating a likely trend towards disease suppression. There was no significant interaction between cacao variety and bacterial treatment.

		Average disease severity by bacterial treatment at two dates[z]									
		Control		A2076 5.1.7		CCAT1858 2.1.2		CUR3 3.1.1		EETht103 2.1.1	
Variety/ Statistics		Sep	Nov	Sep	Nov	Sep	Nov	Sep	Nov	Sep	Nov
A2126	A[y].....	0 a[y]	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
A2634	A......	0 a	0 a	0.5 a	0.8 a	0 a	0 a	0 a	0 a	0 a	0 a
CCN-51	A......	0 a	0 a	0 a	0 a	0 a	0 a	0.3 a	0.3 a	0 a	0.5 a
EET-19	B......	2.0 a	3.0 a	0.5 a	1.8 a	0 a	0 a	0.5 a	0.5 a	0.3 a	0.3 a

[z] Disease severities were measured using the following scale: 0 – no disease present; **1**-infection of one lateral or axillary shoot; **2**-infecton of 2 lateral or axillary shoots or infection of on terminal shoot; **3**-infection of both the lateral and terminal shoots, infection of 3 terminal shoots, or infection of terminal shoot with resulting stem swelling; **4**-infection of 2 lateral shoots with infection of terminal shoot or infection of 4 terminal shoots; **5**–Presence of dry brooms on the plant; 6 – plant killed due to witches' broom

[y] Bacterial treatment means followed by the same lower case letter within columns and cacao variety followed by the same capital letter indicate treatments that are not significant different according to Tukey's HSD (P≤0.05).

Appendix B

Melnick, R.L., Solis, K.S., Vera, D.I., Suárez, C., and Backman, P.A. 2009. Field evaluation of four endophytic *Bacillus* spp with five cacao varieties for management of witches' broom, 2008. Plant Disease Management Reports, 3:V146.

This trial was conducted at the Tropical Experimental Station of INIAP in Pichilingue, Ecuador. The five varieties were the susceptible open pollinated IMC67 and four clonal varieties: susceptible clone EET19, moderately resistant clones A2162 and A2634, and resistant clone CCN51. On 7 Jan five bacterial treatments consisting of endophytic *Bacillus* spp. previously isolated from trees on the INIAP station were applied to one year old plants in the field in a randomized, nested block design with four replicates. Bacterial suspensions were applied to runoff at log 8.0 CFU/ml in 0.1M phosphate buffer with 0.20% Silwet L-77 using a hand-held aerosol sprayer. Bacteria were reapplied on 20 Mar to maintain population levels throughout the rainy season. On 30 May, plants were pruned to remove infected tissue and bacteria were reapplied to trees. Trees were maintained under a canopy of larger trees with high levels of witches' broom infections to provide a naturally timed release of infective basidiospores. The bacterial treatments applied to each variety were: 1) no bacteria, Silwet control; 2) A20; 3) CT; 4) CUR; and 5) ET. Witches' broom severity was determined on 7 Feb, 7 Mar, 21 Apr, and 21 May during the rainy season and on 21 Sep and 3 Dec during the dry season. Area under the disease progress curve was determined using ratings made from 7 Feb to 21 May and the Sep and Dec severity. Mean separation was conducted for both measurements using Tukey's (HSD (α=0.05).

Cacao variety had no significant effect on disease severity throughout the rainy season. Treatment had a significant effect on disease severity, with application of *Bacillus pumilus* ET reducing disease throughout the rainy season when compared to control plants. There was no significance due to clone or treatments during the dry season, except that tolerant nacional variety A2634 had less disease than the susceptible EET-19 in Sep, but not Dec. There was no significant variety x treatment interaction during the rainy or the dry seasons, therefore the AUDPCs presented are averages across all plants of a variety or treatment.

Variety	Rainy Season (Jan 08 - May 08) AUDPC of disease severity	Dry Season (Jun 08 - Dec 08) Disease severity Nov 08
A2126…….	65.7 a[z]	0.94 ab
A2634…….	61.1 a	0.42 a
CCN51…...	110.9 a	0.45 ab
EET19…….	92.3 a	1.50 b
IMC67……	122.7 a	0.42 ab
Treatments		
Control…..	77.4 a	1.10 a
A20……...	104.2 a	0.50 a
CT……...	74.8 a	0.55 a
CUR...…...	103.8 a	0.55 a
ET…..……	32.8 b	0.94 a

[z] Disease severities were measured using the following scale: 0 – no disease present; 1-infection of one lateral or axillary shoot; 2-infecton of 2 lateral or axillary shoots or infection of on terminal shoot; 3- infection of both the lateral and terminal shoots, infection of 3 terminal shoots, or infection of terminal shoot with resulting stem swelling; 4-infection of 2 lateral shoots with infection of terminal shoot or infection of 4 terminal shoots; 5–Presence of dry dead brooms on the plant; 6 – plant killed due to witches' broom

[y] Bacterial treatment and cacao variety means followed by the same lower case letter indicate treatments that are not significant different according to Tukey's HSD (P≤0.05).

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