The Pennsylvania State University

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DEVELOPMENT OF BIOLOGICAL CONTROL STRATEGIES FOR INTEGRATED
MANAGEMENT OF PRE- AND POSTHARVEST DISEASES OF APPLE IN
PENNSYLVANIA

A Dissertation in

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by

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Disease prevention is an essential practice in producing quality fruit. Chemical pesticides are widely used for controlling pre- and postharvest diseases of apple and development of fungicide resistance in pathogen populations has resulted in fewer fungicides available for disease control. Additionally, registration withdrawal and restrictions on postharvest fungicide applications continue to plague the industry. The objective of this research was to develop biological controls for foliar and fruit diseases that can be implemented into existing conventional, reduced risk and potentially organic apple production systems to reduce the use of fungicides needed to maintain a quality product. Specific objectives were to (1) determine the effectiveness of novel indigenous endospore-forming bacterial isolates collected from apple and vegetables to suppress pre-harvest and postharvest diseases of apple; (2) evaluate application strategies for these isolates to optimize suppression of multiple diseases; (3) evaluate growth and survival of these isolates on several apple cultivars; and (4) examine interactions between plant, pathogen, and the biological control agent in order to formulate hypotheses regarding the mechanism(s) of disease suppression.

Healthy leaves and fruit were collected from abandoned, organic, and conventionally managed apple orchards in Adam’s County, PA in 2006 and 2007. Endophytic and epiphytic bacteria were isolated and screened for the ability to form endospores, colonize apple leaves and fruit and the ability to hydrolyze chitin. A total of 75 endospore-forming bacterial isolates were collected from apple and 35% were chitolytic. Isolates were further screened for the ability to reduce pre-harvest diseases; apple scab (Venturia inaequalis), cedar-apple rust (Gymnosporangium juniper-virginianae), flyspeck (Zygophiala jamaicensis), and fire blight (Erwinia amylovora) in growth chamber, greenhouse and field experiments. Isolates were also screened for the ability to reduce severity of the postharvest diseases bitter rot (Colletotrichum acutatum) and blue mold (Pencillium expansum). Following preliminary screens, six isolates were selected for field experimentation.

Four isolates were tested in a two-year field study on biological control of pre- and postharvest diseases at the Penn State Fruit Research and Extension Center in Biglerville, PA. Bacteria were applied to ‘Golden Delicious’ and ‘Rome Beauty’ trees in May or in May +June. Foliar apple scab severity was assessed weekly using a 7-point rating scale to estimate the percent leaf area scabbed. Bacillus megaterium isolate A3-6, B. mycoides isolate A1-1 and B. cereus isolate FLS-5 applied in May or in May+June significantly reduced apple scab severity on ‘Rome
Beauty’ and ‘Golden Delicious’ leaves and fruit. Fruit harvested from this experiment were challenged with the bitter rot pathogen, *C. acutatum*. When applied as postharvest treatments, these isolates significantly reduced mean bitter rot lesion size on both ‘Golden Delicious’ and ‘Rome Beauty’ fruit. A pre-harvest + postharvest application of *B. megaterium* isolate A3-6 resulted in the greatest suppression of bitter rot with an average of 45% and 95% reduction in lesion size compared to no-bacteria controls on ‘Golden Delicious’ and ‘Rome Beauty’ fruit respectively. While a synergistic effect was not observed in treatments combining pre- and postharvest application strategies, there was an additive effect as pre-harvest applications of the biocontrol bacteria were able to reduce foliar and fruit scab. Further, these pre-harvest effects persisted postharvest by suppressing bitter rot, but were enhanced by the addition of a postharvest application of the same isolate.

Five isolates were tested in a field experiment conducted at the PSU Department of Horticulture research farm in Rock Springs, PA to evaluate integration of bacteria with soluble silicon and chitosan for apple scab disease suppression. Application of Chitosan to ‘Cortland’ leaves in May and June did not reduce foliar apple scab severity compared to the non-treated control. All five of the bacterial isolates tested (*B. mycoides* isolate A1-1, *B. megaterium* isolates A3-6 and Ae-1, *B. cereus* isolate FLS-5 and *Brevibacillus laterosporus* isolate FLS-1) applied alone or in combination with chitosan in May and June significantly reduced foliar scab severity on ‘Cortland’. Further research on the concentration of chitosan as a foliar spray and combination with biocontrols is needed to determine the potential for apple scab control.

Application of the potassium silicate product, AgSil alone or in combination with bacteria, to ‘Cortland’ leaves in May and June did not significantly reduce apple scab severity on leaves. No significant reduction in fruit scab was observed for any of the amendment or bacterial isolate treatments.

The combination of bacteria with chitosan was also evaluated for suppression of the postharvest diseases bitter rot and blue mold. Application of chitosan to apple wounds alone or in combination with *B. megaterium* isolate A3-6 or Ae-1, *B. mycoides* isolate A1-1, or *Brevibacillus laterosporus* isolate FLS-1 significantly reduced bitter rot and blue mold lesion size on both ‘Golden Delicious’ and ‘Rome Beauty’ fruit. A synergistic effect was observed in treatments combining chitosan with *B. mycoides* isolate A1-1 or *B. megaterium* isolates A3-6 and Ae-1 on ‘Golden Delicious’ fruit with an average of 81% reduction in bitter rot lesion size compared to the no-bacteria/chitosan control. Application of chitosan with *B. megaterium* isolate A3-6 to wounds resulted in a 78% reduction in blue mold lesion size. Future experiments evaluating
postharvest disease suppression when chitosan is applied in combination with our isolates as a fruit coating (applied as a dip or spray) will provide insights into the level of control that could be achieved in a commercial setting.

The bacteria *B. mycoides* isolate A1-1, *B. megaterium* isolates A3-6 and Ae-1 and *B. cereus* isolate FLS-5 significantly reduced bitter rot lesion size and were able to colonize fruit wounds at room and storage temperatures. Population levels of isolates A3-6 and Ae-1 were typically log 2 lower than isolate FLS-5 at 2°C and log 1 lower at 20°C, suggesting that the mechanism of disease suppression does not involve competition for space or nutrients. Scanning electron microscopy was used to investigate the mechanism of postharvest disease suppression by these isolates. Observations of wounded tissue colonized with *B. megaterium* isolate A3-6 showed attachment of bacterial cells to hyphae of *C. acutatum*. Furthermore, sections of the hyphae where bacterial cells had attached were damaged and collapsed. Further experimentation is needed to more conclusively elucidate the mechanism of bitter rot suppression by isolate A3-6.

The potential for integration of the collected bacterial isolates into existing apple management programs was evaluated. The bacteria *B. megaterium* isolates A3-6 and Ae-1 and *B. cereus* isolate FLS-5 were able to colonize apple foliage treated with sulfur, copper hydroxide or streptomycin sulfate. While *B. mycoides* isolate A1-1 significantly reduced fruit and foliar scab severity in two successive field trials, foliar colonization on non-treated or fungicide treated leaves was low. Although, *B. cereus* is a ubiquitous colonizer of foliar and soil environments, these bacteria are considered an opportunistic animal pathogens and some strains cause food poisoning. Even though only certain strains cause illness, the use of *B. cereus* isolate FLS-5 for biological control on apple may face regulatory issues. Based on the controlled environment, greenhouse and field experiments conducted for this dissertation, *B. megaterium* isolate A3-6 and *B. mycoides* A1-1 are the best candidates for biological control of pre- and postharvest diseases of apple. The combination of our biological control agents with fungicides or antibiotics may provide significant benefits by reducing the variability in disease management associated with biological control agents while reducing the rate of chemical used without compromising disease suppression. The disease management potential of our isolates combined with other BCAs with documented activity against the overwintering or secondary phase of apple scab represents an additional area of research to develop and optimize biological strategies for apple.
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Chapter 1

Literature review and research objectives

Introduction to the crop

The domesticated apple is an interspecific hybrid commonly designated as *Malus* x *domestica* Borkh after the scientist Borkhausen who first described the complex in 1803. The origin and ancestry of the *M. domestica* complex is unknown, however, *Malus sieversii* (Ledeb.) Roem. is regarded as the key species in its origin [55]. The cultivated apple is believed to have originated in central Asia and is currently grown throughout the world [55, 109]. Apples are perennials and a member of the genus *Malus* which is in the Maloideae subfamily of the Rosaceae family. Other members of the Maloideae include pears, quinces, loquats and medlars and are characterized by a hypanthium and gynoecium that remain fused forming an inferior ovary that develops into a fleshy fruit or pome.

Apple is one of the most predominant deciduous tree-fruit crops in the world and is grown in nearly every state in the US and most provinces in Canada [47, 109]. Apples are adaptable to different climates as production also occurs in the Mediterranean zone, as well as tropical and sub-tropical regions. Although production is widespread, successful apple production is limited to areas that meet specific climate, soil quality and water availability requirements. In general deciduous tree fruits such as apple are adapted to cold winters as leaves are shed and the trees enter a dormant state resistant to frost. Leaf senescence and abscission is triggered by temperature and not day length as apple plants appear to lack photoperiodism [64]. Following bud exposure to low temperatures, dormancy is broken with the emergence of leaves and flowers in the spring. Several reports regarding the number of chilling hours required for
dormancy and chilling models have been developed to determine optimal fruit growing areas [64, 78, 97].

Apples are almost always grown as a compound tree consisting of a rootstock, which provides the root system, and a scion, which is the fruiting portion of the tree. Although apple trees can be propagated from seed, the heterozygous nature of *Malus* species result in trees that are highly variable in vigor, habit and fruit characteristics and so seeds originating from a tree with desirable traits will not produce trees that are true to type [122]. Furthermore, many apple varieties are not self-fertile, therefore, vegetative (clonal) propagation via budding or grafting from “mother trees” are the primary means for propagation. Most commercial scion varieties are less productive when grown with their own roots and exhibit poor root formation on cuttings. Rootstock varieties are selected based on their effects on scion vigor, precocity and quality of fruits produced. Rootstocks may also be used to adapt scions to unfavorable climate or soil conditions and protection from aerial and/or soil-borne pests [122]. Many apple rootstock/interstocks reduce the vigor of scions creating dwarfed trees that can be planted closely allowing for greater fruit output per acre [121]. Production with dwarfing rootstocks has several advantages as dwarfed trees are easier to target with sprays and produce fruit precociously. Dwarfing rootstocks often increase numbers of floral clusters per linear branch leading to increased fruit set.

Apple scions are selected primarily for their fruit quality, market demand and productivity [47]. There are thousands of apple cultivars, however, only a fraction are used for commercial production. The two most popular cultivars are ‘Delicious’ and ‘Golden Delicious’ and are grown in most all regions of the world [114]. Other important cultivars include ‘Granny Smith’, ‘Jonagold’, ‘Gala’, ‘McIntosh’, ‘York’ and ‘Fuji’. Apple trees produce flowers and fruit from mixed buds that are usually produced on spurs or on the ends of shoots. Flower buds are occasionally formed in leaf axils on 1-year old shoots, but normally do not set fruit or are smaller
than terminally located fruit. An inflorescence contains five to seven individual flowers where the center bud opens first and produces the largest fruit [14]. The canopy structure of an apple tree varies, containing shoots of different lengths. Rosette-type shoot complexes are short and often referred to as spurs. Longer single shoots with long internodes are referred to as extension shoots and develop from terminal or lateral buds from previous years [64].

In general, apple trees bear many more flowers compared to fruits averaging only 4-10% of the potential fruits. In terms of production, under ideal conditions fruit yields have been reported up to 180 tons per hectare which is equivalent to 30 tons per hectare dry matter. Reported dry matter yields of apple are comparable to that of potato which has the highest C3 crop productivity. The ability to produce high dry matter yields is attributed to the inexpensive energetic costs of the apple fruit compared to other crops such as nuts which contain energetically expensive proteins and oils [64]. Biological yield of dry matter is influenced by several primary components including amount of incident light, fraction of light intercepted, photosynthetic transduction of light energy into fixed energy and the amount of carbon lost to respiration. Management strategies become important in regulating these components to obtain desired effects on yield.

After harvest apples are graded into quality classes based on size, color, shape and presence of surface defects. This type of classification has a major effect on price and thus profit experienced by a grower [47]. Long-term storage has been utilized extensively to extend the marketing season and maximize returns. Apple production systems have also changed significantly over the past several years. High density plantings on dwarfing or semi-dwarfing rootstocks supported on trellis systems have become more popular and are replacing conventional widely spaced plantings. These high density systems require intensive management where pruning is critical in maintaining productivity and quality [114]. Pest management is also important as dense tree canopies provide favorable conditions for growth and survival of many
tree fruit diseases and pests. Pruning, for example, can be used to open the canopy and promote air movement and light penetration. The open canopy will provide for rapid drying after rain (reducing infection periods for pathogens) and allows for more effective spray penetration of the canopy. An open canopy also increases sunlight exposure by leaves leading to greater sugar production and eventually increased fruit color and size [32, 125].

Physiological development of fruit can be divided into three major stages (1) growth (2) maturation and (3) senescence (Figure 1-1). The growth stage encompasses cell division and enlargement. Maturation is described as the stage leading to physiological maturity or the state at which the plant part will continue ontogeny, even when detached [119]. Senescence is defined as the period when anabolic processes slow and catabolic processes predominate leading to eventual death of the organ. Ripening is a term specifically used to describe the later stages of maturation in fruit [126]. In general, ripening marks the end of maturation and the beginning of senescence and is responsible for the transformation of the organ from inedible to edible in terms of color, texture and composition [119]. More specifically, ripening is associated with several physiochemical changes including seed maturation, abscission, ethylene production, tissue permeability, production of volatiles and changes in respiration rate among others [36]. Growth and maturation occur while the fruit remains attached to the tree, while ripening and senescence may take place when the fruit are still attached to the tree or after they have been removed.

Figure 1-1: Growth, respiration and ethylene production patterns of climacteric and non-climacteric plant organs [126].
Fruits are classified as climacteric or nonclimacteric based on the rate of respiration during ripening. More specifically, fruit designated as climacteric exhibit a pronounced increase in respiration that coincides with ripening and leads to an increase in concentrations of carbon dioxide and ethylene [120, 126]. Climacteric fruit are also differentiated from non-climacteric fruit by their production of and response to exogenous ethylene. For example, apple is classified as climacteric and the presence of ethylene will cause an increase in respiration and production of more ethylene. This phenomenon is referred to as autocatalytic.

Diseases of apple

Apples are host to many plant pathogens including fungi, bacteria, phytoplasmas, viruses, and virus-like agents. Although there are over 70 infectious diseases of apple [37], only a small portion cause severe diseases that pose a significant threat to apple production. The majority of diseases on apples are caused by phytopathogenic fungi predominately belonging to the Basidiomycota, Ascomycota and the fungal-like organisms of the Oomycota [37]. Representatives from these groups can cause a variety of diseases including root rots, leaf spots and blights, blossom blights, fruit decay and spots, defoliation and trunk, branch and twig cankers. The most important apple diseases caused by fungi and fungal-like organisms in the northeastern United States are apple scab (*Venturia inaequalis* (Cke.) Wint.), cedar apple rust (*Gymnosporagium Junipei- virginiana* L.), and powdery mildew (*Podosphaera leucotricha*) [54]. Other fungal diseases of apple can be classified into broad categories known as brown-rot diseases, summer diseases, and postharvest diseases. Brown-rot fungi cause blossom wilt, spur dieback, cankering and fruit rot. Summer rots encompass nine diseases that occur in warm, moist regions. Theses rots include; bitter rot, black rot, white rot, black pox, necrotic leaf blotch,
brooks fruit spot, *Alternaria* blotch, sooty blotch and flyspeck [37]. The most important apple disease caused by a bacterium is fire blight (*Erwinia amylovora* (Burrill) Winslow et al) [12].

**Pre-harvest apple diseases**

Powdery mildew can cause potentially serious disease on many hosts throughout the world and is most problematic on apple in nursery production. Leaves, flowers and fruit are susceptible to infection, however new growth is particularly susceptible [114]. This superficial fungus grows on host surfaces and can appear as a solid mat of hyphae on the leaves. Although the fungus does not grow intracellularly, a structure called a haustorium extracts water and nutrients from host cells. Leaf surfaces can become covered with mycelium and a severe infection can reduce tree photosynthesis and transpiration [28]. Spore germination is favored under high humidity and so cultural practices that promote air movement can reduce or help avoid an outbreak. Two basic strategies are used for management of powdery mildew; resistance and fungicides [114]. Fungicides commonly used on susceptible cultivars to control powdery mildew include sulfur, DMIs (sterol inhibitors), benzamidazoles and strobilurins [114]. Sulfur, one of the most economical options, is very effective at reducing powdery mildew, however it must be applied frequently as a protective spray. Traditionally used for insect and mite control, several mineral and plant oils are also effective against powdery mildew [85]. Protective activity of oils is dependent on the type used and the amount of rainfall. Generally, oils have limited kickback activity of about 24 hours.

Apple scab is one of the most economically important diseases of apple and occurs worldwide wherever apples are grown [69]. Although the disease occurs worldwide, it is most severe in temperate regions with cool, moist weather conditions in the spring [69]. Infection and subsequent development of symptoms can occur on leaves, fruit, petioles, sepals, blossoms, and
budscapes. Foliar lesions can develop on both the upper and under surfaces but typically do not lead to alteration of leaf shape. Infected buds or blossoms usually lead to blossom drop or severe infection of the developing fruit. Infections on flowers, petioles and young fruits may lead to fruit drop and subsequent yield reduction [69]. Apple fruit are most susceptible when young and the resulting fruit is often deformed with large lesions (Figure 1-2A). Mature fruits are more resistant with smaller lesions that develop more slowly [69]. Reduction in fruit quality of scabbed apples is the major economic loss resulting from apple scab. This disease may also contribute to losses by reducing fruit set, tree defoliation, and reduction in tree vigor [110]. Due to the agricultural and economic importance of this disease, a substantial amount of research on the biology and epidemiology of the apple scab fungus has been conducted over the past several decades.

![Figure 1-2: (A) Scab lesions on a mature apple. (B) Scab lesions on an apple leaf that overwintered on the orchard floor from the previous season.](image)

The pathogen, *Venturia inaequalis* (anamorph *Spilocaea pomi* Fr.), is a member of the Ascomycota subclass Loculoascomycetidae and order Pleosporales producing ascocarps (pseudothecia) in a stroma [54]. *V. inaequalis* is restricted to the genus *Malus*, and occurs on both cultivated apple varieties and crab apples. Varieties and cultivars of apple exhibit different levels of resistance to the pathogen. The fungus primarily overwinters as pseudothecia initials produced within infected leaves or fruit on the orchard floor (Figure 1-2B). Asexually produced conidia can also overwinter in dormant pustules on shoots or budscapes [69]. Pseudothecia complete their development and ascospores mature with the advent of spring and favorable
conditions (Figure 1-3). Moisture enables the discharge of ascospores, the primary inoculum [34, 41], which are then carried by air currents to apple tissues. Ascospore release periods typically occur before or during bloom [115] and major propulsive discharges take place during distinct intervals corresponding to rain events occurring during daylight hours [70]. Research has demonstrated a steep ascospore dispersal gradient where most ascospores are deposited within 100-200 m from their source [69]. Once in contact with susceptible apple tissues, ascospores germinate and initiate primary infections when kept wet at temperatures from 5-26ºC [54]. A thin film of moisture is required for the initiation of ascospore germination and a relative humidity of approximately 95% is required for germination to proceed beyond initiation [14]. During infection, an appressorium and a penetration peg form and hyphae penetrate the leaf surface extending between the epidermis and the cuticle. Once established beneath the cuticle, the fungus ramifies into a stroma resulting in a visible lesion containing conidiophores bearing conidia. Conidia, which serve as the source of secondary cycles and buildup of the disease during the summer, are disseminated by rain splashing and wind [106] to other tissues within a tree. Conidia can germinate, leading to the development of new lesions on susceptible leaves, buds and fruit. Repeated conidial cycles result in classic polycyclic disease spread within a season. As temperatures drop in autumn, leaf abscission occurs and the fungus enters a saprophytic phase in which mycelia can grow and survive in host tissues (leaves, stems, buds) during the winter months [69].
Management of apple scab has primarily relied on the use of protectant or eradicant fungicides to eliminate the primary ascosporic cycle. Mature leaves and fruit are morphologically resistant to *V. inaequalis*, and new growth is most susceptible to infection [98]. Due to the polycyclic nature of apple scab and susceptibility of new growth, repeated fungicide applications are required over the course of a season, starting at the green tip stage of bud development [53]. Temperature and moisture together are key factors determining the length of the primary infection period as temperature governs the rate of ascospore maturation and rainfall discharges ascospores. Forecasting models are used to time fungicidal sprays to coincide with ascospore maturity and discharge. Based on ascospore germination criteria, infection windows are governed by the number leaf wetness hours and temperature [71]. Mills [80] described the minimum number of hours of continuous leaf wetness required for ascospore infection and compiled a table specifying timing of spraying or dusting with sulfur during rainy periods. Based on Mills’ predictions, discrete infection periods can be forecasted and fungicidal sprays timed appropriately. Mills and Laplante [81] also reported infection periods required by conidia as two-thirds the duration of wetting required by ascospores. Modifications have been proposed to
Mills’ original infection period tables including shorter minimum infection periods for ascospores and conidia at low temperatures, uniform criteria for ascosporic and conidial infection [102] and consideration of the daily periodicity of ascospore discharge [71].

Although a significant amount of research has been conducted on apple scab in the past 100 years, this disease remains one of the most destructive and economically important diseases in apple production today. Most research has focused on the control of primary infections based on weather conditions conducive to ascospore production and predictive models used to time fungicide applications [71]. Attempts have been made toward developing strategies to reduce the overwintering inoculum of *V. inaequalis* and therefore limit primary infections using chemical, cultural [79, 103] and biological strategies [17].

Holb and colleagues [45] point out that less attention has been paid to secondary infections caused by conidia mainly because they are dependent on primary infections. Several studies have demonstrated that scab conidia can overwinter within bud tissues, remain viable and infect exposed sepals in the spring [11, 44]. Research from Holb’s group indicated a significant risk of early scab epidemics initiated by overwintered conidia in orchards with high levels of scab incidence the previous summer [44, 45]. Inoculations of early phenological stages of growth have indicated that sepals could become infected (by conidia) as early as green tip [11]. In integrated and organic apple production systems, scab control may be less effective compared to conventional systems, allowing for more serious late summer and autumn scab epidemics to develop [46]. Under these circumstances control of secondary cycles initiated by conidia are needed in years preceded by high scab incidence. Several studies have shown that treatments such as shredding of leaf litter, urea application [103], and/or application of fungal antagonists [17, 131] can successfully destroy or reduce the amount of overwintering pseudothecia. To achieve acceptable control, strategies preventing infection by overwintering conidia may also be necessary. The recent advances in the understanding of conidial overwintering have opened new
avenues for scab research and development of management strategies. As mentioned previously, cultural and biological control research has focused on eliminating overwintering pseudothecia and initiation of primary infections by ascospores. Limited research has been conducted on organisms antagonistic to the asexual stage of *V. inaequalis* responsible for secondary cycles and the buildup of inoculum within the season. Survival of conidia within the inner bud tissues may also offer a unique niche to a spectrum of resident microflora that may influence conidial germination and infection.

Apple is also host to several rust species, the most common and commercially important belonging to the genus *Gymnosporagium* [114]. Fungi belonging to this genus are classified as heteroecious as they require two hosts to complete their life cycle, a juniper and a rosaceous species. Each of the rusts has similar life cycles but vary in which juniper and rosaceous species they infect [1]. Cedar apple rust (*G. juniper-virginianae*; CAR) is most important in eastern North America where both apple and the alternate host (eastern red cedar) occur, causing significant yield loss due to leaf and fruit infections which can reduce fruit set and quality. The fungus overwinters as mycelium in galls on eastern red cedar which become active in the spring with the arrival of warm, wet weather conditions (Figure 1-5). During wetting periods the galls expand and become jelly-like producing orange-brown telial horns bearing teliospores. The galls eventually dry, but can undergo several wetting-drying cycles during the spring (Figure 1-4). The teliospores are produced during each wetting period and germinate to produce basidiospores. Basidiospores are forcibly discharged during rain events and carried by wind several kilometers without losing their ability to infect apple [90, 114]. Basidiospores are able to infect young leaves and fruit of apple. Fruit are most susceptible to infection during the tight cluster stage and until just after petal fall. Approximately 1-2 weeks after basidiospore infection, pycnia (containing pycniospores and receptive hyphae) develop on the upper sides of leaves or on fruit. Following the fertilization of receptive hyphae by compatible pycniospores, aecia form on the
underside of leaf surfaces or on fruit and produce aeciospores 1-2 months after the formation of pycnia. Aeciospores are released during dry weather in late summer and re-infect nearby young twigs of eastern red cedar.

Figure 1-4: (A) A dried telial gall on cedar (B) Fruit and foliar sign of cedar-apple rust on ‘GoldRush’.

Figure 1-5: Disease cycle of cedar-apple rust, caused by Gymnosporagium juniper-virginianae [1].

Infection periods accounting for time-temperature relationships that influence the production and germination of several spore stages (teliospores and basidiospores in particular) have been investigated and mathematical models developed [89]. These models are used to efficiently time fungicide applications which are currently the primary means for cedar apple rust disease control. Many fungicides used for apple scab are also effective against cedar apple rust.
Removal of nearby eastern red cedar trees is another recommended disease control measure however, due to long distance spread of basidiospores and resistant homeowners, this remains a difficult task. Varying levels of resistance by certain apple cultivars to cedar apple rust have been documented and characterized [2] and use of these cultivars has been suggested as an additional control measure particularly in orchards where eastern red cedar occurs nearby. The most susceptible varieties include ‘Golden Delicious’, ‘Rome’, ‘Gala’, and ‘Gold Rush’ among others [114]. Quince rust caused by *G. clavipes* is also prevalent throughout North America, but unlike cedar apple rust, only infects fruit of apple. Another less common rust disease, hawthorn rust (*G. globosum*) only infects apple leaves and not the fruit [54].

*Postharvest diseases*

Postharvest losses on fruit and vegetables, most of which are caused by microbial pathogens, account for up to 25% of the harvested crop in the United States and approach 50% worldwide [116]. The fungal pathogens *Penicillium expansum*, *Botrytis cinerea*, and *Colletotrichum gloeosporiodes* and *C. acutatum* are the most important postharvest pathogens on apple, causing extensive losses worldwide [52]. Synthetic fungicides are the primary means used to control postharvest diseases and an estimated 23 million kg of pesticides are applied to fruit and vegetables annually [113]. Intensive use of fungicides is considered to be responsible for the development of resistance to several classes of fungicides used on apples, leaving growers with fewer effective control options [29, 96]. Due to the nature of the commodity, most fruits and vegetables are produced and harvested over a relatively short period of time. Most produce, however is stored for weeks or months to avoid exceeding market demand and provide consumers with products year round. In addition to long term storage needs, fresh fruits and vegetables are shipped great distances from producers to supermarkets all over the country. The events
occurring before and after harvest are a vital link in the production chain, ensuring protection from decay, a suitable shelf life, and ultimately a consumer product that is fresh, disease-free, and safe for consumption.

Pathogens causing postharvest decay can be classified into a few broad categories based on (1) the infection strategy and (2) the timing at which infection occurs. In the first category, postharvest pathogens may enter their hosts via either a "wound infection" strategy or by "direct infection". Postharvest decay fungi most commonly utilize wounds to bypass the protective skin of their host [111]. These fungi often invade wounds incited during harvest and handling. Penetration often occurs at the stem-end separation area, where injury commonly occurs when fruit are picked [9]. Fungi able to directly penetrate the host cuticle and epidermis employ specialized infection structures (appressorium and infection peg) to breach these physical barriers. An appressorium, produced shortly after germ tube formation, tightly adheres to the fruit surface and produces several enzymes, such as cutinase, weakening the cuticle. An infection peg then forms penetrating the cuticle, and mycelium ramifies and invades the fleshy tissue [25].

In the second category, postharvest decay fungi are grouped by those which initiate infection primarily after harvest and those that infect fruits preharvest [95]. Fungi in the latter group, initiate infection in the field, but often remain quiescent until after harvest when conditions for growth become favorable and/or host resistance decreases as fruit ripen and tissues senesce [126]. In this case, at some point between arrival of the pathogen and symptom expression, growth of the pathogen is arrested. This lag time between infection and symptom production is often described as a "latent infection" or "quiescent infection". Some variation exists among the postharvest community regarding the use of these two terms and their overlapping definitions [9]. In this review, the term "quiescent" will be used in reference to situations where the pathogen growth is temporarily inhibited [107] and the latent period as the time between pathogen arrival and symptom expression. Quiescence, as defined by Jarvis [50], may run the spectrum from an
ungerminated spore to symptomless internal infections to visible yet non-expanding lesions. Variation in quiescence among the different stages of fungal attack suggests that this phenomenon involves a dynamic equilibrium between the host, the pathogen and the environment [50, 92]. Host physiological, structural or environmental changes may result in an equilibrium shift that allows the pathogen to resume the infection process. There are several theories to explain the establishment of quiescent infections. These theories can be grouped into three categories [117]; (1) Nutritional and energetic requirements of the pathogen (2) activation of pathogen enzymes and (3) the presence or induction of antifungal compounds in the host.

Most postharvest decays that originate in the field are caused by fungi able to directly penetrate fruit surface tissues. For example, fruit infection by the bitter rot fungus (*C. acutatum*) occurs in the orchard throughout the growing season on all developmental stages of apple fruit [104]. During warm (~26°C), wet conditions germinating spores infect by direct penetration of the skin leading to complete decay of fruits which eventually turn into 'mummies' [99]. These mummies produce copious amounts of conidia which serve as inoculum for the next year. The bitter rot fungus can also produce symptoms in storage through infection of wounds made during harvest or packing [9]. In contrast, many storage rots that infect postharvest require wounds or natural openings to infect apple fruit. The causal agent of blue mold rot, *P. expansum*, invades mainly through wounds or bruises although infection via lenticels may occur under favorable conditions [9]. This disease is rarely observed in the field, except on fruit that have fallen off the tree. Infections occur when airborne conidia enter wounds incited during the harvesting process or by spores present on storage bins and packinghouse walls. Waterborne spores are also commonly found in postharvest drench solutions or water flumes used to float apples through the packing lines. Conidia are able to survive from season to season on contaminated bins, picking boxes, and storage room walls [95].
Considerable attention has focused on the potential use of biological control for postharvest disease management of fruits and vegetables [49, 100, 129]. Microbial biocontrol agents are generally target specific, nonhazardous to humans and animals and environmentally friendly. The uniqueness of the postharvest environment offers an advantage where conditions such as temperature and relative humidity can be adjusted to favor survival of biocontrol agents. The challenge to biocontrol is that new technologies must not only be safe, but a viable alternative to current synthetic fungicides. Several microorganisms have been patented for postharvest biocontrol many of which are applied to harvested fruit as a conventional dip or drench. Currently, two strains of *Pseudomonas syringae* are available commercially in the United States for postharvest biocontrol of blue mold and gray mold on apple and pear and are marketed under the name Bio-Save 10 and Bio-Save 11 [23]. Use of these products however, remains limited due to inconsistency in their efficacy, low levels of control as standalone products, and apparent inability to control previously established infections [23]. As described earlier, while appearing healthy at harvest, many fruit may harbor quiescent or latent infections that produce symptoms in storage as ripening occurs and host defenses weaken [25].

