Chapter 3
Bacterial Endophytes of Perennial Crops for Management of Plant Disease

Rachel L. Melnick, Bryan A. Bailey, and Paul A. Backman

3.1 Endophytes

Internal plant tissues are far from a sterile environment, as nearly all tested plant species have been found to be colonized by microbes persisting as epiphytes and endophytes. Endophytes are microorganisms that inhabit the internal tissues of a plant without causing disease (Surette et al. 2003). The term "endophyte" was developed from "endotroph," used to describe the endomycorrhizal association (Frank 1885) and also defines ferns colonized with endophytic algae (Campbell 1908). Endophytes are known to inhabit seeds, ovules, root, stems, leaves, and branches. Endophytes form a unique association with their plant host that can be neutral or beneficial to the plant. Endophytes reduce herbivory (Koh and Hik 2007), promote plant growth (Taghavi et al. 2009), increase mineral uptake (Malinowski et al. 2000), biologically fix nitrogen (Doty et al. 2009; Stoltzfus et al. 1997), suppress disease (Bae et al. 2011; Melnick et al. 2008; Kloepper et al. 2004), and induce plant defense cascades (Bae et al. 2011; Bailey et al. 2006; Kloepper et al. 2004; Melnick et al. 2011).

The occurrence of endophytic bacteria has been known for over 60 years (Hollis 1951). Bacterial endophytes colonizing the intercellular space of plants have been isolated from nearly every plant species sampled (Ryan et al. 2008). Endophytes are associated with perennials such as cacao (Melnick et al. 2011), spruce (Cankar et al. 2005), and diverse Atlantic timber species (Lambais et al. 2006); biennials such as sugar beets (Bargabus et al. 2002) and carrots (Surette et al. 2003); and annuals such

R.L. Melnick (✉) • B.A. Bailey
Sustainable Perennial Crops Lab, USDA Agricultural Research Service, Beltsville, MD, USA
e-mail: rachelmelnick@gmail.com

P.A. Backman
Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802, USA

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as corn (McInroy and Kloepper 1995) and potatoes (Sessitsch et al. 2002), among many other plant species. Bacterial endophytes have been isolated from nearly all plant tissues, including pollen (Madmony et al. 2005) and ovaries (Sugawara et al. 2004). There tends to be higher total endophyte populations in roots than in aerial plant tissues (Rosenblueth and Martínez-Romero 2006; Hallman et al. 1997). Additionally, due to the availability of free water in the phyllosphere (leaf surface), epiphytic leaf populations are typically more numerous than endophytic populations (Beattie and Lindow 1999).

Microbes, in addition to colonizing plants, can also colonize the internal tissues of other microorganisms without causing disease. Bacteria and viruses have been isolated inhabiting the hyphae of fungal endophytes, indicating the potential for beneficial tritrophic endophytic interactions (Hoffman and Arnold 2010; Roossinck 2011). An additional tritrophic interaction is the endophytic colonization of bacterium-induced root nodules by a second distinct bacterium (Bai et al. 2002). Bacterial endophytes that lack the ability to cause nodules were found to inhabit root nodules of red clover (Sturz et al. 1997) and soybean (Bai et al. 2002). *Bacillus cereus* UW85 is not a root nodulating bacterium, but seed treatment of soybeans with this isolate increased root nodules formed by another bacterium, suggesting that there may be synergistic effects of a diverse bacterial community in root nodules (Halverson and Handelsman 1991). Turner and Backman (1991) found similar results for *B. subtilis* (GB03) treated peanut plants. For a more extensive review of bacterial endophytes and their associated hosts, see Hallman et al. (1997), Berg and Hallmann (2006), and Rosenblueth and Martínez-Romero (2006).

Gram-negative endophytic bacteria are easier to isolate, culture, and study, and have therefore been more intensively studied than Gram-positive bacteria. Endospores are formed by some Gram-positive bacteria such as *Bacillus* spp. and allow the organisms to survive in extreme environments (Driks 1999). The spore coat of the endospore is composed predominately of proteins rich in tyrosine and cystine (Driks 1999). Additionally, the DNA is saturated by small acid-soluble proteins protecting the genetic material (Driks 2004). Endospores allow gram-positive bacteria to survive extreme conditions, such as the high radiation zone in the area immediately surrounding the Chernobyl, Ukraine, disaster (Zavilgelsky et al. 1998). Agricultural activities cause stress to native soil microbial communities, and these stresses can also select for endospore-forming isolates. As a result, the dominance of endospore-forming bacteria can be a bioindicator of agricultural history (Nilsson and Renberg 1990). Due to the resistant nature of endospores, they are often sought after for commercial products. Endospore-forming bacteria are easy to formulate, can be combined in formulated products with agrochemicals, surfactants, etc., or during application, and have a long shelf life (Francis et al. 2010; Fravel 2005). Species such as *Bacillus* and *Streptomyces* are readily culturable using traditional microbiological techniques, resulting in the skewed isolation and overrepresentation of this group of bacteria from environmental samples.

3.1.1 Diversity of Bacterial Endophytes

Several other factors can impact the diversity of bacterial endophytes including plant genotype, plant tissue, and plant age. According to Adams and Kloepper (2002), different cotton genotypes had large differences in bacterial population levels and the diversity of the bacterial communities present in seeds, stems, and roots. The radicles of germinating cotton varieties Auburn 56 and Rowden were more highly colonized by endophytic bacteria than were other tested genotypes. Genotype Deltapine 50 had a higher population density of endophytic bacteria than all other tested genotypes when grown in the field, suggesting that the genetic, morphological, or physiological differences are important in determining endophytic bacteria populations (Adams and Kloepper 2002). The community of stem-colonizing bacterial endophytes differed between two thermophilic sweet pepper cultivars. The predominant bacterial genera in cultivar Milder Spiral were *Microbacterium*, *Micrococcus*, *Rhodococcus*, and *Staphylococcus*, while the predominant species in cultivar Ziegenhorn Bello were *Staphylococcus aureus* and *Bacillus* spp. (Rasche et al. 2006).

Plant tissues offer different environments to endophytes due to differences in nutrient availability, environmental fluctuations, etc. The distinct environments in these spatially and physiologically different tissues result in qualitative and quantitative differences in bacterial community membership when niches are compared throughout a single plant. In isolations of bacterial endophytes from Chardonnay grapes, *Curtobacterium* spp., *Pseudomonas* spp., and *Streptomyces* spp. were isolated from cane and leaf tissue, but were absent from the trunk and roots. The trunk and roots were home to a totally different collection of species within the bacterial community (West et al. 2010). Endophytic bacterial communities of *Betula pendula*, *Pinus silvestris*, and *Sorbus aucuparia* differed between roots and leaves/stems (Izumi et al. 2008) of the trees. In addition to differences in composition of communities between plant tissues, some tissues are not readily inhabited by endophytes. Endophytic bacteria and fungi were isolated from leaves of several different cultivars of citrus, but all sampled seeds lacked endophytes (Araújo et al. 2001). The lack of bacterial endophytes in citrus seed is an exception. Bacterial endophytes have been detected in both coffee and cacao seeds (Posada and Vega 2005; Vega et al. 2005). In the coffee study, they also found that of all the coffee berry tissues sampled, seeds had the highest populations of endophytic bacteria, including both gram-negative and gram-positive species. Mundt and Hinkle (1976) isolated endophytic bacteria from the seeds and ovules of 27 different plant species. These seeds did not appear to be home to a diverse range of culturable bacteria, as 93 % of seeds samples were inhabited solely by one culturable species, although this species was variable between seeds of the same and different plants.

