Commercial Bumble Bees as Vectors of the Microbial Antagonist *Clonostachys rosea* for Management of Botrytis Blight in Wild Blueberry (*Vaccinium angustifolium*)

by

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Submitted in partial fulfilment of the requirements
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at

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ABSTRACT

Greenhouse and laboratory experiments in 2011 determined that *Clonostachys rosea* can effectively prevent *Botrytis cinerea* infection in *Vaccinium angustifolium* blossoms. *In vitro* testing demonstrated that *C. rosea* germination was not significantly affected by the presence of Switch®, but was by either Pristine® or Maestro®. Field experiments completed during the summer of 2010 and 2011 indicated that the dispenser designs tested had no significant effects on *Bombus impatiens* foraging behaviours, aside from hive-activity. There was also no difference in the quantity of *C. rosea* applied by each to bees, the distribution of product in the field, or for blossoms exposed to bees from each dispenser to resist infection by *B. cinerea*. However, *B. cinerea* prevalence in blossoms from both treatments was significantly different from the control, with infection reduced by 10-20%. Technical issues with dispensers currently appear to be the limiting factor for application within commercial wild blueberry production.

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Chapter 1. Introduction

1.1 Bees as Vectors for Biopesticides

Biological pesticides (biopesticides), also referred to as microbiological control agents (MCA), microbial antagonists or biological control (biocontrol) agents, are naturally occurring bacteria, fungi, nematodes, and viruses that can be used to manage pathogens and insect pests. In order for a biopesticide to be effective, it must be: (i) genetically stable; (ii) effective at low concentrations; (iii) not fastidious in its nutrient requirements; (iv) able to survive adverse environmental conditions; (v) effective against a wide range of pathogens on different commodities; (vi) amenable to production on inexpensive growth media; (vii) amenable to formulation with a long shelf-life; (viii) easy to dispense; (ix) not detrimental to human health; (x) resistant to pesticides; (xi) compatible with commercial processing (Wilson and Wisniewski 1989). Very few agents currently available can meet all of these requirements, and thus many are limited in both their capabilities and efficacy. As a result, only a select few microbial agents have shown appreciable potential. However, despite current inadequacies, the role of microbial agents in pest management is likely to increase given a number of global, ongoing concerns surrounding the use of synthetic pesticides. For instance, apprehension surrounding the impacts of pesticides on human health has led to increasingly stringent import restrictions on maximum residue limits (European Commission 2011), and limitations on the types of pesticides applied to certain imported products (Wilson and Otsuki 2004). Additional concerns surrounding the detrimental effects of synthetic pesticides on non-target

organisms, such as pollinators (Gels et al. 2002), have cultivated the desire to develop environmentally-conscious integrated pest management (IPM) programs.

Insects have long been known to translocate and disseminate pathogens; from the discovery of anopheline mosquitoes as vectors of malaria (Rees 1900), to the discovery of fungal spore dissemination by pollinators (Leach 1935). Acquisition and translocation of plant pathogens, within an agricultural context, occurs when a pathogen becomes captured on insect body hairs during pollination/feeding, and is then carried to subsequent plants, resulting in the dissemination of the pathogen (Leach 1935). Using these same principles, Peng et al. (1992) performed the first experiments using managed pollinators as vectors of beneficial microorganisms, wherein honeybees (*Apis mellifera* Linnaeus) (Hymenoptera: Apidae) were used to disseminate *Clonostachys rosea* f. rosea Schroers, Samuels, Seifert & Gams (formerly *Gliocladium roseum* Bainier) (Hypocreales: Bionectriaceae), a beneficial endophtyic fungus that demonstrates mycopathogenic capabilities against *Botrytis cinerea* Persoon: Fries (Helotiales: Sclerotiniaceae), into crops of greenhouse and field-grown strawberry. The hive-mounted dispenser designed (Figure 1.1) contained a powdered formulation of the microbial agent. When bees walked through the dispenser as they exited the hive to forage, the powder adhered to the bee's body. *C. rosea* spores present on the bees were gradually lost during pollination, thereby inoculating blossoms with the microbial antagonist. The technique proved successful, with bees providing suppression equivalent to spray application (Peng et al. 1992). Since then, this technique has been examined using different microbial agents and pollinators, within in a handful of crops. For instance, honey bee-vectored Serenade®

(*Bacillus subtilis*; Ehrenberg) Cohn (Bacillales: Bacilaceae) was able to significantly reduce the prevalence of mummy berry (*Monilinia vaccinii-corymbosi* Reade) Honey (Helotiales: Sclerotiniaceae) in fields of rabbiteye blueberry (*Vaccinium ashei* Reade) (Ericales: Ericaceae) (Dedej et al. 2004). The use of *A. mellifera* to disseminate both the fire blight pathogen (*Erwinia amylovora*) (Enterobacteriales: Enterobacteriaceae) (Burril) Winslow et al. and a biocontrol agent for its control (*Pseudomonas fluorescens*) (Pseudomonadales: Pseudomonadaceae) (Flugge) Migula also demonstrated the ability of pollinators to vector both bacterial pathogens and biopesticides into pear and apple crops (Johnson et al. 1993). The success of honey bee-vectored *C. rosea* for *B. cinerea* suppression in strawberry (Peng et al. 1992) eventually led to the discovery that the pathogen could also be suppressed in field-grown raspberry using this same vector-agent combination, while also proving the competency of another commercially available managed pollinator, the eastern bumblebee (*Bombus impatiens* Cresson) (Hymenoptera: Apidae), for this technique (Yu and Sutton 1997). Additional studies have continued work with bumble bee-vectored *C. rosea* in greenhouse and field-grown strawberry, but using a different species of bumble bee (*Bombus terrestris* Linnaeus) (Hymenoptera: Apidae) (Mommaerts et al. 2011a), while others have expanded use with *B. impatiens* and *C. rosea* into greenhouse tomato and sweet pepper (Kapongo et al. 2008). The study by Kapongo et al. (2008) also examined co-vectoring *C. rosea* with the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae). Used in combination, there was a reduction of 46-59% in the incidence of grey mould on foliage and blossoms, with drastic reductions in populations of greenhouse whitefly

(*Trialeurodes vaporariorum* Westwood) (Hemiptera: Aleyrodidae) and tarnished plant bug (*Lygus lineolaris* Palisot de Beauvois) (Hemiptera: Miridae); 49% and 73% mortality, respectively. The use of honey bees to disseminate another entomopathogenic fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), provided 100% control of pollen beetles (Coleoptera: Nitidulidae) in winter oilseed rape (Butt et al. 2008). These studies provide insight into some of the work that has been done with biovectoring work since the early 1990's, demonstrating its potential for increasing the sustainability of agricultural practice. Additional examples can be found in Mommaerts and Smagghe (2011).

There are several potential benefits of using commercial pollinators to deliver biological control agents. In settings where managed bees are being used for crop pollination, the technique capitalizes on normal foraging behaviour as a means to apply the biopesticide. While less than 0.1% of pesticides reach target pests using traditional spray based application (Pimental 1998), in biovectoring most of the agent is delivered to tissues frequented by pollinators, that is the blossoms and proximate leaves, thereby reducing waste to the ground and protecting the fruiting body. The relatively constant delivery during pollination could also minimize reapplications and concurrently provide insect pest and disease suppression during bloom; a time when pesticide application is preferably not performed, due to risk of damage to delicate bloom and detrimental impacts on pollinators. Depending on the level of control provided, fuel, repair costs, and greenhouse gas emissions associated with tractor-based application may be reduced and synthetic inputs minimized.

1.1.1 Dispenser Designs

The relatively recent development and adoption of this technology has resulted in continual emergence and revision of hive-mounted dispenser designs (Mommaerts et al. 2010b). Although several different dispenser designs are available for honeybees (Peng et al. 1992, Gross et al. 1994, Kovach et al. 2000, Bilu et al. 2004, Albano et al. 2009), it is more recently, with the increased use of commercial bumblebees within greenhouse operations, that dispensers for bumble bees have evolved.

Most dispenser designs to-date are similar in layout to either the side-by-side passageway (SSP; Figure 1.2) or the overlapping passageway (OP; Figure 1.3) dispensers (MacCagnani et al. 2005) with slight variations according to hive entrance and exit layout, although unique designs are emerging. In order to be successful, a dispenser must satisfy three factors; 1) sufficient amount of product is loaded onto each bee; 2) the dispenser does not interfere with foraging behaviour; and 3) has a long refill interval (Mommaerts and Smagghe 2011). The ability to load product onto bees seems to be largely determined by the length of the path that bees must take to exit; with the optimal path length being around 20 centimetres, since path lengths in excess of 40 centimetres may lead to self-grooming, which removes the product prior to foraging (Mommaerts et al. 2010b).

Among the newly released dispenser designs is one patented by Dr. Peter Kevan, largely modelled after those used and modified by Yu and Sutton (1997), Al-mazra'awi (2006), and Kapongo et al. (2008); except with a perpendicular orientation to the hive entrance and exit, rather than parallel. The dispenser uses a side-by-side passageway

(SSP) design (Figure 1.2), but arranged vertically, with the one-way exit forcing bees into a lower-level maze, and the upper level allowing returning bees back into the hive through the one-way entrance. A cartridge containing the microbiological control agent (MCA) is placed in the lower level where the legs and ventral surfaces of workers are dusted with the MCA as they traverse the chamber to exit the dispenser. While not the most efficacious design, according to various studies (MacCagnani et al. 2005, Mommaerts et al. 2010b), a sufficient path length (to ensure adequate application) (Mommaerts et al. 2010b) is achieved by the presence of pillars in the MCA cartridge that prevent foragers from following a straight path. A notable modification, to this otherwise classic design, is that the dispenser offers brackets for better attachment to commercial hives and a user-friendly access panel to access the MCA cartridge. The cartridge is changed every three days, rather than refilled, to ensure MCA viability, increase ease of product replacement, and eliminate the need for cleaning. A novel and unique dispenser design produced by Koppert Biological Systems Inc. is mildly similar to the over-lapping passageway (OP) design (Figure 1.3) that has been shown to be superior to some SSP designs (MacCagnani et al. 2005). However, rather than a static tray, this dispenser utilizes an electronic sensor matched to a motorized shutter to intermittently (every 20 bees) refill the exit tube with MCA from a top-loading reservoir. The novel design shows potential, although moving parts are more easily broken.

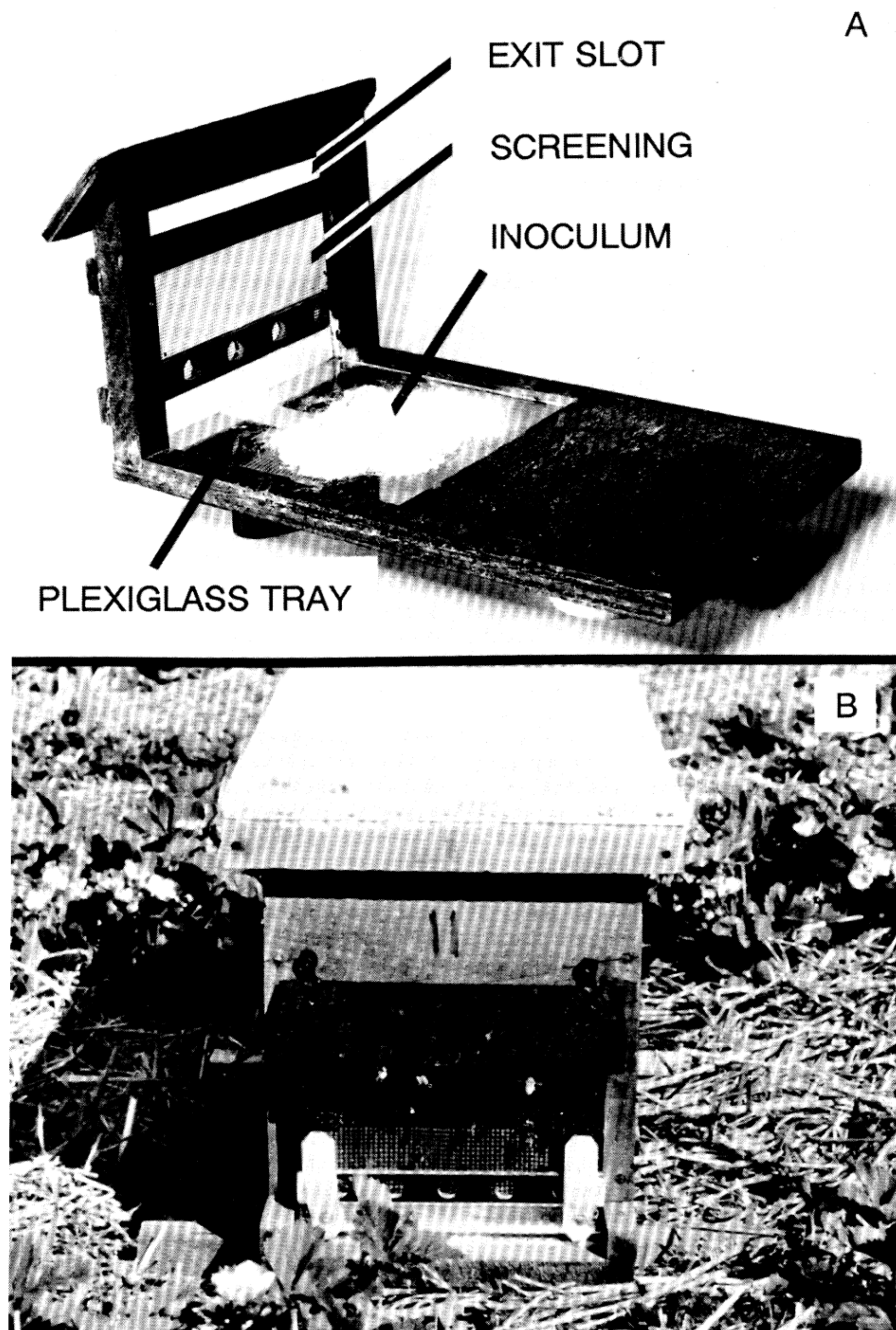

Figure 1.1. The microbial dispenser for *Apis mellifera* hives, used by Peng et al. (1992) to disseminate *Clonostachys rosea* into strawberries for control of *Botrytis cinerea*. Inoculum dispenser shown A) separately; B) attached to a half-size hive. (from Peng et al. 1992)

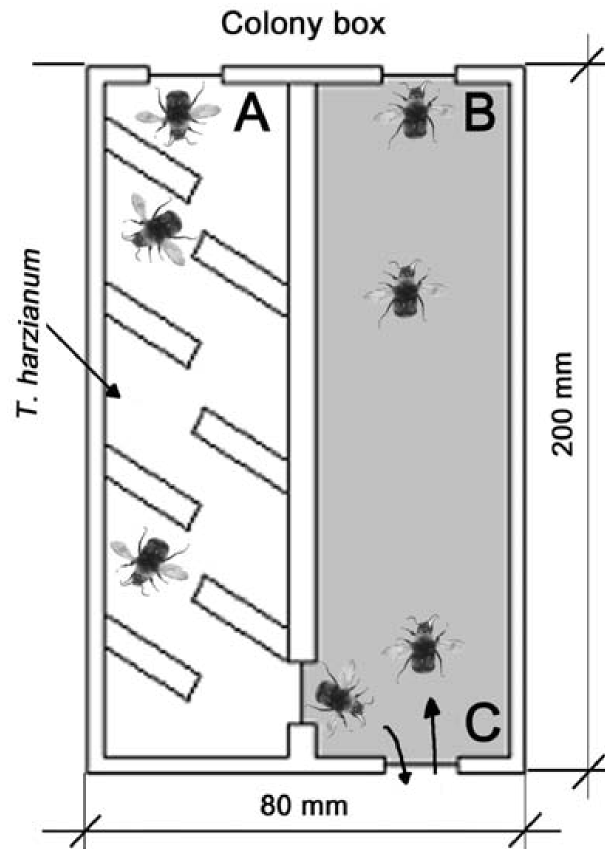

Figure 1.2. The side-by-side passageways (SSP) dispenser design tested for *Bombus terrestris* using the microbial antagonist *Trichoderma harzianum* (MacCagnani et al. 2005). A: One-way exit from hive; B: Entrance to colony box; C: Opening used by outgoing and incoming bees. (from MacCagnani et al. 2005)