Many believe that competition for nutrients and space is the most likely mode of action employed by postharvest biocontrol agents. Others have reported antibiosis and induction of host resistance mechanisms in fruit tissue [18, 128]. Application after harvest however, may be too late for these microbes to effectively compete with decay pathogens that have already established themselves on fruit surfaces in the field. Furthermore, induction of host defense mechanisms after harvest may not be as effective as induction while the fruit are still attached to the tree. Some success has been reported using pre-harvest field application of biocontrol agents on apple and pear [13, 65]. Experiments directly comparing levels of postharvest disease suppression by pre- and postharvest applied biocontrol agents are necessary to determine the most effective application strategy.
Pest management

A successful management program is essential for maintaining a productive, profitable apple production system. Management strategies differ based on the region, economics, consumer preferences and the marketing objective (processing, fresh fruit, organic or conventional). Regular pesticide/herbicide use can result in significant costs associated with disease management and overall production. Expenses include labor, equipment, and cost of the chemical(s). Fungicides are a major component of apple disease management programs and their use dates back to the latter portions of the 1800’s with applications of Bordeaux mixture for control of apple scab. Throughout the past three decades research on apple diseases and efforts to improve disease management programs have focused on equipment, materials, and strategies for using fungicides. With the case of apple scab, a continued dependency on fungicides extended into the 1980’s during which economic levels of apple scab control were achieved solely by applying fungicides [69]. In addition to these issues, public concerns over pesticide use and residues on food has significantly increased since the 1960’s and directly influenced disease management practices on tree fruits. In 1987 the National Academy of Science reported that fungicides constitute 60% of all oncogenic risk among all the pesticides used on food [83]. Legislative banning of many effective fungicides due to health and environmental hazards has left the industry with limited options for disease control. Consumers are increasingly aware of these issues and demand apples free of chemical residues grown using practices that are safe for the environment. As a result, there has been a growing interest in development of safe, effective and economically feasible alternatives. Additionally, movement toward integrated pest management (IPM) and organic apple production has stimulated the need for additional research into alternative pre and post harvest management strategies to control postharvest diseases [43].
Integrated pest management (IPM) can be described as a dynamic, systems approach to crop protection that combines cultural practices, resistant varieties, biological control, and chemical control strategies. Surveillance and forecasting information are also central in the development of sustainable crop protection within an IPM system. Because of the focus on ecological processes and interactions, IPM requires a multidisciplinary approach. Disease development, for example, is influenced by several factors relating to the pathogen, its host, and the surrounding environment. The factors are components of the disease triangle which describes the necessary causal factors for disease development and is of fundamental importance in plant pathology [33]. Each of these three factors must be considered when managing pests or diseases especially in the realm of IPM. Control practices for a particular pathosystem may be aimed directly at the pathogen to reduce survival with the use of protectant fungicides, eradicant fungicides, or sanitation. Practices aimed directly at the cultivar may be used to increase resistance to the pathogen. Practices aimed towards making the environment less favorable for the pathogen may include the use of ground cover, pruning, or proper timing of irrigation. Targeting any one of these components may lead to an acceptable reduction in the amount of disease. However, the reduction in more than one component, with a combination of tactics, will potentially lead to more significant control [69].

The concepts of IPM in apple production was introduced and promoted widely during the 1980’s. Significant advances were made in entomology such as pest monitoring and the establishment of action thresholds. Advances in plant pathology included adjustment of spray schedules based on inoculum levels and exposure [35]. Further refinement of controls developed in preliminary research trials however, were needed in order to achieve success in commercial orchards. Gadoury et al [35] pointed out that management strategies of a specific pest or disease are often developed under highly controlled conditions dealing with pests as discrete units. As a
result, the control measure may reduce the number of fungicide sprays for the specific disease, but sprays may still be required for another pest or disease and the overall amount of fungicide remains unchanged. In order to overcome these issues, IPM control measures for specific diseases must be integrated into an overall management program synchronizing controls for multiple pests and minimizing the number of separate application trips through the orchard. Gadoury et al [35] reviewed the traditional approach to disease management with protectant pesticide application schedules (Figure 1-6) and identified three ‘windows’ where elimination of fungicide applied only for scab control would be possible. The first window corresponds to the period prior to the pink stage of bud development, a time during which other apple diseases and pests are not damaging enough to warrant a fungicide treatment. Gadoury suggests that orchards with a minimal amount of overwintering inoculum may represent prime candidates for the elimination of the early season spray.

Lewis [67] proposed an apple management program based on nine factors; cultivar susceptibility, density of the pathogen population, rainfall and temperature, tree growth; spray equipment, spray timing, application method, fungicide mode of action, and fungicide rate. In 1983, MacHardy and Jeger developed an integrated management program for apple scab based on

![Figure 1-6: The major pest complex for apples in the northeastern United States. Letters at the top indicate the approximate timing of pesticide application in a traditional protectant spray schedule. Each bar indicates the period during which a pest is usually controlled [35].](image)
advances made in Africa by Schwabe and by Olivier in France during the late 1970’s and early 1980’s [69]. MacHardy and Jeger [72] proposed a model that mathematically related the components contributing to disease occurrence and development. Several research papers were published by MacHardy and his coworkers investigating aspects of each component in their model and quantitatively determined its influence on disease progression.

The development of practical and efficient strategies for control of apple diseases and to reduce fungicide applications has been one of the driving forces behind integrated management systems. Development of resistance by key tree fruit pathogens to fungicides and bactericides continues to face the industry as a major obstacle in tree fruit production and disease management [20, 93]. Fewer than half of the fungicides available a decade ago for apple disease management are still available due to registration revocation and/or development of resistance [77]. Intensive use of fungicides, particularly against V. inaequalis, is considered to be responsible for the rapid development of resistance to several classes of fungicides [62]. Resistance to QoI and DMI fungicides, which had previously been regarded as highly effective for use in IPM programs, has been reported and is a significant concern today [73, 105]. Because of these DMI resistant strains of V. inaequalis, other fungicides including dodine and the benzimidazoles are still available for scab management but are presently not widely used in orchards [77]. These issues stress the need for alternative tactics to reduce fungicide usage and pathogen exposure as an anti-resistance strategy [124]. A major objective in the IPM approach is to use a variety of tactics to reduce overall pest and pathogen populations. Pesticides are generally applied only after all other options have been utilized or when their need is justified by established guidelines, pest biology or field monitoring [132]. The need for novel, effective, alternative pest management strategies has stimulated research in several disciplines.
Disease control in organic apple production

In addition to issues regarding pathogen resistance, public concerns over pesticide use and residue has significantly increased since the 1960’s and influenced disease management practices on tree fruits. Rachel Carson’s book, *Silent Spring* brought pesticide issues to the forefront challenging their use without regard for human and wildlife safety. Consumers are increasingly aware of and demand apples free of chemical residues and as a result there has been a growing interest in organic apple production [43]. Retail sales of organic products reached $7.8 billion in the United States in 2000 and it is estimated that 1 out of every four Americans buys organic products [130].

Many of the synthetic products relied upon in conventional systems are prohibited in organic fruit production, making disease control more difficult. The goal of organic farming is to maintain a sustainable balance between crop plants and weeds; unlike conventional systems, complete eradication is not the intention. Control of foliar, soil-borne and post harvest diseases is an important challenge in organic apple production. Among products approved for use in organic apple systems against foliar diseases include sulfur and copper compounds. These formulations however, tend to be more phytotoxic than modern synthetic fungicides [69]. Copper products provide effective control against apple scab, however, due to environmental concerns many countries have banned its use in organic production [42]. Elemental sulfur is considered a contact fungicide and exhibits only weak protective activity and so lime sulfur has received renewed interest [42]. Both sulfur and lime sulfur are among the oldest fungicides used dating back to the latter 1800’s. Holb and Hiejne [43] evaluated lime sulfur products in organic production systems and found lower scab incidences on leaves under heavy disease pressure compared to wettable sulfur alone and applications remained protective after heavy rain. Leaf phytotoxicity was high and so a combination of sulfur and lime sulfur was recommended to reduce phototoxic effects and resulted in control similar to copper. Travis et al. [112] evaluated organically approved
sulfur (Micro-sulf), lime sulfur and copper compounds in combination with alternatives for control of apple scab on mature ‘Rome Beauty’, ‘Golden Delicious’, ‘Cortland’, ‘Stayman’, and ‘Red Delicious’ trees. Alternative treatments tested included Vigor-Cal-Phos, Armicarb +JMS Stylet Oil, and Serenade Max +Nufilm. The sulfur, lime sulfur and lime sulfur+ Vigor-Cal-Phos were the most effective treatments and resulted in levels of leaf and fruit scab control similar to conventional materials tested. Although research has shown that organic apple production is possible in the northeastern US, and sulfur-based spray programs provide adequate scab control, the limited number of management options remains a significant concern. The search for additional control tactics is an important step toward successful organic apple production. Once newer options are identified, the combination of several strategies may reduce the dependence on sulfur compounds and lead to a multifaceted ‘toolbox’ of disease management tactics.

Biological control

The term biological control can be broadly defined as the suppression of the damaging activities of one organism by the activity of one or more other organisms. The term is used across several disciplines most notably in entomology and plant pathology with slightly different meanings. In plant pathology, the term is used to describe the use of microbial antagonists to suppress plant diseases and weed populations [88]. The term 'biological control' is misleading, as biocontrol agents rarely result in 100% control. Biological control agents (BCAs) when successful can reduce disease below an economic threshold and are most successfully utilized as part of an integrated approach utilizing several tactics that collectively are effective. Disease management using biological control is a continual process influenced by many factors beyond just the pathogen and the BCA. As a result, successful development of biological controls
requires integration of knowledge across many disciplines including epidemiology, microbial ecology, entomology, plant pathology, soil science and horticulture.

There are several modes of action leading to biological control. In the broadest sense, pathogens are antagonized by the presence and/or activities of the BCA. Pal and McSpadden Gardener [88] describe three types of interspecies antagonisms leading to biological control: direct antagonism, mixed-path antagonism and indirect antagonism (Table 1-2). Direct antagonism results from the direct parasitism of the pathogen by the BCA. In contrast, mixed-path antagonism occurs when the BCA makes the environment less favorable for pathogen survival through the production of antibiotics, enzymes or volatiles. Indirect antagonism results in suppression of pathogen populations due to induction of host resistance or competition for space and/or nutrients.

**Table 1-1:** Types of interspecies antagonisms leading to biological control of plant pathogens. Adapted from Pal and McSpadden Gardener [88].

<table>
<thead>
<tr>
<th>Type</th>
<th>Mechanism</th>
<th>Examples</th>
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<tbody>
<tr>
<td>Direct antagonism</td>
<td>Parasitism/predation</td>
<td><em>Pasteuria penetrans</em> [21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Trichoderma virens</em> [68]</td>
</tr>
<tr>
<td>Mixed-path antagonism</td>
<td>Antibiotics</td>
<td>Agrosin 84 [94]</td>
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<tr>
<td></td>
<td>Lytic enzymes</td>
<td>Chitinases [58]</td>
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<td></td>
<td></td>
<td>Glucanases [8]</td>
</tr>
<tr>
<td>Indirect antagonism</td>
<td>Competition</td>
<td>Siderophore scavenging [24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Niche exclusion [127]</td>
</tr>
<tr>
<td></td>
<td>Induction of host resistance</td>
<td>PGPR [51]</td>
</tr>
</tbody>
</table>

Often a BCA leads to disease suppression through several mechanisms. *Trichoderma harzianum*, for example has been shown to suppress several plant pathogens using a combination of modes of action including antibiotics, enzymes and induction of host resistance [27].

**Biological control utilizing bacteria**

Biological control using bacteria offers a promising alternative for management of many diseases and promotion of overall plant health [6]. Although several microbes have been the
focus of biological control research, efforts have focused on three main genera, the fungus *Trichoderma*, and the bacteria *Bacillus* and *Pseudomonas* [75]. Many aerobic endospore forming bacteria, *Bacillus* and *Paenibacillus* spp. in particular, act as plant growth-promoting rhizobacteria (PGPR) and are ubiquitous colonizers in bulk and rhizosphere soils [74]. Formation of stress-resistant endospores, able to remain viable for extended periods, makes this class of biocontrol agents particularly useful for large scale production of products with a suitable shelf life. Research on PGPR bacteria, specifically *Bacillus* spp., has demonstrated that these bacteria elicit host defenses leading to induced systemic resistance (ISR) and systemic protection against subsequent pathogen invasion [39, 101, 123]. Research at Montana State University found two strains of *Bacillus pumilis* and one strain of *B. mycoides* able to reduce severity of Cercospora leaf spot of sugar beet [8]. Elicitation of ISR was associated with enhanced peroxidase activity and increased production of a chitinase enzyme [7]. Other mechanisms attributed to control by PGPR bacteria include production of peptide antibiotics and extracellular enzymes [38, 74]. In the 1990’s several PGPR-based products became commercially available in the United States [59]. Yield Shield marketed by Gustafson, LLC as the *B. pumilis* strain GB34 was developed to elicit both plant growth promotion and ISR for use as a seed treatment on soybeans to protect against *Rhizoctonia solani* and *Fusarium* spp.

Reviews presented at a 2004 symposium of the American Phytopathological Society focused on *Bacillus* spp. as biocontrol microbes and emphasized the potential of these organisms as promising agents for use in modern agriculture. The metabolic diversity of *Bacillus* spp. has enabled wide-spread use in industrial processes and production of commercial products such as enzymes, antibiotics, insecticides and food supplements [40]. Production of enzymes such as chitinases and induction of systemic resistance provides new and exciting avenues for biological control research. In an era of increased interest in alternative, specific and environmentally-friendly disease control options, *Bacillus* spp. offer an enormous pool of potential microbial
products to be discovered and utilized in integrated pest management systems. Unfortunately, the use of endospore-forming Gram positive bacteria has received limited attention compared to the plethora of research on the Gram-negative bacteria [30]. Increased research into the potentials of Gram-positive bacteria is necessary to unlock the diversity of disease reducing mechanisms employed by these organisms and generate new products that will ultimately help growers produce disease-free commodities.

**Biological control in the phyllosphere**

Biological control of aerial plant surfaces has been less successful compared to control in the soil/rhizosphere environment. Several reviews on the microbial ecology and microclimate of the phyllosphere suggest that certain differences between these ecosystems may explain the discrepancy in the success of biological control [3, 5, 15]. Exposure to UV radiation, frequent temperature and humidity fluctuations, low water availability and limited nutrients make the phyllosphere a harsh habitat for microbes. On the phylloplane, nutrient sources are scarce and generally originate from non-plant sources such as solutes in rainwater, pollen, aphid honeydew, dead microorganisms and bird or insect excrement [3]. In contrast, the rhizosphere is considered a nutrient rich environment where significant resources originate from the plant as root cap cell are continually sloughed off. Exposure to UV radiation is also significantly greater on the phylloplane. Tolerance to radiation in the near UV (300-400nm) and visible range is important for microbial survival on leaf surfaces [10]. Similarly, alternating patterns of hydration and desiccation strongly affect the ability of added organisms to persist on the leaf and fruit surfaces. These characteristics of the leaf surface environment present a unique set of challenges in the development of biological control of foliar pathogens. More specifically, poor survival and
growth of applied antagonists are two primary factors limiting development of successful biological control in the phyllosphere.

An understanding of the microhabitat in which these organisms reside is essential and should be considered when attempting to select and establish biological control agents. Organisms able to colonize the phyllosphere have developed survival strategies to either avoid or tolerate environmental stresses. A tolerance strategy requires the ability to tolerate direct exposure to stresses and include the production of an extracellular polysaccharide layer [31] which protects bacterial cells from desiccation, or pigment production which can protect cells from UV radiation [10]. An avoidance strategy requires the ability to inhabit or exploit protected sites such as junctions between anticlinal walls of epidermal cells and the base of trichomes. Microorganisms that employ an avoidance strategy may also colonize endophytic sites such as substomatal cavities and vascular tissue [10]. Foreign antagonists introduced from other niches have also been investigated with success in a few circumstances. It seems logical to assume that organisms native to the leaf surface are best suited for foliar biological control. Several articles however, have demonstrated the ability of soil microorganisms to colonize the phylloplane under favorable conditions [66, 87]. *Trichoderma* species are well known soil borne antagonists that have been applied to leaf surfaces to control several foliar diseases. Mechanisms attributed to control by *Trichoderma* spp. include antibiosis, competition, and hyperparasitism. One of the most studied biological control agents is *T. harzianum* isolate T39 marketed as TRICHODEX 20SP which has been reported to control *Botrytis cinerea, Pseudothecia cubensis*, and *Sclerotinia sclerotiorum* on cucumber [27].

Several research groups have investigated the use of organic amendments to improve survival of foliar applied antagonists and increase control efficacy. The underlying premise is to provide a specific nutrient source and create an ecological niche favorable to the antagonist. Andrews [4] reported up to 90% reduction in apple scab severity when ascospores of
Chaetomium globosum were applied to seedlings concurrently with conidia of V. inaequalis. Less than 25% control however, was reported in field tests along with poor epiphytic colonization of C. globosum [16]. Davis et al. [22] found that C. globosum applied to apple leaves in a formulation of colloidal cellulose and a vegetable oil-based spreader-sticker (Soy-Dex, Helena Chemical Co., Memphis, TN) reduced flyspeck severity by 63% and sooty blotch by 79%. Furthermore, the cellulose amendment increased survival and growth of C. globosum where populations on treated leaves were typically log 2 greater than non-treated leaves [22]. In this example cellulose was used as a selective carbon source for the fungal antagonist. Cellulose is an insoluble polymer that can be utilized by only a limited number of organisms possessing the enzymes necessary for its digestion [57]. Previous attempts to supplement antagonists with an organic food base have been unsuccessful due to the use of non-selective materials which do not provide any selective advantage to the applied antagonist. Another insoluble polymer, chitin, is one of the most abundant polysaccharides in nature and is a major constituent of fungal cell walls, insect exoskeletons and crustacean cells [118]. Kokalis-Burelle et al. [61] found that application of chitin as an amendment along with B. cereus strain 304 led to increased populations of chitinolytic bacteria by log 1 and significantly reduced the severity of early leaf spot on peanut caused by Cercospora arachidicola.

Many studies have focused on selecting and testing antagonistic bacteria, specifically those with chitinolytic properties for use as biological control agents [56, 60]. Assays to screen isolates for chitin hydrolysis have relied on conventional plate methods in which colloidal chitin is incorporated into an agar base. Chitinase activity is identified by the development of a clear zone around an inoculated colony [86]. The best known chitinolytic species of bacteria are represented by the genera Aeromonas, Bacillus, Chromobacterium, Myxobacter, Serratia, Vibrio and Streptomyces [19]. Several species of the genus Bacillus have been shown to produce chitinase enzymes including B. circulans [118], B. licheniformis [108], B. macerans [19] and B.
cereus [91]. *B. cereus*, unlike many other chitin-producing bacteria, was found to produce only one chitinolytic enzyme, chitobiosidase which has been also described in *Trichoderma*, *Steptomyces* and *Serratia* species [91]. The exact role of chitobiosidase in fungal antagonism is unclear however, and additional research is needed to better understand and therefore utilize these bacteria and their enzymes in modern agriculture.

Another area of importance regarding successful development of biological control is the screening process utilized to identify new agents. For example, a group in Germany isolated epiphytic bacteria antagonistic *in vitro* to the asexual stage of *V. inaequalis* and identified five strains of *P. fluorescens* and one strain of *B. pumilus* as potential biological control agents [63]. The selection of organisms solely on the basis of *in vitro* tests however, may become problematic as potentially useful organisms, whose mechanism of disease suppression is anything other than antibiosis or direct parasitism will be overlooked. Andrews [3] points out that *in vitro* assays, while quick and inexpensive, are generally poor predictors of field performance. Because field assays are expensive and unrealistic for mass screening, additional methods such as controlled environment bioassys should be considered. The use of *in vitro* assays for screening also fails to identify inducers of ISR and systemic acquired resistance (SAR). According to the literature, the few studies that have focused on the isolation of bacteria antagonistic to secondary cycles of *V. inaequalis* have based their screening programs on *in vitro* assays. It is proposed that the isolation of foliar microflora and screening methods based on *in vitro* and *in vivo* assays will provide valuable information to the scientific community and aid in the advancement of biological control in organic apple production. The combination of biological control agents with different modes of action such as antibiosis, induced resistance and parasitism may also increase the level of disease suppression against a broad spectrum of pathogenic organisms.
Research objectives

Fruits and vegetables are a substantial part of a healthy diet providing essential vitamins, minerals, and disease fighting antioxidants. The dietary recommendations of USDA programs such as the “5 A Day” and the recent “More Matters” programs have increased consumer awareness and consumption of fruits and vegetables. Pesticides remain the primary means for controlling diseases of apple. Chemical revocation, pathogen resistance, exposure of consumers to pesticide residues, and environmental impacts are beginning to change how we view disease control tactics in modern agriculture. Food items where the sprayed surface is directly consumed are of particular concern to consumers and regulatory agencies. Young children and infants, for example, incur significant pesticide exposure as their diets are predominantly fruits and vegetables [82]. The search for new and innovative ways to reduce pesticide use has emerged as an important shift towards environmentally conscious food production. The development of alternative disease management strategies is essential for the maintenance of sustainable apple production systems, improved consumer confidence in food safety and financial gain by growers and the apple industry. The use of biological control approaches as antimicrobial preservatives for harvested commodities to reduce the risk of pesticide exposure has been documented [26, 100].

Research has shown that *Bacillus* spp. have successfully colonized and reduced disease on several crop species [7, 48, 76, 84]. Based on this success, we hypothesized that *Bacillus* isolates known to suppress disease on vegetable crops and native *Bacillus* spp. from apple would also be capable of suppressing apple diseases. The overall objectives of this research were to investigate the ability of bacteria, collected from apple and vegetable crops, alone or in combination with organic amendments to suppress pre-harvest and postharvest diseases on susceptible apple varieties. Specific research objectives include:
1. To collect and screen bacterial isolates from apple and vegetables using hydrolytic enzyme assays, dual-plate cultures, and growth chamber studies to identify isolates for further experimentation;

2. To determine the effectiveness of bacterial isolates selected in objective 1 as biological control agents for pre and postharvest diseases under field conditions compared with traditionally used control measures;

3. To evaluate application strategies to optimize suppression of multiple diseases and survival of bacterial treatments on apple leaves and fruit;

4. To document bacteria-pathogen-plant interactions and investigate potential modes of action using cytological methods.

The overall goal of this research was to develop, validate and deliver alternative management tools that will reduce the dependence on pesticides for disease control and ultimately decrease human exposure to harmful residues on produce.

References


Chapter 2

Development of apple scab biocontrol assays using *Bacillus* spp. isolates from vegetable crops

Introduction

Concern among the general public and scientific community regarding the use of synthetic pesticides for plant disease control has prompted an interest in organic and sustainable agriculture. Specific concerns include pesticide residues in food, groundwater contamination and non-target effects of broad-spectrum chemicals [27]. As a result of this enhanced environmental awareness, there is an increasing consumer driven demand for the development of biologically based alternatives to chemical pest control products. In addition to environmental concerns, the continued dependence on chemical pesticides has resulted in the development of pesticide resistance among many insect, weed and pathogen populations and a subsequent reduction in pesticide efficacy. Alternatives are needed to supplement existing disease control strategies to achieve acceptable levels of control. Biological control of plant diseases using microbes offers a promising alternative for management of many diseases and promotion of overall plant health.

Plants have numerous fungal and bacterial symbionts associated with their aboveground and belowground tissues [29, 36, 40]. These plant-microbe associations range from pathogenic to mutualistic [31]. In the latter case, both participants benefit where the plant may provide a protective habitat or nutrient source, while the microbe offers protection from pests or promotion of overall plant health. Non-pathogenic microbes are common inhabitants on a variety of plant tissues including leaves, stems, roots, seeds and fruits [36]. Some bacteria and fungi are able to colonize plant tissues internally without causing harm and are collectively referred to as endophytes as opposed to parasites, which can cause disease and reduce the fitness of their host.
plant [21]. Disease however, is generally considered the exception as most plant-associated microbes are neutral or beneficial in terms of plant health. Endophytes offer a range of benefits to their plant hosts including growth promotion [14, 18, 38], reduction in disease severity [9, 19], induction of plant defenses [2, 5, 8], production of anti-herbivory products [33, 34], nitrogen fixation [7], and enhanced plant mineral uptake [12, 18]. Endophytes gain entry into their hosts through the root zone or through sites in the phyllosphere. Endophytes typically enter aerial portions of the plant via nectaries, stomates, hydathodes or as a result of foliar damage [20]. Once inside the plant, endophytes may colonize intercellular spaces, within plant cells, or in the vascular system. Endophytes may either colonize locally at the point of entry or spread throughout the plant [13].

Research has documented biological control of plant diseases utilizing phyllosphere- and rhizosphere-inhabiting organisms including yeast, filamentous fungi [15] and both gram negative [35] and gram positive [10] bacteria. The potential for biological control often arises from the fact that endophytes and plant pathogens colonize similar ecological niches [13, 28]. Bacterial endophytes in particular have received attention as potential biocontrol agents and several reviews have focused on their use in agriculture [1, 13, 30, 36]. Research has shown the potential for bacteria to control fungal diseases such as *Fusarium oxysporum* f.sp. *vasinfectum* on cotton [6], *Gaemmannomyces graminis* var. *tritici* on wheat [22] and *Botrytis cinerea* on grapevine [4]. Bargabus [2] showed that *Bacillus mycoides* isolate BacJ, isolated from sugar beet leaves, was able to reduce Cercospora leaf spot (*C. beticola*) of sugar beet by 38-91%. Disease control was attributed to induction of systemic resistance and the isolate was found to elicit the production of β-1,3-glucanase and chitinase enzymes. During subsequent screening for biocontrol agents, Bargabus [3] showed that *B. pumilus* (currently re-described as *B. mojavensis*) isolates 203-6 and 203-7 reduced *C. beticola* symptoms more than 50% when spatially separated from the fungal pathogen. The isolates 203-7 and BacJ have further been shown to delay disease onset, total
disease, and spore production of *Glomerella cingulata* var. *obiculare*, the causal agent of anthracnose on cucumber [26]. Assays of apoplastic proteins showed that isolates BacJ and 203-7 increased β-glucanase activity by 135% and 73% respectively. Additionally isolate 203-7 increased peroxidase activity by 79% compared to the water control.

Research has shown that *Bacillus* spp. successfully colonize and reduce disease on several crop species [17, 19]. *B. cereus* isolates BT8 and BP24 reduced foliar diseases on tomato, pepper, potato and pecan (Backman, unpublished). The objective of this study was to investigate the ability of *Bacillus* spp. isolated from vegetable crops to colonize apple foliage and to suppress apple scab disease severity. *B. mycoides* isolate BacJ, *B. mojavensis* isolate 203-7 and *B. cereus* isolates BT8 and BP24 were chosen based on their abilities to reduce disease on several vegetable crops including sugar beet, tomato and potato [2, 3].

**Materials and methods**

**Preparation of plant material and bacterial isolates**

'Macintosh' and 'Cortland' seeds (extracted from fruit grown at the PSU Fruit Research and Extension Center in Biglerville, PA) were surface sterilized with 10% bleach for 5 minutes and rinsed (3x) with sterile distilled water for 2 minutes. The seeds were then stratified in sterile sand at 4°C for 3 months. Germinating seedlings were planted in 115 ml Cone-tainers® containing Sun Gro Redi-earth plug and seeding mix (Sun Gro Horticulture Canada Ltd) and maintained in a growth chamber (Environmental Growth Chambers M series, Chagrin Falls, OH) with a 12 hour photoperiod, 20°C and 70% relative humidity. Four different *Bacillus* spp. were evaluated for biological control activity on apple: *Bacillus mojavensis* strain 203-7 isolated from germinating sugar beet seeds [3], *B. cereus* strains BP24 from potato and BT8 from tomato (D. Ploper, Auburn University, unpublished data), and *B. mycoides* strain BacJ from sugar beet leaves.
All bacterial strains were stored short term on yeast dextrose calcium carbonate (YDC) and subcultured every 3-4 weeks. For long term storage, isolates were stored in tryptic soy broth (Difco) with 20 % glycerol at -18°C. For inoculation of apple leaves, all bacterial strains were grown in 100 ml of sterile tryptic soy broth in 250 ml Erlenmeyer flasks. Flasks were incubated for 7 days at 28°C and 120 rpm on a rotary incubator shaker (New Brunswick Scientific, Edison, NJ). The bacterial suspensions were adjusted to a concentration of $1 \times 10^8$ CFU/ml.

In all colonization and biocontrol experiments, the bacterial isolates were mixed with the non-ionic organosilicone surfactant Silwet L-77 (G.E. Silicones, Tarrytown, NY). To determine the optimal concentration of Silwet L-77 for infiltration of the bacterial solutions into stomates (while minimizing phytotoxicity), apple seedlings were sprayed with bacterial solutions containing 5 concentrations of Silwet L-77 (0%, 0.05%, 0.1%, 0.15%, and 0.2%). Following application, leaves were evaluated on a light box to verify infiltration into substomatal cavities [41]. Leaves were also evaluated 24 hours after application for signs of phytotoxicity. The concentration of 0.1% Silwet L-77 (v/v) resulted in infiltration with the least amount of leaf burn and was used in all subsequent experiments. The bacterial/surfactant suspension was sprayed onto apple seedlings using an aerosol sprayer (Crown Spra-tool power pack, Aerove Industries, Gardnerville, NV) until runoff.

**Determination of colonization of apple seedlings by Bacillus isolates**

This experiment consisted of six treatments with 11 replications. Individual plants served as experimental units. Leaves of three-week old 'Cortland' seedlings were sprayed to runoff with each of the four bacterial isolates mixed with 0.1% Silwet L-77 (v/v), or the no-bacteria control, in a vented fume hood. The hood surfaces were sterilized with 70% ethanol between treatment applications. Controls included non-inoculated seedlings and seedlings sprayed with a 0.1M
potassium phosphate buffer solution containing 0.1% Silwet L-77. The concentration of bacteria applied was determined by measuring the OD$_{600}$ and plating serial diluted solutions in triplicate on yeast extract dextrose (YED) medium. Plates were stored at room temperature and colonies counted 48 hours after plating. After treatment and when the leaves were visibly dry, the seedlings were returned to the growth chamber in a randomized block design. One plant receiving each of the six treatments represented a block. The Cone-tainers® were spatially arranged in 98 cell trays to avoid leaf contact among neighboring plants (Figure 2-1).

Figure 2-1: 'Cortland' seedlings in a growth chamber at 20°C and 12 hr photoperiod. Seedlings were planted in 115 ml Cone-tainers and arranged in 98 cell trays to prevent contact with neighboring plants.

Foliar colonization by each isolate was assessed 1, 4, 10, 17, 26, 33 and 43 days post inoculation (dpi). At each sampling date, the third fully expanded leaf was collected from each treatment from 3 replicate plants. Two 78 mm$^2$ leaf discs were removed from each leaf using a number 7 cork borer (for a total of 157 mm$^2$ of leaf tissue). The two discs from each leaf were placed in sterile 1.5 ml microfuge tubes with 900 µl of sterile 0.1M potassium phosphate buffer and ground using pellet pestles (Kimble Chase, Vineland, NJ). Fifty µl of the homogenized tissue solution was plated in duplicate on YED using a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA). Plates were incubated at 20°C for 48 hours. Following incubation, total bacteria were enumerated using a Spiral Biotech counting grid. Bacterial populations were recorded as CFU/cm$^2$ of leaf tissue. The minimum detectable population level for this methodology was log 1.5 CFU/cm$^2$. 
**Assessment of epiphytic and endophytic colonization of apple seedlings**

An experiment was conducted to evaluate methods for enumeration of epiphytic and endophytic foliar colonization by each of the bacterial isolates. This experiment was conducted using isolates BP24 and BT8 prepared and inoculated onto apples leaves as outlined earlier. Three methods were evaluated to determine the optimal strategy for removal of epiphytic colonists from leaves prior to determining the population of endophytic colonists. Methods evaluated included: (1) no treatment (2) wash treatment and (3) UV light treatment. For all methods, step one and two of the procedure were the same. In step one, the third fully expanded leaf was collected from three replicate plants and placed in sterile 101 mm x 152 mm stomacher filter bags (Secure-T 80; Labplas, Sainte-Julie, Quebec) with 10 mL of sterile 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 seconds using a stomacher blender (Bagmixer 100 MiniMix, Interscience St. Nom, France). In step two, the supernatant was removed from the bags and 50 µl was plated in duplicate on YED to determine the population of epiphytic colonists. For step three of the "no treatment" method, another 10 ml of phosphate buffer was added to the stomacher bag and the sample was agitated without leaf disruption for 30 seconds. The supernatant was removed and 50 µl plated in duplicate on YED to determine the population of epiphytes remaining on the leaf after the initial agitation (step one). Next, the leaves were removed from the bags and two 78 mm² discs were cut from the leaves using a number 7 cork borer and placed in 1.5 ml microtubes with 900 µl of 0.1M phosphate buffer. The discs were ground using pellet pestles and 50 µl of the homogenized tissue plated in duplicate on YED to determine the population of endophytic colonists. For step three of the "wash treatment" method, leaves were removed from the stomacher bags (following agitation in step one and plating epiphytes in step two) and placed in 50 ml Erlenmeyer flasks with 25 ml 0.1M phosphate buffer. The flasks were placed in a rotary shaker and agitated at 120 rpm for 15 minutes. The buffer was then pipetted off and the leaves were placed in a new stomacher bag with 10 ml of
0.1M phosphate buffer and agitated in the stomacher for 30 seconds at 7 strokes per second. The supernatant was removed and 50 µl plated in duplicate to determine the population of epiphytes remaining after the wash. Two 78 mm² leaf discs were cut from the leaves, ground in microtubes and 50 µl plated in duplicate to determine the population of endophytic colonists. For step three of the UV light treatment, leaves were removed from the stomacher bags and placed under UV light for 2 minutes on each side. The leaves were then placed in new stomacher bags with 10 mL of 0.1M phosphate buffer and agitated in the stomacher for 30 seconds. The supernatant was plated in duplicate to determine the population of epiphytes remaining after UV exposure. Two 78.5 mm² leaf discs were then cut from leaves, ground in microtubes with phosphate buffer, and the homogenate was plated in duplicate to determine the population of endophytic colonists per cm² of leaf tissue.