3.1.2 Classification of Endophytes

Endophytes can be classified into several categories: pathogens of other hosts, avirulent pathogens of the same host, nonpathogens of other hosts, and nonpathogens of the same host. Nonpathogens of other hosts are endophytes isolated from one asymptomatic plant species that have the ability to colonize another species, while nonpathogens of the same host can be reintroduced into the species they were originally isolated from. Commercial products containing nonpathogenic endophytes isolated from another host combined with nonpathogens from the same host have been developed (Bargabus et al. 2002; Brannen and Kenney 1997; Bacon et al. 2001; Zeriouh et al. 2011). Endophytic relationships involving pathogens of other hosts can be beneficial to plants, as the nonhost may still recognize the microbe as a potential pathogen and activate its defenses even though the endophyte is incapable of causing disease in this nonhost. Endophytic colonization of cabbage with the cotton pathogen *Xanthomonas campestris* pv. *malvacearum* reduced the severity of black rot, caused by the pathogen *Xanthomonas campestris* pv. *campestris* throughout the growing season (Backman and Tuzun 1999). This same strain of *X. campestris* pv. *malvacearum* was capable of endophytically colonizing cacao leaves for a month and induced the expression of cacao defense-related transcripts (de Mayolo 2003). Endophytic relationships involving avirulent pathogens often have similar effects as endophytic relationships involving pathogens of other hosts. Inoculation of broccoli with an avirulent strain of *Pereonospora parasitica* reduced disease caused by pathogenic strains of *P. parasitica* (Monot et al. 2002). Inoculation of canola with an avirulent race of the blackleg pathogen *Leptosphaeria masculans* activated the hypersensitive response, while remaining incapable of eliciting disease symptoms (Li et al. 2006). Although not capable of causing disease, avirulent pathogens can often rapidly activate the same plant defense pathways as pathogenic organisms that cause disease (Pieterse et al. 1998).

The capacity to colonize and protect plants in a range of pathosystems is often considered essential for the success of a commercial bioproduct in the agricultural market. The ability to apply a product for management of multiple diseases means a wide range of growers are able to buy and utilize the product. A nonpathogenic endophytic *Bacillus* spp. from tomato was capable of endophytic colonization of cacao leaves and reduced disease in a detached leaf assay (Melnick et al. 2008). *Burkholderia* sp. strain PJN, originally isolated from onion, promoted grapevine growth and inhibited the growth of the noble rot pathogen *Botrytis cinerea* through endophytic colonization (Barka et al. 2002; Compant et al. 2005). Additionally, *Bacillus cereus* UW85, originally isolated from alfalfa seedlings (Handelsman et al. 1990), colonized the roots and rhizosphere of soybeans (Halverson et al. 1993) and increased soybean yield in field trials (Osburn et al. 1995).

It is generally accepted that nonpathogenic endophytes isolated from the same host are highly suited for biological control since they are adapted to the environment from which they were isolated. Plant-associated *Bacillus* spp. from apple were able to colonize apple leaves and fruit preharvest as well as apple fruits in the

postharvest environment (Poleatewich et al. 2011). Melnick et al. (2011) isolated 69 endophytic Gram-positive bacteria from cacao tissues. Of these isolates, inundative application of chitinolytic *Bacillus pumilus* ET to cacao leaves utilizing a Silwet adjuvant for stomatal penetration reduced lesion diameter of *Phytophthora capsici* in growth chamber studies (Melnick et al. 2011). Preliminary field experiments in young plants indicated *B. pumilus* ET also reduced witches' broom, caused by *Moniliophthora perniciosa* (Melnick et al. 2009). *Bacillus subtilis* stain L25 suppressed the growth of chestnut blight pathogen *Cryphonectria parasitica* (Wilhelm et al. 1998). Similar research isolating native endophytes and testing them as biological control agents has been conducted in citrus (Lacava et al. 2004), poplar (Taghavi et al. 2009), carrots (Surette et al. 2003), sugar beets (Bargabus et al. 2003), and a wide range of other plant species.

3.1.3 Endophyte Responses to the Environment

Endophytes are better protected from stress caused by changes in the environment than are epiphytes due to their position within plant tissues. Research by Melnick et al. (2008) demonstrated dynamic changes over time in the relative levels of inoculated *Bacillus* sp. populations in the epiphytic and endophytic environments over a 2-month period. It was suggested that there was a correlation between endophytic population levels and level of ISR as demonstrated by lesion expansion caused by *P. capsici*. In this study, they observed that endophytes were increasingly suppressed as disease suppression approached maximum levels. Later, as endophytic populations were at minimal levels (approaching undetectable), disease suppression also began to recede. These data point to the strong probability that falling endophyte populations were probably a result of suppression by plant defense products. It was further observed that when ISR was at its highest levels, the majority of CFUs of the inducing *Bacillus* were endospores, while when defenses were lowest, a large majority were found as vegetative cells. The ratio of vegetative cells to spores in the epiphytic environment was very stable throughout the experiment, but interestingly, these epiphytic populations may well have provided the reservoir population for restocking the endophytic population as the level of ISR receded and the internal leaf environment became more conducive to the growth of the *Bacillus* sp. (Melnick et al. 2008).

One likely major component influencing the fluctuation of population levels is the movement of bacteria between epiphytic and endophytic environments. In bean plants, internal populations of pathogenic *P. syringae* pv. *syringae* B782a increased at 15 days after bacterization due to inward movement through stomates (Sabaratnam and Beattie 2003), leaving open the possibility that endophytic bacteria may also move in a similar manner. Additionally, endophytic rhizobium species were shown to exit the stomates of tobacco leaves to colonize the epiphytic phyllosphere, indicating that the movement can occur in both directions (Ji et al. 2010). Work with grapevines demonstrated that epiphytic bacteria could enter the internal plant tissues by natural

or human-based means, such as pruning (West et al. 2010). Endophytic *Burkholderia* sp. PsJN readily colonized the epiphytic and endophytic portions of *Vitis vinifera* roots in tissue culture, using endoglucanase to degrade cell walls to internally colonize roots (Compant et al. 2005). Similarly, aerial tissue was colonized through the vascular transport of the root endophyte *Pseudomonas aureofaciens* of corn (Lamb et al. 1996). The bacteria spread through the transpiration stream to colonize stems and leaves driven by water movement (Compant et al. 2005). Endophytes can potentially be vertically transmitted in tissue culture. Propagation of endophyte colonized material can lead to colonization of the derived plantlets.

Work with endophytic *B. cereus*, originally isolated from tomatoes and applied to young cacao plants, demonstrated that populations of these bacteria in the endophytic environment fluctuated between log 2.5 and log 5.5 CFU/cm^3 leaf tissue, while epiphytic populations remained relatively stable during the 68-day sampling period (Melnick et al. 2008). Additionally, endophytic population levels of *B. cereus* BP24 were below the detection threshold 25 days after inoculation, yet recovered 33 days after inoculation. The epiphytic population may have been the source for endophytic colonists (Melnick et al. 2008). Despite the suggested movement of bacteria between the epiphytic and endophytic environments, little research has focused on the fluctuations and interactions of these communities.