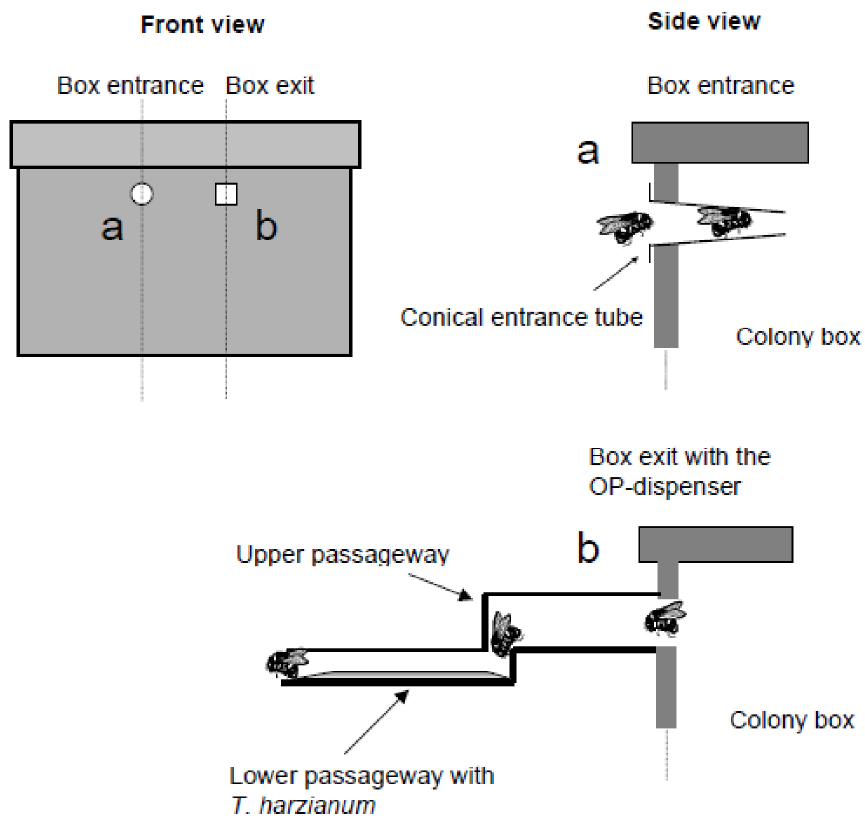

Figure 1.3. The overlapping-passageways (OP) dispenser design tested for *Bombus terrestris* using the microbial antagonist *Trichoderma harzianum* (MacCagnani et al. 2005). A: Entrance to colony box; B: One-way exit from hive. (from MacCagnani et al. 2005)

1.1.2 Impact on Vectoring Pollinators

Due to the many colony health issues already afflicting honey bees, pollinator health has remained a priority during the development of this technology. Several studies have shown that there is no measurable impact of most microbial agents on the pollinators (Al-mazra'awi et al. 2006, Butt et al. 2008, Kapongo et al. 2008, Mommaerts et al. 2009), with only a few biopesticides (Botanigard® and Serenade®) affecting pollinator mortality or other outcomes (Kapongo et al. 2008, Mommaerts et al. 2009). The additives present within powder formulations are also carefully evaluated for their safety, in order ensure that product stability and functionality does not compromise pollinator health (Mommaerts et al. 2011c, Mommaerts et al. 2011b). While exposure to toxic pesticides can influence foraging behaviour in *B. terrestris* (Mommaerts et al. 2010a), exposure to most biological control agents tested, except Botanigard®, seem to have no significant effect (Mommaerts et al. 2009). However, the impacts of other microbials, as well as the added ‘duty’ of vectoring, on pollinator behaviour has not been as thoroughly examined, specifically for *B. impatiens*.

1.2 Wild Blueberry

Wild blueberry (*Vaccinium angustifolium* Aiton; Ericacae), also referred to as lowbush blueberry, is a low growing (7-38 cm) woody deciduous shrub that can sprout from seed, but typically spreads via rhizomes, forming genetically identical clonal patches (Chiasson and Argall 1996). It is an economically important crop in eastern Canada and the state of Maine in the United States. In 2010, Nova Scotia produced 16

395 marketable tons, with a farm gate value of over 22 million dollars (Morin et al. 2011). The expansion of wild blueberry production in Canada was responsible for nearly half of the 'fruit-bearing acreage' increase in 2010 (Morin et al. 2011). Wild blueberry is found from Newfoundland west to southern Manitoba and Minnesota, as well as south to northern Illinois, Pennsylvania, and Delaware, and in the mountains to Virginia (Vander Kloet 1988). *V. angustifolium* is drought tolerant and prefers well-drained, acidic soils (pH 4.6-5.2). It prefers full sunlight (Chiasson and Argall 1996), but is shade-tolerant (Camp 1945).

Although most fields of wild blueberry are composed primarily of *V. angustifolium*, *Vaccinium myrtilloides* Michaux may be present in varying amounts (Aalders and Hall 1961). Cross-pollination of the two species may result in fruit abortion and/or reductions in yield (Aalders and Hall 1961), however some natural hybrids do occur (Darrow and Camp 1945).

V. angustifolium flowers are receptive to pollination for at least 7 days after opening, but thereafter attract fewer pollinators (Wood 1965). The receptivity of flowers to fertilization decreases with blossom age, although the magnitude of this decrease varies between clones (Wood 1962). Due to the predominant self-sterility of most clones (Aalders and Hall 1961), *V. angustifolium* is highly dependent on bees for cross-pollination and fruit set, with managed bees being required in most locations to achieve adequate yield (Whidden 1996). Three species of managed bees are commonly used for wild blueberry pollination in the Atlantic Provinces: *A. mellifera*, *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) and *B. impatiens*. However, loyalty of each

species to blueberry flowers (Whidden 1996) and pollination efficiency (Javorek et al. 2002), is variable. For instance, the *V. angustifolium* flowers have poricidal anthers that typically require sonication to release pollen tetrads, a behaviour best developed in bumble bees (Javorek et al. 2002). Blossom handling time is also highly variable and when compared to *B. impatiens*, *A. mellifera* spend on average 3-fold longer at each flower (Stubbs and Drummond 2001).

The abundance of *B. impatiens* in environments with rainy, cool weather (Heinrich 1981), may suggest the increased suitability of this pollinator for locations with similar conditions, such as the Atlantic Provinces (Stubbs and Drummond 2001). *B. impatiens* has also shown a much higher degree of fidelity to wild blueberry pollen, with nearly 75% of returning workers carrying pure (>90%) *Vaccinium* loads (Whidden 1996). Despite *B. impatiens* only having an average of 120 workers foraging per commercial colony, in comparison to an average of 14,200 foraging workers in a commercial *A. mellifera* colony, pollination between the two species has been shown to be quite comparable (Page and Fondrk 1995). Although honey bee hives can still be rented, bumble bee hives are also now commercially available for purchase by growers through companies such as BioBest Inc. and Koppert Inc., with per unit prices becoming increasingly comparable, making them a feasible alternative for managed pollination (Stubbs and Drummond 2001, Drummond 2012).

Although managed fields were traditionally burned, due to rising fuel costs and permit restrictions, they are now typically pruned by mowing every other year to maintain high fruit production (Kinsman 1993). While more environmentally conscious, the

reduction in burning has important implications for the quantity of disease inoculum present in the abundant litter layer. Pruned or 'sprout' fields constitute a phase of vegetative growth and initial floral bud development. After a cold dormancy period, growth resumes in the 'cropping' phase where floral buds swell that will bloom in 3-4 weeks. If successfully pollinated, these flowers will form berries (Chiasson and Argall 1996).

1.3 Grey Mould / Botrytis Blight

Wild blueberry is afflicted by a number of fungal diseases including Monilinia blight, Botrytis blight, Septoria leaf spot, Valdensinia leaf spot and leaf rust (Delbridge et al. 2011). *Botrytis cinerea* is the causative agent of grey mould and Botrytis blight in wild blueberry. However, *B. cinerea* is a generalist pathogen that can cause pre or post-harvest infection in over 235 plant species, especially if the plant is stressed or damaged (Jarvis 1977). The life cycle of *B. cinerea* (Figure 1.4) can vary greatly within a season, ensuring successful proliferation of the pathogen. For instance, asexual reproduction can occur in *B. cinerea* through the production of sclerotia, which develop within senescing host tissues. These reproductive bodies are covered in a melanised rind to protect the encased mycelia and sclerotia from dessication, UV light and microbial attack (Backhouse et al. 1984). Sclerotia often begin growing in the spring, producing conidiophores and multinucleate conidia to serve as primary inoculum for infection of new hosts (Williamson et al. 2007). However, phialides in aging cultures may also produce microconidia to serve as spermatia for sexual reproduction (teleomorph *Botryotinia*

fuckeliana). Spermatization of sclerotia leads to the growth of apothecia and asci with eight binucleate ascospores (Williamson et al. 2007). Although the high level of genetic variability in *B. cinerea* suggests that sexual reproduction is common, documentation of its occurrence is lacking in most crops (Williamson et al. 2007); including wild blueberry.

In *V. angustifolium* fields, *B. cinerea* overwinters as dormant mycelium or sclerotia in plant debris (Lambert 1990). Conidia are dispersed to plant tissues in the spring primarily by wind (Harrison and Lowe 1987) and splash from rain droplets (Jarvis 1962), although insects may potentially also serve as inadvertent vectors of conidia (Silow 1933). Germination typically occurs on the expanded corolla, starting at the F5 floral stage and increasing to 98% germination during the F7 (full-open) floral stage (Hildebrand et al. 2001). The extent of infection was found to be dependent on inoculum load, temperature, and length of wetting period (Hildebrand et al. 2001). Infections cause reductions in yield, likely by means of premature abscission (Hildebrand et al. 2001), while infected foliar tissues are destroyed by the grey mould, thereby reducing photosynthetic efficiency and acting as a source for infective inoculum (Howatt 2005).

Severe *B. cinerea* infections can cause major economic losses for wild blueberry producers, with infection levels as high as 35% (Howatt 2005). Botrytis blight and grey mould are common concerns throughout the Atlantic provinces and are a particularly serious problem in areas that are prone to fog or high humidity (Lambert 1990), such as the Parrsboro shore area of Nova Scotia. While limited published literature exists regarding the economic threshold for fungicide application, due to the complexity of *B. cinerea* infection risk, it is estimated to be between 7.5% and 15% for wine grapes,

depending on crop value and cultivar susceptibility (Ellison et al. 1998). However, the risk to grapes is much higher, with the potential losses in excess of 50% (Gubler et al. 1987), and thus economic thresholds may be higher for blueberry. In wild blueberry production, growers in regions with a history of the disease will apply fungicides as a preventative measure, even in the absence of disease pressure (Hildebrand 2010).

While *V. angustifolium* produces its own array of phenolic compounds, including tannins (phenolic polymers), to defend against pathogens and herbivory, these natural defences have variable effects on different organisms; with complete tolerance by some (Levin 1976, Duy 1999). *B. cinerea* infection is typically controlled using an early spray of Pristine® or Switch®, although Maestro® is occasionally used as well, depending on market allowances (Delbridge et al. 2011). Select fungicides display some curative capabilities, however, recommended use for fungicides is strictly preventative (Rosslenbroich and Stuebler 2000). The evolutionary capabilities of *B. cinerea* (Williamson et al. 2007) have proven especially problematic, as it has enabled the pathogen to quickly develop resistance to many pesticides over the years (Rosslenbroich and Stuebler 2000, Williamson et al. 2007). As such, a variety of management strategies are necessary to ensure adequate control (Williamson et al. 2007).

Pristine® contains two active components, boscalid, a group-7 respiratory inhibitor, and pyraclostrobin, a group-11 methoxy-carbamate respiratory inhibitor. Switch® also contains two active components, cyprodinil, a group-9 protein synthesis inhibitor, and fludioxinil, a group-12 signal inhibitor. Maestro® contains Captan, a pthalimide that has multi-site activity. A spray regimen of Pristine® and Switch®

typically reduces Botrytis blight of blossoms in highbush blueberry by about 44% at peak bloom (Elmhirst and Smith 2010).

While fungicides, such as these, with multiple modes of action are less prone to resistance, *B. cinerea* has already developed resistance to many synthetic fungicides, including some components of these mixtures (Northover and Matteoni 1986, Myresiotis et al. 2007). In addition to resistance issues, sprays often require very specific application times to ensure fungicidal activity will coincide with infection periods (Delbridge et al. 2011), yet they provide incomplete coverage, and thus incomplete protection (Pimental 1998). The toxicity of synthetic pesticides also raises concerns regarding human and environmental health (Pimental 1998). Suppression with biological pesticides (biosuppression) is a preferable alternative, as they presumably pose less risk to human and environmental health (Wilson and Wisniewski 1989). Since many biopesticides employ a variety of mechanisms, such as antibiotic production, nutrient competition, direct parasitism, and may possibly also induce resistance in the plant or change conditions sufficiently to reduce pathogen access, resistance is much less likely to occur (Baker 1987, Wilson and Wisniewski 1989).

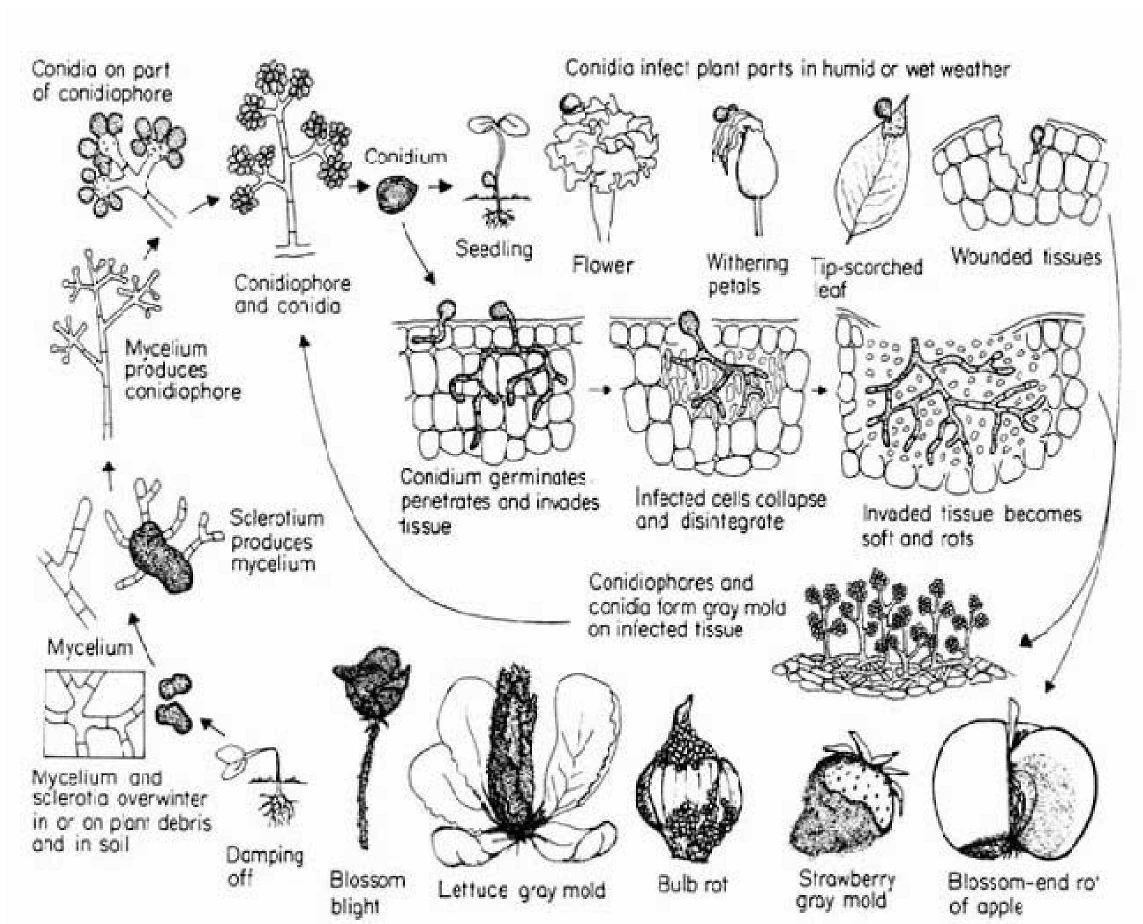

Figure 1.4. The life cycle of *Botrytis cinerea* (Agrios 1997).