Upon optimization of methods to remove epiphytes, a second experiment was conducted to characterize the distribution and persistence of inoculated bacteria within colonized apple leaves. The populations of epiphytic and endophytic bacteria on 'Cortland' seedling leaves were assessed 24 hours after inoculation and 6, 13, 19, 26 and 35 days post inoculation (dpi). Bacteria were prepared and applied to seedlings as described previously with the exception of isolate BacJ. A lyophilized formulation of BacJ cells (1 x 10¹¹ CFU/g) was obtained from Montana Microbial Products (Missoula, MT) and used for this experiment. The BacJ spray solution was prepared by suspending the powder (0.001g/ml) in sterile Milli-Q water for a final concentration of 1 x10⁸ CFU/ml. The surfactant Silwet L-77 was added to a concentration of 0.1%. To enumerate epiphytic bacteria, the third fully expanded leaf was collected from each treatment from each of the 3 replicates. Leaves were placed in stomacher filter bags with 10 ml of sterile 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 seconds using a stomacher blender. After agitating, 50 µl of the buffer solution was plated in duplicate on YED. The remaining buffer in the stomacher bag was pipetted out and discarded.
To estimate the population of endophytic bacteria, a second aliquot of 10 ml of phosphate buffer was added to the stomacher bag and agitated for 30 seconds and the buffer was discarded. Two 78 mm² leaf discs were cut from the leaves using a number 7 cork borer (for a total of 157 mm² leaf tissue). The leaf discs were placed in sterile 1.5 ml microfuge tubes with 900 µl of 0.1M potassium phosphate buffer and ground using pellet pestles. Fifty µl of the homogenized tissue suspension was plated in duplicate on YED. As noted previously, plates were stored at 20°C and colonies counted 48 hours after plating.

**Evaluation of biocontrol activity on apple seedlings**

This study was conducted in two replicate experiments. Experiment one consisted of six treatments with 10 'Cortland' seedlings per treatment. Treatment one consisted of non-sprayed plants and treatment two was the surfactant control, sprayed with a phosphate buffer solution of 0.1% Silwet L-77. Treatments 3-6 included 0.1% Silwet L-77 plus BP24, BacI, 203-7 or BT8 at 1.0 x 10⁸ CFU/ml. Following bacterial application, seedlings were arranged in a randomized block design and maintained in an environmental growth chamber (EGC M-series, Chargin Falls, OH). Experiment two was performed as a repeat of experiment one and was conducted in a different growth chamber (Conviron model no. PGR15, Winnipeg, Canada). Growth chambers used for experiment one and two were programmed to maintain a 14-hour photoperiod, 20°C and 70% relative humidity.

Apple seedlings were challenge inoculated with conidial suspensions of the apple scab pathogen, *Venturia inaequalis*, seven days after the bacterial treatments were applied. For the *V. inaequalis* inoculum, scab-infected 'Golden Delicious', 'Red Delicious' and 'York' leaves were collected from unsprayed trees in Adams County, PA. Infected leaves with visible lesions were placed on paper towels in layers of 10, wrapped in waxed paper, sealed and stored at 4°C until
needed. To prepare the inoculum, approximately 10 sporulating lesions were cut from leaves and placed in a beaker with 100 ml distilled water and stirred on a magnetic stir plate for 5 minutes. This mixture was then filtered through a screen to remove leaf debris. The concentration of conidia in the solution was determined using a hemocytometer (American Optical, Buffalo, NY) and adjusted to approximately $3.0 \times 10^4$ conidia/ml. One drop of the polysorbate surfactant, Tween-20, was added to the conidial suspension to minimize spore clumping. Conidia were applied to the seedlings with an aerosol sprayer to runoff in a fume hood. Immediately following inoculation, seedlings were incubated in a dark dew chamber at 20°C and 100% relative humidity. After 48 hours, seedlings were removed from the dew chamber and returned to the growth chamber with 14 hours daylight, 20°C and 70% relative humidity. The seedlings were monitored daily for symptom development and were rated for apple scab severity once lesions were observed. Seedlings were evaluated based on percent leaf area symptomatic using a 5 point rating scale (0=no disease, 1=less than 25%, 2=25-50%, 3=50-75%, 4=75-100%, and 5=total leaf collapse). Where appropriate, intermediate values were recorded for all leaves evaluated. Upon completion of data collection, the rating scores were converted to percentage infected tissue based on the 5 point scale. Disease progress curves were constructed using the mean severity at each evaluation date. The area under the disease progress curve (AUDPC) was also calculated for each treatment [24]. Data were analyzed for statistical significance using the mixed linear model of SAS (SAS Institute, version 9.2, Cary, NC). Statistical significance was assessed at $P \leq 0.05$ and a Tukey test was used to separate means at each evaluation date and also for comparison of calculated mean AUDPCs.

A third apple scab experiment was conducted on 'Cortland' seedlings to evaluate the effects of bacterial applications on the development and expansion of individual scab lesions apple leaves. This experiment consisted of a 5x2 factorial with five bacterial treatments (BT8, BacJ, BP24, 203-7, and none) and two $V. inaequalis$ application schedules (7 dpi and 14 dpi).
'Cortland' seedlings in 115ml Cone-tainers were maintained in a growth chamber (Environmental Growth Chambers M series, Chagrin Falls, OH) with a 14 hour photoperiod, 20°C and 70% relative humidity. Immediately prior to application of the bacteria, the last fully expanded leaf was marked with tape. Bacteria were spray inoculated as described previously in a 0.1% Silwet L-77 solution. Non-inoculated controls were treated with 0.1 M phosphate buffer containing 0.1% Silwet L-77. Seedlings were challenged with *V. inaequalis* 7 dpi or 14 dpi of the bacterial treatments. The *V. inaequalis* inoculum was prepared as described previously and adjusted to 5.0 x 10⁴ conidia/ml. Twenty ml of this solution was mixed with 100 ml of sterilized 0.25% water agar. Seven days after the bacteria were applied to the leaves (7dpi), two 10 µl drops of the conidia/agar suspension were pipetted onto the tagged leaf (last fully expanded leaf at the time of bacterial application). Fourteen days after bacterial application (14 dpi), two 10 ul drops of conidia were pipetted on the tagged leaf of the respective seedlings. Two additional 10 µl drops were pipetted onto the last fully expanded leaf on seedlings of the 14 dpi treatment. This newly formed leaf was not directly sprayed with bacteria (because it emerged during the 14 day period after the bacteria were applied). Three plants were inoculated per treatment for a total of six replicates. Immediately following *V. inaequalis* application, the seedlings were placed in a dew chamber at 25°C and 100% relative humidity. After 48 hours, seedlings were returned to the growth chamber and arranged in randomized block design. Lesion diameters were measured 14, 18, 23, 27 and 34 days post scab challenge for the 7 dpi treatment and 12, 17, 21 and 28 days post scab challenge for the 14 dpi treatment.

**Evaluation of biocontrol activity on potted apple trees**

Forty 'Gala' and forty 'Golden Delicious' trees grafted onto M26 rootstock were obtained from Adam's Country Nursery in March 2006. The trees were planted in two gallon pots, pruned
to approximately two feet above the graft union and maintained in a greenhouse under natural light for six weeks. The trees were initially fertilized with Osmocote 14-14-14 and an additional 400 ppm 20-20-20 liquid fertilizer was applied every three weeks thereafter until August 2006.

This experiment consisted of 4x2x2 factorial with four bacterial treatments (BP24, BT8, BacJ and none), two sulfur treatments (sulfur and no sulfur) and two varieties (‘Gala’ and ‘Golden Delicious’). Treatments were applied to the potted trees six weeks after they were received from the nursery and potted in the greenhouse. The trees were sprayed until runoff with each of the bacterial treatments in a 0.1% Silwet L-77 solution using a 1L handheld spray bottle. The OMRI approved sulfur product, MircoSulf was applied to respective treatments at a rate of 0.15 lbs/gal four days after the bacteria were applied. Immediately following sulfur application, the trees were moved outside and placed in the test orchard on April 28, 2006 shortly before full bloom (which occurred on 6-May). The orchard site used for this experiment was located at the Penn State Fruit Research and Extension Center (FREC) in Biglerville, PA. The orchard selected for this experiment was also the site of an ongoing experiment testing the effects of alternative treatments for apple scab control. The potted trees were placed under untreated "control" trees that served as inoculum sources of the apple scab fungus *V. inaequalis*. The 'Golden Delicious' potted trees were placed under mature 'Golden Delicious' trees and the 'Gala' potted trees were placed under mature 'Red Delicious' trees due to the lack of mature 'Gala' trees at the test site. A single replication (eight trees) was placed under each tree for a total of eight trees. All mature trees at the test site were on Malling 26 rootstocks spaced at 35 x 10 x 8 ft. Scab incidence on the untreated mature 'Golden Delicious' and 'Red Delicious' trees was recorded by the FREC summer staff by observing all leaves on 25 shoots per tree (4 replicates) on 21 Jun, 25 Jul and at harvest (26-Sept). Colonization of the potted tree foliage was assessed 14 days post inoculation with bacteria on 8-May (10 days after moving the trees into the orchard). Two leaves from four replicate trees were collected, packed in zip-lock bags in ice and transported back to the
University Park lab. The next day, two leaves from each tree were placed in sterile stomacher bags with 20 ml phosphate buffer and agitated in a stomacher for 30 seconds. The leaf tissue was further triturated using a pestle, the supernatant diluted 10-fold and plated on YED in duplicate using the spiral plater.

The potted trees remained in the orchard for two weeks and were watered daily when necessary. Weather monitoring and primary scab infection periods were recorded with a Campbell Scientific and a Field Monitor Weather System (Sensor Instruments Co., Inc.) using the Mills modified apple scab infection model [23]. Following exposure to several infection periods, the potted trees were moved back into the greenhouse on 15-May. Overhead mist irrigation was applied at hourly intervals to maintain humidity in the greenhouse. The trees were monitored daily for symptom development and the severity of foliar scab symptoms were evaluated 15-June (32 days after exposure to an infection period). Leaves on two branches per tree were evaluated based on the estimated percent leaf area symptomatic using a 5-point rating scale (0=no disease, 1=less than 25%, 2=25-50%, 3=50-75%, 4=75-100%, and 5=total leaf collapse). Where appropriate, intermediate values were recorded for all leaves evaluated. Upon completion of data collection, the rating scores were converted to percentages based on the 5-point rating scale. Data were analyzed for statistical significance using the mixed linear model of SAS (SAS institute, version 9.2, Cary, NC). Interactions between cultivar, bacteria isolate and sulfur were tested and statistical significance was assessed at \( P \leq 0.05 \). The slice option of the lsmeans statement was used to test the three-way interaction and compare levels of each factor while holding the other two constant. A Tukey test was also used to separate means.
Results

Colonization of apple seedlings by Bacillus isolates

Immediately after spray application of the bacterial/Silwet solution, seedling leaves developed water soaked regions, indicating sub-stomatal infiltration by the treatments. One day after application minor necrosis was observed at the leaf margins on the youngest leaves treated with Silwet-L77 alone or in combination with bacteria. The damaged young leaves continued to expand and developed into mature leaves that were slightly misshapen at the margins. Application of the bacterial isolates did not appear to have any negative effect on plant growth as compared to the non-treated control plants. Additionally, no symptoms or hypersensitive response was observed on any of the seedlings used in this colonization experiment. All four of the Bacillus isolates tested were able to successfully colonize apple seedling leaves under growth chamber conditions (Figure 2-2). Isolate 203-7 was able to colonize apple leaves up to 26 dpi, but declined rapidly thereafter and was undetectable at 33 dpi. Isolates BP24, BacJ and BT8 colonized apple leaves throughout the duration of the experiment (43 days). Isolate BP24 maintained the highest population levels fluctuating between log 4.9 and log 3.7 per cm$^2$ leaf tissue. Population levels of the isolates BT8 and BacJ slowly declined over the duration of the experiment from log 5.1 to log 2.2 and log 3.4 to log 2.2 per cm$^2$ leaf tissue respectively.
Assessment of epiphytic and endophytic colonization of apple leaves

Evaluation of methods to remove epiphytic colonists from leaves prior to sampling for endophytic colonists indicated that all methods were able to remove 97-99% of the epiphytic bacteria (Table 2-1). The UV method resulted in 100% removal of BP24 and BT8 epiphytic colonists. The use of UV light on leaves colonized with BT8 however, resulted in an average endophytic population of 1.9 CFU/cm² with a standard error of 1.9. In this case, only a single BT8 colony (on one plate) was observed suggesting UV light may have also killed endophytic colonists. The isolate BT8 has been shown to endophytically colonize other plants such as cacao and tomato [25]. The wash method resulted in 99.5% and 98.8% removal of BP24 and BT8 epiphytes respectively. Based on the data collected in this experiment, it appears that the initial agitation step using the stomacher removes approximately 98% of the epiphytic colonists and the wash or UV treatments only confers an additional 1-3% removal. As a result, all subsequent experiments were conducted using two 30 second agitation steps prior to grinding leaf tissue for enumeration of endophytic colonists.

Figure 2-2: Mean total bacterial colonization of 'Cortland' seedling leaves sprayed with 0.1% silwet + log 8.0 bacteria. Initial colonization of leaves was on day 0 and colonization subsequently measured 1, 4, 10, 17, 26, 33 and 43 days post inoculation. Error bars represent standard errors of the mean. The horizontal dashed line indicates the minimum detection level of the experiment.
A second experiment was conducted to provide more information regarding the
distribution (epiphytic vs. endophytic) of the four bacterial isolates within colonized apple leaves.

As observed in the first colonization experiment (Figure 2-2), all the bacterial isolates colonized 'Cortland' leaves throughout the duration of the experiment (35 days). Colonization by the isolate BacJ however, was more stable using the lyophilized formulation, with total population levels fluctuating between log 3.7 and log 4.8. Although Silwet L-77 was used to facilitate sub-stomatal infiltration and thus endophytic colonization by the bacteria, colonization was observed in both the epiphytic and endophytic environments. Furthermore, colonization was predominately epiphytic through the duration of the experiment with the exception of BacJ, which maintained similar epiphytic and endophytic population levels (Figure 2-3). Generally endophytic populations of isolate BT8 showed a greater decline over 34 days then did the epiphytic populations.

Table 2-1: Comparison of BT8 and BP24 epiphyte removal by three methods; no treatment, UV light and distilled water wash. Bacterial counts are reported as CFU/cm² leaf tissue. The percent removed column represents the total percent of epiphytes removed in the experiment (initial agitation + wash or UV exposure). Bacteria on leaves receiving UV light exposure were below the detection limit (BDL).

<table>
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<tr>
<th>Isolate</th>
<th>Treatment</th>
<th>Total bacteria</th>
<th>Total epiphytes</th>
<th>Epiphytes after treatment</th>
<th>Total endophytes</th>
<th>Percent removed</th>
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<td>No treatment</td>
<td>$4.9 \times 10^4$</td>
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<td>BDL</td>
<td>BDL</td>
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<td></td>
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<td>$1.7 \times 10^1$</td>
<td>$1.7 \times 10^1$</td>
<td>98.8</td>
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</table>
Evaluation of biocontrol activity on apple seedlings

Seedlings inoculated with isolate 203-7 and isolate BT8 had significantly lower disease severity from 19 to 45 dpi compared to the Silwet L-77 control in experiment one (Figure 2-4A). Isolates BacJ and BP24 did not have a significant effect on scab disease severity (P<0.05) at any of the evaluation dates in experiment one. Additionally the AUDPC for seedlings treated with 203-7 and BT8 were significantly less than the AUDPC for the Silwet control in experiment one (Table 2-2). In experiment two, seedlings treated with isolate 203-7 again had significantly lower disease severity from 30 dpi to 49 dpi compared to the silwet control (Figure 2-4B). Disease severity on seedlings treated with BT8 in experiment two however, were not significantly
different from the control (Table 2-2). As observed in experiment one (Figure 2-4A), isolates BacJ and BP24 did not have a significant effect on disease severity in experiment two (Figure 2-4B). Overall, the disease severity observed on silwet control plants was lower in experiment two (74% at 45 dpi) compared to experiment one (55% at 49 dpi).

Figure 2-4: (A) Disease severity, measured as percent leaf area diseased on leaves challenged with *Venturia inaequalis* (experiment 1). Bacterial isolates were applied to leaves 7 days prior to scab challenge at day 0. Error bars represent standard errors of the mean. (B) Disease severity (experiment 2). Experiment 2 was designed as a replicate of experiment 1.
The third seedling experiment was conducted to evaluate the effect of bacterial colonization of apple seedlings on the development of individual scab lesions (two 10 µl drops of conidia placed on seedlings leaves). The average lesion size on leaves, colonized 7 days before challenge with scab, with isolates BacJ, BP24 or 203-7 were significantly smaller (P < 0.05) after 34 days compared to the lesions on the Silwet control leaves (Figure 2-5A). However, when challenged with scab 14 days after bacterial inoculation, none of the bacterial treatments resulted in a reduction in lesion size on the colonized leaves (Figure 2-5B). The average size of lesions on tagged leaves challenged with scab 14 days after application of the bacteria were generally smaller (0-25 mm²) than the lesions on leaves challenged 7 days after bacterial application (0-60 mm²). The older leaves challenged 14 dpi may have matured to the point where ontogenic resistance was a factor contributing to the overall smaller lesion size observed. No significant differences in lesion size were observed on the newly unfolded (non-colonized) leaves on seedlings where bacteria were applied 14 days before scab challenge (Figure 2-5C). This observation suggests that the reduction in apple scab lesion size is dependent on the presence of the bacteria on or in the challenged leaf.

Table 2-2: Mean area under the disease progress curve (AUDPC) for 'Cortland’ seedling leaves challenged with *Venturia inaequalis* for experiment one and two. Data were analyzed for significance using the linear mixed model of SAS. A Tukey test was used to separate mean AUDPC values. Within columns, means followed by the same letter are not significantly different at the 95% confidence level.

<table>
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<tr>
<th>Isolate</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Mean AUDPC</td>
<td>Standard error</td>
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<td>164.9</td>
</tr>
<tr>
<td>BacJ</td>
<td>1493.8 bc</td>
<td>238.9</td>
</tr>
<tr>
<td>BP24</td>
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<td>Silwet</td>
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</table>
Evaluation of biocontrol activity on potted apple trees

This experiment was conducted to evaluate biological control of apple scab in an orchard using natural inoculum sources. Prior to placement of the potted apple trees in the test orchard, 4 scab infection periods occurred between 21-April and 24-April resulting in ascospore release. During the two week period (28-April to 15-Jun) the potted 'Golden Delicious' and 'Gala' trees were in the test orchard several scab infection periods occurred. There were 4 severe infection periods and 3 moderate infection periods resulting in ascospore release (Figure 2-6A). The primary scab period for the 2006 season occurred from 27-March to 15-Jun. Scab incidence on

Figure 2-5: Mean apple scab lesion area (mm$^2$) on leaves sprayed with bacteria and challenged with V. inaequalis (A) 7 dpi (B) 14 dpi with bacteria and (C) unsprayed leaves on seedlings with lower leaves sprayed with bacteria 14 days before scab challenge. Error bars represent standard errors of the mean.
the untreated mature 'Golden Delicious' and 'Red Delicious' trees (used as sources of natural inoculum) as of 21-Jun 2006 on shoot leaves was 61%.

All three of the *Bacillus* isolates were able to colonize foliage of potted apple trees in the orchard. After 10 days in the orchard (14 dpi) the epiphytic populations of isolate BP24 on the potted trees ranged from log 4.9 on Gala to log 4.0 (per cm$^2$ leaf tissue) on 'Golden Delicious'. Isolate BT8 ranged from log 3.6 on 'Gala' to log 3.4 on 'Golden Delicious'. Populations of isolate BacJ ranged from log 3.4 on 'Gala' and log 4.0 on 'Golden Delicious'. As observed in the seedling experiments, minor leaf margin necrosis was observed on the youngest leaves treated with Silwet-L77 alone or in combination with bacteria. The damaged young leaves continued to expand and developed into healthy, mature leaves. There were no significant differences in population levels for any of the bacterial isolates on sulfur versus non-sulfur treated leaves.

Figure 2-6: (A) Apple scab infection periods at the PSU FREC in Biglerville, PA from April 19-May 15 2006. Infection periods were determined using the Mills modified model. Potted trees were placed in the orchard on April 28, 2006. (B) Average rainfall in inches and leaf wetness hours.
Initial symptoms (chlorotic lesions) were first observed on 9-June 2006 (46 dpi with bacteria). Apple scab disease severity on the potted trees was evaluated on 15-June 2006 (32 days after the trees were removed from the orchard) immediately following a moderate infection period. The onset of symptom and sign development observed in this experiment (32 days) was longer in the field/greenhouse setting compared to the growth chamber experiments which averaged 14 days. Data analyses indicated a significant interaction between cultivar, bacterial isolate and sulfur treatments \( (P = 0.009) \). Slicing the three-way interaction indicated that the varieties responded differently to application of sulfur and bacteria in regards to apple scab disease severity. For example, there was a significant effect of sulfur in combination with isolate BP24 on ‘Gala’ \( (P = 0.02) \), but not on ‘Golden Delicious’ \( (P = 0.4) \). Disease severity on potted 'Golden Delicious' trees treated with isolate BP24 alone was significantly lower than severity on the sulfur and no-sulfur control trees (Figure 2-7A). Isolate BP24 with or without sulfur did not significantly reduce scab severity on 'Gala' (Figure 2-7B). All isolates (with or without sulfur) significantly reduced scab severity compared to the no-sulfur control on 'Golden Delicious' trees but not on 'Gala' trees. Scab severity on potted 'Gala' trees treated with isolate BT8 alone was significantly lower compared to the sulfur treated control but not the no-sulfur control.

![Graph A](image1.png)

![Graph B](image2.png)

Figure 2-7: Mean foliar scab severity on ‘Golden Delicious’ (A) and ‘Gala’ (B) potted trees 32 days after exposure to seven infection periods. Trees were spray inoculated with 0.1% Silwet + log 7.0 bacteria. Gray bars represent treatments receiving 0.15 lbs/gal sulfur. Error bars represent standard errors of the mean. A Tukey test was used to separate means. Bars with the same letter are not significantly different \( (P<0.05) \).
Discussion

Colonization of apple seedlings by Bacillus isolates

The colonization experiments demonstrated that *Bacillus* spp. from annual crops are capable of colonizing apple foliage under growth chamber and field conditions. The Silwet L-77 surfactant used at 0.1% to infiltrate the bacteria did not negatively affect seedlings grown in the growth chamber or leaves of potted trees growing in the orchard. The *Bacillus* spp. tested here have differential abilities to establish long term colonization on or in apple foliage. Vegetable isolates BP24, BT8 and BacJ were each able to colonize apple leaves for a long period (> 6 weeks) while colonization by isolate 203-7 was more transient. The formulation of the bacteria may also have played a significant role in colonization of apple foliage. Colonization of isolate BacJ applied as a wettable powder was greater and more stable over time compared to colonization by cells grown in broth culture. Further experiments directly comparing broth grown cultures and the wettable powder formulation of BacJ are needed to determine the effects of formulation on colonization and disease suppression.

Biocontrol of foliar apple scab by Bacillus spp.

Growth chamber experiments conducted on apple seedlings provided for evaluation of the biocontrol potential of four *Bacillus* isolates to suppress foliar apple scab severity under controlled conditions. Data from replicate experiments resulted in some inconsistencies between different experiments. Isolates 203-7 and BT8 significantly reduced apple scab severity in the initial seedling experiment (Figure 2-4A). In the second replicate experiment however, isolate BT8 did not reduce disease compared to non-treated controls. When comparing individual lesion size, isolates 203-7, BP24 and BacJ each suppressed lesion expansion when *V. inaequalis* spores
were applied 7 dpi with bacteria. Isolate 203-7 was the only bacterium that consistently reduced disease severity on apple seedlings. Additionally, the variability among replicates both within and between experiments was high leading to the inability to separate means. Scab severity on silwet treated control seedlings was also variable within each experiment and inconsistent between repeated experiments.

The observed variability in the growth chamber experiments may be attributed to the plant material used and the *V. inaequalis* inoculum source. Due to heterozygous nature of *Malus* species, seedlings are heterogeneous expressing variable genetic traits [32, 37]. Additionally most apple varieties are not self fertile and thus seeds are not true to type [16]. As a result, in commercial nursery production apple is clonally propagated via budding or grafting from mother trees. Although a scab susceptible cultivar was selected, the seedlings used in this study may represent variable levels of susceptibility to *V. inaequalis*. Additionally, several pollenizing cultivars (which also differ in scab susceptibility) may be represented in the batch of seeds used in this study. Realizing this issue, clonally propagated potted trees were obtained and used for further field and greenhouse experiments to reduce the variability associated with the host plant. The scab inoculum used in this study is another potential source of variability. Research as shown that genetic diversity within scab populations can be high [39] and several physiological races may be present within an orchard or on a single tree. Inoculum used in the growth chamber experiments was prepared by harvesting conidia from naturally infected leaves collected from an experimental orchard. It is likely that this inoculum contained a mixture of scab isolates with varying levels of virulence on apple. This combination of genetic variation in the plant material and the pathogen inoculum most likely accounts for most of the variation observed in this study. To minimize variably related to the pathogen, single spore *Venturia* isolates will be used to produce inoculum in future growth chamber and greenhouse experiments.
The experiment designed to evaluate bacterial effects on scab lesion size on colonized and non-colonized leaves suggests that a reasonable population of bacteria must be present for disease suppression to occur. Isolates 203-7, BacJ and BP24 significantly reduced lesion size on colonized leaves when *V. inaequalis* was applied 7 days after bacterial application. Scab suppression was not observed on non-colonized seedling leaves. Additionally, suppression of lesion size was not observed on colonized leaves when the pathogen was added 14 days after the bacteria. This observation is most likely due to development of ontogenic (age-related) resistance of the colonized leaves which were fully expanded at the time of pathogen challenge. Due to the high variability in lesion size within treatments (likely due to the previously described reasons), it is anticipated that an increased number of replicates and a more uniform scab inoculum might resolve treatment effects and expose any differences in lesion size on non-colonized leaves.

Results from the potted tree experiment indicate that the *Bacillus* spp. tested in this study can be combined with the protectant inorganic fungicide, MicroSulf to suppress foliar scab on 'Golden Delicious'. Specifically, isolate BP24 combined with sulfur resulted in disease suppression greater than that observed on trees treated with sulfur alone. Overall, scab levels on the potted trees were low ranging from 5-30% leaf area diseased. Overhead mist irrigation was used to maintain humidity in the greenhouse, however, the temperatures in the greenhouse frequently reached 24-27ºC on sunny days which are higher than optimal temperatures for scab infection. Perhaps the two week period that the trees remained in the orchard was not long enough to provide sufficient exposure to scab inoculum. Additionally, since the trees were placed in the orchard at the beginning of the primary scab period, the ascospores that represented the inoculum during that period, may have not reached full maturity.
Potential of Bacillus spp. as biological control agents for apple scab

One factor in determining the success of a biological control agent, especially those employing competition, exclusion, or host defense gene induction as mechanisms of disease suppression is the ability to colonize and survive on the target crop. Biocontrols utilized during the growing season must tolerate the spray regimes or other elements of the pest management program in order to be successfully integrated with other control options. It is therefore promising that the Bacillus isolates used in this study were able to colonize apple leaves when applied with the commonly used fungicide, sulfur. Additional experiments to determine their compatibility with other pesticides (copper and streptomycin) are presented in chapter 5. The use of endospore forming bacteria offers an advantage over some yeasts and Gram negative bacteria which are sensitive to environmental extremes and have a shorter shelf life as formulated products. Many organisms, while successful in research trials, are difficult to formulate and many never reach the marketplace [10]. Some organisms possess certain aspects of their life stage that are easier to formulate than others, such as endospore-forming bacteria, yeasts and resting spore stages of certain fungi, which are dehydrated to produce a dry product [11]. For example, endospore-forming gram-positive bacteria such as Bacillus spp. and Streptomyces spp. produce spores resistant to heat and desiccation that can easily be formulated as a dry product, tolerate, and be co-applied with pesticides. By contrast, Gram-negative microorganisms, such as the commonly used Pseudomonas spp. are formulated as frozen pellets that must be kept cold until use [10].

Overall the Bacillus isolates 203-7 and BP24 were effective in reducing apple scab disease severity in multiple experiments. Isolate 203-7 however, was not an effective long-term colonist on apple foliage and proved difficult to grow in large quantities in liquid culture and was not used in 2007 field experiments. Additional experiments using clonal apple trees and monoconidial isolates for Venturia inoculum to reduce variability are needed to further
investigate the ability of these bacteria as potential biocontrol agents for apple scab. The results from this study (using isolates from annual crops) suggest that native apple isolates that are endospore-forming bacteria may have better potential to suppress apple scab and several other important diseases of apple as well. Future experiments focusing on isolation and screening endospore-forming bacteria from apple using the procedures developed in this study are presented in chapter 3.

References


Chapter 3

Isolation and screening of bacteria collected from apple for potential biocontrol agents

Introduction

Biological control of the apple scab pathogen, *Venturia inaequalis* has eluded scientists for several decades. In 1949, Cinq-Mars was the first scientist to isolate microbes from apple leaves and test them for suppression of apple scab [6]. Ross [26] was the first to conduct *in vitro* assays to demonstrate suppression of pseudothecial development on apple leaves in 1953. Ross is considered a pioneer in biocontrol research, introducing the idea of using antagonists themselves instead of the antibiotics they produce [6]. Because *V. inaequalis* spends a portion of its life cycle as a saprophyte in leaf litter, Ross and subsequent researchers in the 1960's screened organisms colonizing dead and decaying leaves. This approach was pursued by scientists during the 1980-1990's and research on biocontrol of apple scab has continued to target the primary ascosporic phase of the disease cycle to reduce disease pressure in the spring. The use of fungal antagonists has focused on the reduction of ascospore development and maturation when applied to overwintering leaves [3, 7, 9, 11, 31] with some success. Heye and Andrews [11] reported complete suppression of ascospore production on leaves treated with the Basidiomycete, *Athelia bombacina* under field conditions. Further studies revealed that this suppression only occurred when high doses of the antagonist were used and lower rates resulted in 60-70% reduction in ascospore production [21]. To date, this organism has not been commercialized for use on apple. There are few reports in the literature focused on suppression of ascospore infection, conidial production or conidial infection of leaves within the growing season. Andrews [2] reported up to 90% reduction in apple scab severity when ascospores of *Chaetomium globosum* Kunze ex. Fries
were applied to seedlings concurrently with conidia of *V. inaequalis*. Less than 25% control however, was reported in field tests along with poor epiphytic colonization of *C. globosum* [4]. Köhl et al [16] achieved up to 61% reduction in conidial production by *V. inaequalis* under orchard conditions with *Cladosporium cladosporioides* H39. Effects of the introduced antagonist however, were not sustained throughout the season and viability of *C. cladosporioides* H39 conidia diminished toward the end of the experiment.