Seasonal temperature changes altered the species diversity and abundance of the bacterial community of *Ulmus* spp. (Mocali et al. 2003). Chilling of heat tolerant sweet peppers resulted in an altered bacterial community as assessed by the diversity, complexity, and/or abundance of stem endophytes (Rasche et al. 2006). Despite the endophytic habitat protecting bacterial endophytes from environmental stresses, both short- and long-term changes in endophytic communities can result from environmental fluctuations. Populations of native endophytic *Bacillus* spp. in cacao leaves from field grown trees were variable, fluctuating by approximately 1.0 log CFU/cm^3 (Melnick et al. 2011). Populations of isolates derived from cacao were more stable than endophytes from other hosts. The native bacterial and *Bacillus* communities of cacao leaves sampled after 3 months following inundative application of endophytic *Bacillus* spp. had similar species abundance and diversity to those before application of endophytes. Despite having observed fluctuations of singular species in growth chamber studies, bacterial communities in field grown cacao trees returned to pre-application diversity levels from an endophytic population shift (Melnick et al. 2011) when given enough time.

3.2 Biological Control

In agriculture, biological control is the use of beneficial microorganisms to reduce plant pests, such as disease and insects. The term “biological control” itself can be misleading, as microbes only suppress plant disease and are rarely capable of controlling disease. Russian scientists started work on “bacterial fertilizers” to enhance plant growth in the 1950s (Backman et al. 1998). During this period, it is

estimated that famers were treating millions of hectares of crops with bacterial fertilizers (Cooper 1959). Dunleavy (1955) utilized *B. subtilis* to suppress damping off of sugar beets. Early work by Broadbent et al. (1971) found that 40 % of 3,500 isolated soil-inhabiting bacteria were antagonistic to nine pathogens on agar growth medium, with only 4 % being antagonistic to the pathogens in the soil. This work set forth the precedent of screening large numbers of isolates as well as the notion that only a small portion of environmental isolates may be effective in biological control. In 1974, Baker and Cook wrote the first book solely about biological control of plant pathogens, which summarized the early work in the field (Baker and Cook 1974).

3.2.1 Modes of Action

The mode of action of biological control agents can be divided into direct, indirect, and mixed-path antagonism. Direct modes of action include parasitism such as hyperparasitism and mycoparasitism. Indirect modes of action include competition and induction of plant defenses. Mixed-path modes of action include antibiosis, antagonism, and other less characterized mechanisms. Mycoparasites are parasites of fungi, and have been utilized in biological control to suppress fungal pathogens. In terms of commercial success, *Trichoderma* spp. are the most researched and commercialized mycoparasites (Harman et al. 2004). *Trichoderma* spp. form intimate contact during mycoparasitism and can be found coiling around plant pathogenic fungi (Inbar et al. 1996). Once contact is established, the *Trichoderma* spp. can directly penetrate the hyphae of plant pathogens through combined appersorium formation and production of cell wall degrading enzymes, such as chitinase and glucanase (Harman et al. 2004). The key to the success of mycoparasitism in biological control is that the biological control agent (BCA) must come in direct contact with the targeted pathogen and must persist in the same environment as the pathogen. In terms of bacteria, bacterial BCAs are not known to be mycoparasites of fungi. However, bacterial species have been found to be parasites of nematodes. Gram-positive *Pasteuria penetrans* effectively reduces damage of the root-knot nematodes through direct colonization and parasitism of nematodes resulting in lysis and death of the pest (Davies et al. 1988). Commercial production has been difficult since *P. penetrans* in an obligate symbiont and the bacterium must be produced on nematode-infected tomato roots.

Competition in terms of biological control occurs when a BCA obtains resources faster than a pathogen within a shared habitat. Several commercial BCAs operate primarily through the mechanism of niche displacement. *Pseudomonas fluorescens* A506 (BlightBan A506) colonizes apple and pear blossoms and prevents *E. amylovora* from reaching adequate populations for quorum sensing by excluding resources required for the pathogen (Wilson et al. 1992; Wilson and Lindow 1993). Additionally, colonization of trees with *P. fluorescens* A506 prevents frost damage, as the BCA outcompetes ice nucleating bacteria for nutrients, reducing their

population by nearly 100-fold (Lindow et al. 1996). BlightBan A506 is registered to protect almond, cherry, pome fruits, potato, strawberry, and tomato from frost damage (Stockwell and Stack 2007). Colonization of several crops and protection against multiple diseases have resulted in the continued success of BlightBan.

Another mixed-path mode of action is antagonism. Antagonism can be broken down into production of lytic enzymes or antimicrobials by the BCA to make the shared environment inhospitable for pathogens (McSpadden-Gardener 2002). Antagonistic compounds impair pathogen growth, reproduction, sporulation, and infection processes to reduce disease. Traditionally dual-culture *in vitro* assays conducted on Petri dishes have been a key initial screen to identify organisms producing antagonistic compounds. Increased knowledge of the multiple modes of action used by BCAs has reduced the use of this method as a primary selection step since an *in vitro* screen on agar does little to mimic the natural environment and readily eliminates potential BCAs that utilize other modes of action. NoGall is an example of commercial success using antibiosis as a primary mode of action. *Agrobacterium radiobacter* K1026, the active microbe in NoGall, produces the bacteriocin agrosin 84 which kills the crown gall pathogen *Agrobacterium tumefaciens* (Reader et al. 2005). The close genetic relationship of these two species allows them to colonize the same niche. Similarly, *Bacillus* spp. found in several commercial products produce antibiotics and secrete them in the environment shared with plant pathogens (Gupta and Utkhede 1986; Toharisman et al. 2005; Stein 2005). An advantage of *Bacillus* produced antibiotics is that they are often effective against a range of plant pathogens (Kloepper et al. 2004). Additionally, bacteria including *Bacillus* spp. are known to produce lytic enzymes such a chitinase and glucanases which degrade the cell walls of fungal pathogens (Chernin et al. 1995; Frändberg and Schrnürer 1998; Kishore and Podile 2005; Kobayashi et al. 2002; Kokalis-Burelle et al. 1992; Pleban et al. 1997). Some bacteria can produce phytohormones and nutrient solubilizing enzymes that produce plant-growth promoting rhizobacteria (PGPR) effects. These traits coupled with the ability to effectively colonize and dominate the rhizosphere are largely responsible for the beneficial effects of *Bacillus subtilis* GB03, commercially sold as Kodiak (Brannen and Kenney 1997).

The last and most recently recognized mode of action is induction of host defenses, more commonly known as induced resistance. When understanding plant-associated microbes, it should be recognized that plant disease is a rare circumstance in which the pathogen avoids early recognition by the host and early induction of host defenses or does not elicit a timely host response during the initial interaction with the plant (Zehnder et al. 2001). Some beneficial microbes can activate plant defense cascades, resulting in disease suppression (Pieterse et al. 1996). Although effective in reducing disease, activation of plant defense can be costly to the plant, due to the energy required for the production of proteins and plant metabolites (Heil 2001). Some beneficial microbes overcome this high cost by priming the plant for defense. Priming occurs when BCAs do not fully activate plant defense cascades, but instead stimulate slight changes in gene expression and/or metabolism, preparing the plant to rapidly hyperactivate specific defense

response in the presence of the pathogen (van Hulten et al. 2006). In other words, primed plants have a slightly increased expression of key defense genes that allows them to have a faster and stronger defense response upon infection by a pathogen (van Hulten et al. 2006). The advantage of priming over full induction of plant defense is that there is a reduced biological cost to the host plant in the absence of the pathogen (van Hulten et al. 2006).