1.4 *Clonostachys rosea*

While a variety of organisms, including some species of *Trichoderma* (Harman et al. 1996) and *Penicillium* (Peng and Sutton 1991), as well as *Cladosporium herbarum* and *Pullularia pullulans* (Bhatt and Vaughan 1962), have shown promise for biological control of *B. cinerea* in some berry crops, their use is often limited to greenhouses, as they have poor tolerance to adverse climatic conditions. *Clonostachys rosea* f. rosea, a beneficial endophytic fungus, has shown considerable success against grey mould (*Botrytis cinerea*) (Peng and Sutton 1991, Kapongo et al. 2008), as well as a variety of other fungi (Sutton et al. 1997). Residing within the leaf after germination, endophytes encounter less extreme conditions than their epiphytic counterparts, and thus are often capable of withstanding more adverse environmental conditions (Tronsmo 1992). *C. rosea* has demonstrated this, with greater resilience to adverse environmental conditions (Morandi 2008). Although the mechanisms behind *C. rosea*'s plant protection capabilities are not fully understood, it is believed that pre-occupation of tissues inhibits germ tube growth of *B. cinerea*, likely through the production of fungal wall degrading enzymes (Mamarabadi et al. 2008). However, there is evidence that *C. rosea* also induces the production of various systemic plant resistance proteins within the plant itself, in the presence of a pathogen (Roberti et al. 2008). Other mycopathogenic capabilities, such as hyphal penetration (Turhan 1993) and parasitisation of conidiophores (Morandi et al. 2001) have also been documented in established *B. cinerea* infections. In instances where *B. cinerea* is already present before *C. rosea*, the pathogen's sporulation is greatly reduced once the antagonist arrives (Morandi et al. 2001). The manner by which *C. rosea*

colonizes tissues is poorly understood, however it has been shown to colonize a variety of niches including soil, seeds, roots, and leaves (Lubeck et al. 2002). *C. rosea* is able to and is capable of germinating and growing in the presence of different fungicides (carboxin, guazatine, prochloraz, thiram and triticonazole) (Roberti et al. 2006), suggesting potential utility within IPM programs. Isolation of local isolates is on-going (Peng and Sutton 1991), leading to continual discovery, culturing, and reproduction of desirable strains. Formulations for spray application and vector-use are produced by university, government and industrial laboratories, with products commercially available through companies such as Adjuvant Plus Inc. (Endofine®).

1.5 Research Scope and General Objectives

A limited number of studies have examined the utility of the pollinator-vector technique for pest management, and its efficacy in many cropping systems is yet to be evaluated. The effects of vectoring microbial control agents on the pollinators also remains relatively unexamined, but may have implications for efficiency of dissemination and the level of control that it can provide without compromising pollination service. As an emerging technology, novel dispenser designs for applying biopesticides to pollinators are continually emerging, which require testing in order to determine designs that are user friendly, will maximize product distribution, and have minimal impact on hive health and behaviour (MacCagnani et al. 2005, Mommaerts et al. 2010b, Mommaerts et al. 2012).

The focus of this Masters of Science in Agriculture thesis project was to evaluate the effectiveness of *B. impatiens* mediated vectoring of *C. rosea* for *B. cinerea*

management in wild blueberries, while concurrently examining the influence of dispenser design on dissemination and various pollinator behaviours. The hypotheses tested were:

1. *Clonostachys rosea* can prevent, but not cure, *Botrytis cinerea* infections in *Vaccinium angustifolium* blossoms, based on the premise that its primary mode of action is inhibition by niche occupation
2. *Clonostachys rosea* germination will not be significantly affected by the presence of Switch® or Pristine®, but will be affected by Maestro®, based on the relatively high toxicity of the latter fungicide and multiple-site activity.
3. Dispensers will not significantly affect foraging behaviour, but will differ in their ability to apply products and provide disease control; consistent with previous published findings
4. Using hive-mounted dispensers, *Bombus impatiens* can vector *Clonostachys rosea* into wild blueberry fields, in sufficient amounts to significantly reduce *Botrytis cinerea* infection, as has been demonstrated in other cropping systems

Chapter 2. Efficacy and Compatibility of *Clonostachys rosea* for Integrated Management of Botrytis Blight in Wild Blueberry (*Vaccinium angustifolium*)

2.1 Introduction

Wild blueberry, also referred to as lowbush blueberry (*Vaccinium angustifolium*), is an economically important crop of eastern Canada and Maine. In 2010, Nova Scotia produced 16 395 marketable tons, with a farm gate value of over 22 million dollars (Morin et al. 2011). Current management of insect pests, weeds and diseases in the crop is accomplished mainly through the use of synthetic pesticides (Delbridge et al. 2011). However, due to increasingly stringent import restrictions on maximum residue limits (European Commission 2011) allowable products (Delbridge et al. 2011), and a general desire to develop integrated pest management (IPM) programs for wild blueberry, there is growing interest in incorporating biological pesticides (biopesticides) into management practices.

Botrytis cinerea Persoon:Fries, the causative agent of grey mould and Botrytis blight, is a commonly encountered pathogen in wild blueberry production. In *V. angustifolium* fields, *B. cinerea* overwinters as dormant mycelium or sclerotia in plant debris (Lambert 1990) or weed species (Hildebrand 2012a). Conidia are dispersed to plant tissues in the spring primarily by wind (Harrison and Lowe 1987) and splash from rain droplets (Jarvis 1962), although insects may potentially also serve as vectors of conidia (Silow 1933). Germination typically occurs on the expanded corolla, starting at the F5 floral stage and increasing to 98% germination during the F7 (full-open) floral stage (Hildebrand et al. 2001). The extent of infection was found to be dependent on

inoculum load, temperature, and length of wetting period (Hildebrand et al. 2001). Infections cause reductions in yield, likely by means of premature abscission (Hildebrand et al. 2001), while infected foliar tissues are destroyed by the grey mould, thereby reducing photosynthetic efficiency and acting as a source for infective inoculum (Howatt 2005). Severe *B. cinerea* infections can cause major economic losses, with infection levels as high as 35% in some areas (Howatt 2005). Although the economic threshold for fungicide application is around 10% (Ellison et al. 1998), many growers will apply fungicides as a preventative measure, even in the absence of disease pressure (Hildebrand 2010), since the risk for explosive outbreaks associated with missing a spray is too great (Hildebrand 2012a). Currently there is no established program for monitoring *B. cinerea* risk, although alternate hosts such as sheep sorrel (*Rumex acetosella* L.; a weed commonly found in blueberry fields) have shown potential as an indicator species (Hildebrand 2012a).

B. cinerea infections are typically prevented using an early spray of Pristine® (boscalid + pyraclostrobin), Switch® (cyprodinil + fludioxonil), or less commonly Maestro® (captan) (Delbridge et al. 2011). Growers have reported reasonably good control, with preliminary controlled environment experiments showing reductions in conidiophores production of 50%, 92%, and 50%, for the respective aforementioned fungicides (Hildebrand 2012b). A spray regimen of Pristine® and Switch® reduces Botrytis blight of blossoms in highbush blueberry by about 44% at peak bloom (Elmhirst and Smith 2010).

The microbial antagonist *Clonostachys rosea* (syn. *Gliocladium roseum*) has shown good efficacy for control of *B. cinerea* in a variety of fruiting crops and ornamentals (Peng and Sutton 1991, Yu and Sutton 1997, Morandi et al. 2000, Kapongo et al. 2008). Although a similar agent, *Gliocladium catenulatum*, is capable of protecting high-bush blueberry (*Vaccinium corymbosum*) from *B. cinerea* infection (Verma et al. 2006), it is unknown whether *C. rosea* is able to colonize and protect *V. angustifolium*. *C. rosea* has shown tolerance to a number of fungicides (Roberti et al. 2006), but its compatibility with fungicides commonly used for *B. cinerea* management in wild blueberry (Delbridge et al. 2011) is unknown, and thus its suitability for inclusion into an IPM program within this cropping system is unclear.

Herein I describe experiments evaluating the ability of *C. rosea* to prevent or cure *B. cinerea* infection in blossoms of *V. angustifolium* under greenhouse conditions. I also examined the *in vitro* susceptibility of *C. rosea* to three fungicides commonly used in *Botrytis* management.

2.2 Material and Methods

Plants and Cultures

Locally grown 3-year-old mixed clones (syn. cultivars) of *V. angustifolium* in 7.62 cm diameter pots, as well as a wild blueberry isolate of *B. cinerea* (B94.a1), were obtained from P.D. Hildebrand [1] in March, 2010. All plants were kept in cold storage (4°

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[1] Dr. Paul Hildebrand, Research Scientist,

C) until the start of experiment in mid-May, 2010. Plants were placed in a greenhouse (25° C ± 2° C, 16:8-:L:D) with treatments randomly assigned to the three plants within each block. Plants were watered daily and achieved >70% bloom after approximately 8 days. Every 4-6 days another group of plants was moved to the greenhouse in order to block the experiment over time, for a total of 5 blocks.

Working cultures of *B. cinerea* were derived from stock cultures, being grown and maintained on Kings B – Glucose Media, consisting of 38 g/L Pseudomonas agar F media, 10 g/L of dextrose – D-glucose (substituted for 10 g/L glycerine to reduce time to sporulation), mixed in distilled water (Hildebrand et al. 2001). Cultures were kept under fluorescent lighting at 20° C, and reached maturity after 5-8 days. Cultures were used for experiments when 8-11 days old.

A powder formulation of *C. rosea*, suitable for spray application, was provided by Dr. Peter Kevan at the University of Guelph in late March, 2010. The agent was refrigerated (4° C) between uses.

Treatment

A control plus two *C. rosea* treatment regimes (preventative and curative) were evaluated for inhibition of *B. cinerea* infection. The treatments were as follows:

1. Control: 10 µl *B. cinerea* suspension (10^5 spores/ml) applied to each flower (10^3 spores/flower) followed 24 h later with 10 µl per flower of sterile distilled water.

Atlantic Food and Horticulture Research Centre,
Agriculture and Agri-Food Canada,
Kentville, Nova Scotia

2. Preventative: 10 µl *C. rosea* suspension (1g/L) applied to each flower followed 24 h later with 10 µl of *B. cinerea* suspension (10^5 spores/ml) to each flower (10^3 spores/flower).
3. Curative: 10 µl *B. cinerea* suspension (10^5 spores/ml) applied to each flower (10^3 spores/flower), followed 24 h later with 10 µl of *C. rosea* suspension (1g/L) to each flower.

Attached flowers were inoculated by injecting the suspension into the open end of the flower using a P20 micropipette inside a biosafety cabinet. Plants were then placed in plastic sleeves, to prevent contact between plants, and translocated to a fogging box (61 cm x 32 cm x 42 cm) inside of a growth chamber (Conviron Inc., Model E15, Winnipeg, Manitoba). Unopened blossoms were marked and excluded from subsequent analysis. Plants were subjected to 8 hours of cool fog (sterile distilled water; Zoo med Repti Fogger™, San Luis Obispo, California) and kept in the chamber at 20° C, dark (Hildebrand et al. 2001). Plants were removed after 24 h, given the second treatment, and then returned to chamber for another 48 h at 20° C dark. Following the final exposure, blossoms were removed from plants and placed on Whatman #1 filter paper in 10 cm Petri dishes (minimum 5 millimetres between each blossom; maximum of one plant per plate), moistened with sterile distilled water. Plates were kept in a separate section of the chamber at 20° C, dark, and analyzed for growth of *B. cinerea* after three days. Blossoms were deemed positive for *B. cinerea* infection when both mycelia and conidiophores were present, as determined by examination of blossoms by specimen microscope (20-40x total

magnification). The numbers of exposed blossoms with, and without, infection were recorded for each plant.

The *B. cinerea* spore concentration and technique used to inoculate flowers were based on Mommaert et al. (2011a). Fogging exposure, temperature and spore concentration used were ideal for obtaining *B. cinerea* infection in blossoms at stage F7-F8 (Hildebrand et al. (2001).

Fungicide Tolerance

Media bottles (1 L) containing 19.5 g of Difco Potato Dextrose Agar, topped to 488 g with distilled water, were mixed and autoclaved, then cooled to 60° C in a water bath. Prior to pouring, 100 ppm streptomycin sulphate and 2000 ppm Triton X-100 were added to each bottle (Peng et al. 1992), then each was amended with one of four different treatments. Fungicides, reflecting suggested manufacturer maximum application rates, were topped to 10 g using autoclaved distilled water to dissolve them before being added to the media, followed by a 2 g rinse of the container using autoclaved distilled water to remove residues; for a total weight of 500 g. Treatments were as follows:

1. 10 g autoclaved distilled water (control)
2. Maestro 80 DF (80% captan)

   = 2250g/ha / 1000L/ha = 2.25g/L = 1.125g
3. Switch 62.5 WG (37.5% cyprodinil + 25% fludioxinil)

   = 975g/ha / 200L/ha = 4.875g/L = 2.435g
4. Pristine WDG (25.2% boscalid + 12.8% pyraclostrobin)

   = 1600g/ha / 208L/ha = 7.69g/L = 3.845g

Four replicate 1:10 *C. rosea* dilution series' were prepared, blocking over time, (Roberti et al. 2006, Luz et al. 2007) using 50 ml volumetric flasks starting from the recommended concentration of 1g/L. After all dilution series were created, each was plated independently using a sterile stainless steel spreader, with 100 μl aliquots of the dilution series' plated on the amended-media. Once inoculated, plates were stored at 22° C, dark. The numbers of colony-forming units (CFUs) present on each plate were counted starting on the fourth day after plating, although plates with no growth were kept as some treatments showed delayed growth. Only one count of each plate was performed, once colonies were of adequate size. Plates with counts inside the statistically acceptable range (30-300 CFU) were used, in combination with the dilution factor, to calculate the number of CFUs of *C. rosea* for each treatment (Madigan et al. 2003).

Statistics

All results were analyzed using analysis of variance (ANOVA) ($\alpha = 0.05$), blocking by replicate. Data were normal and met the assumption of homoscedasticity, except for the colony-forming unit variances, which were highly variable due to the magnitude of the response variable for select treatments. Multiple mean comparisons for significant ANOVA results were performed using Tukey-Kramer HSD. All data was analyzed using JMP 9.0.2 (SAS Institute Inc. 2011). Time to germination, although documented, was not included in analysis.

2.3 Results

The percentage of blossoms of plants that became infected with *B. cinerea* was significantly affected by treatment (F (2, 8) = 42.28, P< 0.0001) (Figure 2.1). However, infection prevalence in the control and curative treatments did not differ significantly. For the fungicide-amended media experiments, there was a delay in germination, and the number of colony-forming units germinated (Figure 2.2) was significantly affected by the fungicide present in the media (F (3, 9) = 12.72, P = 0.0014). There was a mildly significant effect of block (significant at α = 0.1). The numbers of colony-forming units germinated were significantly different for the media amended with either Pristine or Maestro, compared to the control or Switch.

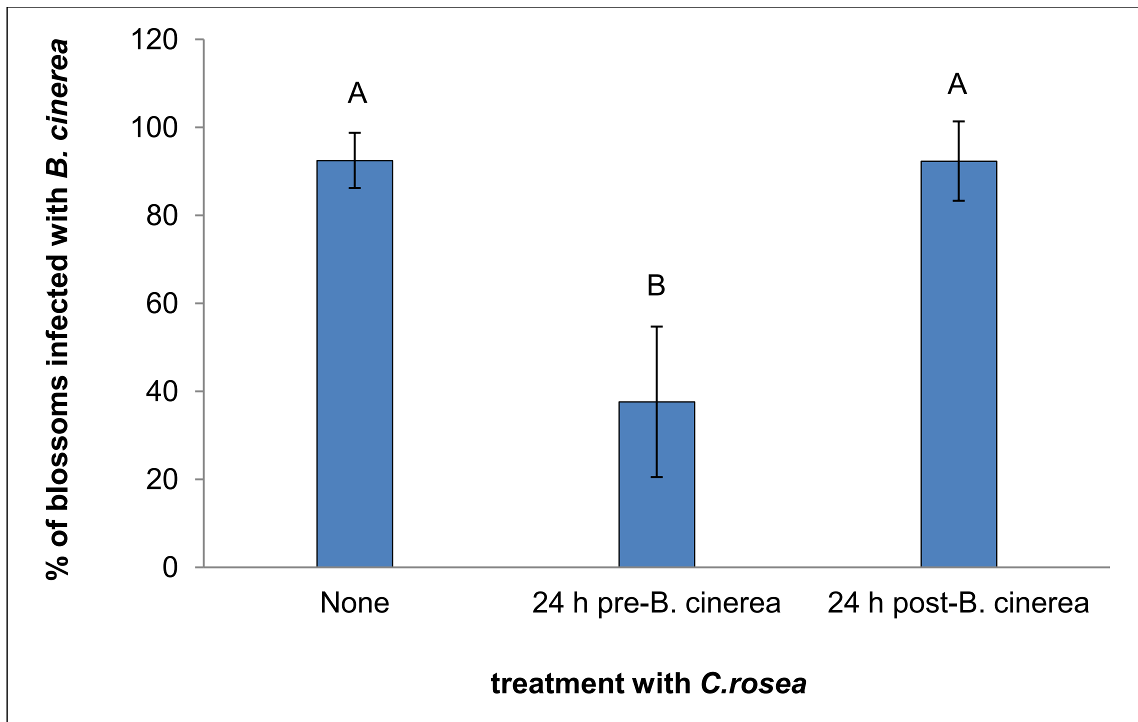

Figure 2.1. Percentage of blossoms infected with *Botrytis cinerea* (±SD) 3 days after incubation (22° C; dark), according to treatment with *Clonostachys rosea*. Bars with different letters are significantly different (α = 0.05; Tukey Kramer HSD).