Studies aimed at finding novel biocontrol agents often rely heavily on *in vitro* assays to quickly screen hundreds of microbes for antagonistic activity to a pathogen of interest. For example, researchers in Germany isolated epiphytic bacteria antagonistic *in vitro* to the asexual stage of *V. inaequalis* and identified five strains of *Pseudomonas fluorescens* and one strain of *Bacillus pumilus* as potential biological control agents [18]. The selection of organisms solely on the basis of *in vitro* tests, however, may become problematic as potentially useful organisms, whose mechanism of disease suppression is anything other than antibiosis or direct parasitism, will be overlooked. Andrews [1] points out that *in vitro* assays, while quick and inexpensive, are generally poor predictors of field performance. For example, Burr et al [5] found no correlation between *in vitro* antibiosis and the ability of bacteria and yeasts to suppress apple scab on seedlings. Because field assays are expensive and unrealistic for mass screening, additional methods such as controlled environment bioassays should be considered. The use of *in vitro* assays for screening also fails to identify inducers of induced systemic resistance (ISR) and systemic acquired resistance (SAR). According to the literature, the few studies which have focused on isolation of microorganisms antagonistic to *V. inaequalis*, based their screening programs on *in vitro* assays. It is proposed that the isolation of foliar microflora and screening methods based on *in vitro* plus *in vivo* assays will provide valuable information to the scientific community and aid in the advancement of biological control in apple production systems.
Many studies have focused on selecting and testing antagonistic bacteria, specifically those with chitinolytic properties for use as biological control agents [13, 14, 17]. Assays to screen isolates for chitin hydrolysis have relied on conventional plate methods in which colloidal chitin is incorporated into an agar base. Chitinase activity is identified by the development of a clear zone around an inoculated colony [22]. The best known chitinolytic species of bacteria are represented by the genera *Aeromonas, Bacillus, Chromobacterium, Myxobacter, Serratia, Vibrio* and *Streptomyces* [8]. Several species of the genus *Bacillus* have been shown to produce chitinase enzymes including *B. circulans* [30], *B. licheniformis* [28] *B. macerans* [8] and *B. cereus* [25]. Chitin is a major constituent of fungal cell walls and several chitinolytic microorganisms have demonstrated potential as biological control agents active against several fungal plant pathogens [13, 15, 30].

Biological control on aerial plant surfaces has been less successful compared to control in the soil/rhizosphere environment. The phyllosphere is generally considered a harsh environment as compared to the rhizosphere due to UV radiation exposure, frequent temperature and humidity fluctuations, low water availability and limited nutrients [1]. Because of their popularity as biocontrols for soil-borne diseases, many researchers have attempted to employ soil/root-inhabiting *Bacillus* strains against foliar diseases without proper consideration of their epiphytic fitness. Considering these limitations, the present research has focused on obtaining and screening foliar inhibiting bacteria already adapted to the phyllosphere, thereby increasing the likelihood of long term colonization of apple leaves and efficacious control of scab. The specific objectives of this research were to collect native bacteria from apple fruit and foliage from orchards in Pennsylvania and screen these isolates using hydrolytic enzyme assays, dual-plate antagonism, and growth chamber studies to evaluate disease suppression and to ultimately identify isolates for further experimentation.
Material and methods

Collection and isolation of bacteria from apple leaves and fruit

Healthy leaves and fruit were collected randomly from three managed orchards (maintained under standard fungicide, reduced risk or certified organic) at the Penn State Fruit Research and Extension Center (FREC) and three abandoned orchards (two at 1 year unmanaged and one at 5 years) in Adam’s County, PA in September 2006 and June 2007. Within the conventionally managed orchards at the FREC, samples were collected from 'Golden Delicious', 'Rome Beauty' and 'Red Delicious' trees grown on Malling 26 (M.26) rootstock. Samples were also collected from 'GoldRush' and 'Enterprise' grown on M.26 rootstock in an organic demonstration orchard established in 2004 at the FREC. Samples were placed in labeled plastic bags and stored in a cooler filled with ice during transport to the laboratory. The samples were stored in the lab overnight at 4ºC until processing the following day.

For isolation of epiphytic colonists, two leaves were placed in sterile 101 mm x 152 mm stomacher filter bags (Secure-T 80; Labplas, Sainte-Julie, Quebec) with 10 ml of 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 seconds in a stomacher blender (Bagmixer 100 MiniMix; Intersciences St. Nom, France). Fifty µl of the suspension was plated on each of two media, tryptic soy agar (TSA) amended with Benomyl and apple juice yeast extract agar (AJYE) using a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA). To select for endospore-forming bacteria, three 1 ml aliquots of the agitated solution were pipetted into 1.7 ml microtubes and heat treated in a 75°C water bath for 15 minutes. The heated solutions were plated on TSA and AJYE as before. To isolate endophytic colonists, leaves were agitated in 10 ml of 0.1 M phosphate buffer for 30 seconds and the suspension discarded (to remove epiphytes remaining on the leaf surface). This step was repeated a second time. A third aliquot of 10 ml of phosphate buffer was added to the stomacher bag and the leaf tissue (from two
leaves) was ground using a pestle. The supernatant was heat treated in a 75ºC water bath for 15 minutes, plated in duplicate on TSA and AJA, and stored at room temperature for 48 hours. After incubation, bacteria from the mixed cultures were streaked onto yeast extract dextrose calcium carbonate agar (YDC) to obtain individual colonies that were selected for pure culture.

**Preparation of plant materials**

'Cortland' seeds (extracted from fruit grown at the PSU FREC station in Biglerville, PA) were surface sterilized with 10% commercial bleach for 5 minutes and rinsed (x3) with sterile distilled water for two minutes. The seeds were stratified in sterile sand at 4°C for 3 months. Germinating seedlings were planted in 115 ml Cone-tainers® (Ray Leach, Canby, OR) containing Sun Gro Redi-Earth Plug and Seedling Mix (Sun Gro Horticulture Canada Ltd, Vancouver) and maintained in a growth chamber (Environmental Growth Chambers M series, Chargin Falls, OH) with 14 hours daylight, 20°C and 70% relative humidity.

Bare root 'Gala' and 'Golden Delicious' trees grafted onto M26 rootstock were obtained from Adam's Country Nursery in March 2006. The trees were planted in 7.5 L pots and used for experiments during the 2006 field season. In November, the trees were placed in a cold room (1°C) and stored for four months receiving approximately 2900 chilling hours. Bare root 'Red York' trees grafted onto Budogovsky 9 (BUD 9) rootstock were obtained from Adam's County Nursery in January 2007. The trees were planted in 11 L pots with a 1:2 sand:ProMix Bx mix (Premier Horticulture; Quakertown, PA). One tablespoon of Osmocote 14:14:14 was added to the pots when 3/4 full with the potting mix. The pots were then filled to the pot edge with the remaining potting mix. The potted trees were maintained in a greenhouse with drip irrigation.
Preparation of bacterial inoculum

For inoculation of apple seedlings in the growth chamber, all bacterial isolates were grown in 100 ml of sterile tryptic soy broth in 250 ml Erlenmeyer flasks. For inoculation of apple leaves in greenhouse and field studies, bacteria were grown in 500 ml of sterile tryptic soy broth in 2800 ml Fernbach flasks. Flasks were incubated for 7 days at 28°C and 120 rpm on a rotary incubator shaker (New Brunswick Scientific Model M1024-000, Edison, NJ). Bacterial cells were harvested by centrifugation at 3800 rpm for 15 min at 4°C using a Sorvall RT7 centrifuge (Thermo Scientific). The supernatant was discarded and the bacterial pellets re-suspended in 0.1M potassium phosphate buffer and adjusted to a concentration of $1 \times 10^7$ CFU/ml. In all colonization and biocontrol experiments, the bacterial isolates were mixed with the non-ionic organosilicone surfactant Silwet L-77 at 0.1% (G.E. Silicones, Tarrytown, NY).

Source and preparation of pathogen isolates

The fire blight pathogen, *Erwinia amylovora* strains 581 and 273 were obtained from Dr. T. McNellis in July 2007. The bacteria were maintained on Luria Broth (LB) agar at room temperature until needed. For inoculum, plates of *E. amylovora* strains 581 and 237 were flooded with sterile 0.05 M phosphate buffer and the liquid poured into sterile 100 ml flasks for a total volume of 60 ml. The bacterial suspensions were adjusted using a Spectronic 20 to an optical density of 0.4 at 460 nm.

*Colletotrichum acutatum* was isolated from symptomatic ‘Golden Delicious’ and ‘Rome Beauty’ fruit at the PSU FREC. These isolates were re-inoculated into wounds on ‘Golden Delicious’ fruit to ensure pathogenicity. Isolates that produced symptoms and formed conidia in culture were selected for use in subsequent experiments. *C. acutatum* isolates were maintained on potato dextrose agar (PDA) stored at 4°C.
Eight single spore cultures of *V. inaequalis* were obtained from Dr. M. Jimenez Gasco. These isolates were previously collected from scab infected leaves from the PSU FREC in Biglerville, PA and the PSU Russell Larson agricultural research center at Rock Springs, PA. A method for obtaining *V. inaequalis* conidia in culture was adapted from Parker [23]. PDA plugs of single spore isolates were transferred and grown on PDA for 2-3 weeks or until colonies reached about 5 cm in diameter. Cellophane discs 78 cm² were soaked in Milli-Q water overnight. After soaking, the discs were layered between filter paper discs (Whatman #4) in a glass Petri dish. Alternate layers of filter paper and cellophane not exceeding 10 of each were moistened with Milli-Q water. The glass Petri dish was wrapped in aluminum foil and sterilized in an autoclave for 15 min at 121°C. Immediately prior to use, sterile forceps were used to place a single cellophane disc onto the surface of the solidified PDA. Forceps were used to gently smooth the surface and remove any air bubbles between media and disc. Using a sterile scalpel, a *V. inaequalis* colony (approximately five cm in diameter) was cut from the agar and placed in a sterile stomacher bag with five ml of sterile Milli-Q water. The mycelium was trituated for 30 seconds at 9 strokes per second (level 9) in a stomacher blender (Bagmixer 100 MiniMix; Intersciences St. Nom, France). This procedure macerated the fungal mycelia into pieces small enough to pipet without clogging a pipet tip. Using a P1000 micropipette, 500 µl of the mycelial slurry was pipetted onto cellophane covered PDA plates. The plate was tipped slightly to evenly distribute the slurry across the surface of the cellophane. The inoculated cellophane plates were wrapped with parafilm and stored in an incubator at 20°C with continuous light for 5-7 days. Once conidia were observed on the cellophane plates, the fungus-covered disc was removed from the agar using sterile forceps and placed in a beaker with 250 ml sterile Milli-Q water and a stir bar. The solution was stirred on a magnetic stir plate for five minutes. The conidial/mycelial suspension was filtered through 3-4 layers of sterile cheesecloth into a sterile 1 L bottle or 50 ml centrifuge tube depending on how much inoculum was needed. The concentration of the
inoculum was adjusted by dilution using a hemacytometer (American Optical, Buffalo, NY) to $3.0 \times 10^4$ conidia/ml. Immediately before scab inoculations, one drop of Tween 20 was added to the conidial suspension.

**Screening bacterial isolates for chitin hydrolysis**

The bacteria isolated from apple leaves and fruit were screened for the ability to hydrolyze chitin using conventional plate methods. Colloidal chitin was prepared using a modified method of Kokalis-Burelle [17]. Sixty g of flaked pure crustacean chitin (Sigma-Adrich; St. Louis, MO) was hydrolyzed in 600 ml of concentrated hydrochloric acid at room temperature (20ºC) in a fume hood. This mixture was stirred intermittently and allowed to sit for 30 minutes before adding 4 L of distilled water. After settling overnight, the suspension was decanted and discarded. Fresh distilled water rinses were repeated until the mixture reached a pH $> 7.2$. The percent chitin (w/v) of the suspension was determined using the oven dry weight of a 10 ml aliquot of the wet suspension. Aliquots of the settled chitin suspension were then blended for 30 seconds in a sterilized Waring laboratory blender (Model 7010S: Waring laboratory Science, Torrington, CT) and the pooled solutions were diluted to a 1% (w/v) stock using 0.1 M potassium phosphate buffer. This stock suspension was stored at 4ºC and diluted to the desired concentration prior to use. Bacterial isolates were streaked onto 0.2% and 0.4% chitin nutrient agar (CNA) and isolates which produced a clearing zone were selected for further evaluation.

**Screening for members of the Bacillus cereus group clade using selective media**

A selective agar was prepared to determine whether any of the collected bacterial isolates are members of the *Bacillus cereus* group clade. For the *B. cereus* medium (BCM), an egg yolk emulsion was prepared as follows. Fresh eggs were washed with a stiff brush and then soaked in
70% ethanol for 1 hour. The ethanol was drained and the eggs allowed to air dry for 5 minutes. The eggs were cracked and the albumen discarded. Egg yolks were removed from the shells with a sterile 25 ml pipette and mixed with equal volume of a sterile 0.85% saline solution in a 50 ml centrifuge tube. The emulsion was stored at 4°C until needed. The medium was prepared by suspending 20.5 g of the *B. cereus* base (HiMedia Laboratories, Mumbai, India) in 475 ml of Mill-Q water and autoclaved for 15 minutes at 121°C. The medium was cooled to 50°C in a water bath and 25 ml of the sterile egg yolk emulsion was added. Polymyxin B (10,000 units/ml) was added to the cooled media and plates poured immediately. Bacteria were streaked onto BCM and incubated at 20°C for 48 hours. After incubation, plates were inspected for growth, colony color the presence of a halo or a precipitate surrounding the colony. A positive for *B. cereus* was indicated by the appearance of dull blue-turquoise colonies.

**Screening for antagonism using an in vitro plate assay**

An *in vitro* plate assay was used to screen isolates for the ability to reduce growth of *V. inaequalis* and *C. acutatum*. Experiment one was conducted with *V. inaequalis* and a second experiment was conducted with *C. acutatum*. In experiment one, a mycelial plug of *V. inaequalis* was placed on PDA in a 100 mm Petri dish and incubated at 20°C for seven days. After incubation, a streak of the candidate bacterial isolate was placed 5 cm from the mycelial plug. Control plates consisted of the mycelial plug without bacteria. Three replicate plates were prepared for each pathogen-bacterial isolate combination. Plates were incubated at 20°C and radial growth of *V. inaequalis* was measured 0, 7, 14, 21 and 28 days post inoculation (dpi) of plates with *V. inaequalis*. Experiment two was conducted exactly as experiment one with a few modifications due to the faster growth of *C. acutatum* in culture compared to *V. inaequalis*. For *C. acutatum*, the streak of bacteria was made immediately after placement of a mycelial plug on
the agar. Growth of *C. acutatum* was measured every two days for ten days. The measurements were used to calculate the radial growth rate of the fungus as mm/day. The final colony diameter perpendicular to the bacterium at the end of the experiment was also determined. Data were analyzed for statistical significance by PROC MIXED followed by a Dunnett's test using SAS (SAS Institute, version 9.2; Cary, NC) to determine if the bacteria were able to significantly suppress fungal growth compared to the control.

**Screening bacterial isolates on apple seedlings and potted trees**

The bacterial isolates that tested positive for chitinase production using the plate assay were then screened for the ability to colonize apple seedlings, and also to determine if they cause any pathology on seedlings under growth chamber conditions. This step was taken to eliminate transient colonizers from moving forward in the screening process. Leaves of six week old 'Cortland' seedlings were sprayed individually to runoff with one of the bacterial strains in a 0.1% Silwet L-77 solution. Controls included non-inoculated seedlings and seedlings sprayed with a 0.1 M phosphate buffer solution containing 0.1% Silwet L-77. The concentration of bacteria sprayed was determined by measuring the OD$_{600}$ in a spectrophotometer and plating serial diluted solutions in triplicate on YED medium using the spiral plater as described earlier. Plates were stored at room temperature and colonies counted 48 hours after plating. Total bacterial populations (epiphytic and endophytic) of each isolate were assessed 10 days post inoculation (dpi). During sampling, the third fully expanded leaf was collected from each treatment for three replications. Two 113 mm$^2$ leaf discs were removed from each leaf using a sterilized number 9 cork borer (12 mm diameter). The two discs from each leaf were placed in sterile 1.7 ml microfuge tubes with 900 µl 0.1 M phosphate buffer and ground using pellet pestles (Kimble Chase; Vineland, NJ). Fifty µl of the undiluted and 10 fold diluted homogenized tissue solution
was plated in duplicate on YED using the spiral plater. Plates were incubated at 20°C for 48 hours. Following incubation, bacteria were enumerated using a Spiral Biotech counting grid. Bacterial populations were recorded as CFU/cm² of leaf tissue. The minimum detectable level for this methodology was log 1.9 CFU/cm².

Colonization by the bacteria was also assessed on potted 'Red York' trees in the greenhouse that had been treated as previously described for the 'Cortland' seedlings. Epiphytic and endophytic populations were determined 7, 14 and 30 dpi. Two leaves from each treatment for 3 replications were sampled and processed immediately. Two 4.2 cm² leaf discs were removed from each leaf using a #15 cork borer (2.3 cm diameter). To determine populations of epiphytic colonists, the four discs (2 from each leaf sampled equaling 16.6 cm² of tissue) were placed in a 101 x 152 mm stomacher filter bag (Secure-T 80; Labplas, Sainte-Julie, Quebec) with 10 ml of 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 s in a stomacher blender. The supernatant was plated undiluted and 10 fold diluted in duplicate on YED using a spiral plater. The remaining supernatant was discarded. To determine the population of endophytic colonists, a second aliquot of 10 ml of buffer was added to the stomacher bag and the sample was agitated at 9 strokes per second for 30 s. The supernatant was discarded and a 3rd aliquot of 10 ml of buffer was added to the stomacher bag and the sample agitated for 30 s (for a total of two wash steps). The discs were then further triturated using a pestle against the stomacher bag until well triturated, and the supernatant plated undiluted and 10 fold diluted in duplicate on YED using a spiral plater. A 900 µl sample of the epiphyte and endophyte supernatant was collected and heat treated in 1.7 ml microtubes in a 75°C water bath for 15 minutes and 50 µl plated on YED to quantify the presence of endospores using a spiral plater. All plates were incubated for 24 hours at room temperature and enumerated using a Spiral Biotech counting grid following the manufacturer's instructions. The minimum detectable population for this methodology was log 1.12 CFU/cm².
Three experiments were conducted to screen the bacterial isolates on potted apple trees during the 2007 field season. A field study was conducted to evaluate the ability of selected bacterial isolates to colonize leaves and suppress apple scab disease severity on potted 'Golden Delicious' and 'Gala' trees under field conditions. Two greenhouse studies were conducted to screen for suppression of fire blight on potted 'Golden Delicious' and 'Gala' trees and suppression of apple scab on potted 'Red York' trees.

In the first greenhouse experiment, the potted 'Red York' trees were arranged in a bidirectional randomized block design to account for proximity to outside walls and walkways (Figure 3-1). A mist irrigation system was installed in the greenhouse to increase the relative humidity in the room during the scab experiment. Baumac ULV extra fine fogger nozzles (MicroMist Systems; Yucaipa, CA) were screwed into 1/4" PVC pipe laid on the gravel floor between each row of trees (Figure 3-2). Nozzles were placed so that each block contained six nozzles. The study consisted of 6 blocks of 12 treatments. Treatment one was a surfactant control and treatment two was the scab control, both sprayed with a phosphate buffer solution of 0.1 % Silwet L-77. Treatments 3-12 included 0.1% Silwet L-77 plus the test isolate. For bacterial application, trees were moved into the adjacent room grouped by treatment and the bacteria applied using a 1.5 L handheld spray bottle. Leaves were allowed to dry before moving the trees back into the test area. The trees were challenge inoculated with a conidial suspension of *V. inaequalis* (prepared as described earlier) seven days after the bacterial treatments were applied. The scab inoculum was applied in the evening (18:00 h) when the temperature in the greenhouse was the coolest of the day (approximately 18ºC). Immediately after pathogen application, each tree was covered with a 13 gal white plastic bag which was attached to the tree with a twist tie. The misters were turned on three hours before inoculation to raise the humidity in the greenhouse.
After completing scab inoculations, the misters remained on continuously overnight. The bags were removed from the trees 14 hours post inoculation.

A second greenhouse experiment was conducted to screen isolates for suppression of fire blight on potted 'Golden Delicious' and 'Gala' trees. This experiment consisted of a 2x2x6 factorial with two apple cultivars, two *E. amylovora* strains (581 and 273) and six candidate biological control bacteria treatments (five isolates plus a no-bacteria control). Controls consisted of non-inoculated trees sprayed with 0.1% silwet L-77. Trees were temporarily moved outside by treatment and the biocontrol bacteria were applied using 1 L hand held spray bottles. Leaves were allowed to dry before the trees were moved back into the greenhouse to minimize cross contamination among treatments. Trees were challenge inoculated with the two strains of *E. amylovora* seven dpi with the biocontrol bacteria. Two actively growing shoots per tree of each

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**Figure 3-1:** Map of greenhouse experimental layout. (A) The experiment consisted of six blocks each with twelve treatments. (B) Within each block, treatments were randomly assigned using random permutations.

**Figure 3-2:** Potted 'Red York' trees in the greenhouse. Fogger nozzles were installed on 1/4” PVC on the gravel floor between each row of trees.
variety were chosen for inoculation. Sterilized scissors were dipped in the respective *Erwinia* strain inoculum (prepared as described earlier) and used to cut the growing tip and the tips of 2-3 of the youngest, unfolding leaves. Sterile cotton swabs were dipped in the inoculum and the cut surfaces were swabbed. Immediately following *Erwinia* inoculation, the shoots were sprayed with distilled water using a hand held spray bottle and the shoots covered with clear plastic bags and secured with twist ties. The plastic bags were removed 24 hours after *Erwinia* inoculation. Three weeks after inoculation, shoots were evaluated for blight symptoms. The length of the blighted tissue and total shoot length of inoculated shoots were recorded and the percent blighted shoot length was calculated. Data were analyzed for statistical significance using the mixed linear model of SAS 9.2 (SAS Institute, Cary, NC). Statistical significance was assessed at $P \leq 0.05$ and a Tukey test was used to separate means of significant interactions. Upon termination of the experiment, the potted trees were pruned using pruners sterilized in 10% commercial bleach to remove blighted tissue and prevent the bacteria from spreading throughout the vascular system of the tree.

A third potted tree experiment was conducted to screen for suppression of apple scab under orchard conditions. This experiment consisted of a 6x2x2 factorial with six bacterial treatments (five isolates and one no-bacteria control), two sulfur treatments (sulfur and no sulfur) and two cultivars ('Gala' and 'Golden Delicious'). ‘Gala’ is considered highly susceptible to apple scab and ‘Golden Delicious’ is susceptible. Treatments were applied to the potted trees 8 weeks after removal from cold storage. The trees were sprayed until runoff with each of the bacterial treatments in a 0.1% Silwet L-77 solution using a 1L handheld spray bottle. The OMRI approved micronized wettable sulfur product, MircoSulf® (Nufarm Americas Inc. Burr Ridge, IL) was applied to respective treatments at a rate of 0.15 lbs/gal six days after the bacteria were applied. Following sulfur application, the potted trees were moved outside and placed in the test orchard on May 10, 2007 shortly after full bloom (7-May). The orchard site used for this experiment was
located at the Penn State Fruit Research and Extension Center (FREC) in Biglerville, PA. The orchard selected was also the site of an ongoing experiment testing the effects of fungicide and alternative treatments for apple scab control. The potted trees were placed under untreated "control" trees that served as ascospore inoculum sources of the apple scab fungus *V. inaequalis*. The 'Golden Delicious' potted trees were placed under mature 'Golden Delicious' trees and the 'Gala' potted trees were placed under mature 'Red Delicious' trees due to the lack of mature 'Gala' trees at the test site. A single replication of a potted cultivar (12 trees) was placed under each inoculum source tree for a total of eight trees. All mature trees at the test site were on M 26 rootstock spaced at 35 x 10 x 8 ft. Scab incidence on the untreated mature 'Golden Delicious' and 'Red Delicious' inoculum source trees was recorded by the FREC summer staff by observing all leaves on 25 shoots per tree (4 replicates) on 19-Jun and 23-Jul 2007.

Colonization of the potted tree foliage with bacteria was assessed 26 dpi on 30-May (20 days after the trees were placed into the orchard). Two fully expanded leaves that had been directly treated were removed from each of four replicate potted trees, packed in zip-lock bags, stored over ice and transported back to the University Park lab. The next day, two 4.2 cm² discs were cut from these two leaves using a #15 cork borer (16.8 cm² total leaf tissue). The discs were placed in sterile stomacher bags with 10 ml of 0.1 M phosphate buffer and agitated at 9 strokes per second in a stomacher for 30 seconds. The leaf tissue was further ground using a pestle and 50 µl of the supernatant was plated undiluted and diluted 10-fold on YED in duplicate using the spiral plater. The remaining supernatant was heated in a 75ºC water bath for 15 minutes and 50 µl was plated in duplicate on YED to quantify the presence of endospores. Plates were incubated for 24 hours at room temperature and enumerated using the Spiral Biotech counting grid. The minimum detectable population using this methodology was log 1.4 CFU/cm².

The potted trees remained in the orchard for four weeks and were watered daily when necessary. Weather monitoring and primary scab infection periods were recorded with a
Campbell Scientific and a Field Monitor Weather System (Sensor Instruments Co., Inc.) using the Mills modified apple scab infection model [20]. Following exposure to several infection periods, the potted trees were moved back into the greenhouse on 6-June. Overhead mist was applied at hourly intervals for five minutes to maintain humidity in the greenhouse. The trees were monitored daily for symptom development and the severity of foliar scab symptoms were evaluated 8-June. Leaves on two branches per tree were evaluated based on the estimated percent total leaf area scabbed using a five point rating scale (0=no disease, 1=20%, 2=40%, 3=60%, 4=80%, and 5=100% or total leaf collapse). A visual diagram developed by Tehon and Stout was used as a guide when assigning a rating value [29]. Where appropriate, intermediate values were recorded for all leaves evaluated. Data were analyzed for statistical significance using the mixed linear model of SAS 9.2 (SAS Institute, Cary, NC). Statistical significance among treatments was assessed at $P \leq 0.05$ and a Tukey test was used to separate means.

**Screening for suppression of foliar and fruit disease on branch inoculated trees**

Branches of non-treated mature ‘Rome Beauty’ trees on M.26 in the FREC test orchard were tagged and spray inoculated with ten biocontrol bacteria on 13-June and 13-July 2007 using handheld 1 L spray bottles. A total of four branches per tree were inoculated with each isolate on each of four replicate trees. Foliar scab severity was evaluated on two shoots per branch on 30-Aug 2007 using the five point rating scale described earlier. The number of cedar apple rust lesions per leaf on each shoot was recorded and the number of infected leaves per shoot was determined. Fruit were harvested from the tagged branches on 27-Sept 2007. Immediately following harvest, fruit were evaluated for all diseases present. The numbers of apple scab lesions on each fruit were counted and the severity of fruit scab was estimated using a five point rating scale. The numbers of sooty blotch and flyspeck lesions were also counted per fruit. Data
were analyzed for statistical significance using the mixed linear model of SAS 9.2 (SAS Institute, Cary, NC). Statistical significance was assessed at P ≤ 0.05 and a Tukey test was used to separate means.

**Screening for suppression of postharvest disease on fruit**

'Golden Delicious' and 'Rome Beauty' fruit were harvested from the experimental orchard at the FREC from treatments receiving a spray program on 10-14 day intervals from 11-Apr (1/2 in green) to 23 Aug (7th Cover). Fruit were harvested from trees receiving a rotation schedule of Captan 80WDG (3 lb/100gal) and MicroSulf (3 lb/100 gal) from the 2nd-5th cover spray. Treatments were applied dilute to both sides of the trees with a boom sprayer at 100 gal/acre operating at 400 psi. A standard maintenance program for insects was also applied with an airblast sprayer when needed. Fruit were harvested on 10-Oct 2007 and held in a 2°C room until needed. The ‘Golden Delicious' and 'Rome Beauty' fruit were used to screen a total of 17 isolates. Screens were conducted on each cultivar in separate experiments. Each experiment consisted of 10 bacterial treatments (nine isolates) and two application methods. For application method #1 candidate biocontrol bacteria were spray inoculated onto the fruit one week prior to pathogen challenge and inoculated directly into wounds (using a micropipette) one hour prior to challenge in the laboratory. For application method #2, the bacteria were applied directly to wounds (using a micropipette) one hour prior to pathogen challenge (with no whole fruit spray). Experiment one, conducted on 'Golden Delicious' fruit consisted of 25 replicates and experiment two on 'Rome' fruit consisted of 12 replicates. For both cultivars, the fruit were washed in a 5% commercial bleach solution and lightly scrubbed with a sponge and rinsed (x2) with tap water.

For application method #1, fruit were placed on sheets of brown paper grouped by treatment and spray inoculated with one of the bacterial isolates using a 1 L spray bottle. Fruit
were allowed to air dry for 30 minutes and then placed on molded cardboard trays. The trays were stacked and stored in cardboard boxes at 2°C for one week. Seven days later, all fruit were wounded at the equator to a depth of 4 mm using a sterilized six pence nail. Nails were mounted through a rubber stopper to ensure uniformity of wound depth. Immediately after wounding, the wounds were inoculated with 20 µl of the candidate biocontrol bacterial isolate or a sterile distilled water control. A total of 1000 fruit (100 fruit per treatment) were inoculated. One hour after bacterial application, wounds of 500 fruit (50 per treatment) were inoculated with 20 µl of a 4.0 x 10⁴ conidia/ml (800 spores/wound) spore suspension of *C. acutatum*. A hemacytometer was used to adjust the concentration. Pathogen inoculum was prepared by flooding a 10 day old culture of *C. acutatum* on PDA with 10 ml of sterile Milli-Q water. A rubber policeman was used to dislodge the spores from conidiophores into suspension. The water was poured off the plate and into a 50 ml beaker with an additional 10 ml of sterile Milli-Q water and stirred on a magnetic stir plate for five minutes. The solution was filtered through 4 layers of sterile cheesecloth into a sterile 15 ml centrifuge tube. The remaining 50 fruit per treatment (not inoculated with *C. acutatum*) served as bacterial controls to determine if the bacteria alone cause symptoms on the fruit. Once the droplets applied to the wounds had dried, the fruit were placed on 20-count molded cardboard trays grouped by application method in a randomized block design with all bacterial treatments represented on each tray (Figure 3-3). The trays were stored in a dark walk-in growth room at 22°C on plastic shelves and were arranged on the shelves in pairs so that trays of application method #1 and #2 were represented on each shelf level equally. Fruit were evaluated for symptom severity seven days after pathogen challenge. To assess disease severity, the lesion diameter was measured laterally and horizontally across the wound site and the total lesion area was estimated using the average of the two measurements.
A third postharvest experiment was conducted on 'Rome Beauty' fruit to screen additional bacterial isolates. The experiment was conducted exactly as described above except using only application method #1. This experiment consisted of 10 treatments (nine isolates and a water control) with 18 replicates. Fruit were randomly arranged in 20-count molded trays as depicted in Figure 3-3. Fruit were evaluated seven dpi by measuring lesion diameters and the mean lesion area for each treatment was calculated. For all postharvest experiments data were analyzed for significance using the mixed procedure of SAS 9.2 with tray as the random variable followed by a Tukey test (α=0.05) to identify differences among mean lesion areas as affected by application method.

**Results**

*Isolation and screening bacterial isolates from apple*

A total of 75 bacterial isolates were collected from conventional and abandoned orchards in 2006 and 2007. Several (35%) of the isolates were positive for chitin hydrolysis as indicated by the production of a clearing zone surrounding the colony on 0.2% and 0.4% CNA. The clearing zones were easier to identify on the 0.4% CNA and so this concentration was used for all.
subsequent cultures. From the 75 isolates, 38 (50%) were epiphytic and unable to hydrolyze chitin, 15 were epiphytic and hydrolyzed chitin, 11 were endophytic and unable to hydrolyze chitin while 11 were endophytic and able to hydrolyze chitin (Figure 3-4). From the total isolates collected 35% were chitinolytic. Of the isolates screened 44% tested positive for *Bacillus cereus* on BCM. Four of the isolates that tested positive could be identified based on morphology as *Bacillus mycoides*, a species in the *B. cereus* clade.

**Plate antagonism assay**

Isolates A1-1, A3-6, A3-2, A3-3, FLS-1, FLS-5, WGD-5, WS-1 and WS-3 significantly (P < 0.0001) reduced radial growth of *C. acutatum* on PDA. Isolates A1-1, FLS-5, FO-1 and WS-1 resulted in the greatest suppression in radial growth of *C. acutatum* compared to no-bacteria control plates, ranging from 41% to 64% reduction in total colony size after the ten day experiment. Isolates A1-1, FO-1 and A3-F1 significantly (P < 0.002) reduced radial growth of *V. inaequalis* on PDA (data not presented). Isolates A1-1 and FO-1 significantly reduced the growth of both pathogens assayed.