There are several advantages to induced resistance. Induced resistance is often effective again a broad range of pathogens since it utilizes plant defenses (van Wees et al. 1999) evolved for broad-spectrum activity. Induced resistance can also act systemically, impacting disease in an area spatially separated from the BCA. Rhizosphere colonizing bacteria have been shown to reduce foliar diseases through production of systemic defense signals (Kloepper et al. 2004; Cartieaux et al. 2003; Heil and Bostock 2002; Zehnder et al. 2001). Colonization of cucumber roots with combinations of plantgrowth promoting rhizobateria (PGPR) reduced the severity of cucumber mosaic virus on the foliage, despite lack of colonization in leaves (Jetiyanon and Kloepper 2002). Colonization of tobacco roots with *Bacillus* spp. reduced the severity of cucumber mosaic virus (Kloepper et al. 2004). Overall, the key to understanding the modes of action utilized by BCAs is to understand that BCAs can readily utilize multiple modes of action to reduce disease. Developing BCAs with multiple modes of action by having one robust organism or multiple organisms may provide more success in disease management.

3.2.2 Biological Control of Perennial Plant Diseases with Bacterial Endophytes

Biological control in woody perennial crops offers many challenges that are not encountered with annual crops. One of the largest issues to overcome with perennial crops is the absence of crop rotations to reduce levels of pathogen inoculum. For example, an apple tree may be in an orchard for 30 years. Pathogens of perennial crops can overwinter on debris, but also overwinter/off season on the plants themselves. Cleistothecia of powdery mildew of grapevine (*Uncinula necator*) overwinter in leaf scars and crevices of bark while the mycelium can also overwinter in dormant buds (Pearson and Gadoury 1987). During spring, ascospores are discharged from the cleistothecia to infect developing leaves and perpetuate the disease into the next growing season (Pearson and Gadoury 1987). Similarly, the conidia of *Venturia inaequalis*, causal agent of apple scab, overwinter in inner bud tissue (Holb et al. 2004). Mummified non-abscised fruit serve as a refuge for overwintering conidia of *Monilinia fructicola*, causal agent of peach brown rot (Landgraf and Zehr 1982).

Tropical crops are under constant disease pressure, and managing inoculum sources in tropical woody perennials is even more difficult than for temperate perennials. Vascular wilt of the perennial tropical crop Naranjilla (*Solanum quitoense* L.), caused by *Fusarium oxysporum*, has decimated production in

Ecuador (Ochoa et al. 2001). Farmers faced with this pathogen by simply abandoning plantations or replanting elsewhere every few years in order to continue producing the crop (Ochoa et al. 2001). Guava is widely grown throughout the tropics, but succumbs to many fruit rots, such as *Pestalotiopsis* species (Keith et al. 2006) and *Phomopsis destructum* (Rao et al. 1976). Since both of these crops are actively growing year round, there is always a potential for disease to occur. Despite the perennial nature of their hosts, inoculum for most tropical diseases is not consistent through the year. For example, the mushrooms of *Moniliophthora perniciosa*, causal agent of witches' broom of cacao, require an alternating wet/dry cycle typically found during the rainy season (Meinhardt et al. 2008). While far more mushrooms form during the rainy season, infections still occur during the dry season, forcing farmers to scout for disease and conduct phytosanitatry practices year round.

3.2.2.1 Delivery and Entrance of Endophytes

A key step to use of bacterial endophytes in biological control is the development of methods to facilitate colonization of the plant. Most commercial BCAs are formulated so that they can be applied in a manner similar to fungicides through aerial sprays, seed treatments, roots dips, and incorporation into soil mixes (Fravel 2005). One challenge to use of bacterial endophytes with woody plant species is physically getting the endophytes into the plant. In work with cotton, endophytes were introduced into the stems by puncturing the stem with a needle (Chen et al. 1995). This method allowed the bacteria to survive in the stems for nearly a month, although the bacteria did not move further than 5.0 cm from the inoculation point (Chen et al. 1995). Another method is using surfactants, such as Silwet, which reduce the surface tension of the solution, allowing for substomatal infiltration (Melnick et al. 2008). Additionally, timing of application can be essential for effective management of disease. The key success with the use of BlightBan A-506 (*Pseudomonas fluorescens*) on tree fruit is application timing. Since the bacterium colonizes internal flower parts (stigma, nectaries, etc.), farmers must time the application of BlightBan with flower opening, allowing the bacterium to colonize the flowers before the pathogenic *Erwinia amylovora* bacterium. The shortcoming of this strategy is that flowers are opening every day for 2 weeks, and all flowers are susceptible to *Erwinia* when they open (Bubán et al. 2003). Timing sprays to coordinate with flower opening, including prediction of rain events that deliver the bacterium, is critical to success (Wilson et al. 1992; Wilson and Lindow 1993).

For some tree species, endophytes naturally spread through seeds, grafting, or bud wood. These types of vertical transmissions are most commonly seen with fungal endophytes of grasses (Afkhami and Rudgers 2008). Vertical transmission has been suggested for bacterial endophytes, but this is an under-researched topic. Although not as well studied as in perennial grasses, vertical transmission has been found in woody perennial plants. Natural bacterial endophytes were detected in

Eucalyptus seeds and the same species were detected in developing seedlings (Ferreira et al. 2008). Ferreira et al. (2008) created gfp-labeled strains of the endophytic bacterium, *Pantoea agglomerans*, and tracked the movement of the species from seeds into the roots of developing *Eucalyptus* seedlings to confirm that vertical transmission occurred. We need a better understanding of how widespread and effective vertical transmission of bacterial endophytes is in plants.

3.3 Case Study: Biological Control of Cacao Pod Diseases

Theobroma cacao L. is an economically significant crops, as the beans (seeds) produced in pods (fruits) are processed into chocolate and cocoa products. Cacao is a tropical crop grown in countries located between the tropics of Capricorn and Cancer. Approximately 70 % of cacao is grown by smallholder farmers. Traditionally, cacao trees have been intercropped with timber and fruit trees, which provide shade for the cacao trees and additional income for the farmers. Additionally trees were often planted from seed, leading to significant genetic diversity within a field. In recent years, improved clones have been introduced and are being planted in monoculture under full sun and in large hectarage farms, changing disease management needs (Hebbar 2007).