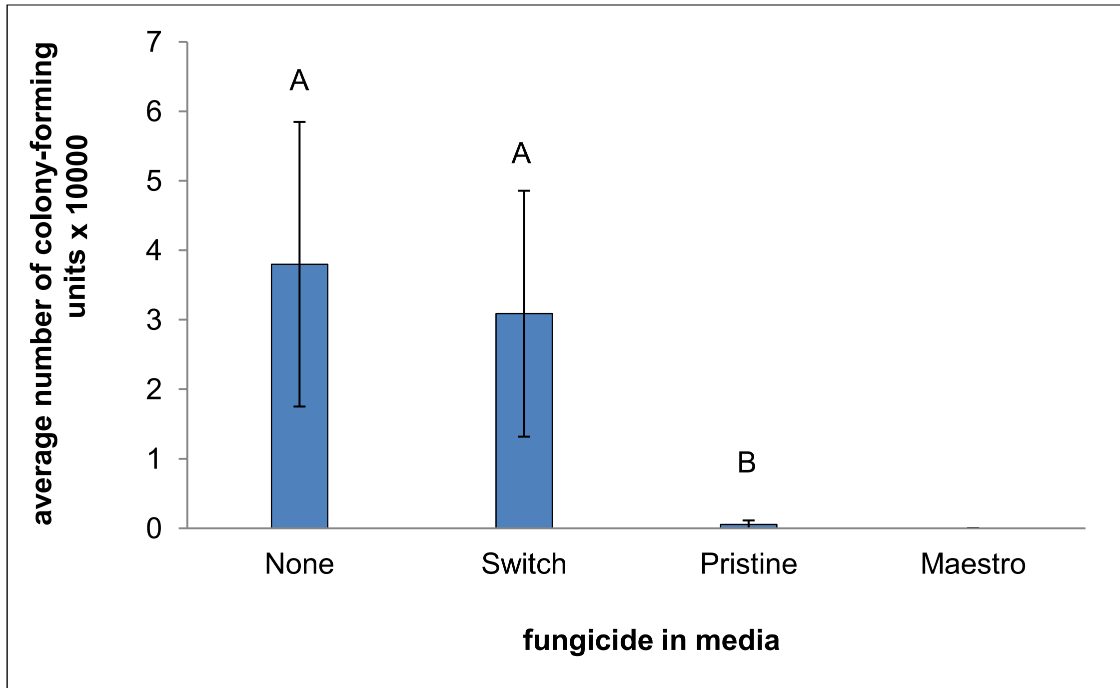

Figure 2.2. The number of colony-forming units of *Clonostachys rosea* germinated (±SD) according to the fungicide present in Kings B-glucose media. Bars with different letter groupings are significantly different ($\alpha = 0.05$; Tukey Kramer HSD).

2.4 Discussion

Consistent with previous findings (Sutton 2009), the results of the greenhouse experiment demonstrate that *C. rosea* must be present prior to plant attack in order to prevent infection by *B. cinerea*. This is in accord with the belief that *C. rosea* prevents *B. cinerea* infection through niche occupation, present as a latent infection. Although not examined in these experiments, it has been suggested that mychorrhizal associations of *C. rosea* may increase plant vigor by increasing nutrient uptake, resulting in increased yields in some crops. Prolonged use could theoretically increase resident populations of this beneficial fungus and thus this could potentially be an added benefit, although further research is required within this system. Since the antagonist proliferates during times of plant stress, and only sporulates when tissues die (Sutton et al. 1997), it also stands to reason that protection should be afforded for the entire season. Although *C. rosea* primarily inhibits infection by occupation, some mycopathogenic capabilities, such as hyphal penetration, have been documented (Turhan 1993), and thus the agent may reduce the severity of infections. In addition to endophytic activity, *C. rosea* is commonly found in soils demonstrating saprobiotic activity, where *B. cinerea* spores may overwinter (Schroers et al. 1999). The potential for pathogen – antagonist interactions within the soil community deserves examination.

As with synthetic fungicides currently registered for *B. cinerea* management in wild blueberry, *C. rosea* is also limited to prophylactic protection, being unable to cure established infections in blossoms. A disease reduction of ~50%, under ideal *B. cinerea* infection conditions, is comparable to the reduction provided by *C. rosea* in greenhouse

grown sweet pepper (Kapongo et al. 2008), and similar to the reduction in disease prevalence found for highbush blueberry at peak bloom using a spray regimen of Pristine and Switch (Elmhirst and Smith 2010). However, further research is needed to compare synthetic treatments to *C. rosea* under similar conditions.

The apparent compatibility of *C. rosea* with Switch *in vitro* has valuable implications. Exposure *in vitro* represents a worst-case-scenario exposure for biopesticides, as they come into continuous direct contact with nutrients that are bathed in the fungicide. As such, it seems that *C. rosea* could likely be applied as tank mix in conjunction with Switch, in order to reduce synthetic inputs and establish antagonist populations earlier in the season; although further testing to ensure viability of *C. rosea* would be required. Since preliminary tests have suggested that Switch is superior to the other two fungicides examined (Pristine and Maestro), with regard to inhibition of conidiophore production (Hildebrand 2012b), and the reduction in disease is comparable between Switch and *C. rosea*, it stands to reason that an IPM program utilizing both constituents has considerable potential. For such a program, an early spray of Switch could be supplemented during bloom with *C. rosea*, due to its safety to pollinators (Kapongo et al. 2008), thereby providing continuous protection throughout the growing season. Application during bloom could be performed as a spray, although there has also been considerable success using commercial pollinators as vectors of this agent for disease management (Peng et al. 1992, Yu and Sutton 1997, Kapongo et al. 2008, Reeh 2012).

Despite the significant effect of Pristine on the germination of *C. rosea*, the presence of some colony-forming units may indicate the possibility for generating tolerant strains. Additionally, although combination fungicides with multiple modes of action are preferred (in order to prevent resistance development), single component fungicides are still used. As such, further testing should be performed to elucidate whether single components from Pristine (Boscalid or Pyraclostrobin) may be compatible with *C. rosea*, and thus potential options for IPM inclusion.

The marginally significant effect of replicate during colony-forming unit determination suggests that the longer exposure of *C. rosea* to room temperatures while in solution may have had some adverse effects on the viability of spores. This should be more closely examined, as it has important implications for spray application in large operations.

C. rosea is permitted for use for pest management within organic production systems in Canada (Government of Canada 2011). The extent of its biosuppression of *B. cinerea* provides hope for both conventional and organic blueberry production in the face of increasingly stringent import regulations. The efficacy of *C. rosea* for management of other fungal pests of wild blueberry, including Valdensinia leaf spot and Septoria leaf spot, deserves further examination.

A second partially-redundant control, wherein blossoms would be inoculated with water then *B. cinerea* 24 h later, was excluded due to space limitations. This control would have served to demonstrate that the second incubation period (with only high RH) would also provide high *B. cinerea* infection levels. However, there was ample mention

throughout the literature that RH levels of >95% were more than sufficient for high *B. cinerea* infection levels.

In conclusion, the biopesticide *Clonostachys rosea* is an effective, organically compatible, option for management of *Botrytis cinerea* in wild blueberry production. The compatibility of this antagonist with at least one synthetic fungicide also used in *B. cinerea* management, and its safety to pollinators, indicates an excellent opportunity for the development of an integrated pest management program for *B. cinerea* in wild blueberry.

Chapter 3. Bumble bee Biovectoring for Disease Management in Wild Blueberry

3.1 Introduction

Insects have long been known to translocate and disseminate pathogens (Rees 1900, Leach 1935). Acquisition and translocation of plant pathogens occurs when they become captured on insect body hairs during pollination/feeding (Leach 1935). This transportation has recently been exploited by using managed pollinators as vectors of biological pesticides to control various crop pests (Peng et al. 1992). The technique is intriguing since it may provide direct delivery of the product to targets (blossoms and leaves) with little waste, reducing hazards from traditional application (e.g. over-spray, drift, etc.). If effective for field-crops, growers could also be spared significant time and money associated with conventional tractor-based pesticide application. The feasibility of this technique for disseminating powder formulations of different microbial control agents (MCA) using various managed pollinators for management of diseases and pests has been demonstrated in some greenhouse (Al-mazra'awi et al. 2006, Kapongo et al. 2008, Mommaerts et al. 2011a) and field crops (Johnson et al. 1993, Gross et al. 1994, Yu and Sutton 1997, Kovach et al. 2000, Dedej et al. 2004, Al-mazra'awi et al. 2006, Butt et al. 2008, Albano et al. 2009). However, less is known about the utility of the technique for other cropping systems and the effects of vectoring such products on pollinators.

Wild (syn. `lowbush`) blueberry (*Vaccinium angustifolium* Aiton) is an important crop in eastern Canada that is highly dependent on bees for cross-pollination and fruit set (Aalders and Hall 1961). Although both honey bees (*Apis mellifera* Linnaeus) and alfalfa leafcutter bees (*Megachile rotundata* Linnaeus) are commonly used for pollination in this

crop (Javorek et al. 2002), the eastern bumble bee (*Bombus impatiens* Cresson) has recently seen increased use due to commercial availability, high affinity for wild blueberry flowers (Whidden 1996), efficient transfer of pollen tetrads (Javorek et al. 2002) and ability to work in the sometimes 'less than ideal' weather conditions encountered in the Atlantic provinces (Stubbs & Drummond 2001).

Wild blueberry is afflicted by a variety of insect, bacterial and fungal pests (Delbridge et al. 2011). Among them is *Botrytis cinerea* Pers.:Fr., a necrotrophic fungal pathogen which infects over 235 plant species (Jarvis 1977). In *V. angustifolium* fields, *B. cinerea* overwinters as dormant mycelium or sclerotia in plant debris (Lambert 1990). Conidia are dispersed by wind (Harrison and Lowe 1987) and splash from rain droplets (Jarvis 1962) to plant tissues in the spring. Germination typically occurs on the expanded corolla, starting at the F5 floral stage and increasing to 98% germination during the F7 (full-open) floral stage (Hildebrand et al. 2001), coinciding with peak pollination activity (Wood 1965). Blossom infections cause reductions in yield, likely by means of premature abscission (Hildebrand et al. 2001), while infected foliar tissues are destroyed by the grey mould, reducing photosynthetic efficiency and acting as a source for infective inoculum (Howatt 2005). Severe *B. cinerea* infections can cause major economic losses for wild blueberry producers in the Maritimes (Lambert 1990). Some wild blueberry production areas may experience infection levels as high as 35% (Howatt 2005). Although no economic threshold for fungicide application has been established for *B. cinerea* on wild blueberry, growers in regions with a history of the disease will typically apply fungicides as a preventative measure, even in the absence of disease pressure (Hildebrand 2010).

Clonostachys rosea Schroers, Samuels, Seifert & Gams (formerly *Gliocladium roseum* Bainier) is an endophytic fungus that has shown the ability to protect plants from pathogens by pre-colonizing mature tissues, or those stressed by herbicides or disease (Sutton et al. 1997). It has shown considerable success in preventing grey mould (*Botrytis cinerea*) in some crops (Peng and Sutton 1991, Kapongo et al. 2008) and ornamentals (Morandi et al. 2000, Morandi 2008). Residing within the leaf after germination, *C. rosea* is more protected from the elements than other microbials, and thus has demonstrated tolerance to adverse environmental conditions (Morandi 2008). The antagonist is also capable of germinating and growing in the presence of a variety of fungicides (Roberti et al. 2006, Reeh 2012), suggesting potential utility within integrated pest management (IPM) programs. This biocontrol agent has been safely used for a number of pollinator-vector experiments in greenhouses (Kapongo et al. 2008, Mommaerts et al. 2011a) and a few small-acreage fruit crops (Peng et al. 1992, Yu and Sutton 1997).

Herein I describe experiments completed during 2010 and 2011 that examined vectoring of *C. rosea* by *B. impatiens* in wild blueberry fields. Several hypotheses were tested: (1) *C. rosea* will be disseminated over the full forage range of *B. impatiens*. If the MCA is recovered over the full range, it will provide evidence that the technique may be useful for field cropping systems; (2) Blossoms exposed to *B. impatiens* carrying *C. rosea* will have lower incidence of Botrytis blight. This would provide evidence that the MCA was delivered to blossoms in sufficient amounts to prevent infection; (3) Dispenser design will affect the distribution of the MCA and incidence of *B. cinerea* in blossoms, but not

foraging or aggression. Such would indicate that one dispenser design was superior to the other in terms of dissemination and disease control, yet that neither affected pollinator behaviour; (4) The presence of the MCA in a dispenser will not affect foraging or aggression. As such, the formulation of *C. rosea* does not affect measured pollinator behaviours.

3.2 Materials and Methods

3.2.1 2010 Field Experiment

Experiments were performed on a commercial wild blueberry field in Mt. Stewart, Prince Edward Island (46.377089°, -62.897620°). At ~5% bloom commercial *B. impatiens* hives (Koppert Canada Ltd., Scarborough, ON) were distributed in a randomized block design (n = 4), with each treatment separated by 150 m around the perimeter of the blueberry field. Individual hives were composed of a plastic nesting box with a Styrofoam piece beneath surrounding a bag of supplementary sugar solution, all tightly fit into a cardboard sleeve with ventilation holes. In order to protect the hives from rain and moisture, each was covered by a 64 cm x 76 cm piece of plywood attached to four wooden stakes using small L-brackets and screws. The cover was approximately 45 cm high at the front and 40 cm high at the back to provide about 5 cm clearance over the hive/dispenser and assist with run-off. Corrugated plastic was placed beneath the hives as a moisture barrier to the ground and cotton string attached to a plastic stake on each side of the hives anchored them in place.

The following biopesticide dispensers were randomly assigned within each block to hives: (1) empty wooden dispenser; (2) wooden dispenser with biocontrol agent; (3) Koppert dispenser with biocontrol agent. Wooden dispensers were mounted to the front of hives using zip-ties, while Koppert dispensers were mounted to the top using packing tape. The wooden dispenser (Figure 3.1) was made primarily of 6 mm thick plywood, except for the sides which used 12 mm thick plywood. The front entrance panel and divider between the upper and lower level were made of 3 mm Plexiglas. It was modelled after the original Yu & Sutton (1997) dispenser, with modifications by Al-mazra'awi et al. (2006) and Kapongo et al. (2008). Additional modifications were made in our lab, including switching the position of the return hole from the left to the right, and the tube to the lower tray vice-versa, since the dispenser was originally used with BioBest Canada Ltd. (Leamington, ON) bumblebee hives whose entrance/exit is reversed from the Koppert hives. The clear Plexiglas divider between the upper and lower chambers was also covered with black construction paper on the underside, to prevent disorientation of bees exiting or returning to hives. This dispenser had a lower level zigzag passage through which bees had to pass to exit the hive, and an upper level passage that bees had to use to enter the hive. The lower level maze contained the biopesticide, which bees became dusted with (primarily on their legs and ventral surfaces) as they exited the dispenser. The second dispenser ('Koppert') (Figure 3.2) was manufactured by Koppert Biological Systems Inc. (Netherlands). This plastic dispenser utilized an electronic sensor matched to a mechanical shutter that intermittently (every 20 bees) refilled the exit tube with powdered biopesticide fed from a top-loading reservoir. As bees exited the hive via

the exit tube, their legs and ventral services were similarly dusted with the MCA. Hives were outfitted with dispensers in the field and allowed to acclimate for five days before the addition of biopesticides.

A commercially produced powder formulation of *C. rosea*, Origro's Endophyte®, was used in the experiment. The product was received mid-May, 2010 and was kept refrigerated (4° Celsius) between uses. Ten g aliquots of the microbial agent were weighed into sterile 20 ml scintillation vials in the lab, the contents were added to dispensers in the field. Dispensers were cleaned and refilled with fresh product every 3 days thereafter. For the wooden dispenser, single aliquots were poured into the tray, and then lightly shaken to obtain a uniform depth throughout the tray (~0.5 cm). Aliquots were poured directly into the top reservoir of the Koppert dispensers. Hives were put into the field on June 5, 2010 and removed July 8, 2010. However, as bloom had ended, dispensers were not refilled after June 23, 2010.