Figure 3-4: Bacterial isolates collected from conventional and abandoned orchard in Adam’s county, PA. Slices in green represent percentages of bacteria isolated from leaf or fruit surfaces (epiphyte) and slices in gray represent those isolated from internal leaf or fruit tissues (endophyte). Lighter colors represent percentage of chitinolytic isolates as indicated by a clearing zone on 0.4% chitin nutrient agar.
Colonization of apple seedlings and potted tree leaves

Isolates that tested positive for chitinase production were evaluated for their ability to colonize apple leaves. Several of the apple isolates exhibited poor growth in typical solid and liquid culture and were not used for further testing. Of the 21 apple isolates tested, none of the bacteria resulted in noticeable negative effects on seedling health relative to non-inoculated and surfactant controls. Isolates varied significantly however, in their ability to colonize apple foliage in growth chamber and greenhouse tests. Several isolates applied to seedling leaves could not be re-isolated 10 dpi, or were recorded at low populations (< log 1.0 CFU/cm²). Isolates FO-1, FLS-5, A1-11, A2-4 and BacJ were isolated at the highest population levels ranging from log 4.5 to log 4.0 CFU/cm² leaf tissue of seedlings. Isolates exhibiting poor colonization on seedlings were not tested on potted trees. Isolates able to colonize seedlings were also able to colonize potted 'Red York' trees grown in the greenhouse (Figure 3-5A-B). Isolate A1-3 initially colonized leaves at log 2.9 CFU/cm² but populations declined over the 30 day sampling period below the detectable level. Isolates WS-3 and WGD-5 were poor colonizers of apple foliage in growth chamber and greenhouse studies and were not further pursued in biocontrol experiments.

Figure 3-5: Mean foliar epiphytic (A) and endophytic (B) colonization by bacterial isolates of potted 'Red York' in the greenhouse. Bacteria were applied on day 0 and colonization assessed 7, 14 and 30 dpi. Error bars represent the standard error of the mean. The dashed line indicates the minimum detectable level.
Screening for biocontrol activity on potted trees

Two greenhouse experiments were conducted to screen isolates collected in 2006 from apple for suppression of apple scab and fire blight. In the first greenhouse experiment, there were no significant differences (P > 0.05) in foliar scab severity between treatments on the potted 'Red York' trees. Overall scab severity on the potted trees was very low with most leaves showing no symptoms and those with symptoms averaged less than 10% diseased leaf area. Although the experiment was conducted in the greenhouse from 29-Mar to 12-Apr when outside temperatures were cool, there were few cloudy/rainy days and the sunlight would quickly warm the greenhouse to 21-26 ºC. The greenhouse used for this experiment does not have any cooling capability with the exception of wall fans for ventilation, and the ambient temperatures probably exceeded those that would allow for infection in the control treatments.

The second greenhouse experiment was conducted to screen apple isolates for suppression of shoot blight caused by *E. amylovora*. Two isolates collected from annual crops (BP24 and BacJ) were also evaluated in this experiment. The mean percent shoot blight was significantly less (P < 0.05) on 'Gala' shoots treated with BacJ, BP24, FLS-5 and FO-1 compared to the Silwet control when challenged with *E. amylovora* strain 273 (Figure 3-6A). 'Golden Delicious' shoots colonized with isolate FLS-5 had significantly less blighted tissue compared to the Silwet control when challenged with *E. amylovora* strain 581 (Figure 3-6B). There was no significant reduction in percent blight on 'Golden Delicious' challenged with strain 273. There was no significant reduction in percent blight on 'Gala' challenged with strain 581.
A field experiment was conducted to evaluate biological control of apple scab in an orchard using natural inoculum sources. Prior to placement of the potted apple trees in the test orchard, five scab infection periods occurred between 25-April and 29-April resulting in ascospore release from overwintering debris from the previous season. During the four week period (10-May to 8-June) that the potted 'Golden Delicious' and 'Gala' trees were in the test orchard numerous primary scab infection periods occurred. There were seven severe infection periods and five moderate infection periods (Figure 3-7A). The primary scab period for the 2007 season occurred from 15-March to 15-Jun. Overall, scab ‘disease pressure’ in the test orchard was relatively light due to the dry weather. Rainfall for Apr, May and Jun was 3.81, 1.91 and 2.34 in respectively. Scab incidence on the untreated 'Golden Delicious' and 'Red Delicious' trees (used as sources of natural inoculum) as of 19-Jun 2007 on shoot leaves was 51% and 59% respectively.

Figure 3-6: Mean percent shoot blighted by *E. amylovora* isolate 273 (A) and isolate 581 (B). Blue bars represent blight tissue on cv. Gala and the grey bars represent cv. Golden Delicious. A Tukey test was used to separate means. Within cultivar, bars with the same letter are not significantly different (P > 0.05). Error bars represent the standard error of the mean.
There were no significant differences in foliar scab severity observed between treatments in this experiment. Overall scab severity on the potted trees was low, averaging less than 5-10% leaf area scabbed, with many trees showing no symptoms. Leaf samples collected 20 days after the potted trees were moved into the field (26 dpi with bacteria) indicated that isolates FLS-5, FO-1, BacJ and BP24 were able to colonize leaves under field conditions (Figure 3-8A-B). There was no significant difference in bacterial colonization levels on 'Golden Delicious' and 'Gala' trees so the data were pooled. Bacterial populations were generally lower on trees receiving a MicroSulf application. Isolate FLS-5 for example, averaged log 3.9 CFU/cm² on non-sulfur treated leaves and log 2.4 on sulfur treated leaves.

Figure 3-7: (A) Apple scab infection periods at the PSU FREC in Biglerville, PA from May 1-June 10 2007. Infection periods were determined using the Mill's modified model. Potted trees were placed in the orchard on May 10, 2007. (B) Average rainfall in inches and leaf wetness hours.
As mentioned earlier, overall apple scab disease severity was low throughout the 2007 growing season at the FREC. There were no significant differences in foliar scab severity on branches among treatments. The average number of foliar cedar apple rust lesions was significantly less (\(P < 0.05\)) on branches treated with isolates FLS-5, A1-1, A1-11 and A2-4 compared to the no-bacterial controls. The vegetable isolates, BP24 and BacJ had no significant effects.

**Screening for suppression of diseases on inoculated branches**

Figure 3-8: Mean colonization of apple leaves 26 dpi on non-treated (A) and sulfur treated (B) potted 'Golden Delicious' and 'Gala' trees. Red bars represent the total population (vegetative cells + endospores) of each isolate. Yellow bars represent the portion endosporic. Means were not significantly different between cultivars. Error bars represent the standard error of the mean. Means with the same letter are not significant based upon Tukey's test (\(\alpha=0.05\)). The dashed line indicates the minimum detection level.

Figure 3-9: Mean number of rust lesions on leaves from branches treated with bacteria on 13-Jun and 13-Jul 2007. Error bars represent the standard error of the mean. Means with the same letter are not statistically significant based upon Tukey's test (\(\alpha=0.05\)).
Apple scab severity was significantly less (P < 0.05) on 'Rome Beauty' fruit harvested from branches treated in June and July with isolates A1-1, A1-5 and BacJ compared to fruit from the Silwet L-77 control branches (Figure 3-10A). Scab incidence on fruit treated with A1-1, A1-5 and BacJ was 25%, 9% and 13% incidence compared to 65% on Silwet L-77 control fruit and 64% on the no-treatment control fruit. Incidence of flyspeck lesions per fruit was also significantly less (P < 0.05) on fruit treated with isolate A1-1 and BacJ, but not for A1-5 compared to the Silwet L-77 controls (Figure 3-10B).

**Screening for biocontrol activity of postharvest disease on harvested fruit**

Three postharvest experiments were conducted to screen bacteria collected in 2006 and 2007 from apple. Two *Bacillus* isolates collected from perennials (described in Chapter 2) were also evaluated in this experiment. The bacterial isolate treatment was significant (P = 0.01) however, there was no significant difference (P = 0.63) in lesion size on fruit receiving method #1 or #2 for application of the bacteria and so the data were pooled. Additionally, there was no significant interaction between bacterial isolate and application method (P = 0.79). Three of the isolates tested resulted in significantly (P < 0.04) smaller bitter rot lesion areas 7 dpi on 'Golden
Delicious' fruit and six were significant on 'Rome Beauty' fruit (Figure 3-11A-B). Isolates A3-1 and A3-3 reduced lesion size by 73% and 61% respectively and isolate BacJ resulted in a 41% reduction of lesion size on 'Golden Delicious' fruit (Figure 3-11A). Isolates A3-1 and A3-3 also reduced lesion size by 25% and 24% on 'Rome Beauty' fruit compared to the water controls (Figure 3-11B). The no-pathogen bacterial controls could not be evaluated on 'Golden Delicious' fruit due to contamination by fruit flies in the growth room. The presence of *C. acutatum* in these bacteria-only control wounds showing symptoms was confirmed by plating wound tissue on PDA. It is assumed that fruit flies moved the pathogen from wound to wound during the experiment. In experiment two, conducted on cv. Rome Beauty, pairs of method #1 and #2 trays were placed in clear plastic bags to keep the fruit flies out of the wounded tissue. No symptoms were observed on the no-pathogen bacterial controls in this experiment.

A third postharvest experiment was conducted on 'Rome Beauty' fruit to screen additional isolates and confirm significant results found with isolates in postharvest experiments one and two. Because application method did not have a significant effect on lesion area in the first experiments, only application method #1 was used to prepare fruit for this experiment. Results indicate that all bacterial isolates resulted in significantly ($P < 0.01$) smaller mean lesion areas.

![Graph A](image1.png)

![Graph B](image2.png)

Figure 3-11: Suppression of Bitter rot lesion area by bacteria isolated from apple and vegetable crops on 'Golden Delicious' (A) and 'Rome Beauty' fruit (B). The average lesion diameter was used to calculate the total diseased area ($\pi r^2$) on each fruit. Error bars represent the standard error of the mean. Means with the same letter are not statistically significant based upon Tukey's test ($\alpha=0.05$).
compared to the water controls (Figure 3-12). Fruit treated with isolate A3-6 had the smallest lesions, resulting in an 81% reduction in mean lesion area from the buffer control.

**Discussion**

This study represents one of a limited number of reports on the isolation and screening of bacteria from apple for biological control of apple scab. Burr et al. [5] isolated bacteria and yeasts from abandoned and commercial orchards in New York. From 931 bacteria, an isolate of *Pseudomonas syringae* (isolate 508) prevented conidial germination *in vitro* and suppressed scab on seedlings by 80%. Kucheryava et al. [18] isolated epiphytic bacteria from an orchard in North Germany and reported five strains of *Pseudomonas fluorescens* and one strain of *Bacillus pumilis* as potential biocontrol agents based exclusively on *in vitro* screens. Further research based on Kucheryava's initial study, demonstrated inhibition of conidial germination [12] and induction of pathogenesis-related proteins in cv. Holsteiner Cox by *P. fluorescens* BK3 [19]. With the exception of a few studies mentioned here, the breadth of research on biological control of apple scab has focused on isolation of yeasts and fungi [2, 3, 24]. Additionally, a majority of scab...
biocontrol research has targeted overwintering inoculum (pseudothecia producing ascospores) in leaf litter [7, 11].

This study is the first known report specifically targeting endospore forming bacteria as biocontrol agents for apple scab. This strategy was selected as a means to quickly narrow down the field of organisms for further screening. Strict selection criterion were important to achieve our goal to use in vivo methods to screen isolates, which can be more time consuming and require large amounts of plant material and space compared to in vitro assays. Another benefit in selecting endospore forming bacteria is colonization stability and ease of formulation. These bacteria have the potential to tolerate extremes in environmental conditions and survive when applied with standard tree-fruit spray regimes, ensuring long-term survival on leaf and fruit surfaces. Boudreau and Andrews [4] reported 90% reduction in scab severity using C. globosum, however, less than 25% control was observed in the field. Further investigation revealed that the antagonist did not colonize leaves and antibiotics from C. globosum ascospores were likely degraded abiotically. Production of endospores also allows for a relatively straightforward process to formulate the biocontrol agent into a product with a suitable shelf life compared to products made with yeast or Gram-negative bacteria such as Pseudomonas spp. These heat and desiccation resistant spores of Gram-positive Bacillus and Steptomyces can be readily formulated as a wettable powder or flowable [27]. Gram-negative microbes such as P. syringae on the other hand, are formulated as frozen pellets that must be kept cold until application to maintain viability of the product [10].

Using endospore formation as a selection criterion (by heat treatment of samples), produced a total of 75 bacterial epiphytes and endophytes isolated from orchards in Adam's County, PA in 2006 and 2007. Chitinase production was an additional criterion used to determine which endospore producing isolates to test for colonization of apple in growth chamber and greenhouse experiments. Of the 19 chitinase positive isolates collected in 2006, 7 were
easily maintained in culture, colonized apple foliage endophytically and were also capable of maintaining a total population above log 2.5 CFU/cm$^2$ under greenhouse conditions. These isolates were then screened for scab suppression on potted trees in the greenhouse using single spore \textit{V. inaequalis} isolates and natural inoculum in the field. Unfortunately sub-optimal conditions for scab infection in the greenhouse likely contributed to the observed low apple scab incidence and severity on potted trees. The field trials using potted trees were also unsuccessful due to low apple scab pressure and little rainfall during the spring and summer months of 2007. The use of potted trees also proved to be problematic due to the level labor required for maintenance. The lack of rain required FREC crew to hand water each of the 120 pots at least every two days which were spread out over the 372 m long orchard. When dry, the potted trees often fell over comprising the experiment. Although scab levels were low, significant differences in fruit scab and flyspeck disease severity were observed on fruit harvested from the branch inoculation experiment. One isolate, A1-1, significantly reduced foliar rust incidence, apple scab severity on fruit and also flyspeck incidence on fruit. Isolate FLS-5 significantly reduced shoot blight caused by \textit{E. amylovora} strain Ea 273 on cv. Gala and the \textit{E. amylovora} strain Ea 581 on cv. Golden Delicious. Isolate FLS-5 was also able to provide long-term colonization of apple foliage endophytically in the greenhouse at log 3.1-4.4 CFU/cm$^2$ over the 28 day sampling period. Isolate FLS-5 was also able to colonize foliage of potted apple trees grown in the field at a level of log 3.9 CFU/cm$^2$ for 26 dpi.

The challenges experienced in our greenhouse and field studies reduced our ability to efficiently screen and select for superior performing isolates from our 2006 collection of spore forming bacteria. Upon collection of additional apple isolates in 2007, postharvest experiments were used as an alternate strategy to screen bacterial isolates. Using \textit{C. acutatum} as a model pathogen, postharvest experiments allowed us to screen isolates quickly compared to seedlings and potted tree experiments. Postharvest experiments were also less labor intensive. Five of the
tested isolates significantly reduced bitter rot lesion size by at least 50% in postharvest experiments: A3-6, A3-1, A3-3, A3-2 and A3-F1. It should be noted that all of these isolates were collected from an abandoned orchard (five years unmanaged). Field experiments are needed to determine if suppression of *C. acutatum* in fruit wounds correlates to suppression by the bacteria of foliar diseases such as apple scab and cedar apple rust. Based on the *in vitro*, colonization, greenhouse, field and postharvest studies, apple isolates FLS-5, A1-1, A3-6, A3-1, A3-2 and A3-F1 were selected for future field studies on biological control of pre and post-harvest diseases of apple. Overall, these studies have identified several isolates that have significantly suppressed multiple diseases in different environments. They also have frequently demonstrated prolonged persistence in the epiphytic and/or endophytic environments of several apple cultivars. Although the results of field and greenhouse experiments were often compromised by inabilities to control the environment, these results allowed us to greatly reduce the number of candidate bacteria to carry forward to field and postharvest experiments.

Future research with these apple isolates, which is presented in later chapters, was focused to determine the effectiveness of the isolates as biological control agents and to evaluate application strategies to optimize suppression of multiple diseases of apple. Although potted trees offered a solution to the variability associated with seedlings described in chapter 2, mature trees should offer a more manageable (less labor intensive) approach to biocontrol experiments. Experiments presented in chapters 4 and 5 focused on testing selected apple isolates on mature trees for their abilities to reduce pre and postharvest diseases under field conditions when combined with standard insecticide and reduced rate fungicide spray programs.

**References**


Chapter 4

Field assessment of biological control of pre- and postharvest diseases of apple in Pennsylvania

Introduction

Production of fresh fruits and vegetables presents a unique challenge as most produce is harvested over a relatively short period of time and then stored for weeks to months, to avoid exceeding demand and provide consumers with a product year round. Postharvest decay by microorganisms represents a significant limitation in our ability to store fruit. The economic losses incurred from storage diseases are considerable given the initial investment related to crop production and management of pathogens and pests in the field prior to harvest. To date, the use of synthetic fungicides (as pre- or postharvest treatments) remains the primary means for managing postharvest decays [8]. Development of fungicide resistant strains has been documented for several postharvest pathogens [24, 41]. Human health concerns, due to pesticide residues on surfaces directly consumed as food, have also limited the available options for control of postharvest pathogens [30]. Several countries have banned or significantly restricted postharvest applications of fungicides [11]. As a result, there is an ongoing effort to develop alternative control methods to reduce fungicide dependence, reduce environmental risks and improve consumer confidence in food safety.

Considerable attention has focused on the potential use of biological control for management of postharvest diseases of fruits and vegetables and there have been several review articles on the topic over the past 20 years [8, 14, 31, 33, 38-40]. The uniqueness of the postharvest environment offers an advantage where environmental parameters can be adjusted to favor survival of biocontrol agents (BCAs). The challenge is that new technologies must not only
be safe, but a viable alternative to current synthetic fungicides. Several microorganisms have been patented for postharvest biocontrol, many of which are applied to harvested fruit as a conventional dip or drench. Over the past two decades postharvest biocontrol has evolved and matured into a significant area of research. Pioneering work conducted by Wilson and Pusey [29, 38] on biocontrol of brown rot on peach with a strain of *Bacillus subtilis* in the 1980's soon lead to a wealth of research and development of commercial products. Although numerous products were pursued, most were met with limited success [8]. Currently, there are only two commercial products available for postharvest use in the United States, each with limited markets. Two strains of *Pseudomonas syringae* are available as Bio-Save™ 10 and 11 (JET Harvest Solutions, Longwood, FL) [9] in the USA for control of blue mold and gray mold on apple and pear, but are more frequently used for control of postharvest sweet potato and potato diseases. More recently, Shemer™ (*Metschnikowia fructicola*) was registered in Israel for pre- and postharvest application on several fruit and vegetables but is mostly used for control of sweet potato and carrot storage diseases [2]. The two yeast-based products, Aspire™ (*Candida oleophila*) and YieldPlus™ (*Cryptococcus albidus*) for use on citrus and pome fruits in the U.S. are no longer available [8].

Commercialization and use of most postharvest biocontrol products remains limited due to inconsistency in their efficacy, low levels of control as stand-alone products, and apparent inability to control previously established infections [9]. Infection by postharvest pathogens frequently occurs either during the growing season and/or during harvest and packing when mechanical injury commonly occurs providing entry points for the pathogens. While appearing healthy at harvest, many fruit harbor quiescent or latent infections that over time produce symptoms in storage as ripening occurs and host defenses weaken [10]. In a recent review article, Janisiewicz [13] stated that the main challenge for the next generation of postharvest biocontrol products is control of latent infections. Successful disease control in both the field and postharvest, depends on the appropriate application of a BCA in time and space [4]. Research on
postharvest disease management has typically focused on application of BCAs after harvest. Application after harvest however, may be too late for the BCAs to effectively compete with decay pathogens that have already established themselves on or in fruit tissues in the field. Furthermore, induction of active host defense mechanisms after harvest may not be as effective as induction while the fruit are still attached to the tree. Application of BCAs prior to harvest could allow establishment of the BCA on leaf and fruit surfaces in advance of pathogen arrival. In addition to postharvest protection, application of BCAs with broad antifungal activity (perhaps capable of inducing non-specific host defenses) during the growing season may confer additional benefits of suppression of foliar diseases. In apple, this could mean suppression of apple scab, powdery mildew, and/or cedar apple rust.

Some success has been reported using pre-harvest field applications of biocontrol agents on apple and pear [1, 22, 23]. Nunes et al. [28] found that a 50:50 mixture of *Candida sake* CPA-1 and *P. syringae* CPA-5 applied two days before harvest reduced blue mold incidence by 56% on apple and 90% on pear. Leibinger et al. [23] reported a significant suppression of storage rots comparable to the sulfamide fungicide Euparen, by a mixture of *Aureobasidium pullulans* and *Rhodotorula glutinis* applied approximately four weeks and two weeks before harvest. Although pre-harvest applications of these BCAs resulted in disease suppression, experiments comparing pre- versus postharvest applications were not reported. Lahlali et al. [22] reported an 87.7% reduction in blue mold incidence when *Pichia anomala* strain K was applied 12 days before harvest compared to 0% reduction when applied postharvest. Unfortunately however, contradicting results were obtained in the second year of the study. The authors suggested that the variable meteorological conditions may have contributed to the considerable variation in population densities observed for *Pichia anomala* between the two study years, thus affecting the antagonist's efficacy. In one of the few studies directly comparing combinations of pre- and postharvest BCA applications, Teixido et al. [36] reported a 50% reduction in incidence and an
80% reduction in diameter of lesions caused by *Penicillium expansum* when treated with a postharvest application of *C. sake*. However, no additional advantages were reported when apples were treated with the BCA both pre- and postharvest.

As briefly reviewed here, a large majority of the postharvest biocontrol research reported in the literature has focused on application of BCAs after harvest. There are a limited number of reports on pre-harvest application strategies. Furthermore, pre-harvest application research has focused on a limited time frame, usually from two days to two weeks before harvest. There have not been reports of the effects of early or mid-season applications of BCAs on preharvest and postharvest disease severity on pome fruits. Given the available research, the question still remains, when is the best time for biocontrol application(s)? Experiments directly comparing levels of postharvest disease suppression by pre- and postharvest applied biocontrol agents are necessary to answer this question. The objectives of the present research are to investigate the potential for biocontrol of several apple diseases using endospore-forming bacteria collected from apple and to evaluate application strategies to optimize suppression of pre- and postharvest diseases of apple.

**Material and methods**

*Preparation of bacterial inoculum*

Four bacterial isolates (A1-1, A3-6, FLS-5 and A3-F1) were evaluated as potential biocontrol agents of pre- and postharvest diseases in field experiments. The individual isolates were screened and selected in preliminary experiments (see chapter 3). The isolates were grown in 500 ml of sterile tryptic soy broth in 2800 ml Fernbach flasks. Each isolate was grown in two Fernbach flasks for a total volume of 1L. The flasks were incubated for 7 days at 28°C and 120 rpm on a rotary incubator shaker (New Brunswick Scientific Model M1024-000, Edison, NJ).
Bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C using a Sorvall RC5C Plus centrifuge (Thermo Scientific). The supernatant was discarded and the bacterial pellets were re-suspended in 0.1 M potassium phosphate buffer. In all biocontrol experiments, the bacterial isolates were mixed with the polysilicon surfactant Silwet L-77 (GE Silicones, Tarrytown, NY) immediately before application at 0.1% (v/v).

**Field sites**

Field experiments were conducted at the Penn State University Fruit Research and Extension Center (FREC) in Biglerville, PA and at the Russell E. Larson Agricultural Research and Education Center at Rock Springs, PA. A total of three experiments were conducted in FREC orchards in 2008 and 2009. Experiments one and two were conducted in the 5C orchard and University Drive research orchard on mature 'Golden Delicious' and 'Rome Beauty' grafted on M.26 rootstock spaced 35 x 10 x 8 ft. Experiment three was conducted on 'GoldRush' located in the buffer row of an organic demonstration orchard established in 2004 and certified organic in 2006. Maintenance programs for insects were applied with an airblast sprayer with applications as needed in all orchards following commercial production practices.

**Field experiments to test application schedule on pre-harvest disease suppression on 'Golden Delicious' and 'Rome Beauty'**

Experiment one was conducted in 2008 and 2009 to evaluate the timing of bacterial application on suppression of pre-harvest and postharvest disease severity on 'Golden Delicious' and 'Rome Beauty'. Both cultivars received a dormant copper spray but did not receive any other fungicide treatments during the 2008 season. High scab pressure in 2008, from this low-input program, lead to a limited number of fruit available for the postharvest phase of the 2008
experiment. Trees in the 2009 experiment therefore received a copper spray at ½ in green (10-Apr) and 5 lbs/100 GPA sulfur + 1.5% lime sulfur at 14 day intervals from 1st cover (22-May) to 5th cover (8-Jul) in 2009. Environmental conditions were recorded with electronic monitoring systems (Campbell Scientific and Spectrum Weather Systems). The pre-harvest phase of this experiment consisted of a 4x2x4 factorial in 2008 with four bacterial isolates, two cultivars and four timings of bacterial application. The four bacterial isolates tested in 2008 were A1-1, A3-6, FLS-5 and A3-F1. The 2009 experiment consisted of a 3x2x4 factorial with three isolates (A1-1, A3-6 and FLS-5), two cultivars and four timings of bacterial application. Branches were tagged and utilized for pre-harvest applications of bacterial isolates and as a source of fruit for postharvest applications. The four timings of bacterial application included; one application in May, an application in May plus June, one postharvest application or no application. At the time of bacterial inoculation (in May and June) the last fully expanded leaf of two shoots per branch were marked with red tape (Figure 4-1).

Figure 4-1: The last fully expanded leaf of two shoots per branch was tagged with red tape at the time of bacterial application in May and June in the 2008 and 2009 field experiments.

In 2008, each bacterial isolate was applied to branches on two replicate trees of each cultivar located in the 5C and University Drive orchards (Figure 4-2). Bacteria were applied to run-off using 1L handheld spray bottles on 10-May (May and May+June treatments) and 18-June (May+June treatment). The 500 ml bacterial stock solutions of $1.0 \times 10^7$ CFU/ml were added to 500 ml of distilled water in each bottle for a total volume of 1L (final concentration of approximately $5.0 \times 10^6$ CFU/ml). Silwet L-77 was added to each bottle for a final concentration
of 0.1%. Individual trees were inoculated with a single bacterial isolate and each of the four application treatments were applied to four replicate branches per tree (16 branches per treatment). At the time of application branches designated as non-treated or as postharvest application, were covered using 50 gal plastic bags to avoid drift contamination and the bags were removed immediately afterwards.

**Figure 4-2**: 2008 experimental layout at the PSU FREC (A) 5C and (B) University Drive research orchards. Four bacterial isolates were tested; A1-1, A3-6, FLS-5 and A3-F1. Each isolate was applied to branches on two replicate trees of 'Golden Delicious' and 'Rome Beauty'. Trees are indicated by an X. Cultivars in each plot are indicated by letters A = 'Rome Beauty' B = 'Golden Delicious' C = 'Cortland' D = 'Stayman' and E = 'Red Delicious'.

In 2009, each of the three isolates tested were applied to branches of four replicate trees of each cultivar located in the University Drive orchard (Figure 4-3). Bacteria were applied using an 11 L Solo backpack sprayer (Solo, Newport News, VA) on 7-May (May and May+June treatments) and 19-June (May+June treatment). Two backpack sprayers were used, one for bacteria and the second for the Silwet L-77 control applications. The 1 L bacterial stock solutions of 1.0 x 10^7 CFU/ml were added to 4 L of tap water with 0.1% Silwet L-77 in the spray tank for a total volume of 5 L (final concentration of approximately 2.0 x 10^6 CFU/ml bacteria). Individual trees were inoculated with a single bacterial isolate and each of the two application timings (May or May+June) were applied to four branches per tree for a total of eight sprayed branches per tree with 32 branches per cultivar and 64 branches total per bacterial isolate (Figure 4-4). Bags were
not used to cover untreated branches as described in 2008 because controls were located on separate trees in the 2009 experiment.

Foliar colonization of sprayed 'Golden Delicious' and 'Rome Beauty' trees was assessed one week and seven weeks after the initial application of the bacteria in May. For each cultivar, two leaves were collected from each of two shoots (one and two leaves below the red tag) from two replicate trees. Leaves were removed and placed in plastic bags and stored in a cooler with ice for transport to the lab. The next day, two 4.2 cm² leaf plugs were excised from each of two leaves (for a total of 16.8 cm² leaf tissue) and placed in a 101 x 152 mm stomacher filter bag.
(Secure-T 80; Labplas, Sainte-Julie, Quebec) with 5 ml of 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 s in a stomacher blender (Bagmixer 100 Minimix; Intersciences St. Nom, France). Fifty µl of the supernatant was plated undiluted and diluted 10 fold in duplicate on YED using a spiral plater (Autoplate 4000l Spiral Biotech Inc., Norwood, MA). All plates were incubated for 24 hours at room temperature and enumerated using a Spiral Biotech counting grid following the manufacturer's instructions. The minimum detectable population for this methodology was log 1.0 CFU/cm².

Starting in May 2008 and 2009, apple scab and rust disease severity were assessed weekly until terminal bud stage was reached and new susceptible tissue was no longer available. Disease assessments were taken on leaves (equal to or younger than the red tagged leaf) of two shoots per sprayed branch on all 'Golden Delicious' and 'Rome Beauty' trees (32 shoots per treatment in 2008 and 64 shoots per treatment in 2009). To evaluate rust, the numbers of rust lesions per leaf were counted for all leaves on two shoots per branch. To evaluate apple scab, percent leaf area scabbed was estimated on all leaves on two shoots per branch using a seven point rating scale where 0 = No visible symptoms, 1< 2%, 2= 2-7%, 3=7-15%, 4=15-25%, 5=25-40%, 6=40-60%, and 7>60%. Visual diagrams adapted from Tehon and Stout [34] and Croxall et al [5] were used to classify each leaf to a 'scale class' corresponding to the diagram it most closely resembled (Figure 4-5). Data were analyzed for significance using the mixed procedure of SAS 9.2 (SAS Institute; Cary, NC). The model statement included isolate, application timing and cultivar with tree (replicate) as the random variable. A Tukey-Kramer test was conducted (α=0.05) to compare interaction means and determine if bacteria were able to significantly reduce disease severity at each sampling date.
All fruit were harvested from all tagged branches on 25-Sep and 28-Oct from 'Golden Delicious' and 'Rome Beauty' trees respectively in 2008 and 1-Sept and 6-Oct in 2009. During harvest, all fruit were labeled with the tree and branch number using a permanent marker. Following harvest, fruit were evaluated for incidence and severity of all diseases present. Apple scab, rust, sooty blotch and flyspeck (*Schizothyrium pomi*) lesions were counted on all fruit from each treatment. Apple scab severity on fruit was also estimated using a 0-6 rating scale based on a visual key (Figure 4-6). Fruit were assigned a value based on the corresponding picture it most clearly resembled. Fruit data were analyzed for significance using the Kruskal-Wallis test of SAS 9.2 (SAS Institute, Cary, NC).

Figure 4-5: Foliar apple scab rating scale used to estimate the percent leaf area scabbed in 2008 and 2009 field experiments. Visual diagrams were adapted from Tehon and Stout [34] and Croxall et al. [5] as reprinted by MacHardy [25].

Figure 4-6: Diagram for assessment of apple scab severity on fruit. Each fruit was assigned a value corresponding to the picture it most clearly resembled. Fruit lacking visible lesions were assigned a 0.
Postharvest evaluation of bacteria application schedule on biocontrol of bitter rot on 'Golden Delicious and 'Rome Beauty'

 Marketable fruit (rated a 0 or 1 as shown in Figure 4-6) from each treatment were selected for use in the postharvest phase of experiment one. As described in the previous section, four bacterial isolates were evaluated in 2008 (A1-1, A3-6, FLS-5 and A3-F1) and three in 2009 (A1-1, A3-6 and FLS-5). To determine the effects of bacterial application schedule on postharvest disease suppression, eight treatments were tested as described in Table 4-1. Treatments 1 and 2 received pre-harvest applications of the bacteria (May or May +June), treatment 3 was the untreated control, treatments 4 and 7 received only a postharvest application of bacteria while treatments 5 and 6 received both pre and postharvest applications of the bacteria. Treatments 1-6 were challenged with the bitter rot pathogen, *C. acutatum*. Treatments 7-8 were not challenged with the pathogen and served as bacteria and wound controls.