Many pods are lost early during development to a physiological condition known as cherelle wilt. The physiological initiation of cherelle wilt is unknown, but the trigger causes xylem occlusion by a mucilage-like substance which results in wilting and pod death (Nichols 1961). Even if developing pods survive past the critical period for cherelle wilt (<70 days), they can still be lost from the destructive effects of three major pod rotting diseases. Black pod is caused by several *Phytophthora* spp. (Guest 2007). Symptoms of pod infection are dark necrotic lesions and rotten beans. Frosty pod rot and witches' broom are caused by two distinctive agaric *Moniliophthora* spp. Symptoms of frosty pod rot, caused by *Moniliophthora roreri*, are pod malformation and the development of necrotic lesions which rapidly become covered with mycelium bearing powdery white meiospores (2*N* spores) (Evans 1981). Infections of maturing pods by *M. perniciosa*, causal agent of witches' broom disease, may not be seen until the pod is opened to expose the rotten beans. Additionally, infection of young pods and flower cushions leads to the development of parthenocarpic fruit known as cherimoyas, which later become necrotic and remain on the tree as a source of basidiocarps. Management of these cacao diseases consists of phytosanitation to remove disease branches and pods in order to reduce inoculum and planting of tolerant clones. Fungicides can improve yield, but are often too costly for small-holder farmers as well as pose risks to the health of the applicator and the environment. For these reasons, there has been increased research on biological control as a sustainable disease management option.

Previous work by Melnick et al. (2008) found that an endophytic *Bacillus cereus* from tomatoes could colonize cacao foliage and suppress *P. capsici* in detached leaf assays. Although *Bacillus* sp. BT8 was capable of reducing disease in detached leaf assays, the isolate was not native to cacao growing regions, making release of these

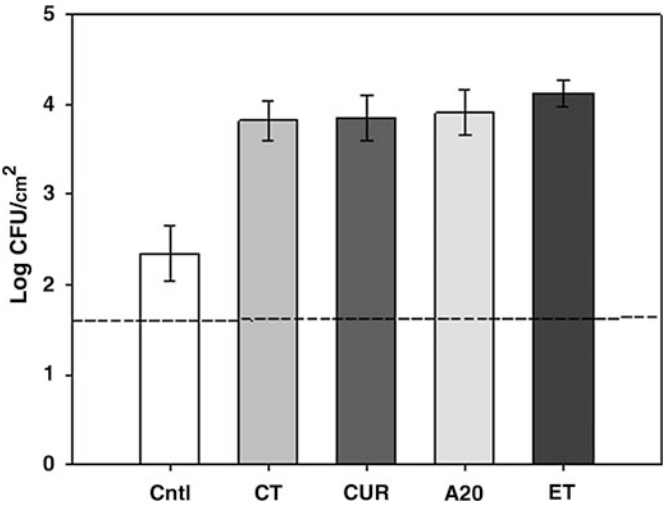

Fig. 3.1 Mean initial epiphytic colonization of immature pods (log CFU/cm^2) found on "Nacional" cacao pods at 24 h after treatment when sprayed with either 0.2 % Silwet control or 0.2 % Silwet + log 8.0. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET Control pods had low levels of naturally occurring endospore-forming bacterial endophytes. The *dotted line* indicates the minimum detectable level of log 1.8 CFU/cm^2. *Bars* extending from means indicated the standard error of that mean

isolates in cacao production areas difficult. Melnick et al. (2011) expanded on this work by isolating native endophytic endospore-forming bacteria from cacao flower cushions, pods, leaves and branches from trees grown in Ecuador. Isolates were screened to determine whether they possessed attributes of BCA, such as chitinase production, antagonism toward the three cacao pods diseases, ability to colonize cacao seedlings, and ability to reduce *Phytophthora* lesion expansion in a detached leaf assay by 34–44 % (Melnick et al. 2011).

Field trials were conducted to test how elite bacterial isolates (*Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET) affected cacao disease when applied to immature trees grown under two cacao farming strategies (monoculture and intercropped) typical of what is found in Ecuador. Intercropped cacao trees (cacao grown under shade of forest trees) were "Nacional" trees having been derived from several cacao clones. We found that application of bacteria with the organo-silicon surfactant Silwet (0.2 %) did not alter normal pod development. Twenty-four hours after application, applied bacteria were detected in both the epiphytic (Fig. 3.1) and endophytic environments (Table 3.1).

Endophytic bacteria survived in both the epiphytic and endophytic environments. There were no detectable endophytic colonists in control pods immediately after application. Despite control pods initially lacking endophytic endospore-forming bacteria, they were detected in some control pods pieces 3 months after bacterization. Endophytic colonists were not equally dispersed throughout the pod, as endospore-forming bacteria were not isolated from all pod sections.

Table 3.1 Mean percentage of sampled pods with endophytic endospore-forming bacterial colonists

Treatment	Initial	3 mai
Control	0	33.3
CT	100	100
CR	100	66.7
A20	100	66.7
ET	100	33.3

Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET. Initial indicates the percentage of pods with endophytic colonization 24 h after inoculation with bacteria, while 3 mai indicates the percentage of pods with any heat stable endophytic colonists tested 3 months after inoculation (mai)

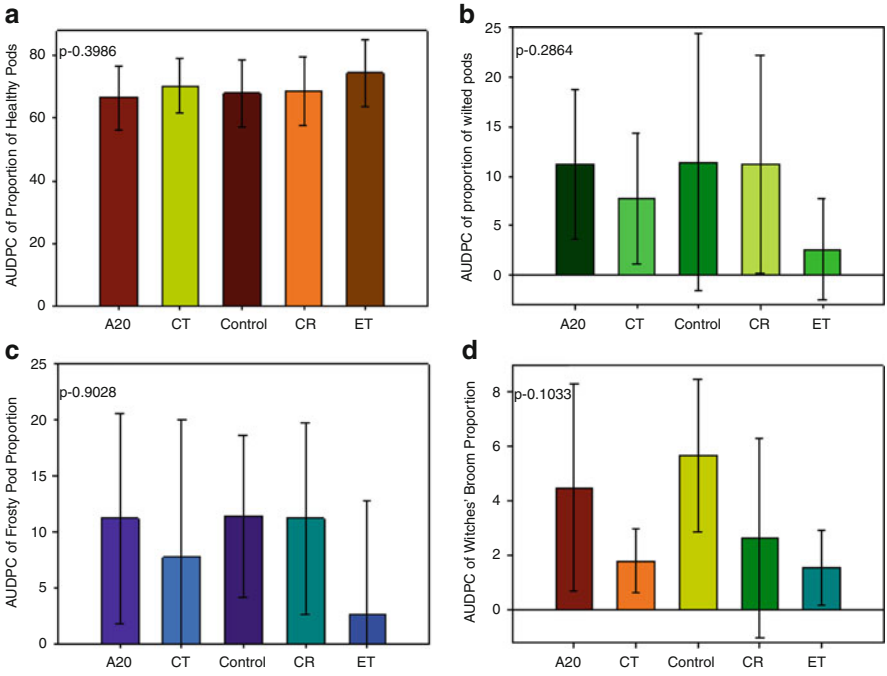

Fig. 3.2 Mean AUDPC of incidences of (**a**) healthy pods, (**b**) cherelle wilt, (**c**) frosty pod, and (**d**) witches' broom out of 78 "Nacional" pods per treatment blocked by location at the INIAP-EET. Control pods were sprayed with 0.20 % Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU.cm^2 solution of bacterial isolate + 0.20 % Silwet L-77. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* 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

Pods were counted and disease was assessed monthly. In the intercropped "Nacional" trees, *B. pumilis* ET significantly increased the number of healthy pods 1 month after bacterization ($p = 0.0262$) (Fig. 3.2a), but not at later time

points. Pods treated with *B. pumilis* ET also had less pods lost to cherelle wilt at 1 month after bacterization ($p = 0.044$), after which, pods were physiologically resistant to cherelle wilt. Therefore, the incidence of cherelle wilt did not increase beyond that point. Despite an increase in the number of healthy pods early in the season, no isolate reduced losses to frosty pod rot or witches' broom.