Field Distribution. To examine distribution of the microbial agent in the field, blossom and leaf tissues were collected along transects at distances of 3, 30, 75, 150, and 250 m from each hive. Since hives were only spaced 150 m apart, it would not be possible to tell which bees were foraging at the 250 m mark. However, observations were not expected at this distance, and the marking was only included to detect anomalies outside the normal forage range of *B. impatiens*. Samples were collected five, 11 and 17 days after the first addition of the product. Along each transect, 20 full-open blossoms and 20 leaves (proximate to blossoms) were collected from random stems at each distance on each sampling date. Sterile tweezers and dissection scissors were used to remove tissues. Both

were cleaned using 70% ethanol between each distance. Samples of blossoms and leaves from each distance were stored in labelled sterile 1.5 ml snap-top vials. All vials remained at ambient temperature during transportation from the field, but were refrigerated at 4° C immediately upon return to lab. Tissues were subsequently surface-sterilized and cultured onto paraquat-chloramphenicol agar, with two plates of ten blossoms and two plates of ten leaves per replicate, distance, and sampling date, in the dark at 22° C for five days using the methodology of Peng et al. (1992). *C. rosea* growth was qualified by the presence of both verticillate and penicillate conidiophore growth (Schroers et al. 1999, Lubeck et al. 2002, Chatterton et al. 2008) when viewed under a light microscope (100 x). The percentage of blossoms and leaves with *C. rosea* growth were recorded for each replicate, distance, and sampling date. Data met the assumptions of normality of residuals and homescedasticity and were analyzed using analysis of variance with repeated measures for sampling date, incorporating fixed factors of treatment, block, distance, and the interaction of these factors.

Bee Aggression. Bee aggression was measured 11 days after introducing the product. This was performed between 1 p.m. and 3 p.m. on a temperate (24° C), sunny day when refilling the dispensers with the antagonist. Mock refill was performed on hives with dispensers containing no product. Aggression was assessed using a scale ranging from 0 to 3; with 0 representing no aggression and 3 representing extreme aggression. No aggression was recognized as no attempt by exiting bees to attack the experimenter during the refill; mild aggression was categorized as one to two bees hovering around the veil during refill; moderate aggression was categorized as greater than two bees hovering

around the veil and/or 1 attempting to sting through the veil or gain access beneath the veil during refill; extreme aggression was categorized as two or more bees attempting to sting through any surface and/or find access to bare skin. The resulting scores were compared among treatments using a Kruskal-Wallace test ($\alpha = 0.05$).

Forager Pollen Load. To determine if foraging loads differed among bees returning to hives with different dispensers, bees returning to hives were collected directly into individually labelled scintillation vials that were immediately frozen using dry-ice to preserve specimens for later analysis. Collections were done between 1 p.m. and 3 p.m. on June 23 (day 17), under sunny and warm (22.5° C) conditions. After transportation on dry-ice, specimens were kept frozen at -24° C. Pollen was subsequently scraped off the corbiculae of individual specimens into clean plastic weigh-boats using toothpicks, and pollen load per bee (g) was recorded. Total pollen weight attached was divided by the number of corbiculae it was removed from (1 or 2) per bee, as in some instances bees collected only had pollen on one leg. Pollen weight (g/leg) data were transformed using a square-root transformation in order to normalize residuals. The transformed data was analyzed using analysis of variance to compare pollen weight between treatments ($\alpha = 0.05$). Back-transformed data are presented in results.

3.2.2 Semi-Field Experiment

In 2011, pollination tunnels (Figure 3.3) were used to provide a semi-natural environment, while maintaining bees within designated treatments. Tunnels measured 1.83 m wide x 1.83 m high (at the centre) x 9.14 m long, and were constructed of galvanized tube framing anchored to a wood base (Multi Shelter Solutions, Palmerston,

ON). The tunnels were setup at the Wild Blueberry Research Station in Debert, Nova Scotia (45.443094°, -63.449229°), and arranged into four blocks. At the onset of bloom (<5% open blossoms), 30% shade cloth was placed over the tunnels to prevent bees from entering or escaping treatment plots. The shade cloth was removed on June 28, after all experimental tissues and data from the tunnels were collected. After the placement of shade cloth, bumble bees were allowed to forage freely on uncovered areas of the blueberry field for three days, in order to replenish colony stores and allow bees to adjust to the dispensers, and then randomly assigned to tunnels. Treatments (n = 4) consisted a commercial *B. impatiens* hive (Koppert Biological Inc., Scarborough, ON) with one of the following: (1) no dispenser (control), (2) a Koppert dispenser, and (3) a PK dispenser. Commercial hives were the same types as those used in 2010.

The Koppert dispenser described in the 2010 experiment and a new 'PK' dispenser patented by Dr. Peter Kevan (University of Guelph) were used in this experiment. The PK dispenser (Figure 3.4) was similar in design to the wooden dispenser previously described except it was oriented perpendicular to the hive entrance and exit and was constructed entirely of 3 mm thick opaque plastic. A cartridge used to contain the microbiological control agent replaced the lower level corridor of the wooden dispenser. Several posts within the cartridge prevented foragers from following a straight path to the exit, which was intended to maximize pick-up of the MCA by bees. Another modification was that the dispenser had mounting brackets that allowed better attachment to commercial hives, and a user-friendly access panel to access the MCA cartridge. The cartridge was changed every three days, rather than refilled, to ensure MCA viability,

increase ease of product replacement, and eliminate the need for cleaning. Although the Koppert dispensers were the same style as those used in 2010, batteries were replaced and attachment points between plastic components were re-enforced using hot glue to counter durability issues experienced with the units in 2010.

Dispensers were filled with 10 g of a powder formulation of *C. rosea* using the same technique as 2010, which was replaced every 3 days. Pre-packaged cartridges were not available for the new dispenser, so the existing trays were manually cleaned and refilled. A formulation of *C. rosea* produced at Guelph University was used, and stored under the same conditions as in 2010.

Since tunnels provided insufficient forage for a bumble colony, hives were contained within vented Plexiglas boxes (Figure 3.5), supplemented with a small weigh-boat of water, a 10 ml vial of 50% honey solution (mixed with distilled water, and containing a dental wick), and 10 g of freeze-dried pollen (Cosman and Whidden Honey Ltd., Wolfville, NS). Supplements were refreshed every two days, and closed during the alotted foraging periods. Plexiglas boxes were opened to the tunnel for an experimental foraging period of 20 minutes each day to prevent over-foraging, based on the observations of Mommaerts et al. (2011a), since over-foraging can damage flower receptacles and inhibit fruit set. The Plexiglas cages also provided protection from rain and wind, replacing the plywood covers and posts used in 2010.

Handling Time. In order to assess potential impacts of dispensers on foraging behaviour of *B. impatiens*, the amount of time required for bees to process flowers during feeding was examined. During the daily 20 minute experimental periods that bees were allowed to

forage in tunnels, personnel followed the paths of bees exiting the hive and used stopwatches to time the amount of time (seconds) spent at each of the first 5 flowers visited. Flower visit time was measured on three days (6, 8 and 17 June 2011) between 10 a.m. and 2 p.m. Data were not used if a bee could not be tracked directly from the hive to all five flowers. Tracking was performed on days where temperatures allowed sufficient hive activity (17° C - 22° C). Handling time values (seconds) were transformed to obtain normality using an exponent of -0.5, based on the results of a Cox-Box plot. Transformed values were analyzed using analysis of variance ($\alpha = 0.05$).

Field Control of Botrytis Blight. Greenhouse experiments (Chapter 2) indicated that treatment with *C. rosea* could significantly reduce the ability of *B. cinerea* to infect wild blueberry blossoms. In order to determine whether *C. rosea* could be vectored by *B. impatiens* to prevent *B. cinerea* infection of blossoms in the field, all open blossoms on randomly selected stems (totalling 50 blossoms), were inoculated with 10 µl of *B. cinerea* suspension (10^5 spores/ ml sterile distilled water; 10^3 spores/flower) (Mommaerts et al. 2011a). Since the number of blossoms present was variable, the number of stems treated varied slightly between treatment dates. Inoculation was accomplished by inserting a sterile 200 µl pipette tip (P20 micropipette) into the opening of the blossom. Pipette tips were changed intermittently, and closed blossoms were marked with Sharpie marker so as to not be included in analysis. Inoculations were performed on day 2 of the tunnel experiment (June 6, 2011), day 5 (June 8, 2011), and day 14 (June 17, 2011), with 50 different blossoms each date. Multiple inoculation dates were used to evaluate changes in protection and/or infection over the bloom period. The *B. cinerea* isolate (B94.a1) used

for inoculations was obtained from local wild blueberry leaves by P.D. Hildebrand [2], and maintained on Kings B – Glucose agar (Hildebrand et al. 2001). Cultures reached maturity in 4-5 days and were used to create suspensions when 8-11 days old. Since the Debert area does not typically receive sufficient humidity for appreciable *B. cinerea* infection, immediately following each inoculation small transparent plastic bags were placed over the blossoms and sealed at the stem using a twist-tie to ensure high humidity (Mommaerts et al. 2011a). Stems were flagged by date of inoculation after bagging. Once blossom abscission was complete (June 27, 2011), bags containing the blossoms were removed. Any blossoms that had not yet dropped into the bag were carefully removed and placed into the bag using sterile tweezers. Once returned to lab, blossoms were refrigerated (4° C) until plating. Blossoms not coloured with marker were plated in 10 cm diameter Petri plates on Whatman #1 filter paper moistened using autoclaved distilled water, with a maximum of 10 blossoms per plate. Plates were incubated at 22° C in the dark for 3 days then analyzed under a dissection microscope for the presence of *B. cinerea* on blossoms. *B. cinerea* infection was qualified by the presence of conidiophores (Kendrick 2000, Williamson et al. 2007, Plant Disease Diagnostic Clinic 2011). The percentage of inoculated blossoms showing *B. cinerea* infection was recorded. Data met the assumptions of residual normality and equivalency of variance, and were analyzed

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[2] Dr. Paul Hildebrand, Research Scientist,
Atlantic Food and Horticulture Research Centre,
Agriculture and Agri-Food Canada,
Kentville, Nova Scotia

using analysis of variance to compare treatments with repeated measures for the different inoculation dates ($\alpha = 0.05$).

Hive Activity. After all blossom tissues were collected, the Plexiglas hive cages were moved directly outside their respective tunnels and opened to allow free foraging of bumble bees on the few remaining blossoms not contained within the tunnels. At this time, hive activity was measured to determine if the presence of dispensers impacted hive activity. On June 23 and 24, hive activity was assessed by counting the number of bees exiting the hive per ten minutes in the morning (between 9 a.m. and 10 a.m.) and afternoon (between 2 p.m. and 3 p.m.). Conditions were sunny with fair temperatures (temperature range 16° C – 18° C and 22° C – 23° C for both days, for morning and afternoon respectively). Observations were performed from a distance so as to not elicit defensive behaviours. The number of exiting bees per ten minutes were averaged between the four observation periods and compared among blocks using analysis of variance, ($\alpha = 0.05$). Data displayed normality of the error terms and equivalency of variance.

Bee Self-Grooming. During hive activity counts, occurrences of self-grooming while exiting the dispenser were recorded as a proportion of the total number of bees exiting the hive. Self-grooming or preening was defined as brushing of the head using the fore tarsi, followed by cleaning the tarsi using the maxillae.

Colony Forming Units by Dispenser. In order to determine the average number of colony forming units (CFUs) of *C. rosea* present on bees exiting each dispenser, 3-4 bees exiting each hive/dispenser in the field were captured directly into sterile 20 ml scintillation vials, then brought back to the laboratory where they were refrigerated until

analysis 3 days later. Often, multiple bees (2-3) could be captured into a single vial, although, some vials only contained 1 specimen. Captures were performed on July 5, 6 and 7, to coincide with fresh MCA after the refill on day 1, and existing MCA on day 2 and 3 (last day before the next refill). Bees died as a result of hypoxia in sealed vials. The procedure performed replicated the methods used by Yu and Sutton (1997). Bees from each replicate hive/date (3-4 bees per hive) were poured into respective sterile 500 ml Erlenmeyer flasks, then 100 ml of sterile distilled water was used to rinse vials. The 100 ml of wash water was added to the respective flasks. This resulted in a final volume of 100 ml plus bees in each flask for each hive on each day. Fifty µl of surfactant (Triton X-100) was added to each flask, and then swirled to mix. All flasks were placed on a rotary shaker at 110 rpm for one hour. Four - $1/10^{th}$ serial dilutions were created using the resultant bee+water+surfactant solutions after agitation, of which three subsample volumes of 100 µl were plated on respective Petri plates containing potato dextrose agar, amended with 100 ppm streptomycin sulphate and 200 ppm Triton X-100. This was performed using a sterile stainless steel spreader within a laminar flow hood. Plates were incubated at 22° C ± 2° C in the dark for 4-5 days until colonies were of sufficient size for counting. Plates with colony counts within the statistically appropriate range (30-300 colonies) (Madigan et al. 2003) were used to calculate the number of colony forming units per bee. CFU counts were averaged using the three subsample platings. Data from two samples from Koppert dispensers were excluded from statistical analysis (Day 1, Block 1; Day 3, Block 3), since exiting pollinators pushed mounds of the MCA powder from the exit tunnel into vials during collection resulting in highly inflated values. Data

were normal with equal variance. Analysis of variance with repeated measures for sampling day was used to compare treatments, blocking by block (α = 0.05).

Berry Maturity, Size and Yield. On August 8, prior to commercial harvest of the field, three 1 m^2 subsamples were harvested from all pollination tunnels using hand-rakes, avoiding stems that were used for *B. cinerea* inoculation experiments whenever possible. Three 1 m^2 subsamples were also harvested from one area outside the tunnels to compare pollination adequacy within the tunnels. All berries were counted and sorted, and the ripe berries weighed (by subsample), to obtain data on the percent of berries harvested that were ripe, the number of ripe berries per square-metre and the average ripe berry weight. Berries were considered ripe if at least dark pink (colour code: #aa0033). To normalize the data, the yield of ripe berries (per square-metre) was transformed using a square-root transformation. The proportion of ripe berries was also transformed, except using an arcsin transformation. Berry weight had normal residuals. Analysis of variance was performed on each variable to compare among treatments, (α = 0.05).

For all analyses, assumptions of normality and homoscedasticity were checked using Minitab 16 (Minitab Inc. 2010). Multiple mean comparisons for significant analysis of variance results were performed using JMP 9.0.2 (SAS Institute Inc. 2011). A Tukey-Kramer HSD means separation test was used for all experiments, except for *B. cinerea* suppression experiments which utilized LSD due to the expectation of higher experiment-wise error. Analyses that required repeated measures were analyzed using SAS 9.1.2 (SAS Institute Inc. 2006). All graphs are presented using back-transformed data.

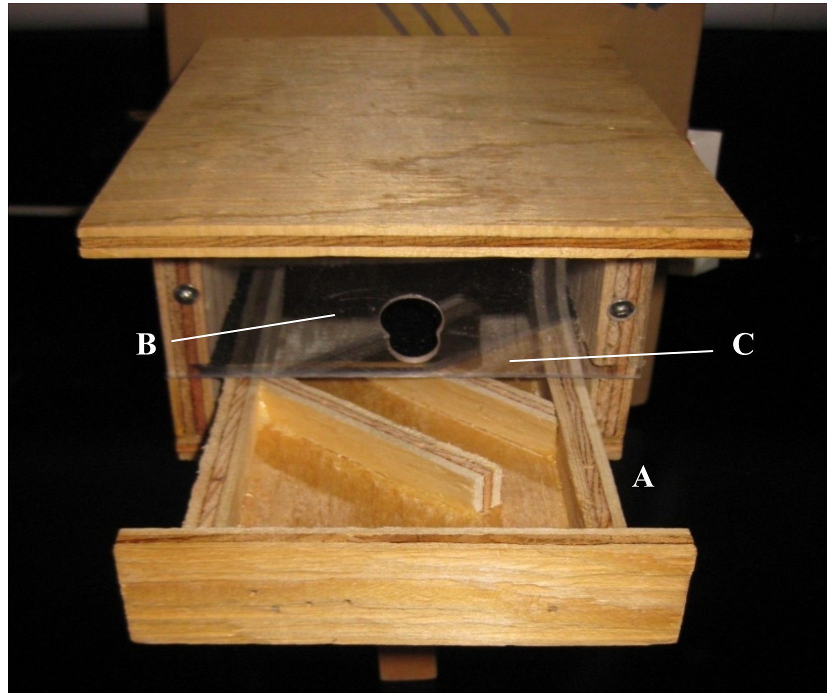

Figure 3.1. Wooden dispenser for mounting on a bumble bee hive for bee vectoring of microbial biological controls. (A) Lower-level maze (filled with powdered biopesticide) – closed except during re-fill; (B) Upper-level return to hive; (C) Gap in the partition allowing exit from lower-level maze to the upper level (other side of Plexiglas).

Figure 3.2. Koppert Biological Inc. top-loading mechanical biopesticide dispenser attached to a bumble bee hive for bee vectoring of biopesticides. (A) Dispenser exit tunnel wired with electronic sensor, and filled with biopesticide; (B) Return entrance to hive; (C) Top-loading reservoir for biopesticide, attached to gate mechanism and battery pack with electronics.