Table 4-1: Treatments used to determine the effects of bacterial application schedules on postharvest disease suppression. Bacteria were applied to 'Golden Delicious' and 'Rome Beauty' branches on 10-May and 18-June in 2008 and 7-May and 19-June in 2009. Treatments 1-6 were challenged with the bitter rot pathogen *C. acutatum* (+). Treatments 7-8 were not challenged and served as the bacteria and wound controls (-).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial application</th>
<th>C. acutatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>May</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>May + June</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Non-treated</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Postharvest</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>May + Postharvest</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>May + June + Postharvest</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Postharvest</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Non-treated</td>
<td>-</td>
</tr>
</tbody>
</table>

'Golden Delicious' and 'Rome Beauty' fruit were challenged with *C. acutatum* in separate experiments. Experiments conducted on 'Golden Delicious' consisted of 10 replicates in 2008 and 21 replicates in 2009. Experiments conducted on 'Rome Beauty' consisted of 10 replicates in 2008 and 20 replicates in 2009. All fruit were wounded to a depth of 4 mm using a sterilized 6 pence nail to simulate stem punctures. Nails were mounted through a rubber stopper to ensure
uniformity of wound depth. Wounds on fruit receiving postharvest application of bacteria (treatments 4-7) were inoculated with 20 µl of the respective bacterial isolate at a concentration of $10^7$ CFU/ml (2.0 x $10^5$ CFU/wound). One hour after bacterial application, wounds of fruit receiving treatments 1-6 were challenge inoculated with 20 µl of a 4.0 x $10^4$ conidia/ml spore suspension of *C. acutatum*. Pathogen inoculum was prepared by flooding a 10 day old culture of *C. acutatum* on PDA with 10 ml of sterile Milli-Q water. A rubber policeman was used to scrape the mycelia and spores into solution. The water was poured off the plate and into a 50 ml beaker with an additional 10 ml of sterile Milli-Q water and stirred on a magnetic stir plate for five minutes. The solution was filtered through four layers of sterile cheesecloth into a sterile 15 ml centrifuge tube. A hemacytometer (American Optical, Buffalo, NY) was used to adjust the concentration to 4.0 x $10^4$ conidia/ml. Once the droplets applied to the wounds had dried, the fruit were arranged on 20-count molded cardboard trays by bacterial isolate in 2008 with each tray consisting of two replications. In 2009, fruit were placed in 20-count plastic ornament trays (Figure 4-7) with five holes drilled in the plastic lids to allow for air exchange. The trays were stored in a dark walk-in growth room at 20ºC on plastic shelves. Fruit were evaluated for symptom severity three days post inoculation (dpi) and daily thereafter until eight dpi. To assess disease severity, the lesion diameter was measured laterally and horizontally across the wound site and the total lesion area was estimated using the average of the two measurements. Data were analyzed for significance using SAS mixed model repeated measures with the Toeplitz covariance structure to analyze the repeated measurements of lesion area over the eight day period. Tray was the random effect in the model and fruit was the subject (experimental unit). The statistical model contained bacterial isolate, application schedule, dpi and the interaction term bacterial isolate x application schedule. For each isolate, single degree-of-freedom contrasts were used to partition treatment sums of squares to compare specific application treatments (Table 4-1).
or groups of treatments as well as interactions of interest. A Tukey-Kramer test ($\alpha=0.05$) was also used to separate means.

![Image](image_url)

**Figure 4-7:** (A) 20-count molded cardboard trays used for 2008 experiments and (B) 20-count molded plastic trays used for 2009 experiments.

**Determining the effect of a late-season application of bacteria on postharvest disease suppression**

Experiment two was conducted at the FREC in 2009 to evaluate a single late-season spray (of trees receiving a standard fungicide program) on suppression of postharvest disease. Two cultivars ('Golden Delicious' and 'Rome Beauty') and four bacterial treatments were tested (A1-1, A3-6, FLS-5 and none). Each of the three isolates tested were applied to fruit on branches of four replicate trees of each cultivar located in the 5C and University Drive orchards (Figure 4-8). Each isolate was applied using a 7.6 L pump sprayer (Gilmour model # T200, Gilmour Louisville, KY) 19-Aug 2009. The 1 L bacterial stock solutions of $10^7$ CFU/ml were added to 2 L of tap water with 0.1% silwet in the spray tank for a total volume of 3 L (final concentration of $3.0 \times 10^6$ CFU/ml bacteria). Individual trees were inoculated with a single bacterial isolate applied to five branches per tree for a total of 20 branches per cultivar and 40 branches total per bacterial isolate.
Golden Delicious' fruit were harvested on 1-Sept (two weeks post inoculation) and 'Rome Beauty' fruit were harvested on 6-Oct (six weeks post inoculation) and stored at 2ºC. To determine the effect of bacterial application schedule on postharvest disease suppression, five application schedules were tested for each of the three bacterial isolates. Treatment 1 received a pre-harvest application of the bacteria (19-Aug), treatment 2 received both a pre-harvest and a postharvest application of the bacteria, treatment 3 received only a postharvest application of bacteria and treatments 4 and 5 did not receive bacteria pre or postharvest. Treatments 1-4 were challenged with the bitter rot pathogen, C. acutatum. 'Golden Delicious' and 'Rome Beauty' fruit were challenged with C. acutatum in separate experiments. All fruit were wounded and inoculated with the bacteria (treatments 2 and 3) and pathogen as described earlier. One fruit from each of the 15 bacteria x treatment combinations (three isolates x five application schedules) were randomly placed in a single 20-count plastic tray (Figure 4-7B), where a tray represented a replicate. Experiments conducted on both cultivars consisted of 20 replicates. The trays were stored in a walk-in growth room at 20ºC on plastic shelves. Fruit were evaluated and lesions measured as described in experiment one. Data were analyzed to test for significance using SAS.
mixed model repeated measures with an autoregressive covariance structure. Tray was coded as
the random variable and fruit as the subject (experimental unit). Analyses were conducted to
analyze for bacterial isolate x application schedule interactions and a Tukey-Kramer test ($\alpha=0.05$)
was used to separate means. For each isolate, single degree-of-freedom contrasts were also used
to compare application treatments or groups of treatments as well as interactions of interest.

Field experiments to test suppression of pre-harvest and postharvest diseases on 'GoldRush'

Experiment three was conducted in the buffer row of the organic ‘GoldRush’ orchard at
the PSU FREC in Biglerville, PA in 2008 and 2009. This experiment consisted of five treatments
(four bacterial isolates and a non-bacteria control) with five replications (Figure 4-9). The four
isolates tested in 2008 were A1-1, A3-6, A3-F1 and FLS-5 and isolates A1-1, A3-6, Ae-1 and
FLS-5 were tested in 2009. Bacteria were applied as described in experiment two on 8-May, 11-
July and 15-Aug in 2008 and 10-July and 19-Aug in 2009. The 1 L bacterial stock solutions of
$10^7$ CFU/ml were added to 2 L of tap water with 0.1% Silwet L-77 in the spray tank for a total
volume of 3 L (final concentration of $3.0 \times 10^6$ CFU/ml bacteria). Starting in May, four branches
per tree (total of 20 branches per treatment) were evaluated every two weeks for cedar apple rust
lesions. The numbers of lesions per leaf and the number of infected leaves per branch were
recorded. ‘GoldRush’ is an apple scab resistant variety, and no scab disease developed.

Figure 4-9: Experiment three layout at the PSU FREC ‘GoldRush’ organic research orchard. Five bacterial
treatments were tested; A1-1, A3-6, Ae-1, FLS-5 and none. Isolates were applied to five replicate trees on
8-May, 10-July and 15-Aug 2008 and 11-Jul and 19-Aug 2009 with 0.1% Silwet. Trees not inoculated are
indicated by an ‘X’ and posts are indicated by an ‘O’. Trees receiving treatments are indicated by an ‘H’.
Fruit were harvested on 28-Oct in 2008 and 3-Nov in 2009. To determine the effect of bacterial application schedule on postharvest disease suppression, five application schedules were tested for each of the four bacterial isolates (see experiment two for treatment descriptions). All fruit were wounded and bacteria applied as described previously. One hour after bacterial application, wounds of fruit receiving treatments 1-4 were challenge inoculated with 20 µl of 4.0 x 10^4 conidia/ml of *C. acutatum*. Pathogen inoculum was prepared as described earlier. One fruit from each of the bacteria x treatment combinations (4 isolates x 5 application schedules) were randomly placed in a single 20-count plastic tray (Figure 4-7B), where a tray represented a replicate. The trays were stored in a walk-in growth room at 20ºC on plastic shelves. Fruit were evaluated for symptoms as described in experiment one. Data were analyzed for significance using SAS mixed model repeated measures with tray as the random variable and fruit as the subject (experimental unit). A Tukey-Kramer test (α=0.05) and single degree-of-freedom contrasts were used to separate treatment means.

**Determination of bacterial colonization on 'Golden Delicious' leaves**

An experiment was conducted to assess bacterial colonization of leaves under field conditions in 2009 at the plant pathology farm located at the Penn State Russell Larson Agricultural Research Center in Rock Springs, PA. Three isolates (A1-1, A3-6 and FLS-5) were tested for colonization on 'Golden Delicious' leaves. Bacteria were applied to five branches on two replicate trees for a total of 10 branches per bacterial treatment. Immediately prior to bacterial inoculation, the last fully expanded leaf on each shoot was marked with red tape (Figure 4-1). Eight leaves (immediately below the tagged leaf) from each treatment on two replicate trees were sampled 2, 7, 14 and 30 days post inoculation. Leaves were placed in zip-lock bags and transported to the lab on ice and processed immediately. Two (4.2 cm^2) leaf discs were cut from each of two leaves (total leaf area of 16 cm^2) and placed in a 101 x 152 mm stomacher filter bag
(Secure-T 80; Labplas, sainte-Julie, Quebec) with 5 ml of 0.1 M potassium phosphate buffer and agitated at 7 strokes per second for 30 s in a stomacher blender (Bagmixer 100 Minimix; Intersciences st. Nom, France). Fifty µl of the supernatant was plated undiluted and diluted 10 fold in duplicate on YED using a spiral plater (Autoplate 4000 Spiral Biotech Norwood, MA). All plates were incubated for 24 hours at room temperature and enumerated using a Spiral Biotech counting grid following the manufacturer's instructions. The minimum detectable population for this methodology was log 1.0 CFU/cm².

**Determination of bacterial colonization in fruit wounds**

An experiment was conducted to determine bacterial colonization of wounds on 'Golden Delicious' fruit. Three bacterial isolates were tested (A1-1, A3-6 and FLS-5). Colonization was assessed at five time points (4, 24, 48, 72 and 96 hours post inoculation). All fruit were wounded as described earlier and 20 µl of a log 7.0 CFU/ml solution of each isolate was pipetted into wounds of four replicate fruit per sampling point (a total of 20 fruit per isolate). One replicate fruit of each treatment was placed in 20-count plastic trays and stored at room temperature (20ºC) for the duration of the experiment. To assess colonization, at each time point the wounded tissue from 4 replicate fruit was excised using a # 4 (7.5 mm diameter) cork borer to a depth of approximately 6 mm. A single core was placed in a 101 x 152 stomacher filter bag with two ml of 0.1 M potassium phosphate buffer and triturated in a stomacher blender for 30 s. The solutions were diluted 10x and 100x and 40 µl was plated in duplicate on YED. Plates were incubated at room temperature for 48 hours then enumerated. Bacterial populations are reported as CFU/wound. The minimum detectable population level for this method was log 1.7 CFU/wound.
Results

Effect of bacterial application on foliar disease severity

Overall, apple scab pressure was high in the FREC test orchard in 2008. A severe risk of infection persisted during the primary scab period from 15-Mar to 15-Jun. During the primary period, eight severe infection periods and 10 moderate infection events were recorded (Figure 4-10). Rainfall for April through August 2008 was 4.97 in., 6.3 in., 2.49 in., 3.7 in., and 2.44 in. respectively. Due to the high scab pressure in 2008 and the limited availability of scab-free fruit for postharvest evaluation, bacterial treatments were combined with a reduced rate fungicide program in 2009. Apple scab pressure was also high in 2009 and several infection periods occurred during the primary period from 16-Mar to 15-Jun. There were six moderate infection events that occurred over 11 days and included 1-4 continuous days of wetting and nine severe infection events that ranged from 2-5 continuous days of wetting (Figure 4-10). Rainfall for April though August 2009 was 4.4 in., 6.9 in., 4.42 in, 4.98 in. and 2.75 in. respectively.

![Graph of apple scab infection periods at the FREC in Biglerville, PA in 2008 and 2009. Weather data obtained courtesy of Noemi Halbrendt. Apple scab infection periods were determined using the modified Mill's table [26].](image)

In 2009, isolates FLS-5 and A3-6 colonized apple foliage in the field (Figure 4-11). Differences in colonization on 'Golden Delicious' and 'Rome Beauty' were not statistically significant and so the data were pooled. Isolate FLS-5 had the highest populations both one and
seven weeks post inoculation ranging from log 4.4 to log 3.5 CFU/cm$^2$. Isolate A1-1 remained below detectable levels throughout the sampling period.

![Colonization of apple foliage 1 and 7 weeks post inoculation of bacteria. The dashed line indicates the minimum detectable level. Leaves were sprayed with log 7.0 CFU/ml of each isolate + 0.1% Silwet on 7-May 2009. The error bars indicate the standard error of the mean.](image)

In both 2008 and 2009 study years of experiment one, the May application treatment versus the May+June application treatment were not significantly different with regards to foliar scab so the data were combined for statistical analyses. In the 2008 study, isolates A1-1 and FLS-5 significantly (P < 0.05) reduced foliar scab severity on 'Golden Delicious' and 'Rome Beauty' from four weeks (6-Jun) post application of the bacteria through eight weeks (16-Jul) when disease assessments were discontinued since leaves were mature and no longer susceptible (Figure 4-12). Isolate A3-6 significantly reduced foliar scab severity weeks 4-8 on 'Rome Beauty' but was not significantly different from the control until week five on 'Golden Delicious'. Leaves treated with isolate A1-1 resulted in reductions in mean leaf area scabbed by 65% and 58% on 'Golden Delicious' and ‘Rome Beauty' respectively compared to control leaves at the end of the experiment (eight weeks post inoculation of the bacteria). Leaves treated with isolate A3-6 resulted in reductions in mean leaf area scabbed of 41% and 65% on 'Golden Delicious' and ‘Rome Beauty' leaves respectively compared to control leaves at the end of the eight week experiment. Isolate A3-F1 did not significantly reduce disease severity on either cultivar tested in 2008. As a result isolate A3-F1 was not included in the 2009 FREC experiments.
Figure 4-12: Mean percent leaf area scabbed on (A) 'Golden Delicious' and (B) 'Rome Beauty' in 2008. Bacterial treatments were applied on 10-May and 18-June 2008. Non-treated controls were treated with 0.1% Silwet L-77. Leaves on 32 shoots per tree were evaluated weekly for scab severity 3 weeks through 8 weeks post application of the bacteria. Error bars represent the standard error of the mean.
In 2009, all three of the isolates tested (A1-1, A3-6 and FLS-5) significantly reduced foliar scab severity compared to the controls from three weeks to eight weeks post inoculation with the bacteria (Figure 4-13). Most likely due to the addition of a sulfur program (as a treatment applied to all test trees) in 2009, the overall mean percent area scabbed on all treatments was lower in 2009 compared to 2008. At eight weeks post inoculation of the bacteria (two weeks after the second application), leaves treated with isolate A1-1 averaged 13.7% leaf area scabbed on 'Rome Beauty' compared to 25.2% on control leaves, representing a 45% reduction in disease severity. Application of isolate A3-6 on 'Rome Beauty' leaves resulted in a similar level of disease suppression, with a 42% reduction in severity when compared to the control. On 'Golden Delicious' isolates A1-1 and A3-6 resulted in a 48% and 45% reduction in mean percent leaf area scabbed respectively.

Figure 4-13: Mean percent leaf area scabbed on (A) 'Golden Delicious' and (B) 'Rome Beauty' at the FREC in 2009. Bacterial treatments were applied on 7-May and 19-Jun 2009 (six weeks post inoculation). Control trees were treated with 0.1% Silwet L-77. Leaves of 64 shoots per tree were evaluated weekly for scab severity from three weeks to eight weeks post inoculation of the bacteria. Error bars represent the standard error of the mean.
Field evaluation of bacterial application schedule on fruit diseases

Fruit were harvested from tagged branches of experiment one in both study years and evaluated for all diseases present. Fruit harvested from branches treated with isolates A1-1 and FLS-5 had significantly lower scab severity on 'Golden Delicious' (P < 0.001) and 'Rome Beauty' (P < 0.01) fruit in 2008 (Figure 4-14). Isolate A3-6 reduced fruit scab severity on 'Golden Delicious' (P < 0.03) but not on 'Rome Beauty' in the 2008 study year. Mean fruit scab incidence on control fruit was 97% on 'Golden Delicious' and 93% on ‘Rome Beauty’ in 2008. Fruit harvested from branches treated with isolate A1-1 had the lowest scab incidence on 'Golden Delicious'; 76% and 64% from the May and May+June treatments respectively (Table 4-2). Mean flyspeck incidence on control fruit was 32% on 'Golden Delicious' and 21% on 'Rome Beauty'. Fruit harvested from branches treated with A3-6 had the lowest fliespeck incidence of 2.6% and 3.8% from the May and May+June treatments respectively on 'Golden Delicious'. On 'Rome Beauty', fruit from branches treated with isolate A1-1 had the lowest fliespeck incidence of 0% from both the May and May+June treatments in 2008.

Figure 4-14: Apple scab severity on (A) 'Golden Delicious' and (B) 'Rome Beauty' fruit in 2008. The fruit were harvested from branches sprayed with bacteria on 10-May (May), 10-May+19-June (May+June) or non-treated (None). Apple scab severity was evaluated using a 0-6 visual scale. Within each bacterial treatment, means with the same letter are not significantly different (α=0.05) according to the Tukey-Kramer test.
In the 2009 study year, isolates A1-1 and A3-6 significantly reduced the mean fruit scab severity compared to controls on fruit of 'Golden Delicious' (P < 0.01) and 'Rome Beauty' (P < 0.003) (Figure 4-15). Fruit from branches treated with isolate FLS-5 had significantly lower scab severity on 'Golden Delicious' (P < 0.03) but not on 'Rome Beauty'. Mean fruit scab incidence in 2009 was 87% and 96% on non-treated control 'Golden Delicious' and 'Rome Beauty' fruit respectively (Table 4-3). As observed in 2008 (Table 4-2), fruit harvest from branches treated with isolate A1-1 had the lowest incidence on fruit scab of 53% and 63% from the May and May+June treatments respectively on 'Golden Delicious fruit in 2009 (Table 4-3).

Table 4-2: Mean proportion of fruit with apple scab, flyspeck and rust symptoms/signs on 'Golden Delicious' and 'Rome Beauty' fruit in 2008. Incidence of all diseases shown here is represented as the number of fruit with at least one lesion divided by the total number of fruit observed.

| Bacteria | Treatment | 'Golden Delicious' | | 'Rome Beauty' | |
|----------|-----------|--------------------|------------------|------------------|
|          |           | Scab | Flyspeck | Rust | Scab | Flyspeck | Rust |
| FLS-5    | May       | 0.94 | 0.45    | 0.00 | 0.77 | 0.23    | 0.00 |
| FLS-5    | May+June  | 0.76 | 0.39    | 0.00 | 0.88 | 0.20    | 0.00 |
| FLS-5    | None      | 1.00 | 0.64    | 0.00 | 0.97 | 0.26    | 0.00 |
| A3-6     | May       | 0.95 | 0.01    | 0.00 | 0.86 | 0.41    | 0.00 |
| A3-6     | May+June  | 0.96 | 0.03    | 0.00 | 0.74 | 0.05    | 0.00 |
| A3-6     | None      | 0.98 | 0.10    | 0.00 | 0.90 | 0.26    | 0.00 |
| A1-1     | May       | 0.76 | 0.13    | 0.00 | 0.91 | 0.00    | 0.00 |
| A1-1     | May+June  | 0.66 | 0.38    | 0.00 | 0.90 | 0.00    | 0.00 |
| A1-1     | None      | 0.97 | 0.11    | 0.00 | 0.92 | 0.17    | 0.00 |

Figure 4-15: Apple scab severity on (A) 'Golden Delicious' and (B) 'Rome Beauty' fruit in 2009. Fruit were harvested from branches sprayed with bacteria 10-May (May), 10-May+19-June (May+June) or non-treated. Means with the same letter are not significantly different (α=0.05) according to a Tukey-Kramer test.
There were no significant differences in flyspeck incidence on either cultivar in 2009.

Fruit harvest from branches treated with isolates A3-6 and A1-1 in May+June had significantly lower incidence of rust lesions (0%) compared to the untreated controls (11%) on 'Rome Beauty' fruit in 2009 (Table 4-3).

Table 4-3: Mean proportion of fruit with apple scab, flyspeck and rust symptoms/signs on 'Golden Delicious' and 'Rome Beauty' fruit in 2009. Incidence of all diseases shown here is represented as the number of fruit with at least one lesion divided by the total number of fruit observed. A minimum of 50 fruit were evaluated for each treatment.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Treatment</th>
<th>'Golden Delicious'</th>
<th>'Rome Beauty'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Scab</td>
<td>Flyspeck</td>
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<tr>
<td>FLS-5</td>
<td>May</td>
<td>0.82</td>
<td>0.000</td>
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<tr>
<td></td>
<td>May+June</td>
<td>0.85</td>
<td>0.000</td>
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<tr>
<td>A3-6</td>
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<td>0.013</td>
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<tr>
<td></td>
<td>May+June</td>
<td>0.94</td>
<td>0.026</td>
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<tr>
<td>A1-1</td>
<td>May</td>
<td>0.53</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>May+June</td>
<td>0.63</td>
<td>0.000</td>
</tr>
<tr>
<td>none</td>
<td>May</td>
<td>0.88</td>
<td>0.000</td>
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<tr>
<td></td>
<td>May+June</td>
<td>0.85</td>
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**Effect of bacterial application schedules on postharvest biocontrol on 'Golden Delicious' and 'Rome Beauty' fruit**

Marketable fruit harvested from bacteria-treated and non-treated branches of experiment one trees were challenged with *C. acutatum* to determine the effect of application schedule on postharvest disease suppression in 2008 and 2009. As mentioned previously, scab pressure was high in the FREC research orchards in 2008. Few 'Rome Beauty' fruit (most noticeably in control treatments) were suitable for use in the postharvest phase of the experiment. There were no significant differences in mean lesion size on 'Rome Beauty' fruit in 2008. Statistical analysis indicated a significant interaction between bacteria and application schedule (P < 0.001) on ‘Golden Delicious’ fruit. Single degree-of-freedom contrasts indicated that the effect of adding a postharvest application to either the May or May+June application treatments was similar for all isolates (P > 0.07). When applied postharvest to 'Golden Delicious' fruit, isolates A1-1 and A3-6...
significantly reduced bitter rot lesion size in 2008 (Figure 4-16). Fruit receiving the May + postharvest application of isolate A3-6 resulted in a 48% reduction in lesion size, while fruit receiving only a postharvest application of bacteria resulted in a 28% reduction compared to untreated controls eight days post inoculation. There was no statistically significant difference however, between treatments combining a pre-harvest and postharvest application of bacteria compared to the postharvest alone treatment for isolates A1-1, A3-F1 or FLS-5 over the eight day sampling period (P > 0.3). Application of isolate A3-6 May + postharvest however, resulted in smaller lesion size at the end of the experiment (on the eighth day) compared to the postharvest application alone (P = 0.008). Fruit receiving a postharvest application of isolate A1-1 or A3-6 did not develop symptoms until six dpi whereas non-treated control fruit typically developed symptoms five days after treatment indicating a one day delay in the onset of symptom development. There were no significant reductions in lesion size on 'Golden Delicious' fruit harvested from branches treated pre-harvest with isolates A1-1, A3-6 or A3-F1 (not receiving a postharvest application of bacteria) compared to the non-treated control lesions in the 2008 study year.
In the 2009 study year, all three of the tested isolates significantly (P < 0.05) reduced bitter rot lesion area compared to the non-bacteria treated controls on 'Rome Beauty' and 'Golden Delicious' when applied postharvest (Figure 4-17). Statistical analysis indicated a significant effect of bacterial isolate (P < 0.0001), application schedule (P < 0.02) and an interaction between bacterial isolate and application schedule on both cultivars tested (P < 0.0008). Single degree-of-
freedom contrasts indicated there was a similar effect of adding a postharvest application to either the May or May +June application schedules for isolates A1-1 and FLS-5 (P > 0.3) on ‘Rome Beauty’ fruit. On ‘Golden Delicious’ fruit, the effect of adding a postharvest application (to the May or May+June schedule) was similar for isolates A3-6 and A1-1. Postharvest application of isolates A3-6 and FLS-5 provided a significantly greater reduction in bitter rot lesion size compared to pre-harvest applications on ‘Rome Beauty’ (P < 0.001) and ‘Golden Delicious’ (P < 0.04). Postharvest application of isolate A1-1 however, resulted in a greater reduction in lesion size compared to pre-harvest applications on ‘Rome Beauty’ (P < 0.0001), but not on ‘Golden Delicious’ fruit (P = 0.14). Isolate A3-6 provided the greatest reduction in bitter rot lesion area. Fruit receiving a May+June+postharvest application of isolate A3-6 resulted in a 41% and 95% reduction in lesion area on ‘Golden Delicious' and 'Rome Beauty' respectively, compared to the non-bacteria controls.
Figure 4-17: Mean lesion area (mm$^2$) on (A) 'Golden Delicious' and (B) 'Rome Beauty' fruit inoculated with C. acutatum in 2009. Three bacterial isolates were tested; A1-1, A3-6 and FLS-5. Fruit were harvested from non-treated and branches treated 7-May and 18-June 2009. Data were analyzed using the SAS mixed repeated measures. Treatments with the same letter are not statistically significant as determined by single-degree-of freedom contrasts ($\alpha=0.05$).
Overall, rust incidence in the organic buffer row was low in 2008 and 2009. All four isolates tested however, had significantly lower incidence of lesions compared to the non-bacteria controls in 2008. At the end of the assessment period, four of the five control trees had rust lesions on at least one branch. None of the trees treated with isolate A1-1 developed any rust lesions on the leaves. One of five trees treated with isolate A3-6 and A3-F1 had rust lesions (on one of 20 branches assessed). Although three of the five trees treated with isolate FLS-5 developed rust, there were significantly fewer infected leaves compared to the controls. There were no significant differences in rust incidence in 2009.

When applied postharvest isolate FLS-5 significantly reduced bitter rot lesion size compared to the non-bacteria controls in 2008. Repeated measures analysis of 2009 data indicated a significant interaction between bacterial isolate and application schedule (P < 0.001). When applied postharvest, isolates A1-1, A3-6 Ae-1 and FLS-5 significantly (P < 0.001) reduced bitter rot lesion size in 2009 (Figure 4-18). Pre-harvest applications (without a postharvest application) of isolates A1-1, A3-6 and FLS-5 also resulted in significantly (P < 0.001) smaller lesions compared to the non-bacteria controls. Postharvest applications of the biocontrol isolates however, resulted in the greatest level of suppression. Specifically, postharvest application of isolates A1-1 and A3-6 resulted in the greatest reduction in lesion size of 61% and 60% respectively. Combining a pre-harvest and postharvest application of the biocontrol bacteria did not provide an additional level of suppression for isolates A1-1, A3-6 or FLS-5 compared to the postharvest only application. A significant difference was observed however, on fruit treated with isolate Ae-1 as lesions on fruit receiving a pre-harvest + postharvest application were significantly smaller than lesions on fruit receiving a postharvest only application.
Repeated measures analysis indicated a significant effect of bacterial isolate (P < 0.05) and application treatment (P < 0.0001) on bitter rot lesion size. A significant interaction between bacterial isolate and application treatment was observed on ‘Rome Beauty’ (P = 0.009). When applied postharvest, all three bacterial isolates tested significantly reduced bitter rot lesion area on 'Golden Delicious' and 'Rome Beauty' fruit compared to the non-treated control (Figure 4-19).

**Effect of a late-season application of bacteria on postharvest disease suppression**

Figure 4-18: Mean lesion area (mm²) on wounded ‘GoldRush’ fruit in 2009. Bacteria were applied to wounds of fruit receiving a postharvest treatment (post) and fruit receiving a pre-harvest + postharvest treatment (pre+post) at day 0. Wounds of fruit receiving a pre-harvest application of bacteria (pre) or no bacteria (control) received a sterile water treatment at day 0. Fruit were challenged with the bitter rot pathogen, *C. acutatum* one hour after application of the bacteria. The error bars indicate the standard error of the mean.
Application of the bacteria to fruit in August (without a subsequent postharvest application) did not have a significant effect as a pre-harvest treatment compared to non-bacteria controls. Additionally, there was no benefit of combining a pre-harvest and postharvest application of bacteria compared to a postharvest application alone. When applied postharvest, isolate A3-6 resulted in a 50% and 81% reduction in average lesion area (at seven dpi) on 'Golden Delicious' and 'Rome Beauty' respectively, compared to the non-bacteria controls. When applied postharvest, isolate FLS-5 resulted in a 41% and 81% reduction in average lesion area (at seven dpi) on 'Golden Delicious' and 'Rome Beauty' respectively, compared to the non-bacteria controls. Isolate A1-1 resulted in a 41% and 47% reduction on 'Golden Delicious' and 'Rome Beauty' fruit respectively when applied postharvest. The results obtained in this experiment support the results obtained in experiment one.
Bacterial colonization on 'Golden Delicious' leaves

Isolates A3-6 and FLS-5 colonized 'Golden Delicious' leaves throughout the 30 day experiment (Figure 4-20). Isolate FLS-5 maintained populations between log 5.0 and log 4.0 CFU/cm². Isolate A3-6 maintained populations between log 3.5 and log 4.3 CFU/cm². Colonization by isolate A1-1 however, was poor and remained below detectable levels throughout the experiment.

Figure 4-19: Mean lesion area (mm²) on (A) 'Golden Delicious' and (B) 'Rome Beauty' fruit challenged with the bitter rot pathogen, C. acutatum. Bacteria were applied to wounds of fruit receiving a postharvest treatment (post) and fruit receiving a pre-harvest + postharvest treatment (prepost) at day 0. Wounds of fruit receiving a pre-harvest application of bacteria (pre) or no bacteria (control) received a sterile water treatment at day 0. The error bars indicate the standard error of the mean.

Figure 4-20: Bacterial colonization of 'Golden Delicious' leaves at the PSU Russell Larson Research Center in 2008. The dashed line indicates the minimum detectable level. Leaves were sprayed with log 7.0 CFU/ml of each isolate + 0.1% Silwet in June 2009. The error bars indicate the standard error of the mean.
**Bacterial colonization of apple wounds**

Isolates A3-6, A3-F1 and FLS-5 were able to colonize wounds on 'Golden Delicious' fruit over the duration of the 96 hour experiment (Figure 4-21). Isolate FLS-5 had the highest and most stable mean population levels ranging from log 5.8 to log 5.9 CFU/wound. Isolates A3-6 and A3-F1 ranged from log 4.1 to log 5.9 CFU/wound. Colonization by isolate A1-1 was more variable ranging from log 4.5 to log 2.1 CFU/wound after 72 hours. At the end of the sampling period (96 hours), isolate A1-1 was below the detectable level for the methodology used in the experiment.

![Figure 4-21: Mean colonization of wounds on 'Golden Delicious' fruit at 4, 24, 48, 72 and 96 hours post inoculation with bacteria. Each bacterial isolate was inoculated into wounds at hour 0 as a 20 µl solution of log 7.0 CFU/ml suspension. Error bars represent the standard error of the mean. The dashed line indicates the minimum detection level of the experiment.](image)

**Discussion**

This study is the first known report of the evaluation of endospore-forming bacteria for biological control of pre and postharvest diseases of apple when applied as pre-harvest treatments. Studies evaluating pre-harvest applications of BCAs on apple for postharvest disease suppression have generally tested late-season applications ranging from four weeks to two days before harvest. Following harvest, incidence of decay is commonly evaluated after a two week-
A four month incubation period at room temp or following cold storage. A majority of the published experiments report postharvest disease data as incidence (percent fruit infected). Teixido et al. [37] reported both incidence and severity data (average lesion diameters) of fruit treated with \textit{C. sake} two days before harvest. In the current study, the severity of apple scab and bitter rot symptoms was evaluated to determine the effectiveness of the bacterial isolates. Severity data is extremely informative as the incidence of apple scab on leaves may be very similar among treatments, however there is a considerable difference between (for example) 5% and 50% leaf area scabbed. Biological control rarely results in 100% disease control, thus incidence data may not reveal significant differences. Successful BCAs often reduce disease severity below an acceptable threshold or allow for disease control when combined with reduced rates of fungicides [3, 27].