Despite the successes of the bacteria in reducing cherelle wilt in Nacional pods, no treatment caused season-long disease suppression as seen in a reduction in the AUDPC (Fig. 3.2). Fewer pods overall were lost to cherelle wilt or witches' broom. Despite the 83 % reduction in cherelle wilt in *B. pumilis* ET-treated pods, the high levels of variability in the control treatment precluded a significant effect.

3.3.1 Results from a Large Farm Producing Sun-Grown Cacao

All plants on the commercial farm were clonally propagated "CCN-51" clones, known for their high yield and reduced levels of disease. *B. pumilis* ET significantly decreased cherelle wilt on CCN-51 trees throughout the study when compared to both control pods and pods treated with *Lysinibacillus sphaericus* A20. This bacterium appears to have increased resistance to cherelle wilt during the susceptible period. Despite the wilt reduction, the number of healthy pods throughout the season did not increase.

Bacterial treatments did not reduce overall black pod (Fig. 3.3c) or frosty pod (Fig. 3.3d) incidence, but *B. pumilis* ET decreased overall cherelle wilt (Fig. 3.3b) when compared to both controls and *L. sphaericus* A20.

In both "Nacional" and "CCN-51" plots, *B. pumilis* ET reduced cherelle wilt, but did not reduce cacao disease. One confoundment in these experiments was the fact that a higher frequency of pod-set from cherelle wilt suppression provided more pods that might be infected with fungal pod diseases, causing a statistical skew in the data.

3.3.2 Conclusions

These finding are one of the first studies in which application of a BCA suppressed cherelle wilt which had previously been ascribed to "self-thinning." Fungal mycoparasites used against frosty pod in Peru (Krauss and Soberanis 2001) and black pod rot in Africa (Deberdt et al. 2008) had no impact on cherelle wilt in field trials. In the key cacao production zones of central and south America, it is common to see 50–60 % of young pods lost in the first 6 weeks following flower fertilization. The reduction in losses to cherelle wilt by *B. pumilis* ET followed the same trend at both replicated field sites, despite differences in genotype, environment, and field management strategies between the two fields. Reducing the number of pods lost to cherelle wilt could potentially increase the number of healthy pods, thus potentially increasing yield. Loss of "CCN-51" pods to cherelle wilt was reduced by 52 % by

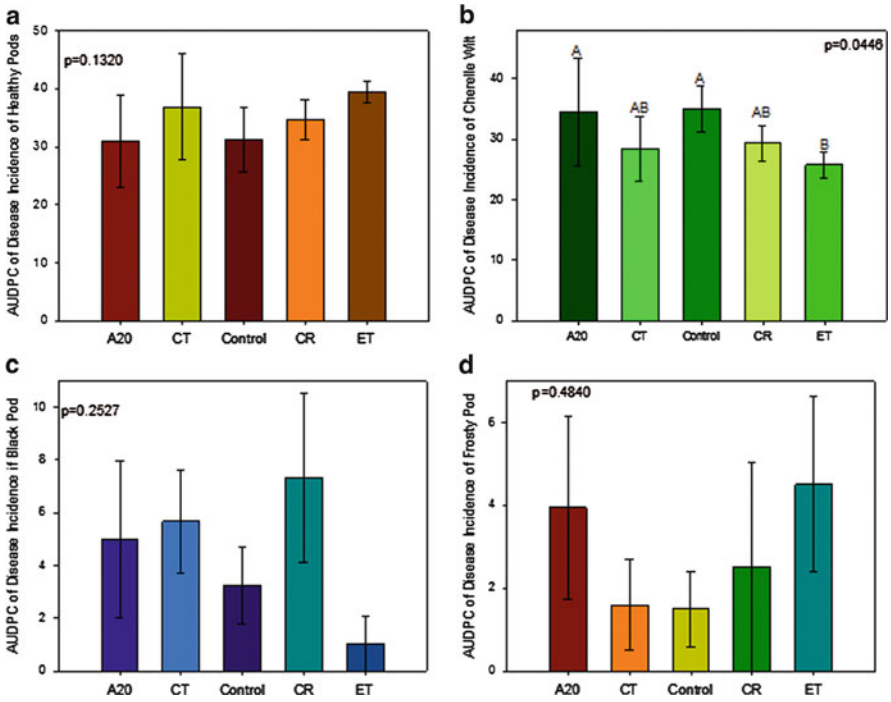

Fig. 3.3 Mean AUDPCs for incidence of (**a**) healthy pods, (**b**) cherelle wilt, (**c**) frosty pod, and (**d**) witches' broom out of 82 "CCN-51" pods per treatment blocked by tree location at the Rio Lindo farm. Control pods were sprayed with 0.20 % Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU/cm^2 solutions of *Bacillus* isolate + 0.20 % Silwet L-77. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* 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

B. pumilis ET, while loss of Nacional pods was reduced by 38 %. If cherelle wilt is simply physiological wilting, then hormone imbalances might initiate xylem occlusion. *Bacillus* spp. are known to have the ability to produce phytohormones (Idris et al. 2007) and also affect plasticity of plant cells (Kerff et al. 2008). If *B. pumilis* ET is found to produce phytohormones in future experiments while colonizing pod tissue, they could potentially prevent cherelle wilt initiation.

The most consistent observation at both sites was that one application of bacterial endophytes was not enough to reduce disease throughout the 4–5-month period from early pod set to harvest. The protection against cherelle wilt required a much shorter term of protection, lasting only until pods were physiologically resistant to cherelle wilt, which was roughly March in these experiments. Although *B. pumilis* ET decreased pods lost to cherelle wilt, it also increased the number of healthy pods which could have become infected, adding a complicating factor to the experiment. The increased numbers of healthy pods warrant additional measures to

protect the pods until harvest. Based upon previous research and the need to protect pods throughout the season, experiments are ongoing to assess the effect of monthly application of bacteria endophytes for control of cacao diseases.

3.4 Case Study: Endophytic *Curtobacterium flaccumfaciens* and Reduction of Citrus Variegated Chlorosis

Xylella fastidiosa is a xylem-limited Gram-negative bacterium which causes diseases in at least 10 woody species including grapevine and citrus (Hopkins 1989). The bacterium is transmitted between plants by xylem feeding insects (Hopkins 1989). *X. fastidiosa* sbsp. *pauca* (*Xfp*) infection of sweet oranges (*Citrus sinensis* L) results in citrus variegated chlorosis (CVC) disease (Lacava et al. 2007a). As the name suggests, the bacterium induces leaf chlorosis as well as gummy lesions on the abaxial surface of leaves, which become necrotic. CVC infection results in smaller fruit with hard rinds, making them unmarketable (Amorim et al. 1987). Additionally, tree growth is stunted and twig and branch dieback occurs (Roberto et al. 1996). All sweet orange cultivars are susceptible to the disease (Lacava et al. 2007a). Since its arrival in Brazil in 1987 (Amorim et al. 1987), production of sweet oranges has been drastically reduced. A difficulty in management of the disease is related to transmission of the pathogen by xylem feeding sharpshooter leafhoppers (Roberto et al. 1996) and the xylem limited nature of the pathogen. Current recommendations are purchasing disease-free nursery stock, insecticidal treatments to manage vectors, and phytosanitation (Almeida et al. 2001). Due to the inability to directly attack the pathogen in the plant, scientists decided to assess the potential of utilizing bacterial endophytes to manage CVC.