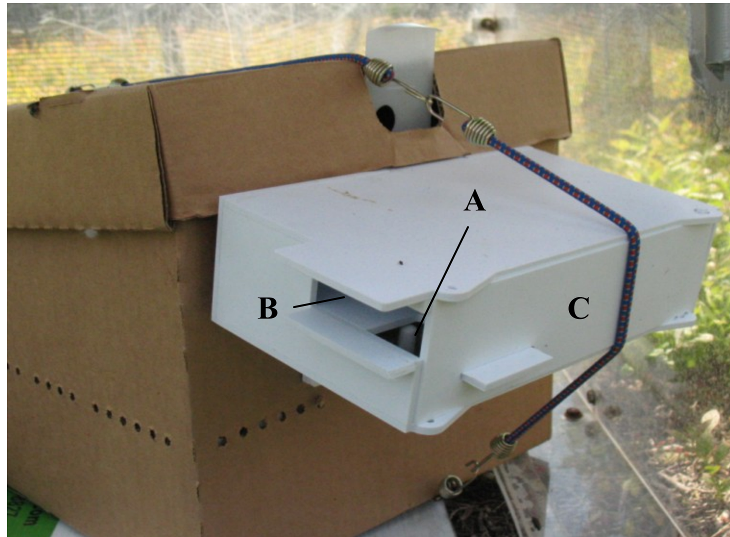

Figure 3.3. PK biopesticide dispenser attached to bumble bee hive for bee vectoring of biopesticides. (A) Exit from lower-level biopesticide cartridge; (B) Return entrance; (C) Access panel to biopesticide cartridge.*Note: Bungee cord was added mid-bloom to maintain a tight fit between the dispenser and hive, due to cardboard sagging.

Figure 3.4. Pollination tunnels (Multi Shelter Solutions, Palmerston, ON) used for 2011 semi-field experiment.

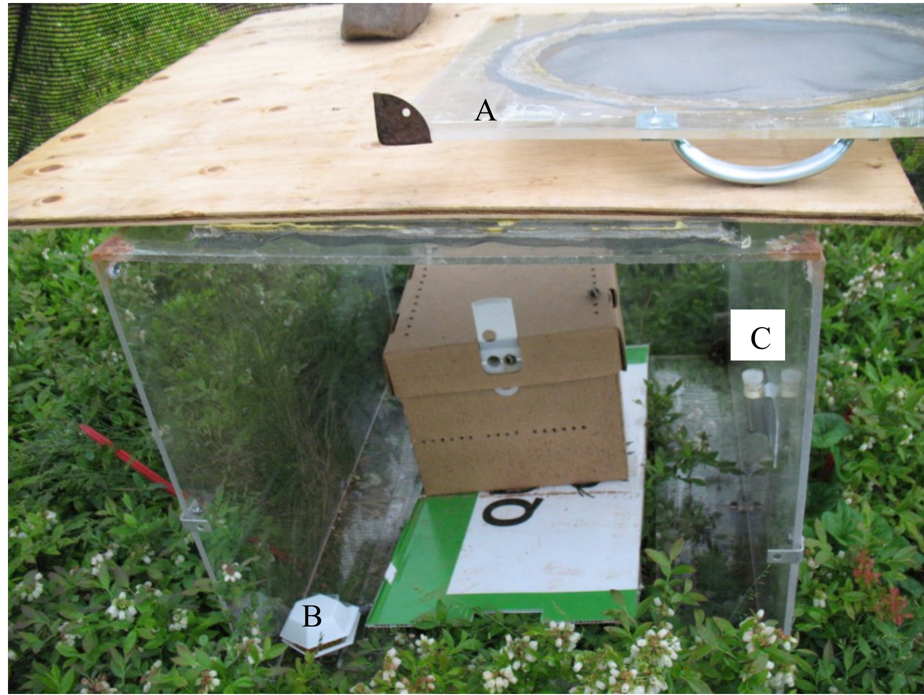

Figure 3.5.Vented Plexiglas cage used to protect bumble hives in semi-field experiment. (A) Vented front panel that slides down over front of hive to prevent bees from accessing the tunnel between experimental pollination periods; (B) Dish of supplemental pollen; (C) Vial of 50% honey solution.

3.3 Results

In 2010 experiments, there was no significant effect of dispenser type on the 'per-leg' pollen weight (Figure 3.6) obtained from foragers returning to hives ($F_{2,22} = 0.25$, $P = 0.784$). Observed aggression based on the subjective scale used was not significantly different between dispenser designs ($H_2 = 4.11$, $P_{adjusted} = 0.128$). Dispenser design had no effect on the incidence of *C. rosea* in leaves ($F_{4,175} = 1.26$, $P = 0.288$) or blossoms ($F_{4,175} = 0.98$, $P = 0.418$) at different distances (Figure 3.7) and also had no significant effect on overall *C. rosea* growth (Figure 3.8) in leaves ($F_{2,177} = 1.40$, $P = 0.25$) or blossoms ($F_{2,177} = 1.54$, $P = 0.217$). However, the percentage of inoculated blossoms did vary significantly with date of sampling ($F_{2,177} = 3.18$, $P = 0.044$) (Figure 3.8). Despite graphical indications, there were no significant interactions between any components.

In the 2011 semi-field experiment, there were no significant differences in flower handling time (Figure 3.9) between treatments ($F_{2,50} = 0.61$, $P = 0.547$). Handling time also did not significantly differ between the five flowers visited ($F_{4,50} = 0.34$, $P = 0.850$). However, the number of bees exiting the hive per minutes (Figure 3.10) was significantly affected by treatment ($F_{2,6} = 6.57$, $P = 0.031$), with approximately 3-fold more bees exiting hives without a dispenser than bees exiting hives outfitted with the PK dispenser. There were no recorded occurrences of self-grooming at any of the dispenser or hive exits during formal observation. *B. cinerea* suppression in blossoms (Figure 3.11) was found to be significantly affected by treatment ($F_{2,28} = 14.53$, $P < 0.0001$), with flowers exposed to pollinators from dispensers having a significantly lower proportion of blossoms infected with *B. cinerea*. However, dispensers were not significantly different from each other. *B.*

cinerea infection of blossoms was also significantly affected by inoculation date ($F_{2,28}$ = 18.97, $P < 0.0001$), with the June 6, 2011 inoculation having lower infection levels than the latter two dates. The number of CFUs of *C. rosea* obtained from bees (Figure 3.12) were not significantly affected by dispenser design ($F_{1,15}$ = 1.30, P = 0.273) or by the date of sampling after introducing the product (Day 1, 2 or 3) ($F_{2,15}$ = 2.18, P = 0.148). Upon harvesting, it was found that there was no significant difference in the proportion of ripe berries ($F_{3,9}$ = 0.67, P = 0.589) or average ripe berry weight ($F_{3,9}$ = 0.80, P = 0.523) among treatments from berries collected from open plots. However, berry yield (Figure 3.13) was significantly affected by the exposure ($F_{3,9}$ = 12.10, P = 0.002), with the open field having significantly higher yield than all experimental treatments.

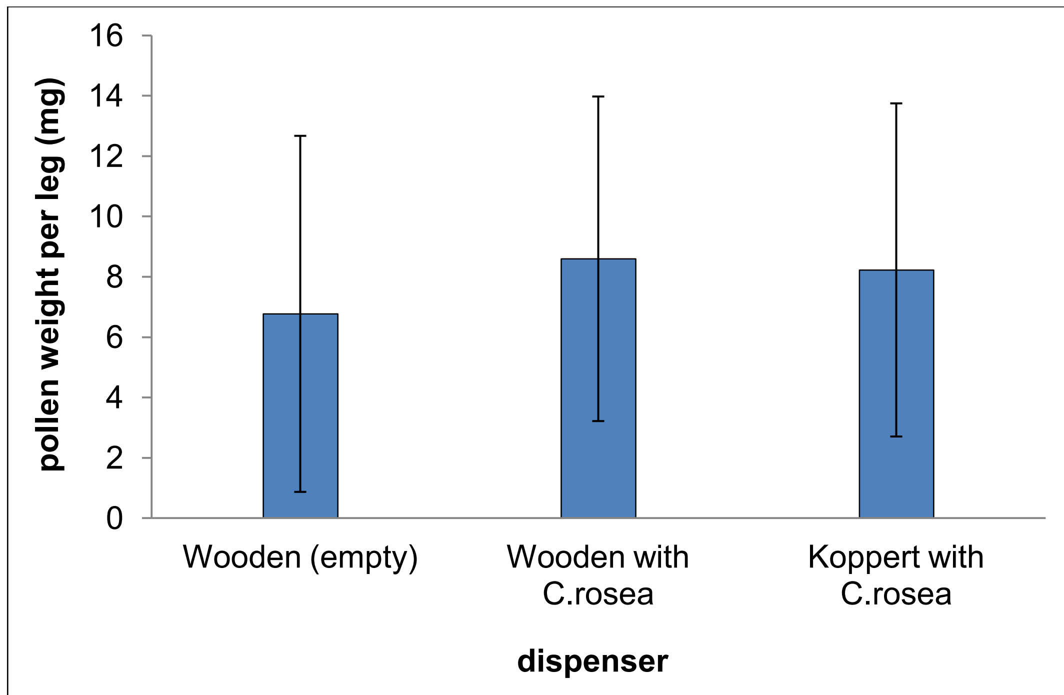

Figure 3.6. Pollen weights (± SD) recovered from *B. impatiens* returning to hives fitted with different dispensers (used to load bees with microbial biopesticides) containing powdered *Clonostachys rosea*. No significant differences ($\alpha = 0.05$).

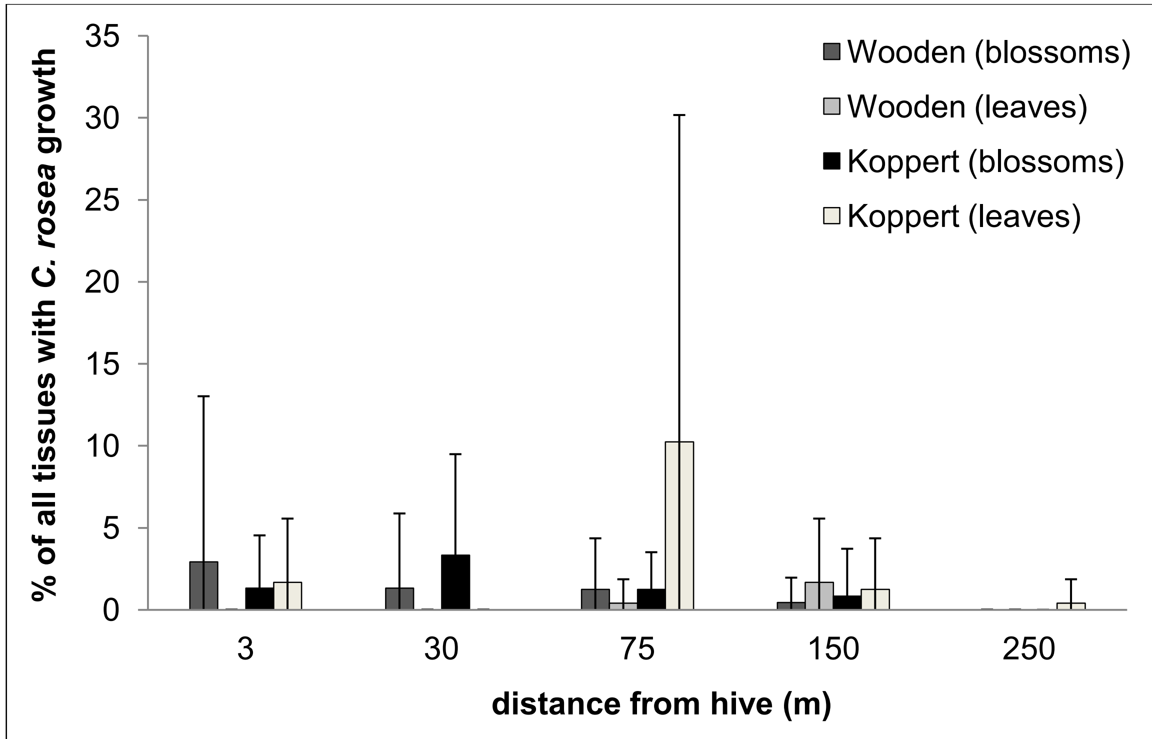

Figure 3.7. Average percent of field-collected blueberry tissues (± SD) with *C. rosea* growth, from transects exposed to *B. impatiens* hives outfitted with wooden or Koppert dispensers containing *C. rosea*. No significant differences or interactions ($\alpha = 0.05$).

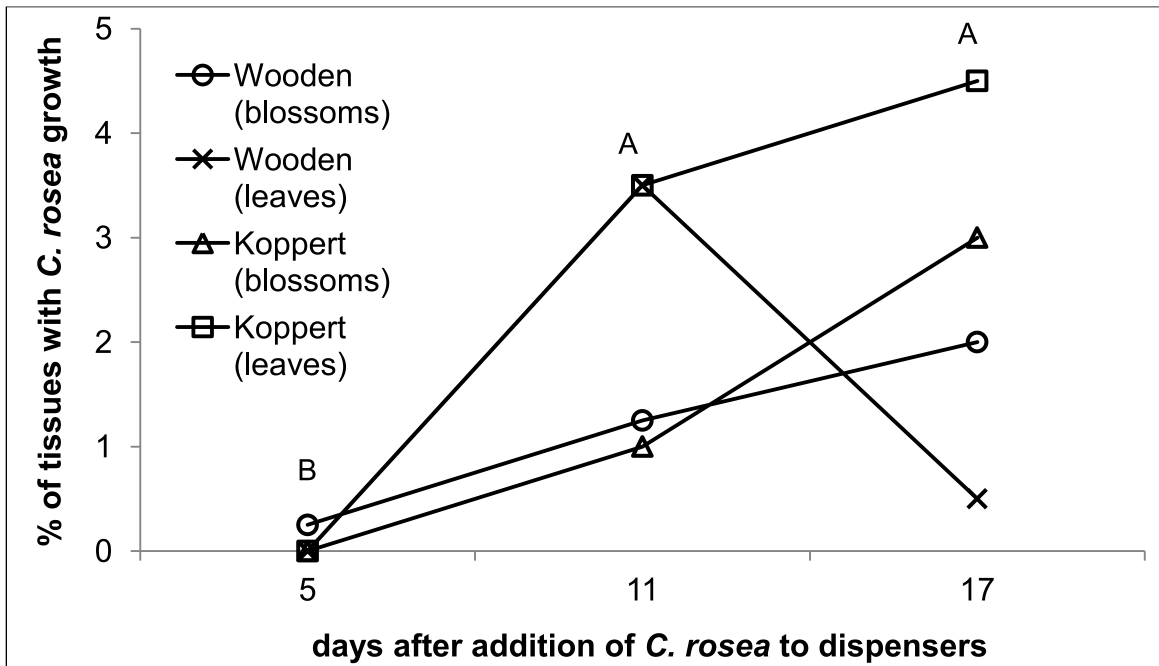

Figure 3.8. Incidence of *C. rosea* in blueberry tissues collected along transects at various distances from *B. impatiens* hives outfitted with wooden or Koppert dispensers containing Origro's Endophyte. Days with different letter groupings are significantly different ($\alpha = 0.05$; Tukey-Kramer HSD). There were no significant interactions. Error bars were omitted to maintain legibility of the figure.

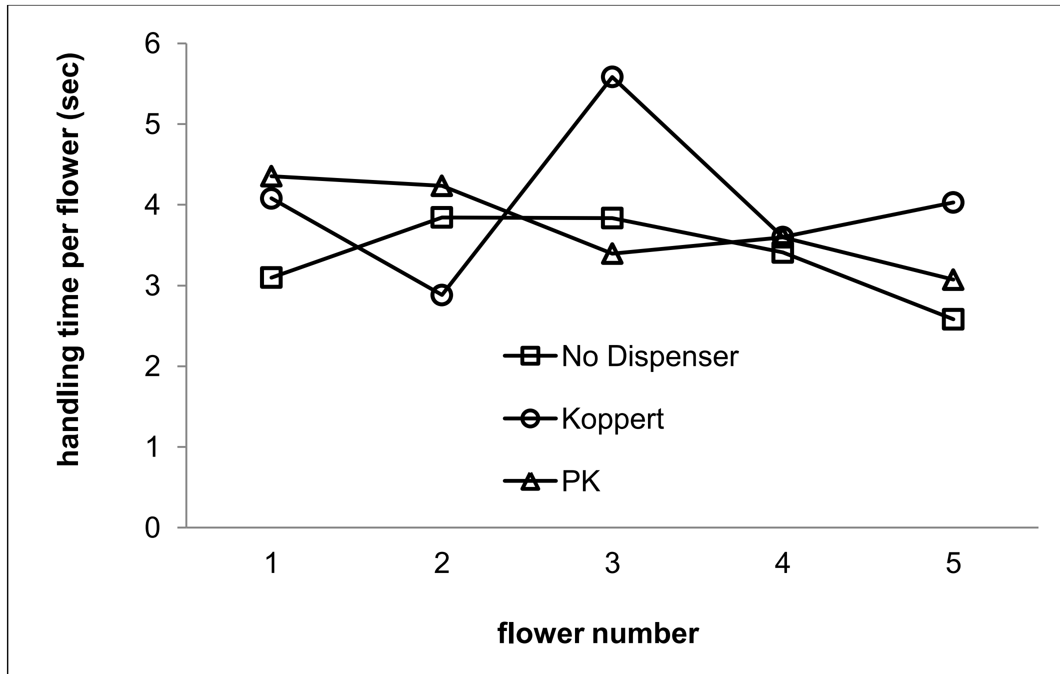

Figure 3.9. The handling time spent per flower by *B. impatiens* foragers, for the first five flowers visited after leaving a hive fitted with either no dispenser, a Koppert dispenser, or a PK dispenser. Dispensers contained the biopesticide *Clonostachys rosea*. No significant differences ($\alpha = 0.05$). Error bars were omitted to maintain legibility of the figure.