A review of the literature indicates that suppression of pre-harvest or summer diseases such as apple scab, sooty blotch/flyspeck rust or powdery mildew by BCAs has not been evaluated in combination with suppression of postharvest diseases on apple. Application of the bacterial isolates A1-1, A3-6 and FLS-5 at bloom combined with a mid-season application significantly reduced foliar and fruit apple scab disease severity in both the 2008 and 2009 study years. Results from the 2008 and 2009 field experiments also indicate that the bacterial isolates A1-1, A3-6 and FLS-5 provided significant scab suppression on unmanaged trees (exp #1 2008) or when combined with a reduced sulfur/copper spray program (exp #1 2009). Isolates A1-1 and A3-6 provided the greatest reduction in foliar and fruit scab in both study years compared to isolates A3-F1 and FLS-5. Isolates A1-1 however, demonstrated poor field colonization of 'Golden Delicious' and 'Rome Beauty' leaves compared to isolates FLS-5 and A3-F1 suggesting that high population levels may not be required for scab suppression by this isolate, or that the method of suppression is independent of population. Season-long suppression of scab severity was observed on both directly sprayed and non-sprayed leaves. It is possible that application of
isolate A1-1 resulted in activation of plant defenses in the new growth, and this induced resistance may have suppressed the eliciting organism. Activation of plant defense proteins by non-pathogenic organisms has been demonstrated in apple. Kürkcüoğlu et al. [20, 21] reported that application of *P. fluorescens* Bk3 to leaves of cv. Holsteiner Cox resulted in up-regulation of several PR proteins in the apoplast including β-1, 3-glucanase, and chitinase. The eliciting organism, *P. fluorescens*, has also been shown to inhibit conidial and mycelial growth of *V. inaequalis* [12, 19].

When applied as postharvest treatments, isolates A1-1, A3-6 and FLS-5 significantly reduced mean lesion area of bitter rot incited by *C. acutatum* on 'Golden Delicious' and 'Rome Beauty' fruit. These results were observed on both 'Golden Delicious' and 'Rome Beauty' trees receiving a reduced spray program, standard fungicide program and unmanaged trees. The pre-harvest application of bacteria alone did not provide significant postharvest protection against *C. acutatum* artificially inoculated into fruit wounds compared to a postharvest application. Furthermore, the combination of pre-harvest and postharvest applications of bacteria did not provide additional benefit over the single postharvest application with regards to bitter rot suppression in wounds. A similar result was reported by Teixido et al. [36] in a study comparing biocontrol efficacy of pre-harvest and postharvest applications of *C. sake* to apples inoculated with *P. expansum*. Pre-harvest applications (two days before harvest) of yeast cells provided significant control, however maximum control was achieved with postharvest application. Teixido et al. [36] also reported no advantage in combining pre- and postharvest application of the yeast antagonist. Since the inoculum dose of *C. acutatum* in our experiments was high (~800 conidia/wound), it may have overwhelmed host induced resistance that would have been a likely mechanism active in a fresh wound on the fruit. Further experiments are needed to determine the effects of pathogen dose on disease suppression by pre- and postharvest applied bacteria.
While a synergistic effect was not observed in our study, for treatments combining pre- and postharvest application strategies, there is an additive effect in that pre-harvest applications of biocontrol bacteria were able to reduce foliar and fruit scab and additional postharvest applications were effective in reducing bitter rot severity. While application of a single organism to control both pre and postharvest disease has not been previously reported for apple, application of several organisms has proven successful in other systems. For example, pre-harvest applications of *Bacillus licheniformis* to mango trees have proven effective in controlling both pre- and postharvest diseases including bacterial black spot (*Xanthomonas campestris* pv. *magenticae*) and soft rot [32]. Pre-harvest sprays of *B. subtilis* on avocado have been reported effective for control of the preharvest diseases black spot (*Pseudocercospora purpurea*) and sooty blotch (*Akaropeltopsis* sp.) and postharvest decay from anthracnose, stem-end rot and the *Dothiorella- Colletotrichum* fruit rot complex [17, 18].

The achievement of successful biological control (of plant pests or pathogens), not only depends on the biocontrol agent itself, but also on the application of the agent(s) when and where they are needed to maximize control [4]. Our study has demonstrated that two field applications of *Bacillus* BCAs were able to reduce fruit and foliar scab severity on apple. Further research is needed to optimize the biocontrol efficacy of our isolates. Additional experiments that would focus on the rate of bacterial application, timing and number of applications, and survival and redistribution of bacteria after application could all lead to more predictable benefits from treatment.

Research has demonstrated the link between colonization and disease suppression of both pre and postharvest diseases on many cropping systems and the ecological competence of a BCA is widely recognized as a key selection factor [15]. Lahlali et al. [22] found that the population density of *P. anomalum* strain K on apple fruit surface in the field affected the biocontrol efficacy of *P. expansum*. Several strategies have been investigated to improve survival of BCAs under
unfavorable conditions. Teixido et al. [37] reported improved colonization of apple fruit and foliage in the field and in cold storage with modified low water activity ($a_w$)-tolerant (desiccation tolerant) cells of *C. sake* compared to unmodified cells. Furthermore, the $a_w$-tolerant cells provided significantly greater suppression of *P. expansum* in fruit wounds compared the unmodified cells. In another study, Teixido et al. [35] successfully improved low $a_w$-tolerance of *P. agglomerans* by subjecting cells to mild osmotic stress during growth. Improvement of stress tolerance such as low water activity has generally been used to develop BCAs that can more readily be dehydrated during the formulation process. Many organisms tend to lose viability during this process making formulation difficult. The use of endospore-forming bacteria offers an advantage due to the environmental stress resistance afforded by the *Bacillus* spore coat [7].

The use of organic amendments has also been investigated as a strategy to improve survival of foliar applied antagonists and increase control efficacy. Davis et al. [6] found that cellulose-producing *C. globosum* applied to apple leaves in a formulation of colloidal cellulose and a vegetable oil-based spreader-sticker (Soy-Dex) reduced flyspeck severity by 63% and sooty blotch by 79% and increased survival and growth of *C. globosum* log 2 greater than non-treated leaves [6]. Kokalis-Burelle et al. [16] found that application of chitin as an amendment along with chitinolytic *Bacillus cereus* strain 304 led to increased populations of bacteria by ten-fold and significantly reduced the severity of early leafspot on peanut caused by *Cercospora arachidicola*. The use of a spreader/sticker and/or selective nutrient sources to improve colonization may also increase the level of pre-harvest and/or postharvest disease suppression with our *Bacillus* biocontrol isolates.

Because biological control systems often fail to provide sufficient control on their own, an integrated approach offers a more reliable strategy for disease control. Implementation into an integrated pest management (IPM) program combining multiple strategies such as sanitation, host resistance, reduced rates of fungicides and biocontrols, may provide for a more effective use of a
BCA. For successful integration, it is important to identify BCAs that are able to survive and reduce disease when combined with commonly used fungicides and insecticides. More information is needed on the colonization of our biocontrol isolates in the field and in storage, particularly when integrated into existing apple management programs. Experiments to investigate the ability of our biocontrol isolates to colonize leaves in the presence of commonly used fungicides/bactericides such as sulfur, copper and streptomycin are presented in chapter 5.

Regarding postharvest biological control, growth and survival at storage temperatures (2-5°C for apple) is important for biocontrol, as many postharvest BCAs are thought to reduce disease through competition for resources. Experiments to investigate colonization of apple wounds by our biocontrol isolates at room and storage temperatures are presented in chapter 5.

References

anthracnose of cucurbits caused by *Glomerella cingulata var. orbiculare*. Biological Control 48(2): 140-146.


Chapter 5

Characterization and integration of bacteria with IPM strategies for management of apple diseases

Introduction

Research on biological control of plant diseases has utilized a broad spectrum of microorganisms and resulted in the discovery of several novel biocontrol agents (BCAs). Like any emerging strategy however, BCAs should be developed for integration with other methods as the complete substitution of synthetic chemicals is unrealistic. The ultimate goal of biocontrol research is to provide additional tools for disease management with modes of action that are different from traditional chemical pesticides [32]. Additionally, farmers are more likely to purchase new products that can be incorporated into their current production systems with little disruption [58]. In order to be successfully integrated, the compatibility of a given BCA must be evaluated during the screening process. Implementation into an integrated pest management (IPM) program combining multiple strategies such as sanitation, host resistance, reduced rates of fungicides and biocontrols, will provide for efficient use of a BCA. The potential to reduce the amount of fungicides needed for disease control has been investigated in several systems in which BCAs are combined with reduced rates [13, 14, 20, 33] or alternated with fungicide sprays [26, 33]. These approaches, when successful, offer an effective means of reducing environmental risks associated with use of synthetic chemicals. Seed treatment is one of the most common uses of BCAs with fungicides. The biocontrol product Kodiak (Bacillus subtilis GB03) is commonly used as a cotton seed treatment in combination with fungicides, providing improved control from that achieved with either treatment alone [10, 36]. Reports on the use of BCAs in combination with fungicides for control of foliar diseases are limited however, compared to their use in the rhizosphere or on seed.
Interest regarding the use of BCAs in combination with natural compounds as components of an integrated strategy has also increased. Chitosan, a deacetylated derivative of chitin, has become one of the more promising compounds due to its documented antifungal activity [1, 22, 46], elicitation of plant defense responses [6, 21, 62] and delays of ripening [22]. An important property related to chitosan’s fungicidal effect is its positive charge in acidic solutions. Research has demonstrated that low molecular weight chitosans (less than 10KDa) in solutions with a pH < 6.5 have greater antimicrobial activity, with yeasts and fungi being the most sensitive [56, 57]. Chitosan has been shown to suppress pre- and postharvest diseases on several important crops. El-Ghaouth et al. [23] reported that supplementation of soil media with 100 µg/ml of chitosan controlled root rot caused by *Pythium aphanidermatum* on cucumber and triggered the induction of host produced antifungal compounds including chitinase, chitosanase and β-1,3-glucanase [25]. El-Ghaouth [22] also reported that a chitosan coating on strawberry fruit reduced decay caused by *Botrytis cinerea* and *Rhizopus stolonifer*. In this study chitosan inhibited spore germination, germ tube elongation and radial growth of *B. cinerea* and *R. stolonifer* in culture. In a study conducted on apple, de Capdeville [18] found that 1% and 2% chitosan was effective in reducing blue mold caused by *Penicillium expansum*. The combination of chitosan with BCAs has also been investigated with promising results. A synergistic effect was observed when 0.2% glycolchitosan, a derivative of chitosan, was applied with the BCA *Candida saitoana*, reducing the incidence of disease caused by *B. cinerea* and *P. expansum* on apple and *P. digitatum* on citrus fruits [24]. This enhancement of BCA performance has also been reported with several organisms on a variety of commodities including grape [48], tomato [5], cucumber [53], and potato [39].

Silicon is another natural compound recognized for its disease reducing capabilities. Silicon, a ubiquitous constituent of plants and their environments, is involved in many important processes in plant physiology [27]. The use of soluble silicon in agriculture for protection against
fungal diseases has been reported, for both monocots and dicots [4, 49]. Research has documented that silicon may enhance soil fertility [17], increase plant growth [64], improve disease resistance [28-30], and reduce abiotic stress [45]. A majority of the research for disease suppression has involved root absorption of silicon from either soil or soilless media. A few studies have shown significant results when applied as a foliar spray. For example, Menzies et al. [49] reported that foliar sprays of potassium silicate reduced powdery mildew severity on cucumber, muskmelon and zucchini squash. Greenhouse studies on grape, have also demonstrated that foliar sprays of potassium silicate can significantly reduce severity of powdery mildew. However, in grape the method of silicon delivery significantly influenced disease suppression. Soil inoculations of soluble Si had no effect on disease severity, however, leaves sprayed with Si one day before pathogen challenge, resulted in substantial reduction of disease development [9]. Application of silicon has also been shown to suppress postharvest diseases in combination with BCAs. A synergistic effect was observed on jujube fruit against *Alternaria alternata* and *P. expansum* when the biocontrol yeast *Cryptococcus laurentii* and *Rhodotorula glutinis* were combined with sodium silicate [63]. In another study, sodium metasilicate enhanced biocontrol activity of *C. laurentii* against *P. expansum* and *Monilinia fructicola* on sweet cherry [55]. Colonization and cytological investigations showed that application of silicon increased population density of the BCA and inhibited pathogen spore germination and germ tube elongation in wounds of sweet cherry. Additionally silicon treatment induced biochemical defense reactions, as activities of polyphenoloxidase and peroxidase increased compared to wounded control fruit.

Although biological control has developed into a significant area of research, the use of microbial BCAs, often fails to provide acceptable levels of disease suppression as standalone products, especially compared to the chemical fungicides that they are intended to replace or compliment [20, 47]. Often, microbial BCAs are utilized for disease control within a chemical
paradigm rather than the more realistic biological paradigm [15]. The BCA *Bacillus thuringiensis*, is an atypical scenario because it works dead or alive (by ingestion of pre-existing endotoxins), perpetuating the perception that BCAs can be used in a similar manner as a chemical. One factor in determining the success of a BCA, especially those employing competition or exclusion as mechanisms of disease suppression, is the ability of the applied BCA to colonize and survive on/in the target crop. BCAs provide a unique advantage with their ability to deliver bioactive products when and where they are needed, compared to the broadcast applications of pesticides by man. Therefore, biocontrols utilized during the growing season must tolerate the spray regimes of the pest management program in order to be successfully integrated. Several endospore-forming bacterial isolates screened in previous chapters have shown considerable potential to suppress several pre-harvest and postharvest diseases of apple. The objectives of this study are to (1) further characterize the efficacy and modes of action of these candidate isolates, (2) evaluate disease suppression when combined with chitosan and soluble silicon and (3) evaluate BCA compatibility with fungicides and antibiotics commonly used in current apple management programs.

**Materials and methods**

**Preparation of bacterial inoculum**

Five bacterial isolates previously found to have disease control potential were further evaluated for biocontrol activity in apple field experiments; A1-1, A3-6, Ae-1, FLS-5 and FLS-1. Each bacterial isolate was grown in 500 ml of sterile tryptic soy broth in 2800 ml Fernbach flasks. Each isolate was grown in two Fernbach flasks for a total volume of 1L. The flasks were incubated for 7 days at 28°C and 120 rpm on a rotary incubator shaker (New Brunswick Scientific Model M1024-000, Edison, NJ). Bacterial cells were harvested by centrifugation at 6000 rpm for
15 minutes at 4°C in 250 ml centrifuge tubes using a Sorvall RC5C Plus centrifuge (Thermo Scientific). The supernatant was discarded and the bacterial pellets were re-suspended in 0.1 M potassium phosphate buffer. In all biocontrol experiments, the bacterial isolates were mixed with the polysilicon surfactant Silwet L-77 (GE Silicones, Tarrytown, NY) immediately before application at 0.1% (v/v). Bacterial isolates evaluated in postharvest experiments were prepared using a similar method; the isolates were grown in 50 ml of sterile tryptic soy broth in 250 ml flasks. The isolates were incubated for seven days as described above. Bacterial cells were harvested by centrifugation at 3800 rpm for 15 minutes at 4°C in 50 ml centrifuge tubes using a Sorvall RT7 Benchtop Centrifuge (Thermo Scientific).

Field experiments to test for integration of bacteria with soluble silicon and chitosan on pre-harvest disease suppression on 'Cortland'

A field experiment was conducted at the Department of Horticulture farm located at the Penn State University Russell E. Larson Agricultural Research Center at Rock Springs, PA. A research area consisting of five rows of 'Cortland' on M.26 rootstock was used for the experiment. A single row of apple scab resistant cultivars ('Coop 37', 'Enterprise' and 'GoldRush') bordered the test site to the north and three rows of 'Pink Pearl', 'StarKrimson Delicious' and 'Mercier Delicious' bordered the plot to the South. All trees were pruned by farm staff in March 2009. Due to the low scab incidence reported at the site in previous years, apple scab inoculum was added to the study site to ensure development of disease across the plot. Venturia inaequalis infected leaf litter was collected on 21-March 2009 from underneath 'Pink Pearl' trees located to the south of the 'Cortland' plot. Leaves were placed in trash bags and stored in a dark cold room (5°C) until needed. The leaf litter was placed under the 'Cortland' trees at the test site on 19-April 2009. Plastic mesh was used to keep the leaves in place and minimize loss of inoculum laded leaves due to wind and rain. One square foot (0.09 m²) of 19 mm mesh was placed under the
west side of each tree, and anchored down at the four corners with metal landscaping pins (Figure 5-1). Leaves were placed under each tree.

Figure 5-1: Placement of *Venturia inaequalis* infected leaf litter under 0.3m squares of 19 mm mesh under the west side of a 'Cortland' tree.

This experiment consisted of six bacterial treatments (A1-1, A3-6, Ae-1, FLS-1, FLS-5 and none) and three amendments (Silicon, Chitosan or none) as a 6 x 3 factorial. Treatments were arranged in a split plot design with amendment as the main plot and bacterial isolate as the subplot (Figure 5-2). At the time of treatment application, the last fully expanded leaf of ten shoots was marked with red tape. The bacteria and amendment treatments were applied using an 11 L Solo backpack sprayer (Solo, Newport News, VA) on 15-May and 16-June 2009. The 1 L bacterial stock solutions of $10^8$ CFU/ml were added to 9 L of tap water with 0.1% Silwet for a total volume of 10 L (final concentration of $10^7$ CFU/ml bacteria). The backpack sprayers were washed with 10% commercial bleach and triple rinsed with tap water after each bacterial treatment. Individual trees were inoculated with a single bacterial isolate for a total of ten trees per bacterial treatment. At the time of application a 3-sided plastic sheeting screen supported with PVC was used to minimize drift to neighboring trees (Figure 5-3). The plastic sides of the PVC screen were washed with tap water using a 7.6 L pump sprayer (Gilmour model #T200, Gilmour, Louisville, KY) between application of each bacterial treatment.
After application of bacterial treatments, the amendments were applied to main plots. There were three replications of each bacteria x amendment combination. The chitosan product ODCTM colloidal chitosan (AgriHouse Inc., Berthoud, CO) was applied at the manufacturer's suggested rate of 20 ml/3.8L. The soluble silicon product AgSil potassium silicate (PQ Corporation, Valley Forge, PA) was applied at 1% v/v.

Starting in May, apple scab and rust disease severity was assessed weekly until terminal bud when new susceptible tissue was no longer available. Disease assessments were taken on...
leaves of six shoots per tree on all replicate trees (18 shoots per bacteria x amendment treatment).

To evaluate rust, the numbers of rust lesions per leaf were counted for all leaves (equal to or younger than the tagged leaf) on two shoots per branch. To evaluate apple scab, percent leaf area scabbed was estimated on all leaves (occurring after the tagged leaf) on six shoots per tree using a seven point rating scale where 0 = No visible symptoms, 1 < 2%, 2= 2-7%, 3=7-15%, 4=15-25%, 5=25-40%, 6=40-60%, and 7>60%. Visual diagrams adapted from Tehon and Stout [61] and Croxall et al. [16] were used to classify each leaf to a 'scale class' corresponding to the diagram it most closely resembled (See chapter 4, Figure 4-5). Data were analyzed for significance using the mixed procedure of SAS 9.2 (SAS Institute; Cary, NC). The model contained amendment, isolate, and the interaction term amendment x isolate. The terms block and block x amendment were the random effects. A Tukey-Kramer test (α=0.05) was used to test significant interactions.

Fruit were harvested from all trees on 3-Oct 2009. During harvest fruit were labeled with the tree number using a permanent marker. Following harvest, fruit were evaluated for all diseases present. Apple scab, rust, sooty blotch and flyspeck (*Zygophiala jamaicensis*) lesions were counted on all fruit from each treatment. Apple scab severity on fruit was also estimated using a 0-6 rating scale based on a visual key (see chapter 4, Figure 4-6). Fruit were assigned a value based on the corresponding picture it most clearly resembled. Intermediate values were assigned when appropriate. Due to the use of an ordinal scale, data were analyzed for significant using the Kruskal-Wallis test of SAS 9.2 (SAS Institute, Cary, NC).

**Determining the potential for integration of biocontrol isolates with sulfur, copper and Streptomycin**

'Cortland' seeds (extracted from fruit grown at the PSU Russell Larson Research Center) were surface sterilized with 10% commercial bleach for 5 min and rinsed (x3) with sterile distilled water for two min. The seeds were stratified in sterile sand at 4°C for three months.
Germinating seedlings were planted in 500 ml pots containing Metro-Mix 360 growing medium (Sun Gro Horticulture Canada Ltd) and maintained in a growth chamber (Environmental Growth Chambers M series, Chargin Falls, OH) with a 12 hour photoperiod, 20°C and 70% relative humidity. This experiment consisted of a 4 x 4 factorial with four bacterial treatments (A1-1, A3-6, Ae-1 and FLS-5) and four fungicide/antibiotic treatments (none, sulfur, copper and streptomycin) for a total of 16 treatments with six replications. At the time of treatment application, the last fully expanded leaf on each seedling was tagged with red tape. Leaves of six week old 'Cortland' seedlings were sprayed individually to runoff with one of the bacterial isolates (undiluted stock solution of 5.0 x 10^7 CFU/ml) in a 0.1% Silwet solution using an aerosol sprayer (Crown Spra-tool power pack, Aerove Industries, Gardenerville, NV). The fungicide/antibiotic treatments were applied 24 hours after bacterial application. All products were applied at the label rate; Ag Streptomycin (Streptomycin Sulfate; Makhteshim Agan, Raleigh, NC) was applied at 100 ppm (0.12g/200 ml), Micro Sulf® (Nufarm Americas Inc, Burr Ridge, IL) was applied at 0.15 lb/gal (3.4g/200 ml) and Kocide® 2000 (Copper Hydroxide; Dupont Wilmington, DE) was applied at 2 lb/100 gal (0.5g/200 ml). The seedlings were arranged in a randomized block design with two replicate seedlings in each of three blocks (Figure 5-4). Eight seedlings were placed in each of twelve trays. The bacterial treatments were applied to all eight seedlings in three replicate trays and each fungicide/antibiotic treatment was applied to two seedlings in each of the twelve trays.
Colonization of apple foliage by the four bacterial isolates was determined 1, 7, 14 and 21 days post inoculation (dpi). One leaf was collected from three replicate seedlings and processed immediately. Two, 4.2 cm$^2$ leaf discs were removed from each leaf using a #15 cork borer (for a total of 8.4 cm$^2$ leaf tissue per replicate). The two leaf discs were placed in a 101 x 152 mm stomacher filter bag (Secure-T 80; Labplas, Sainte-Julie, Quebec) with 5 ml of 0.1 M potassium phosphate buffer and agitated with a stomacher blender (Bagmixer 100 MiniMix, Intersciences St. Nom, France) at 7 strokes per second for 10s. The discs were further ground using a pestle against the stomacher bag until well triturated and 50 µl of the undiluted, 10x and 100x diluted supernatant was plated in duplicate on YED. A 1 ml sample of the supernatant was collected and heat treated in 1.7 ml microtubes in a 75°C water bath for 15 minutes and 50 µl was plated in duplicate on YED to quantify the presence of endospores. All plates were incubated for 24 hours at room temperature and enumerated. The minimum detectable population for this methodology was log 1.1 CFU/cm$^2$.

Figure 5-4: Randomized block design with a 4 x 4 factorial to assess colonization of four bacterial isolates in combination with four fungicide/antibiotic treatments. Each bacterial isolate was applied to eight seedlings in three replicate trays; trays colored in green = A1-1, red = A3-6, blue = FLS-5 and grey = Ae-1. Each fungicide/antibiotic treatment was applied to two seedlings in each tray.
Determining the effect of bacteria and chitosan on postharvest disease suppression

Chitosan was prepared using the following method; five g of low molecular weight chitosan (Sigma-Aldrich) was suspended in 200 ml of sterile Milli-Q water. In a fume hood, the suspension was stirred constantly on a magnetic stir plate and glacial acetic acid was slowly added using a 1 ml dropper until the chitosan was completely dissolved (total of 5 ml glacial acetic acid was added). The solution was stirred for an additional two hours and the volume was brought up to 250 ml with sterile Milli-Q water. The pH was adjusted to 5.0 with 1 M NaOH using a pH meter.

This experiment consisted of a 5 x 2 x 2 factorial with five bacterial treatments (A1-1, A3-6, Ae-1, FLS-5 and none) two chitosan treatments (chitosan, no chitosan) and two pathogens (C. acutatum and P. expansum) for a total of 20 treatments. All fruit were wounded at the equator to a depth of 4 mm using a sterilized six pence nail to simulate stem punctures. Nails were mounted through a rubber stopper to ensure uniformity of wound depth. Wounds on fruit receiving the chitosan treatment were inoculated using a micropipette with 20 µl of a 1% chitosan solution. Wounds were then inoculated with 20 µl of the respective bacterial isolate (sterile water for no-bacteria controls) at a concentration of 10^7 CFU/ml. Two hours after bacterial application, wounds were challenged with 20 µl of either a 3.0 x 10^4 conidia/ml of C. acutatum or a 4.0 x 10^4 conidia/ml of P. expansum. A hemacytometer (American Optical, Buffalo, NY) was used to adjust the concentration of the pathogen inoculum. Pathogen inoculum was prepared by flooding 10 day old cultures of C. acutatum or P. expansum on PDA with 10 ml of sterile Milli-Q water. A rubber policeman was used to scrape the mycelia and spores into solution. The water was poured off the plate and into a 50 ml beaker with an additional 10 ml of sterile Milli-Q water and stirred on a magnetic stir plate for five minutes. The solution was filtered through four layers of sterile cheesecloth into a sterile 15 ml centrifuge tube. All 20 treatment combinations were randomly placed in ten 20-count plastic trays and covered with a plastic lid (containing five holes
for air circulation). The trays were placed on shelves in a dark walk-in growth room at approximately 20°C and 24% relative humidity. This experiment was conducted on 'Golden Delicious' fruit and repeated on 'Rome Beauty' fruit.

Fruit were evaluated for symptom severity three dpi and daily thereafter until eight dpi. To assess disease severity, the lesion diameter was measured laterally and horizontally across the wound site and the total lesion area was estimated using the average of the two measurements. Data were analyzed for significance using SAS mixed model repeated measures with an autoregressive covariance structure to analyze the repeated measurements of lesion area over the seven day period. Tray was the random effect in the model and fruit number was the subject (experimental unit). The model statement contained bacterial isolate, chitosan, and the interaction term isolate x chitosan. The slice test option of SAS was used for mean separation for significant interactions. A Tukey-Kramer test was also used to separate means of significant interactions.

**Colonization of fruit wounds by biocontrol bacteria in cold storage**

An experiment was conducted to assess bacterial colonization of fruit wounds at room temperature and in cold storage. This experiment consisted of a 5 x 2 factorial with five bacterial isolates (A1-1, A3-6, Ae-1, FLS-1 and FLS-5) and two temperatures (20°C and 2°C) for a total of 10 treatments. The experiment was conducted on 'Golden Delicious' fruit and repeated on 'Rome Beauty' fruit. Fruit were wounded as described earlier and 20 µl of 10⁷ CFU/ml of bacteria was added to the two wounds on sixteen fruit per bacterial isolate (2.0 x10⁵ CFU/wound). Eight fruit of each isolate treatment were placed in plastic trays with lids and stored at 20°C and eight fruit of each isolate treatment were stored in a walk-in cold room at 2°C. Wound colonization of fruit stored at 20°C was determined 1, 2, 4 and 8 days post inoculation and 1, 7, 14 and 28 days for fruit stored at 2°C. To assess colonization, wound tissues from two replicate fruit were excised
using a #4 cork borer (44.2 mm$^2$) to a depth of approximately 4 mm (Figure 5-5). A single core was placed in a 101 mm x 152 mm stomacher filter bag with 5 ml of 0.1 M potassium phosphate buffer and triturated in a stomacher blender for 30s. Fifty µl of the undiluted and 10 fold diluted triturate was plated in duplicate on YED. Plates were incubated at 20°C for 24 h and enumerated. The minimum detectable population for this methodology was log 2.0 CFU/wound.

Figure 5-5: To determine bacterial colonization, wound tissue was excised using a #4 cork borer (44.2 mm$^2$) to a depth of approximately 4 mm.

**Cytological investigation of postharvest disease suppression in fruit wounds by bacteria**

An experiment was conducted to observe interactions between the biocontrol bacteria and the postharvest pathogens *C. acutatum* and *P. expansum* in fruit wounds using scanning electron microscopy. This experiment consisted of a 5 x 2 x 2 factorial with five bacteria treatments (A3-6, Ae-1, FO-20, A3-2 and none), two pathogens (*C. acutatum* and *P. expansum*) and two sampling times (24 hours and four days post inoculation). Isolates A3-6 and Ae-1 were chosen for their ability to suppress *C. acutatum* and isolate A3-2 was chosen for its ability to suppress *P. expansum*. Isolate FO-20 has not shown significant suppression of either pathogen in previous experiments and was chosen as a negative control. A sterilized six pence nail was used to make four wounds on each 'Golden Delicious' fruit. Wounds were then inoculated with 20 µl of the respective bacterial isolate (sterile water for no-bacteria controls) at a concentration of $10^7$ CFU/ml. One hour after bacterial application, wounds were challenged with 20 µl of $3.0 \times 10^4$ conidia/ml suspension of either *C. acutatum* or *P. expansum*. A hemacytometer (American
Optical, Buffalo, NY) was used to adjust the concentration of the pathogen inoculum prepared as described earlier. Fruit were placed in 20-count molded plastic trays and placed in a dark walk-in growth room at 20ºC. A 10 ml stock fixing solution was prepared with 5 ml of 0.2M potassium phosphate buffer (pH 7.4), 1.6 ml of 16% paraformaldehyde, 0.6 ml of 25% glutaraldehyde and sterile distilled water added to a final volume of 10 ml. The final concentration of the fixing solution was 25% paraformaldehyde and 1.5% gultaraldehyde. One ml of fixing solution was pipetted into each of ten vials. Wound tissue was excised using a sterilized #4 core borer (44.2 mm²) to a depth of approximately five mm. Tissue samples were collected 24 hours and four days post inoculation and immediately immersed in the fixing solution for 24 hours at 4ºC. Four tissue samples per treatment were placed in each vial (containing 1 ml of fixative).

Following the 24 hour fixation, samples were washed with 0.2 M potassium phosphate buffer (pH 7.4) in three five minute wash cycles. After washing, samples were dehydrated in a gradient ethanol series consisting of 50%, 70%, 85%, 95%, and 100% ethanol for five minutes in each grade at room temperature. Next, samples were critical-point dried using a Bal-Tec CPD030 critical point dryer (Techno Trade, Manchester, NH). Samples were loaded into the pressure chamber in 100% ethanol and the chamber was cooled to 10ºC. The chamber was filled with bone-dry liquid carbon dioxide until the chamber reached 800 psi/54 bar and the liquid media was mixed for five minutes. After mixing, the media mixture was drained and the chamber refilled with the liquid CO². This procedure was repeated 6-7 times until all of the ethanol was replaced by liquid CO². To dry the samples, the chamber was heated to the critical point of 31ºC and 73.8 bar. The chamber was allowed to rise slightly above the critical point to 35.8ºC and 80 bar upon which the chamber was slowly depressurized to atmospheric pressure. The samples were removed and immediately mounted on aluminum stubs (15 mm x 10 mm) using double-sided carbon tape and stored in a dessicator cabinet. The next day samples were sputter coated with 10 nm of gold/palladium (two min at 10 mAmps) using a Bal-Tec SCD-050 sputter coater.
Following sample preparation, tissues were viewed using a JOEL JSM 5400 scanning electron microscope (JOEL; Peabody, MA) at 10 kV, 0 degree tilt and a 20 mm working distance.

A parallel experiment was conducted on 'Golden Delicious' fruit in which wounds colonized with isolates A3-6, Ae-1, A3-2 and FO-20 were challenged with *C. acutatum* or *P. expansum* as described above. Fruit were placed in 20-count molded plastic trays (a total of 17 trays/replicates) and placed in a dark walk-in growth room at 20ºC. Fruit were evaluated for symptom severity three dpi and daily thereafter until seven dpi. Lesions were measured as described previously. Data were analyzed for significance using SAS mixed model repeated measures with tray as the random variable and fruit as the subject (experimental unit). A Tukey-Kramer test (α=0.05) was used to separate means.