Araújo et al. (2001) assessed the diversity of endophytic fungi and bacteria isolated from citrus rootstock. Thirty-six distinct bacterial isolates were isolated from the endophytic portion of the roots, with several of these isolates being inhibitory to the citrus pathogen *Guignardia citricarpa*, causal agent of black spot of citrus (Araújo et al. 2001). This work was then expanded to assess the diversity of endophytes inhabiting the branches. Araújo et al. (2002) compared differences between the bacterial communities and their relationship to *Xfp*. The bacterial communities of healthy, CVC infected and symptomatic, and CVC infected yet asymptomatic plants were compared using traditional isolation techniques and culture-independent technologies. There were no differences in the number of endophytic bacterial strains isolated from these trees, but asymptomatic plants had a higher frequency of *Curtobacterium flaccumfaciens* (Araújo et al. 2002). Additionally, there was a positive correlation between disease intensity and abundance of *Methylobacterium* spp. (Araújo et al. 2002). This observation may be related to the fact that a *M. extorquens* isolate stimulated *Xfp* growth on Petri dishes (Lacava et al. 2004). Other endophytic isolates, in particular, *Methylobacterium mesophilicum* and *C. flaccumfaciens*, were found to be inhibitory to *Xfp* and were further screened for their potential in biological control of CVC (Lacava et al. 2004).

Madagascar periwinkle (*Catharanthus roseus*) has been used as a model plant to study *Xfp* in the greenhouse (Lacava et al. 2007a). This system was utilized to study the interaction of inhibitory bacterial endophytes with *Xfp in planta* (Lacava et al. 2007b). Inoculation of *C. roseus* with citrus *Xfp* reduced flower number, stunted growth, stunted leaf size, and caused wilting (Lacava et al. 2007a), similar to the reaction in citrus. Once the model system was developed, scientist screened *M. mesophilicum* and *C. flaccumfaciens* for their ability to reduce CVC. *M. mesophilicum* was inoculated into *C. roseus* via seed treatment, causing a shift in the native bacterial communities of the plant, particularly in root tissue (Andreote et al. 2006). In its interaction with *Xfp*, some endophytic *Methylobacterium* spp. produce hydroxamate-type siderophores which stimulated *Xfp* growth (Lacava et al. 2008), supporting results from earlier studies in which there was increased *Methylobacterium* colonization with CVC disease (Araújo et al. 2002). Additionally, *M. mesophilicum* preferentially colonized xylem vessels (Gai et al. 2009). The sharpshooter insects *Bucephalogonia xanthophylls*, CVC vectors, were also shown to be able to vector *M. mesophilicum*, suggesting that the insect may also vector endophytic bacteria (Gai et al. 2009). When plants inoculated with *C. flaccumfaciens* were challenged with the pathogen, no disease symptoms developed as *C flaccumfaciens* colonized the same niche and the pathogen and produced bacteriocins effective against *Xfp* (Lacava et al. 2007a). Further work on this bacterium may provide citrus farmers with another management option for CVC.

3.5 Biological Control in the -Omics Age

Traditional microbiology methods often underestimate the diversity of species present in endophytic communities, as approximately 90–99 % of microbes cannot be cultured (Curtis et al. 2002; Pace 1997). Combining culture-based and culture-independent technologies can provide a better picture of endophytic communities present in a host. Additionally, they can be used to determine how inundative application of a BCA affects the community of beneficial and pathogenic microbes. Work by Gilbert et al. (1993) illustrated that inundative application of *Bacillus cereus* UW85 to soybean roots drastically altered rhizosphere bacterial communities. Berg et al. (2005) used terminal restriction length polymorphism analysis to estimate the endophytic and ectophytic bacterial (combined rhizosphere and phyllosphere) community inhabiting different potato tissues and the soil. They found that the rhizosphere and endorhizosphere communities were home to more species of antagonistic bacteria than aboveground plant parts (Berg et al. 2005). Melnick et al. (2011) analyzed community diversity of cacao leaves at three months using automated ribosomal intergenic spacer analysis (ARISA) after application of endophytic *Bacillus* spp. to determine whether inundative application of bacteria could incite long-term shifts in the native microbial communities. Despite application with Silwet adjuvant, followed by robust endophytic colonization immediately following application, the bacterial community had fully recovered from inundative application by the 3-month

sampling date by returning to a similar microbial community as found in nontreated leaves (Melnick et al. 2011).

A range of molecular technologies have been developed to estimate the numbers of individual species in an interaction as well as to determine the diversity of endophytic bacterial communities present in plants. One technique with a diverse range of applications is real-time PCR. Real-time PCR simultaneously amplifies and quantifies gene fragments. Amplification-specific fluorescent dyes are detected by the machine to quantify the amount of DNA amplicons. The detection step can be performed using two methods. One methodology utilizes primers which have a fluorescence reporter label which is only detected once it has hybridized with the target (Arikawa et al. 2008). The other methodology utilizes nonspecific fluorescent dyes that intercalate into the double-stranded DNA amplified from targets (Arikawa et al. 2008).

Real-time PCR is an invaluable technique for rapid diagnosis of plant diseases, especially when the pathogenic organism cannot be cultured, such as viruses (Schaad and Frederick 2002). Knowledge of changes in the genes of different pathovars of a species can help determine the pathovar of isolated organisms without the need to conduct Koch's postulate. With the increasing ease of extracting DNA using pre-assembled kits, diagnosticians can potentially determine causal agents in hours, as they will not have to wait for pathogen sporulation or for the microorganism to grow in media. Additionally, since 96-well plates are typically used, analyses can be simultaneously performed using multiple primers targeting multiple pathogens and utilizing multiple samples.

Quantitative real-time PCR (QPCR) is an invaluable tool for estimating the population levels of endophytes. Tellenbach et al. (2010) developed QPCR primers to estimate the biomass of endophytic fungus *Phialocephala fortinii* inhabiting plant roots. Lacava et al. (2006) used QPCR to access population levels of the bacterial endophyte *Methylobacterium mesophilicum* in the presence of xylem-limited pathogen *Xylella fastidiosa* in the model plant *Catharanthus rosea*. They found the endophytic population increased by nearly 200-fold in the presence of the pathogen compared to disease-free plants (Lacava et al. 2006). Studies using bacteria often combini serial dilution plating with QPCR to confirm findings.