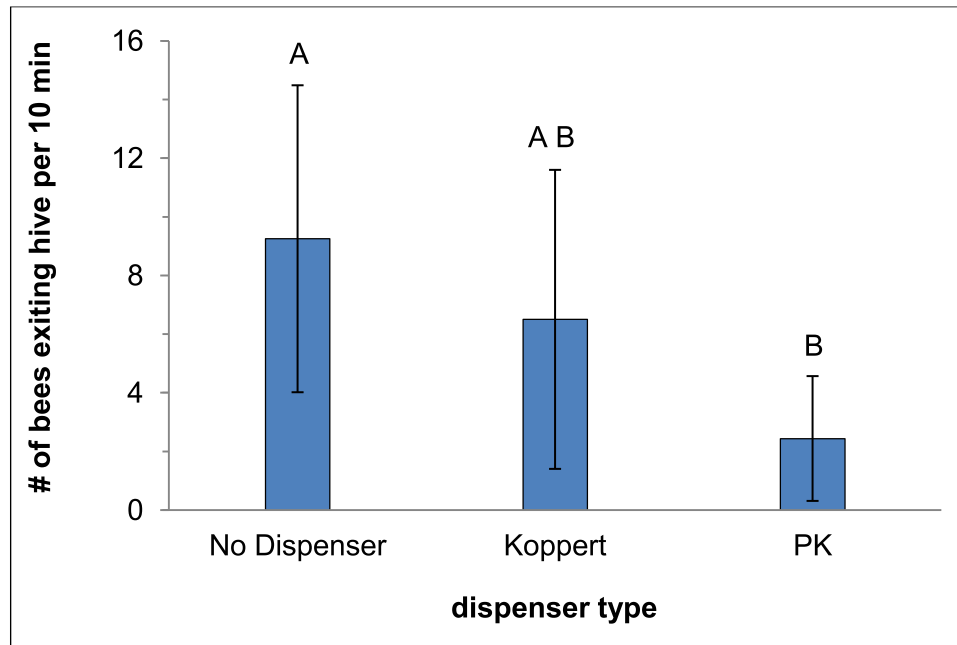

Figure 3.10. Average number (± SD) of *B. impatiens* foragers exiting a hive per 10 minutes according to dispenser. Bars with common letter groupings are not significantly different (α = 0.05; Tukey Kramer HSD).

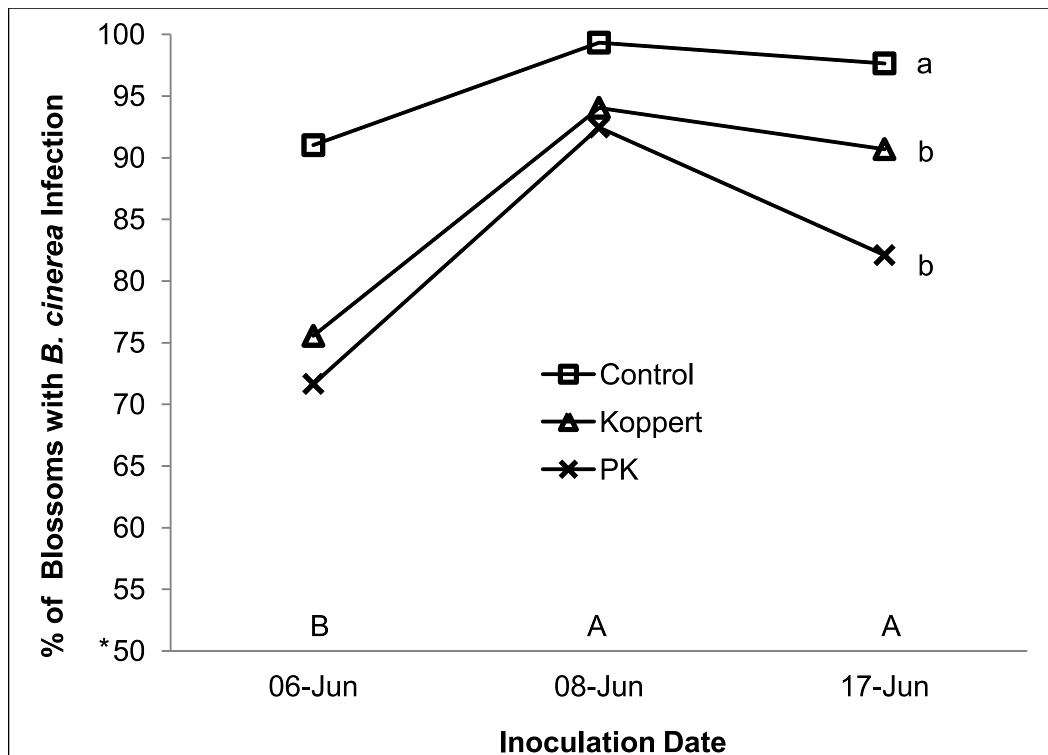

Figure 3.11. Average percent (back transformed) of *V. angustifolium* blossoms inoculated with 1 x 10^3 *Botrytis cinerea* spores that developed an infection after exposure to bumble bee hives fitted with biopesticide dispensers (Koppert, PK) containing *C. rosea*, or control hives with no dispenser. Lines and dates (see x-axis) with different letter groupings are significantly different (α= 0.05; LSD). *Note y-axis scale

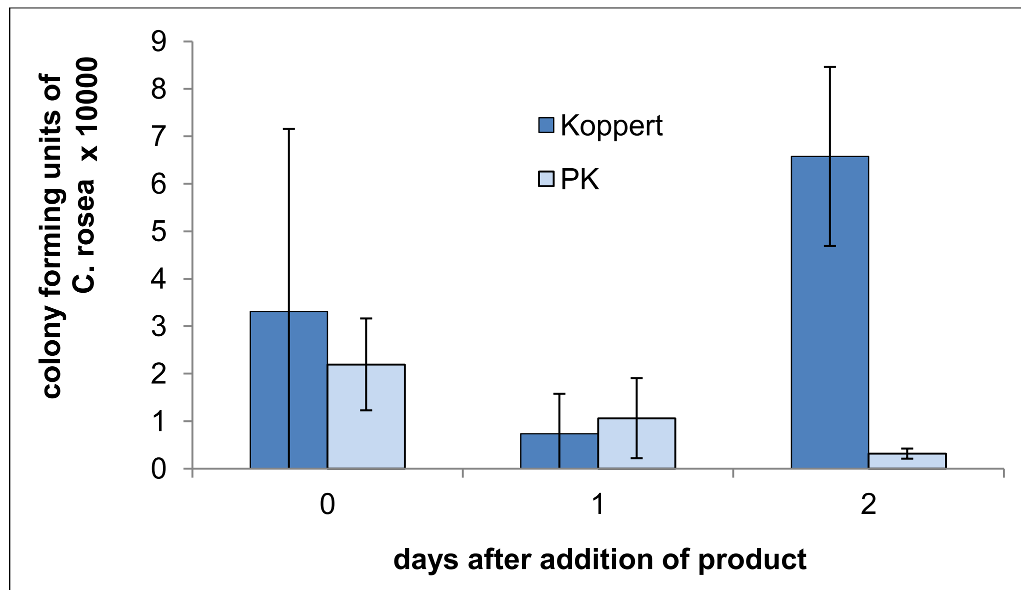

Figure 3.12. Average number of colony forming units (± SD) of *Clonostachys rosea* derived from serial dilutions of washes from bees exiting hives fitted with either the Koppert or PK biopesticide dispenser. No significant difference between dispensers ($\alpha = 0.05$). Note that y-axis scale represents value times 10,000 colony forming units.

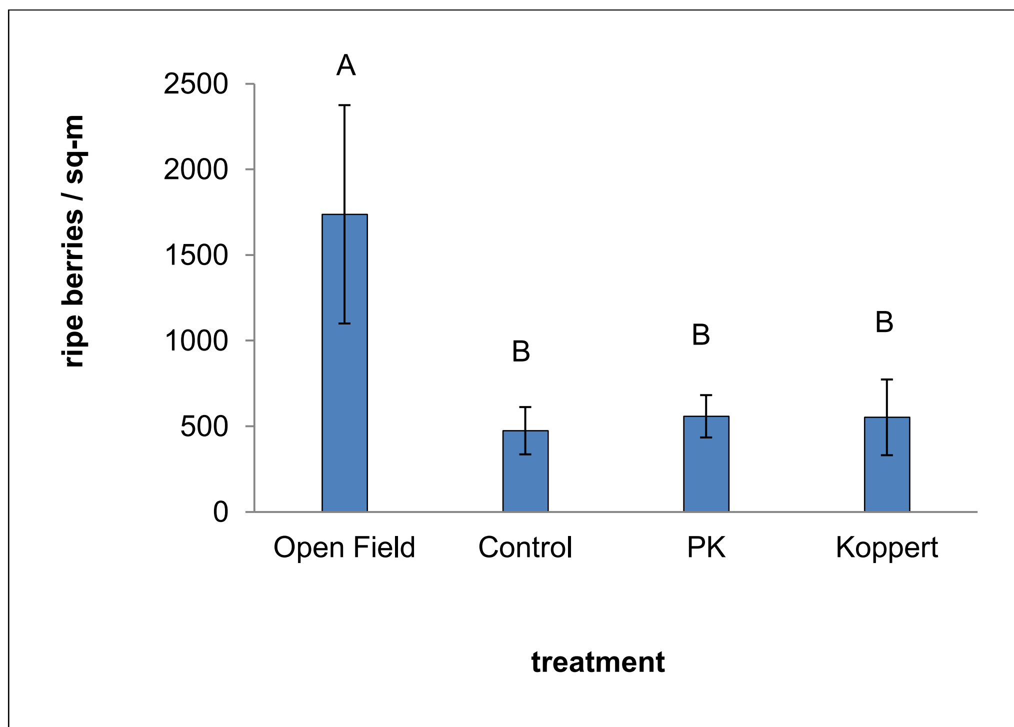

Figure 3.13. Yield of ripe berries (per square-metre) harvested from pollination tunnels with bumble bee hives, with or without dispensers containing *Clonostachys rosea*, and open field. Bars with different letter groupings are significantly different ($\alpha = 0.05$; Tukey Kramer HSD).

3.4 Discussion

Although it has been shown that 95% of the initial biopesticide is lost in the first 60 seconds of flight (Mommaerts et al. 2010b), the microbial antagonist was consistently able to be recovered from blossoms at distances up to 150 metres from hives in the 2010 field experiment, consistent with the typical near-nest foraging patterns of *B. impatiens* (Desjardins and DeOliveira 2006). This demonstrates both that the MCA can be vectored into wild blueberry fields, and that it can be disseminated appreciable distances. Although the tissues collected from the field in 2010 showed low incidence of *C. rosea* growth, the hive densities used were less than 1/30th of those recommended for blueberry production (8-10 hives/ha). The high variability in tissues showing *C. rosea* growth may have been an artefact of low hive densities or due to sampling error as a result of the large acreage of the plots. It is recommended that future field distribution experiments utilize green-fluorescent-protein (GFP) labelled strains of *C. rosea* (Lubeck et al. 2002) in order to allow larger collections and to expedite sample processing.

Pollination tunnel experiments in 2011demonstrated that *B. impatiens* can distribute biopesticides in lowbush blueberry fields in sufficient amounts to provide significant reductions in *B. cinerea* infection of blossoms. I attempted to mimic appropriate hive stocking density in 2011 trials by restricting the amount of time that hives were allowed to forage in the tunnels. However, the experimental forage times were extrapolated from the methods and observations of greenhouse experiments by Mommaerts et al. (2011a); conditions which proved to be quite incomparable. Cool, windy and rainy conditions were experienced during the majority of the bloom period in

the 2011 season and as a result the experimental forage time may have been inadequate, due to reduced foraging activity (Lundberg 1980). Pollination deficit within the tunnels was apparent based on the significant difference in fruit set between the pollination tunnels and the open field. Open field yield (per square-meter) was approximately three times higher than any of the pollination tunnels. If the shade cloth were responsible for the drastic decrease in yield, due to inhibition of photosynthesis, the other parameters measured (berry weight and proportion of berries ripe) would have been likely to significantly vary as well; whereas they did not. Therefore, it is likely that with proper stocking densities even better levels of disease management would be achieved. The significant effect of inoculation date, and only moderate reduction of the proportion of blossoms that showed *B. cinerea* infection, was also likely the result of climatic conditions affecting both pollinator (Lundberg 1980) and antagonist activity (Sutton and Peng 1993). Although temperatures were moderate on the days leading up to the first two inoculations (June 6, 2011 and June 8, 2011; average temperatures of 11.5° to 17.3° C), there were two rainfall events (~5 mm) each and wind speeds of 39 km/h before the second inoculation. Temperatures remained cool (average temperatures of 8.8° to 11.4°C) and wind speed was typically elevated (>31 km/h) on the days leading up to the third inoculation (June 17, 2011; Environment Canada – Debert, NS). Since hives were only open for 20 minutes per day, it is difficult to ascertain the exact conditions experienced during the allotted foraging time, and thus hive activity, each day. While *C. rosea* still provides significant control as low as 10° Celsius, colonization may not be as effective as at higher temperatures (Sutton and Peng 1993).

Although exposure to *C. rosea* has been suggested to increase yield in some crops (Sutton et al. 1997), no such increases were documented in 2011. This may have been the result of a lack of nutrient uptake stimulation in this plant species, inadequate product dissemination, or may merely have been masked by poor pollination levels. However, typically such increases in growth are achieved from root inoculation (Sutton et al. 1997), whereas blossom protection was the primary goal of these experiments.

The two dispenser designs examined, in spite of major differences in design, were comparable in terms of distribution, hive activity, product application and disease management. Although colony forming units appeared to be significantly different, large variation was present for Koppert samples due to the collection method. During the collection, bees often pushed some of the powder from the exit tunnel into the scintillation vial with them leading to an over-exaggeration of CFUs on some individuals, including those excluded from analysis. Intermittent refill of the exit tunnel by the sensor may have also contributed to this variation. Due to the PK dispenser design, these factors were not an issue. Based on these observations, it may be entirely possible that the PK dispenser applies the product more effectively for forager activity, given that comparable counts were obtained despite bees climbing or flying out of the lower tray before being captured. However, this warrants further testing. Despite the disparities in hive activity between hives with PK dispensers and control hives with no dispensers, yield was not significantly different between treatments, suggesting that the difference in activity may not be significantly important. However, this difference may become intensified when foraging is unrestricted.

Interestingly during CFU plating, colony growth on PDSTA was almost entirely *C. rosea*, this either attests to the cleanliness of *B. impatiens*, or suggests that *C. rosea* germination inhibits the growth of contaminant fungi (Peng and Sutton 1991) acquired by the bee. Since contaminants were rarely present in the controls, it seems to attest more so to the former.

The lack of effect of this technology on foraging behaviour, based on handling times and pollen returns, suggests that the added duty of vectoring does not affect pollination service. However, this is not entirely surprising since bumblebees have been known to compromise individual health during peak food availability (Heinrich 1981), and thus the lack of impact could potentially be a result of such behaviour. Given that commercial bumblebee hives are only designed for single-season use, the implications of this possible increase in hive-stress may not be meaningful. These results are consistent with those observed by Mommaerts et al. (2009), when using a similar product (Prestop-Mix®; *Gliocladium catenulatum* Gilman & Abbott) with *Bombus terrestris*; wherein there was no notable change in foraging behaviour when bees were exposed to the product.

While forager preening or self-grooming, upon being dusted with biopesticide, has been documented with some dispenser designs (MacCagnani et al. 2005, Mommaerts et al. 2010b), no such events were documented during formal observations. The only occasions when this behaviour was detected was during captures of specimens for CFU counts when the exit of the dispenser was impeded. This suggests that both dispenser designs were not too elaborate for exiting foragers to navigate (Mommaerts et al. 2010b).