**Sequencing regions of the 16S rRNA gene for species identification of select bacterial isolates collected from apple**

Bacterial isolates were streaked onto fresh yeast extract dextrose agar (YED) one day before PCR amplification. This short growth period was used to ensure that the bacterial cells were in the vegetative state. DNA extraction was not necessary as PCR amplification was performed directly from the bacterial colonies. A small colony was removed from the surface of the YED plate with a 10 µl pipette tip and placed into a PCR tube containing 20 µl of master mix. The master mix contained 2 µl of 10x PCR buffer with 1.5 mM MgCl, 1.6 µl dNTP mic (200 µM each), 0.4 µl 530f primer (910 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). PCR amplification was conducted using an Eppendorf Mastercycler Personal Thermal Cycler (Eppendorf AG, Hamburg, Germany) with the following
cycle; 5 min at 95°C followed by 35 cycles of 94°C for 15s, 72°C for 15s, and a 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 Genomics Core Facility. Sequences from the forward and reverse primers were edited and aligned using Sequencher 4.7 (Gene Codes, Ann Arbor, MI), then the sequences were compared to similar 16s rRNA sequences in the GenBank database (www.ncbi.nlm.nih.gov) using the Basic Alignment Search Tool (BLAST) search algorithm (to determine the species of the isolate). The identities of the five apple isolates were determined by comparison with high-scored rRNA sequences in BLAST searches and sequences from Bacillus, Brevibacillus and Paenibacillus type strains. The 16S rRNA sequence data from the five apple isolates and 37 strains were used to create phylogenetic trees. The phylogenetic trees were constructed using the maximum parsimony and neighbor-joining methods in PAUP 4.0 for Macintosh (Sinauer Associates Inc., Sunderland, MA). Strength of the internal branches of the resulting trees was statistically evaluated by bootstrap analysis with 1000 bootstrap replications.

Results

Effects of integrating biocontrol bacteria with soluble silicon and chitosan on pre-harvest disease suppression on 'Cortland'

Total rainfall at the Rock Springs test site was 2.44, 3.63 and 4.36 inches in April, May and June respectively. Apple scab incidence and severity in the test orchard was high, particularly in the three rows at the south end of the orchard. Scab severity was lowest on trees in the northern edge row bordered by the scab resistant cultivars. Leaves on 'Cortland' trees receiving the 1% AgSil treatment (without bacteria) had the highest percent leaf area scabbed
followed by the non-treated control and chitosan trees. Two applications of bacteria, alone or in combination with chitosan, reduced the mean percent leaf area scabbed at five and seven weeks post application of the bacteria compared to leaves from non-treated control trees (Figure 5-6).

Figure 5-6: Mean percent area scabbed on 'Cortland' leaves 2, 3, 5 and 7 weeks after initial application of the bacteria and amendment treatments. Treatments were applied on 15-May and 16-June 2009 (Week 4). Six bacterial isolates (A1-1, A3-6, Ae-1, FLS-1, FLS-5 and none) and three amendments (Chitosan, 1% AgSil, and none) were tested. Three sulfur sprays were also applied to all trees in the orchard on 29-May,
There was no significant difference in apple scab severity among treatments on harvested 'Cortland' fruit (data not shown). There was no significant difference in fruit scab incidence and a minimum of 98% of the harvested fruit (depending on the treatment) had at least one scab lesion. Fruit harvested from trees treated with the biocontrol isolates A3-6, Ae-1, FLS-1 or FLS-5 (alone or in combination with AgSil or chitosan) had significantly fewer flyspeck lesions compared to the no-bacteria/amendment control (Figure 5-7). The Agsil or chitosan amendment treatments alone (with no bacteria) did not have a significant effect on flyspeck incidence. Fruit harvested from trees treated with FLS-1+AgSil had the lowest percentage of fruit with at least one flyspeck lesion of 14%. Fruit from the control, chitosan and AgSil treatments had 67%, 74% and 64% of fruit with flyspeck lesions respectively.

**Effects of sulfur, copper and streptomycin on bacterial colonization of apple leaves**

The biocontrol isolates A3-6, Ae-1 and FLS-5 colonized apple seedling foliage throughout the duration of the 21 day experiment, while isolate A1-1 remained below the
detectable population level (log 1.1 CFU/cm²). Application of sulfur, copper or streptomycin (on leaves sprayed with biocontrol bacteria) at labeled rates did not negatively affect bacterial colonization of apple foliage under growth chamber conditions (Figure 5-8). Colonization levels on leaves receiving sulfur or copper treatments were higher compared to the bacteria-only controls at each sampling point (except at 1 dpi) for isolates A3-6, Ae-1 and FLS-5. Colonization of leaves treated with streptomycin was slightly lower than colonization on the bacteria-only controls at 7 dpi for isolate A3-6 and at 14 dpi and 21 dpi for isolate Ae-1. Streptomycin did not significantly affect colonization levels on leaves treated with isolate FLS-5.

Figure 5-8: Colonization of apple foliage (reported at CFU/cm² leaf tissue) by the biocontrol isolates A3-6, Ae-1 and FLS-5 in combination with streptomycin, sulfur, copper or no fungicide/antibiotic treatment. Bacteria were applied at 10⁷ CFU/ml on day 0 with 0.1% Silwet L-77. Colonization was measured at 1, 7, 14 and 21 days post application of the bacteria. Error bars represent the standard error of the mean. The dashed line indicates the minimum detection level of the experiment.
Effects of biocontrol bacteria and chitosan on postharvest disease suppression

All four of the bacterial isolates tested significantly (P < 0.0001) reduced bitter rot lesion size caused by C. acutatum compared to the no-bacteria controls on ‘Golden Delicious’ fruit (Figure 5-9A). The 1% chitosan treatment also resulted in a significant (P < 0.0001) reduction (66%) in lesion area compared to the no-bacteria no-chitosan control. Additionally, the chitosan treatment (without bacteria) exhibited a greater level of suppression of bitter rot compared to the bacteria-only treatment for isolates A3-6, Ae-1 and FLS-5. A synergistic effect was observed in treatments combining chitosan with isolates A1-1, A3-6 and Ae-1 with regards to suppression of bitter rot lesion area. The chitosan+A1-1 treatment resulted in the greatest level of suppression of bitter rot with an 81% reduction in lesion area compared to the no-bacteria/chitosan control.

Both the chitosan and chitosan + bacteria treatments significantly (P < 0.0001) reduced lesion size (≥ 46%) in wounds inoculated with the blue mold pathogen, P. expansum on ‘Golden Delicious’ fruit (Figure 5-9B). In contrast to bitter rot, there was no significant difference (P = 0.47) in blue mold lesion area on fruit receiving chitosan compared to chitosan+A1-1. The chitosan+A3-6 and chitosan+Ae-1 resulted in the greatest level of suppression of blue mold with a 78% and 72% reduction in lesion area respectively, compared to the no-bacteria no-chitosan control. The chitosan (without bacteria) treatment resulted in a 61% reduction in blue mold lesion area on ‘Golden Delicious’ fruit.
All four of the isolates tested significantly (P < 0.04) reduced bitter rot lesion size caused by C. acutatum compared to the no-bacteria controls on 'Rome Beauty' fruit (Figure 5-10A). The 1% chitosan treatment also resulted in a significant (P < 0.0001) reduction (56%) in lesion area.
compared to the no-bacteria/chitosan control. For all biocontrol isolates tested, there was no significant difference (P > 0.2) in bitter rot lesion area on fruit receiving chitosan compared to chitosan+bacteria on 'Rome beauty' fruit. Both the chitosan and chitosan+bacteria treatments significantly (P < 0.0001) reduced lesion size (≥ 39%) in wounds inoculated with the blue mold pathogen, *P. expansum* (Figure 5-10B). The chitosan+A1-1 treatment resulted in the greatest level of suppression of blue mold with a 65% reduction in lesion area, compared to the no-bacteria/chitosan control. The chitosan treatment resulted in a 48% reduction in blue mold lesion area on 'Rome Beauty' fruit.
Colonization of fruit wounds by the biocontrol isolates in cold storage

All of the bacterial isolates tested were able to colonize wounds on ‘Golden Delicious’ and ‘Rome Beauty’ fruit at 20°C and 2°C (Figure 5-11A-B). Approximately 2.0 x 10^5 CFU/ml were applied to each wound. Colonization levels by isolates FLS-5 and A1-1 were similar at the two temperature treatments. Isolate FLS-5 averaged between log 5.3 and log 6.8 CFU/wound at 20°C and between log 5.3 and log 6.3 CFU/wound at 2°C. Colonization by isolates A3-6 and Ae-1 was greater at 20°C compared to 2°C on both cultivars tested, ranging between log 3.6 and log 5.0 at 20°C and between log 2.2 and log 3.8 CFU/wound at 2°C.

Figure 5-10: Mean lesion area (mm^2) on wounded ‘Rome Beauty’ fruit caused by (A) C. acutatum and (B) P. expansum. Twenty µl of a 1% chitosan and/or 10^7 CFU/ml of each biocontrol bacterial isolate (BCA) were applied to wounds at day 0. The pathogens, C. acutatum and P. expansum were applied to wounds at 10^4 conidia/ml 1 hour after inoculation with the bacteria and/or chitosan.
Fruit wounds treated with the biocontrol isolates A3-6 or Ae-1 developed lesions that were significantly smaller ($P < 0.05$) compared to the no-bacteria control when challenged with the bitter rot pathogen, *C. acutatum* (Figure 5-12A). Lesions on fruit treated with isolates FO-20 or A3-2 were not significantly different from the lesions on control fruit challenged with *C.*
When challenged with the blue mold pathogen, *P. expansum* however, only fruit treated with isolate A3-2 resulted in a significant suppression in lesion size (Figure 5-12B).

Suppression of symptoms (caused by *C. acutatum* and *P. expansum*) by application of the biocontrol isolates into wounds of 'Golden Delicious' fruit was investigated by scanning electron microscopy. Observations of wounded tissue colonized with the biocontrol isolate A3-6 showed attachment of bacterial cells to the hyphae of *C. acutatum* in samples taken four dpi (Figure 5-13A). Closer examination revealed that sections of the hyphae where bacterial cells had attached were damaged and collapsed (Figure 5-13B) while sections with no attached bacteria appeared normal. Observations indicated that isolate A3-6 did not attach or appear to cause any physical damage to hyphae of *P. expansum* in apple wounds (Figure 5-14A-B). Wound tissue samples inoculated with isolate Ae-1 and challenged with *C. acutatum*, were found to have higher numbers of bacterial cells covering the area inside and around the wound site four dpi (Figure 5-13C). Closer examination indicated that these bacteria did not attach or cause damage to hyphae of *C. acutatum* (Figure 5-13D).
Wound tissue inoculated with *P. expansum* (without bacteria) sampled four dpi was extensively colonized by hyphae and several conidiophores bearing conidia were present (Figure 5-13E-F). Conidiophores were not observed on tissues samples colonized with isolates A3-6 or A3-2, however only isolate A3-2 resulted in significant suppression in blue mold lesion area (Figure 5-13 A-D). Several non-germinated conidia of *P. expansum* were observed in wound tissue inoculated with isolate A3-2.

Figure 5-13: Scanning electron micrographs of biocontrol bacterial cells interacting with hyphae of *C. acutatum* in apple wounds four days after inoculation. (A) attachment of biocontrol isolate A3-6 cells to hyphae of *C. acutatum* at 750X. (B) attachment and degradation of *C. acutatum* hyphae by isolate A3-6 at 1500X. (C) isolate Ae-1 in fruit wounds at 1000X and (D) 2000X.
Figure 5-14: Scanning electron micrographs of biocontrol bacterial cells interacting with hyphae of *P. expansum* in apple wounds four days after inoculation. (A-B) isolate A3-6 at 500X and 1000X (C-D) isolate A3-2 at 500X and 1000X and (E-F) no-bacteria control at 500X and 1000X.
Identification of bacterial species from the 16s rRNA gene

DNA amplification and sequencing was successful for the apple bacterial isolates. The identities of the apple isolates were determined by comparing them with high-scored rDNA sequences in BLAST-N searches and rDNA sequences of other *Bacillus*, *Brevibacillus* and *Paenibacillus* species in the database (Figure 5-15). Using phylogenetic analyses, it was determined that isolates A1-1, A3-6, Ae-1 and FLS-5 belong to the genus *Bacillus* and isolate FLS-1 belong to the genus *Brevibacillus*. Isolate A1-1 and FLS-5 were determined to be members of the *B. cereus* group. Isolate FLS-5 was most similar to *B. cereus* and *B. thuringiensis* and could not be further resolved based on 16S rDNA sequences. Isolate A1-1 was most similar to *B. mycoides* and *B. weihenstephanensis*. Based on the mycoidal colony morphology on nutrient media, isolate A1-1 was concluded to be *B. mycoides*. Isolates A3-6 and Ae-1 were most similar to *B. megaterium* and *B. flexus*. 
Figure 5-15: Phylogenetic relationships of the collected apple isolates and type strains of *Bacillus* species based on the 16S rDNA sequences. The branching pattern, which is rooted by using *Alicyclobacillus acidocaldarius* as the outgroup, was generated by the neighbor-joining method. Branch lengths are proportional to the scale given. Bootstrap values are reported on the branches. The GenBank accession number is shown for each taxon.
Discussion

The five key bacterial isolates collected from apple, that were screened and selected for field evaluation (chapter 3) were identified to species by sequencing regions of the 16S rRNA gene (Figure 4-15). Isolates FLS-5 and A1-1 were determined to belong to the *B. cereus* group which contains seven closely related species, with *B. cereus, B. thuringiensis* and *B. mycoides* being the most common. Phylogenetic analyses indicate that isolate FLS-5 is most similar to *B. cereus* and *B. thuringiensis*. It has been reported that these two species are closely related and their discrimination is difficult by any method, particularly on the basis of the 16S rDNA sequence [3, 52]. Although the 16S rRNA gene is considered most useful for classification of bacteria, including *Bacillus* species, the conserved nature of the gene results in limited variation of sequences between members of closely related taxa [31]. *B. cereus, B. anthracis* and *B. thuringiensis* are opportunistic or pathogenic to insects or mammals. *B. cereus* is found in diverse habitats and is ubiquitous in soil [38]. *B. cereus* can cause two types of food poisoning; a diarrheal type and an emetic type which are caused by very different toxins [60]. Although these illnesses are generally mild and self-limiting, some more serious cases have been reported [60]. Some *B. cereus* strains have beneficial properties and certain strains, producing negligible amounts of toxin at 37°C, are used as probiotics in Europe [60]. Several *B. cereus* strains have demonstrated biological control activity on several crops [41, 50, 59] and many are chitinolytic [34, 50].

Analyses indicate that isolate A1-1 is most closely resembles *B. mycoides* and *B. weihenstephanensis*. Mycoidal growth of isolate A1-1 on nutrient media allowed us to place this isolate in the sub-group *B. mycoides* as *B. weihenstephanensis* has a non-mycoidal colony morphology [44]. Isolates Ae-1 and A3-6 were most similar to *B. megaterium* and *B. flexus*. The species *B. flexus* is often considered a subspecies of *B. megaterium* [54]. *B. megaterium* has been shown to have biocontrol activity, including reduction in postharvest decay of peanut kernels.
caused by *Aspergillus flavus* [42] and septoria tritici blotch of wheat. Isolate FLS-1 was identified as *Brevibacillus laterosporus*, an aerobic spore-forming bacterium. *B. laterosporus* has potential as a biological control agent with documented biocidal activity towards insects [19].

**Integrating biocontrol bacteria with soluble silicon and chitosan for disease suppression**

Chitosan, a polycationic compound, has been shown to have activity against a wide range of fungal pathogens [2]. A majority of the research contributing to our knowledge of chitosan and its prophylactic properties has focused on root application (either as a seed treatment or soil amendment). While foliar applications of chitosan have been documented for suppression of disease on crops including cucumber [49], strawberry [8] and grape [48], field applications have not been reported for apple. Based on the documented dual effects of chitosan (direct antifungal activity and activation of defense responses), an objective of the present study was to determine if foliar applications of chitosan could suppress apple scab disease severity alone or enhance disease suppression when combined with our bacterial isolates. Application of Chitosan at the recommended rate (the ODC™ formulation) to 'Cortland' leaves in May and June 2009 did not reduce foliar apple scab severity compared to the non-treated control. Additionally, while significantly different from the control, a synergistic effect was not observed when chitosan was applied in combination with a biocontrol isolate. Apple scab pressure was high in the 'Cortland' test orchard due to the presence of inoculum and weather conditions conducive to disease development throughout the season. It is possible that the high level of apple scab disease that occurred at our test site masked beneficial effects that chitosan application may have had, simply because the system was overwhelmed. Likewise, the levels of scab suppression on 'Cortland' leaves by the biocontrol isolates, while significant, were still not acceptable for marketing and
may be due to high scab pressure. Perhaps incorporation of chitosan and the biocontrol isolates into a low-input spray program would result in greater disease suppression on apple.

Application of the water soluble potassium silicate product, AgSil to 'Cortland' leaves in May and June (week 1 and week 4) did not significantly reduce apple scab severity on leaves or fruit. Foliar scab severity was actually higher on AgSil treated leaves compared to leaves from non-treated control trees. Interestingly, foliar scab severity was not significant on leaves treated with AgSil in combination with the bacterial isolates A1-1, A3-6, FLS-1 or FLS-5 weeks 2-5, but was significantly less than the control by week 7 (Figure 5-6). The mean scab severity on leaves receiving these treatment combinations did not increase from week 5 to week 7, whereas mean severity on leaves receiving all other treatments did increase. Few studies have been reported in the literature investigating the use of soluble silicon in apple production. Researchers in Belgium, reported that application of 0.1% potassium silicate (March-May) slightly reduced apple scab on fruits, but not on leaves [37]. Based on the results from the present study and reports in the literature, soluble silicon does not appear to be a promising candidate for suppression of apple scab. There are no reports of the effects of a pre-harvest application of Si to fruit for suppression of postharvest decay. When applied to apple wounds postharvest, potassium silicate (alone or in combination with bacterial biocontrol isolates) did not significantly reduce bitter rot lesion size caused by C. acutatum [51].

A second objective of the 'Cortland' experiment was to test the effects of pre-harvest application of the bacteria, silicon and chitosan both alone or in combination on postharvest disease suppression. The fruit harvested for this purpose, were however, 98% symptomatic with apple scab lesions and most were not suitable for postharvest experimentation. Research has demonstrated that pre-harvest sprays of chitosan provide multiple benefits including suppression of postharvest diseases and maintenance of fruit quality [2]. For example, pre-harvest sprays of chitosan on strawberry plants significantly reduced postharvest rot on fruit challenged with B.
cinerea [8]. Additionally, berries receiving chitosan 10 days before harvest were firmer and ripened at a slower rate than berries from non-treated plants. These effects are often seen when chitosan is applied as a coating, which forms a semi-permeable film that regulates gas exchange and reduces transpiration [21]. Meng and Tian reported that spraying the antagonistic yeast C. laurentii in combination with chitosan 10 days before harvest significantly reduced decay of table grape fruit [48]. While postharvest application of chitosan and glycolchitosan have shown potential to suppress postharvest decay on apple (alone or in combination with BCAs), pre-harvest applications has not been reported in the literature. Future experiments to investigate the effect of pre-harvest chitosan applications on apple (in combination with BCAs) compared to postharvest application of chitosan would provide valuable information to optimize the use of chitosan as a valid management strategy and if successful, allow research to make informed recommendations to growers.

**Potential for postharvest biological control**

The bacterial isolates A1-1, A3-6, Ae-1 and FLS-5 significantly reduced bitter rot lesion area in fruit wounds. All of the bacterial isolates tested were able to colonize fruit wounds at both room and storage temperatures, with isolate FLS-5 maintaining the highest population levels. While isolates A3-6 and Ae-1 resulted in significant suppression of lesion size, wound colonization levels were typically log 2 lower than isolate FLS-5 at 2°C and log 1 lower at 20°C. These observations suggest that mechanism of disease suppression by isolates A3-6 and Ae-1 does not involve competition for space or nutrients. This hypothesis was validated in the SEM experiment, in which hyphal tissues of C. acutatum had collapsed where isolate A3-6 had attached to the hyphae. On the other hand, isolate A3-6 (which did not significantly reduce blue mold) did not appear to attach or cause physical damage to hyphae of P. expansum.
Experimentation is needed to further elucidate the mechanism of disease suppression by isolate A3-6. Isolates A3-6 and Ae-1 (identified as *B. megaterium*) did not significantly inhibit growth of *C. acutatum* in dual culture assays. A similar result was reported by Kildea et al. [40] in which *B. megaterium* inhibited development of septoria tritici blotch on wheat, but did not directly inhibit growth of the causal agent *Mycosphaerella graminicola* in dual culture assays. Additional plate assays (detailed in chapter 3) indicated that isolates A3-6 and Ae-1 did not produce a clearing zone on CNA, indicating that the isolates do not produce chitinase. Screening for the production of other hydrolytic enzymes such as chitosanase, cellulase and glucanase will complement our knowledge of disease suppression and help explain SEM experiments. Bertagnolli et al. [7] identified several extracellular enzymes produced by *B. megaterium* strain B153-2-2, including protease and pectin lyase, capable of reducing growth of *Rhizoctonia solani* growth in vitro. In agreement with the present study, Bertagnolli also indicated a lack of detectable chitinase activity by *B. megaterium*.

Application of chitosan to apple wounds alone or in combination with isolates A1-1, A3-6, Ae-1 or FLS-1 significantly reduced bitter rot and blue mold lesion size on both 'Golden Delicious' and 'Rome Beauty' fruit. Although lesion development (of either pathogen) was not completely inhibited, lesion size was reduced by 39-81% after 7 days at room temperature. In general, reductions in lesion size were less on 'Rome Beauty' compared to ‘Golden Delicious’ (in the chitosan experiment). This is contradictory to other postharvest experiments (shown in chapter 4) in which the opposite effect was reported. This observation may be related to the amount of time the fruit had been in cold storage. The chitosan experiment on 'Rome Beauty' was the last postharvest experiment conducted in 2009, and so while 'Rome Beauty' fruit were harvested one month later then the 'Golden Delicious' fruit, they were in cold storage for a longer period of time. Space and resources limited the number of concurrent experiments that could be run. Further experimentation is needed to determine the effects of chitosan on bacterial growth
on fruit surfaces and on wound colonization by our bacterial isolates. In the present study, chitosan was applied directly to fruit wounds. Future experiments evaluating postharvest disease suppression when chitosan or glyclchitosan is applied in combination with our isolates as a coating will provide insights into the level of suppression that could be achieved in a more realistic (whole fruit) scenario.

**Potential for integration of bacterial isolates into existing apple management programs**

The bacterial isolates A3-6, Ae-1 and FLS-5 demonstrated potential for integration into existing apple management programs as they were able to colonize apple foliage treated with sulfur, copper or streptomycin. While isolate A1-1 (*B. mycoides*) significantly reduced fruit and foliar apple scab in two successive field trials (see chapter 4), foliar colonization in controlled and field environments was variable. Because of poor colonization on leaves of non-fungicide treated control seedlings, the effects of sulfur, copper or streptomycin on survival of isolate A1-1 cannot be inferred. Preliminary experiments conducted with another *B. mycoides* isolate, BacJ, indicated that foliar colonization was not inhibited by application of 0.15 lbs/gal sulfur (see chapter 2). In vitro assays could provide more information regarding sensitivity of endospores and vegetative cells of our isolates to the chemicals tested in the present study and the active ingredients of other commonly used fungicides including Rally® (myclobutanil), Inspire® (difenoconazole) and Vangard® (cyprodinil).

Further research also is needed to determine if the application timing of our isolates in combination with a fungicide/antibiotic could influence colonization and/or biocontrol efficacy. Should the biocontrol bacteria be applied before a chemical spray, afterwards, or can the bacteria be tank mixed with fungicides and applied simultaneously? The combination of a fungicide or antibiotic with a biological control agent can provide significant benefit to both systems by
reducing the variability in disease control associated with biological control agents [35] while reducing the rate of chemical used without compromising control [11, 12]. Jacobsen et al. [36] reported that reduction in Cercospora leaf spot on sugar beet with B. mycoides applied with half of the usual rate of tetraconazole was equal to control achieved with the full rate. Buck [11] found that combination of the biocontrol yeast R. glutinis with several fungicides at one-tenth their labeled rate was effective at reducing and/or eliminating disease caused by B. cinerea. Buck further suggests that this reduction in disease can minimize risk associated with the selection of fungicide resistance [11]. Larson [43] studied the prevalence of benomyl, azoxystrobin and tetraconazole resistance of C. beticola and reported that plots receiving B. mycoides isolate BmJ plus fungicide treatments had a significantly smaller percentage of conidia insensitive to 1 ppm of benomyl and tetraconazole compared to plots receiving a rotation of fungicides. The impact of our isolates and other BCAs with documented activity against apple scab on prevalence of fungicide resistant strains in conventional and low-input orchards is an interesting area of further research. This type of research could provide valuable information to extension specialists and growers and perhaps broaden the scope of biocontrol use as an anti-resistance strategy in addition to simple disease suppression.

References


Appendix A


Nine endospore-forming bacteria (7 from unmanaged apple orchards in Adams County, PA and 2 from vegetables in Alabama) were evaluated for the ability to suppress the bitter rot pathogen Colletotrichum acutatum on apple fruit. Effectiveness of the bacteria alone or in combination with AgSil (potassium silicate) was also tested. Tree-ripe Rome Beauty fruit were harvested from trees receiving a standard fungicide program. Following harvest, fruit were washed in a 1% bleach solution and rinsed twice with tap water. Isolates of C. acutatum were obtained from infected fruit and maintained on PDA. The bacterial isolates were grown in tryptic soy broth at 24°C for 7 days. Bacterial cells were pelleted using a Sorvall RT7 centrifuge at 2800 rpm for 20 min, resuspended in 0.1 M potassium phosphate buffer and the concentration was adjusted to $10^8$ CFU/ml. Fruit were inoculated with bacterial isolates by applying suspensions using hand-held spray bottles, placed on cardboard packing trays and stored at 4°C. One week later, fruit were wounded (2 mm diam x 2 mm deep) with a sterile nail. Wounds were treated with a 20 µl bacterial suspension ($10^8$ CFU/ml). Within 1 hr wounds were challenge-inoculated with a 20 µl suspension of C. acutatum conidia. One hour after challenge, respective wounds were treated with 20 µl of a 2% AgSil solution. Wounded fruit were randomly placed on molded cardboard packing trays (20 fruit/tray) so that each tray contained representatives from each treatment. Individual fruit served as one replicate with 18 replications per treatment and trays served as blocks. Trays were stored on plastic shelving at 20°C in a controlled atmosphere chamber. Fruit were evaluated for disease severity 1 wk after pathogen inoculation.

All bacterial treatments (except isolate BacJ alone) significantly reduced lesion area compared to untreated controls. AgSil alone was not effective as it was not significantly different from the untreated control. The presence of AgSil did not have a significant effect when combined with most isolates except with A1-1. A 68% reduction in lesion area compared with untreated controls was observed on fruit treated with the isolate alone, however, only a 30% reduction resulted when combined with AgSil. Isolate A3-6 was most effective, resulting in 89% reduction in lesion area compared with unsprayed controls and 90% compared with AgSil treated controls.

<table>
<thead>
<tr>
<th>Treatment and rate</th>
<th>Mean lesion area (mm$^2$)$^y$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria alone</td>
</tr>
<tr>
<td>Untreated control</td>
<td>268 a$^x$</td>
</tr>
<tr>
<td>A1-1</td>
<td>87 c</td>
</tr>
<tr>
<td>FO-20</td>
<td>151 bc</td>
</tr>
<tr>
<td>BT8</td>
<td>153 bc</td>
</tr>
<tr>
<td>BacJ</td>
<td>195 ab</td>
</tr>
<tr>
<td>A2-4</td>
<td>143 bc</td>
</tr>
<tr>
<td>A3-F1</td>
<td>111 bc</td>
</tr>
<tr>
<td>A3-1</td>
<td>87 c</td>
</tr>
<tr>
<td>A3-6</td>
<td>30 d</td>
</tr>
<tr>
<td>A3-2</td>
<td>119 bc</td>
</tr>
</tbody>
</table>

$^x$Lesion diameters were measured vertically and horizontally across the wound site. The average diameter was used to calculate the total diseased area ($\pi r^2$) on each fruit.

$^y$Rates based on product recommendations

$^x$Within columns, means with the same letter are not significantly different at P=0.05 as determined by the Tukey-Kramer test.
Appendix B


An experiment was conducted to evaluate the timing of preharvest and postharvest bacterial applications on suppression of the postharvest pathogen Colletotrichum acutatum on apple fruit. Four application schedules were tested; 7 May, 7 May+19 June, 7 May+postharvest and 7 May+19 June+postharvest. Preharvest treatments were applied to mature 'Rome Beauty' and 'Golden Delicious' trees on M.26 rootstock using a Solo backpack sprayer. Treatments were arranged in a randomized complete block design with 4 replications and 4 blocks. All trees received a copper treatment at 1/4" green tip. 'Rome Beauty' and 'Golden Delicious' fruit were harvested at commercial maturity. Isolates of C. acutatum were obtained from infected fruit and maintained on PDA. The bacterial isolates were grown in tryptic soy broth at 24ºC for 7 days. Bacterial cells were pelleted using a Sorvall RT7 centrifuge at 3800 rpm for 15 min, suspended in 0.1 M potassium phosphate buffer to a concentration of 10⁷ CFU/ml. For postharvest applications, fruit were wounded (2 mm diam x 4 mm deep) with a sterile nail. Wounds were treated with a 20 µl bacterial suspension (10⁷ CFU/ml). Within 1 hr, wounds were challenged with a 20 µl suspension of C. acutatum conidia (5.0 x 10⁴ conidia/ml). Wounded fruit were randomly placed on molded cardboard packing trays (20 fruit/tray) so that each tray contained representatives from each treatment. Individual fruit served as one replicate with 15 replications per treatment and trays served as blocks. Trays were stored on plastic shelving at 20ºC in a walk-in chamber. Fruit were evaluated for disease severity 1 wk after pathogen inoculation by measuring the average lesion diameter. Mean separation was conducted using the Tukey-Kramer test (α=0.05).

All bacterial isolates applied as a postharvest treatment significantly reduced lesion area compared to untreated controls on Rome Beauty and Golden Delicious fruit. No symptoms were observed in wounds with bacteria application without pathogen challenge. Preharvest application of bacteria (without postharvest) was not as effective as a postharvest application. Combination of preharvest and postharvest application of bacteria did not result in a synergistic effect on reduction in lesion size compared to a postharvest application alone on either variety (except isolate FLS-5 on Golden Delicious). Overall greater disease suppression was observed on Rome Beauty compared to Golden Delicious. Isolate A3-6 was the most effective as a postharvest application, resulting in an average reduction in lesion area of 89% on Rome Beauty and 41% on Golden Delicious compared to the untreated controls.

Table 1: Rome Beauty apples

<table>
<thead>
<tr>
<th>Application schedule</th>
<th>Mean lesion area (mm²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A3-6</td>
</tr>
<tr>
<td>Untreated control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>123 a</td>
</tr>
<tr>
<td>May</td>
<td></td>
</tr>
<tr>
<td></td>
<td>130 a</td>
</tr>
<tr>
<td>May + June</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101 b</td>
</tr>
<tr>
<td>May + Postharvest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 c</td>
</tr>
<tr>
<td>May + June + Postharvest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 c</td>
</tr>
<tr>
<td>Postharvest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 c</td>
</tr>
</tbody>
</table>
Lesion diameters were measured vertically and horizontally across the wound site. The average diameter was used to calculate the total diseased area \((\pi r^2)\) on each fruit.

Within columns, means with the same letter are not significantly different at \(P=0.05\) as determined by the Tukey-Kramer test.

Table 2: Golden Delicious apples

<table>
<thead>
<tr>
<th>Application schedule (^\dagger)</th>
<th>A3-6</th>
<th>A1-1</th>
<th>FLSD-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>260 (a) (^z)</td>
<td>261 (a)</td>
<td>261 (a)</td>
</tr>
<tr>
<td>May</td>
<td>233 (a)</td>
<td>168 (b)</td>
<td>208 (c)</td>
</tr>
<tr>
<td>May + June</td>
<td>254 (a)</td>
<td>171 (b)</td>
<td>159 (b)</td>
</tr>
<tr>
<td>May + Postharvest</td>
<td>149 (b)</td>
<td>205 (b)</td>
<td>149 (b)</td>
</tr>
<tr>
<td>May + June + Postharvest</td>
<td>154 (b)</td>
<td>170 (b)</td>
<td>173 (b)</td>
</tr>
<tr>
<td>Postharvest</td>
<td>158 (b)</td>
<td>206 (b)</td>
<td>215 (c)</td>
</tr>
</tbody>
</table>

\(^z\) Lesion diameters were measured vertically and horizontally across the wound site. The average diameter was used to calculate the total diseased area \((\pi r^2)\) on each fruit.

\(^\dagger\) Within columns, means with the same letter are not significantly different at \(P=0.05\) as determined by the Tukey-Kramer test.
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