QPCR has been used to assess the effects of colonization by endophytes on plant gene expression. Bailey et al. (2006) found that endophytic colonization of cacao seedlings with *Trichoderma* spp. induced the expression of plant expressed sequence tags (ESTs) related to osmotic stress response and defense. The ESTs' induction patterns were *Trichoderma* isolate specific. Additionally, *Trichoderma* had altered expression of ESTs related to nutrient acquisition in a low nutrient environment (Bailey et al. 2006). Pavlo et al. (2011) assessed whether colonization of Arabidopsis with potato endophytes *Pseudomonas* sp. or *Methylobacterium* sp. induced expression of defense genes via QPCR. In the absence of the pathogen *P. syringae* pv. *tomato* DC3000, there was a slight change in gene expression (Pavlo et al. 2011). Plants colonized with endophytes and then challenged with the pathogen had higher expression levels for marker genes for ISR and SAR than plants simply colonized with the endophyte or challenged with the pathogen, suggesting that the endophytes primed the plants for defense (Pavlo et al. 2011).

Estimation of gene expression is not only important in the understanding of plant–microbe interactions, but also in understanding the induction of resistance by endophytes. This technology is not only useful in detecting host gene expression in response to endophytic colonization, but also to estimate gene expression of the plant-associated microbes. Bailey et al. (2006) used QPCR to determine how endophytic colonization of cacao with *Trichoderma* spp. altered gene expression in the plant, indentifying seven cacao ESTs which were induced by the fungus. Additionally, QPCR was used to estimate expression of *Trichoderma* ESTs *in planta* to gain a better understanding of the genes utilized during the endophytic interaction. Similar work was conducted on the interaction of the plant root nodulating bacteria and its legume host using a specialized dual genome microarray (Barnett et al. 2004). Microarrays, often known as gene chips, are specialized slides in which DNA spots of specific sequences are attached or printed on the surface. The target cDNA is then hybridized to the chip. If a gene is expressed by the plant, then the cDNA will form a probe–target hybrid which can be detected with chemiluminescence to estimate the expression levels of the genes in the sample. The advantage of microarrays over QPCR is that tens of thousands of gene are on the chips, allowing scientist to measure shifts in expression of many genes with just one chip as opposed to thousands of QPCR reactions. Verhagen et al. (2004) used the Arabidopsis GeneChip to determine which genes were induced during rhizobacteria-induced systemic resistance in response to colonization of the rhizosphere with *P. fluorescens* WC471r. This work provided researchers with a list of potential genes involved in the ISR pathway. While a very useful technology, scientists are limited by the genes present on the chip and the availability of a microarray for a specific species. Microarrays have been used and are available commercially for perennial *Vitis vinifera* (Waters et al. 2005), citrus (Martinez-Godoy et al. 2008), and poplar (Azaiez et al. 2009).

Another use for microarray technology is the Phylochip. Instead of the microarray having genes of one organism, the DNA bound to the chip is 16S RNA genes from thousands of bacterial species. The Phylochip was used by Weinert et al. (2011) to demonstrate that potato cultivar impacted the abundance of specific plant-associated bacterial genera. Phylochip technology has also been applied to communities of endophytic bacteria. Sagaram et al. (2009) used this technology to study the diversity of endophytic bacteria associated with citrus leaf midribs. Through this study, an increased abundance of nine taxa of bacteria was observed in leaves having symptoms of Huanglongbing disease, caused by "*Candidatus* Liberibacter asiaticus." Pathogen "*Canididatus* Liberibacter asiaticus", which cannot be cultured using current methodologies, was present at 200 times higher population levels in symptomatic leaves than asymptomatic leaves. These results confirmed that Phylochip technology could potentially be used to detect specific pathogens in addition to estimating total diversity of microbes (Sagaram et al. 2009). Although it is a powerful technology, it is only as good as the genes on the chip. Currently, there is no PhyloChip for fungal species.

There are other less costly molecular technologies based upon whole-community fingerprinting that have been utilized to assess microbial communities. One technique is automated ribosomal RNA intergenic spacer analysis (ARISA). 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 region and the fluorescence of the fragment to estimate the diversity and abundance of the microbial community (Fisher and Triplett 1999). ARISA cannot estimate exact counts or identify the organisms present, but can provide useful estimates of diversity and abundance. Bacterial identification can be accomplished only if the amplicons are run on a gel and the individual bands are excised and sequenced. ARISA can be used to assess total bacterial or fungal communities or specific populations of microorganisms, depending on primer design. ARISA has most often been utilized to assess the diversity of bacterial communities in the soil and rhizosphere, but some researchers have utilized this technology for assessment of endophytic communities. Manter et al. (2010) used ARISA to demonstrate difference in the endophytic bacterial community associated with the roots of different potato cultivars. ARISA has also been used to assess endophytic diversity in perennial plants such as cacao (Melnick et al. 2011) and several Brazilian Atlantic forest tree species (Lambais et al. 2006).

Another similar technology is the use of terminal restriction fragment length polymorphism (T-RFLP) analysis. While ARISA primers amplify the intergenic spacer region, T-RFLP primer amplicons amplify a region containing a restriction site. The amplicons are exposed to a restriction enzyme and the resulting fragments are separated via DGGE or capillary electrophoresis. Benıtez et al. (2007) used T-RFLP to assess not only the bacterial soil communities that developed under different soil management strategies, but also to assess differences in pathogenic communities causing damping-off. T-RFLP has been used to assess endophytic bacterial community structure in sweet pepper (Rasche et al. 2006), potato (Sessitsch et al. 2002), wheat (Conn and Franco 2004), poplar (Ulrich et al. 2008), and many other plants.

A further technology is the use of pyrosequencing to assess microbial communities. The concept of pyrosequencing is best described as sequencing by synthesis. The DNA sequence is obtained from the complementary strand as it is sequenced from the target strand. Pyrosequencing is an evolving field with new technologies bring continually released. The technology generates a plethora of data in a fraction of the time required for Sanger sequencing methodologies. Manter et al. (2010) assessed the bacterial endophytes of potato roots using 454 sequencing technology in which primers amplified regions of the 16S rRNA gene. These data were compared to those generated from bacterial ARISA (B-ARISA). Both analyses demonstrated that the bacterial communities differed between cultivars (Manter et al. 2010). The advantage of pyrosequencing is the ease at which species identification can be determined, since the 16S rRNA genes are directly sequenced. In B-ARISA, all individual bands would have to be removed from DGGE gels and then sequenced to determine the exact identification of the species within the microbial community, a process obviously not required by direct sequencing. Relative abundances can be estimated based on the number of amplicons from a specific species.

3.6 Conclusions

Despite years of research on bacterial endophytes as tools to manage plant diseases, new technologies and new crops still leave many areas open for research that will lead to more optimal disease control. While previous research focused on annual crops, utilizing bacterial endophytes for management of diseases in perennial crops is an ever-increasing area of research. Molecular tools have expanded our knowledge on the role of endophytes in managing diseases. Whether it is measuring something as small as expression of a host gene or something as large as determining the abundance and diversity of species in a bacterial community, knowledge of the interaction of bacterial endophytes with their host and other microorganisms in the endosphere will continue to expand our knowledge of the role of bacterial endophytes in nature. High-throughput sequencing can lead to discovery of new groups of microorganisms which may play an important role in biological control, yet cannot be cultured using current methodologies. Understanding gene expression in perennial crops can provide a better understanding of induced resistance in plants. Perennial crops are more complicated than model annual plants. Perennial crops grown in the field support large and diverse microbial communities, certainly more diverse and competitive than those found in plants grown in a laboratory situation. Molecular methodologies have allowed researchers to gain a better understand of the complex nature of the interaction between endophytic microbial communities and their perennial hosts.

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