Measurement of hive aggression, a valuable component for grower safety, showed no significant effect of dispenser design in 2010. Informal follow-up observations in the 2011 pollination season agreed with this, as aggression seemed to vary more greatly between individual hives than treatments, and thus is not likely the result of the dispensers. Although manufacturers try to select for docile queens (Watts 2010), it is possible that the queens present in some hives are more aggressive than others. Much the same as honey bees, smoke may be used to subdue aggressive bumble bee hives (Visscher and Vetter 1995) when refilling dispensers with biopesticide.

Hive integrity was a major quality control issue experienced that could hinder field-use of this technology. As previously described, the hives were composed of a plastic nesting box, with a Styrofoam bottom (with bag of supplementary sugar solution) all within a cardboard sleeve; which held the hives together. The biggest issue is that the cardboard sleeve is not appropriate for outdoor use. In addition to being completely vulnerable to rain, the humidity levels experienced in the field are too high even when hives are protected from the elements. Exposure to moisture led to warping of the sleeve and eventual bee escape. When this issue is resolved, by taping the top of the plastic insert to the cardboard sleeve, bees will start to eat away at the ventilation holes along the sides, through which they can eventually exit. These issues present a major problem for this technology, since dispensers are dependent on the pollinators being forced through the main exit into the powder chamber. In order to increase the viability of these hives for field use, construction materials should be used for the sleeve which are tolerant of rain and humidity.

Dispenser designs also had some operational issues. The initial wooden dispenser used was awkward to attach to hives, although the use of bungee cord ended up being a fairly simple solution for good attachment. There were cleanliness issues with the wooden dispenser, as previously documented by MacCagnani (2005), wherein foragers would frequently contaminate the biopesticide with their excrement while traversing the chamber. This led to product clumping, and thus greatly hindered acquisition by foragers. This cleanliness issue appeared to be resolved in the new version of the wooden dispenser, with little to no evidence of excretion and clumping in the tray. In fact, the only issue with the PK dispenser was that, once the cardboard sleeve of the hive became softened by humidity and moisture, the cardboard was too weak to support the dispenser properly. This led to separation of the dispenser from the hive which foragers utilized to bypass the dispenser. Small bungee cords were used to close this gap, although this invalidated the user-friendly access panel. This would likely become a non-issue if hive materials were altered, or the dispenser was used within a greenhouse. The Koppert dispenser suffered a variety of maladies. Firstly, the gate mechanism malfunctioned on numerous occasions, often remaining open. This led to over-fill of the exit tunnel, and excess product being wasted by over-flowing onto the ground. During the first season of field-use (2010), dispensers were in shambles by the end of the bloom-period (4 weeks) due to weak or insufficient epoxy binding the various plastic components, prompting re-enforcement of dispensers before field use in 2011. Additionally, the advantage of the large top-loading reservoir was negated since high humidity in the field also led to clumping of the product, and thus prevented the dispenser from releasing it properly.

In conclusion, these experiments suggest that bumble bee vectored *C. rosea* may be an effective method for management of *B. cinerea* in wild blueberry production. Moderate reductions in disease may be improved with proper stocking densities, and/or may be appropriate for inclusion into IPM programs for this crop. The dispenser designs examined and vectoring of *C. rosea* appear to have minimal effects on foraging behaviour and aggression, with comparable product loading, dissemination and disease management. Currently, hive suitability for field use is the primary limiting factor for adoption of this technology for commercial use. The PK dispenser provides equal control, while being more ruggedly designed, reliable, and user-friendly. In their current states, the PK dispenser is more suitable for commercial use. Extrapolation to larger scale operations is on-going, which will confirm the viability of this tactic for combined pollination and disease management within commercial organic berry production.

Chapter 4. General Discussion

4.1 Pesticides in Agriculture

The discovery and development of synthetic pesticides ushered in a new era of agricultural productivity and protection from disease vectors. However, indiscriminate use quickly led to the development of resistance in pest populations (Abedi and Brown 1960, Brazzel 1964), and the discovery of worrisome secondary effects (Quinby et al. 1965, Hickey and Anderson 1968). Concerns over the hazardous impacts of liberal pesticide use were voiced in Rachel Carson's "Silent Spring" (1962). The book resulted in tremendous public outcry and increased government scrutiny over pesticide use, and is likely the primary impetus for modern environmentalism and IPM (Lytle 2007).

Prokopy (2003) defined IPM as ". . . a decision-based process involving coordinated use of multiple tactics for optimizing the control of all classes of pests (insects, pathogens, weeds, vertebrates) in an ecologically and economically sound manner." (2003). Tools used within IPM programs include a variety of biological, cultural, physical and chemical management techniques suited toward the ecology of the pest(s) being managed. Included among these tools are microbial antagonists of plant pathogens (Nautiyal 2000, Droby 2006), which have increased in popularity due to wider regulatory acceptance and improved cost-effectiveness (Kapongo et al. 2008). Despite the increased use of biopesticides, it is unclear if there have been any appreciable reductions in pesticide use overall (Ehler 2006). However, significant reductions in pesticide use have been achieved in some crops with comprehensive IPM programs, such as soybean (Dent 2000).

The level of adoption of IPM technologies by growers is highly variable, and poor acceptance usually stems from of a lack of acknowledgement of social factors during development (Dent 2000). For instance, growers and communities are often not included in product development, despite being the end users (Chambers 1983).

However, import restrictions on allowable pesticides and maximum residue levels on crops, imposed by various foreign markets, are beginning to influence the pesticides used by commercial growers (Wilson and Otsuki 2004). Consumer preference towards natural options and public concern over the use of synthetic pesticides on food are similarly restricting the management options of growers (Dent and Waage 1999, Dent 2000). As this selective pressure increases, grower adoption of alternative options is likely to increase as well. This would likely improve the economic stability of biological control manufacturing, and provide the necessary financial security to develop increasingly efficacious microbial strains and control options (Dent and Waage 1999).

Some techniques go beyond IPM, wherein the tool or technique may enhance crop productivity in addition to managing or preventing pest damage. This paradigm, integrated crop management (ICM), includes techniques such as synthetic mulches, which boost crop growth and yield while also reducing pest populations (Vos et al. 1995). Using pollinators to vector MCAs also falls within this category, since pollination improves yield while the delivered agent prevents pest damage. Such techniques may prove especially promising, as the double-benefit realized from a single technique may enhance their attractiveness to growers and investors.

4.2 *Botrytis* Management

Although historical records are imprecise, it is likely that *Botrytis* species have been pests of some crops since the beginning of formal cultivation (Rosslenbroich and Stuebler 2000). However, their impact was likely not severe until crop production intensified (Rosslenbroich and Stuebler 2000). The diverse host range, multiple modes of attack, genetic plasticity, and ability to survive for extended periods in leaf litter as mycelium and/or sclerotia, have established *B. cinerea* as an especially problematic pathogen (Williamson et al. 2007). Integrated pest management programs are important for *B. cinerea* management, as the use of multiple management strategies, including MCAs, reduces dependence on chemical fungicides which in-turn reduces the rate that resistance develops (Wisniewski et al. 2001, Williamson et al. 2007). Although traditional spray application can be used to apply biopesticides, application using managed pollinators has been shown to be equivalently (Peng et al. 1992) or more effective (Archer 2002) than spray application for suppression of *B. cinerea* (Yu and Sutton 1997, Kapongo et al. 2008).

4.3 Rationale and summary of current research

While synthetic pesticides will continue to play a role in pest management, social and regulatory pressures may increasingly limit the extent that they can be used. Environmentally conscious IPM programs are therefore likely to increase in importance. Developing, evaluating, and improving IPM tools and techniques is of value, in order to provide a variety of tools that can be combined with judicious pesticide use to ensure

successful management below economic thresholds. ICM techniques that serve multiple purposes may appear more attractive to growers and increase the likelihood of adoption. Few techniques are capable of this, increasing the attractiveness of the pollinator-vector system, especially to growers who already employ the services of managed pollinators.

The compatibility of MCAs with some synthetic insecticides and fungicides means that the expectation of implementing the pollinator-vector technique need not be complete control. The technique may serve as partial or supplementary control to reduce the number of synthetic applications, and/or reduce inoculum load in the field (in the case of mycopathogenic MCAs). Acknowledging these potential endpoints is important when evaluating the value of its use.

Although the pollinator-vector technique has been tested in a variety of crops, many of the experiments were completed in greenhouses, and thus hives and dispensers were protected from the elements. Few, if any, published experiments, have been completed in cropping systems like wild blueberry. The often cool, windy, and rainy conditions of the Maritimes represent the ultimate field test for this technique.

The focus of this *Master of Science* thesis was to evaluate pollinator-vectoring, using *B. impatiens* and *C. rosea*, as an alternative or supplementary disease management technique for Botrytis blight in wild blueberry. Within this model, emphasis was placed on the effects of different dispenser designs on foraging behaviour, aggression, product loading and distribution, and differential management of Botrytis blight.

4.4 Recommendations

The biopesticide *Clonostachys rosea* was found to be an effective, organically compatible, option for prevention of Botrytis blight in wild blueberry plants. The compatibility of this MCA with at least one synthetic fungicide also used in *B. cinerea* management, combined with the elevated safety of the agent to pollinators, suggests a good opportunity for development of an IPM program for *B. cinerea* management in wild blueberry. Further research to evaluate the capacity of this antagonist to prevent other diseases may increase the utility of this product for a wider range of growers and operations. Microbial activity may be improved to obtain better control by co-vectoring the agent with other antagonists (Guetsky et al. 2001, Guetsky et al. 2002). Synergistic effects may also be elicited by combining the agent with a synthetic fungicide (Zhou et al. 2002, Sugar and Basile 2008, Nallathambi et al. 2009). For example, Zhou et al. (2002) found that while post-harvest treatment of two apple varieties with either cyprodinil or the MCA *Pseudomonas syringae* MA-4 gave good (77 % - 85 %) control of grey mould (*B. cinerea*) independently, a combination of low-rate cyprodinil with the MCA gave complete control. However, interactions can vary between fungicides and MCAs, and thus preliminary research to ensure the safety and efficacy of such combinations should be performed prior to field use. Spray application of *C. rosea*, separately or potentially as a tank mix with Switch, earlier in the season may be an option worth exploring for pre-emptive protection. This may also serve to help establish the endophyte in the field if temperatures are conducive. If applied early enough, sufficient amounts may reach the roots to provide growth stimulation, as seen in other crops (Sutton et al. 1997).

Despite inclement maritime conditions, it appears that *B. impatiens* can be used distribute biopesticides in wild blueberry fields. In my experiments, sufficient amounts were deposited during pollination to provide moderate, but significant reductions in Botrytis blight. It is unclear whether or not these levels of reduction would be of biological or agronomic significance. Proper hive stocking densities (those in our experiment were more than 30-fold below that recommended for blueberry production (Drummond 2012)) would likely result in improved disease control and experiments should be done to test this hypothesis. While not explicitly tested, changes in blossom infection during bloom, combined with the previously acknowledged effects of climatic conditions on forager activity (Lundberg 1980) and microbial colonization (Morandi 2008) may result in varying levels of disease protection within a season.

The dispenser designs and formulation of *C. rosea* examined in this thesis had no significant impact on the foraging behaviours examined, except for a reduction in hive activity between control hives and those fitted with the PK dispenser. However, it is unclear to what extent the observed reduction in forager activity will effect pollination service and crop protection. Additionally, there appeared to be no effect of the dispensers or the MCA on bee aggression. Originally, it was thought that the presence of the man-made device and MCA might agitate the bees, posing a risk to growers during the refill of dispensers. However, no bee aggression was observed after the acclimation period, revealing no significant differences between control hives and those fitted with dispensers. While the rating system used in this study may not have provided the most objective data, I believe that other measures, such as a strike counter (Visscher and Vetter

1995), would find comparable results. Given the difference in design between the dispensers examined, it seems likely that designs that are not overly complex (Mommaerts et al. 2010b) should not have a negative impact on hive aggression after the acclimation period. In terms of the effects of the MCA and its components, these will need to be evaluated on a case-to-case basis, as some carriers may have deleterious effects (Mommaerts et al. 2009, Mommaerts et al. 2011c).

The inability of commercial hives to retain their integrity under field conditions represents a major limitation to the implementation of this technique for commercial wild blueberry growers. The materials currently used are simply not capable of standing up to the rigors of field use. The two dispenser designs examined, in spite of major differences in design, appeared to be quite comparable in the distribution, activity and disease management parameters examined. Apparent discrepancies between dispenser abilities to load bees with the microbial antagonist are likely an artefact of the sampling methodology employed, and dispenser functionality. Operational flaws with dispensers were largely limited to hive integrity and attachment issues, but are aspects that deserve ample consideration before large scale production begins. The PK dispenser was an excellent improvement over the previous wooden dispenser, and promises to be the more reliable and user-friendly option compared to the Koppert dispenser tested. The Koppert dispenser was a much less invasive design, however functionality was unpredictable due to mechanical malfunction, and poor construction made it too delicate for field conditions without additional reinforcement.

In order to make this technique viable for field-use, hives could be constructed out corrugated plastic, a material which is already used to ship quads (packages of four hives – a standard unit of purchase), rather than corrugated cardboard, which did not retain its integrity well in the field, resulting in eventually difficulties with dispenser attachment and performance. Manufacturers could potentially offer to growers the option of a corrugated plastic hive if they anticipate use of biopesticides dispensers. Alternatively, it may be possible to modify the openings on existing quad boxes to allow attachment of dispensers. However, it is unclear if this latter option would suffice.

My experiences suggest that 'ruggedly-simple' dispenser designs, like the PK dispenser, are most likely to be successful. Ideally, dispensers should require no more work or maintenance than absolutely necessary. After all, this technique is intended to save growers time and energy. However, intelligent, unique designs like the Koppert dispenser offer insight into alternative methods. The electronically driven moving parts of the Koppert dispenser are more susceptible to failure or malfunction. However, it may be possible to slightly modify the dispenser, replacing the flawed electronic gate with a wheel mechanism, not dissimilar from a water mill. Rather than bringing material up, the wells of the wheel would deposit small amount of the MCA from the top reservoir into the tunnel. If positioned correctly, the wheel would be turned when exiting foragers brush against it with their ventral surface. As this technique continues to develop, I believe that it will become critical for existing companies like Koppert/Biobest to initiate research collaborations with potentially emerging companies that develop new dispensers. With

such increases in cooperation, I anticipate that we would see better designs that are suitable for a variety of environments and applications.

MCA formulations should be improved to maintain adequate consistency in the presence of high moisture levels. The addition of silica nanoparticles (NPs) has been suggested as a possible solution, as these particles would reduce clumping and have been shown to be quite safe to bees (Mommaerts et al. 2011c). However, their effects on the developing crop and fruit should also be carefully examined, as their potential to bioaccumulate and biomagnify within the ecosystem is not known (Mommaerts et al. 2011c). Rather than incorporating the NPs into the formulations, I suggest that trays or reservoirs could be lined with packages of silica beads to remove excess humidity. While perhaps this would not be as effective, it avoids the ecotoxicology concerns associated with the use of NPs.

Although bumble bees were used as pollinator-vectors for my experiments, honey bees are also commonly used to pollinate wild blueberry (Stubbs and Drummond 2001) and were the first vector to demonstrate the potential of the pollinator-vector technique for disease management (Peng et al. 1992). The level of pollination in *V. angustifolium* afforded by honey bee hives is comparable to that of bumble bee hives, under most stocking densities and conditions (Stubbs and Drummond 2001, Drummond 2012). However, under cool, wet and windy conditions bumble bees are likely to be the superior pollinator (Stubbs and Drummond 2001). Based on the conditions experienced in 2010, pollination of these two species would have been comparable. In 2011, the conditions were much cooler and bumble bees (both managed and native) seemed to be the only

insects flying on many days, with on-site honey bee hives having minimal to no activity. Additionally, bumble bees tend to be more loyal to blueberry pollen than honey bees (Whidden 1996), with forage ranges that are complementary to the field sizes of many blueberry growers (Desjardins and DeOliveira 2006). In times of good weather some larger operations may benefit from the use of honey bees as the vector. However, their ability to distribute MCAs into wild blueberry fields and contribute to disease management has yet to be examined.

In conclusion, bumble bee vectored *C. rosea* is an effective method for management of Botrytis blight in wild blueberry fields. Moderate reductions in disease may be improved with proper stocking densities, and/or may be appropriate for inclusion into IPM programs for this crop. The dispenser designs examined and vectoring of *C. rosea* appear to have minimal effects on foraging behaviour and aggression, as well as comparable product loading, dissemination and disease management. Currently, commercial hives are unsuitable for field use with this technology, and represent the primary limiting factor for adoption by commercial growers. The PK dispenser provides equal control, while being more ruggedly designed, reliable and user-friendly. In their current states, the PK dispenser is more suitable for field use. Extrapolation to larger scale operations is currently being examined by project collaborators, which will confirm the viability of this tactic for combined pollination and disease management within commercial organic wild blueberry production.

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