DETECTION, QUANTIFICATION AND BIOLOGICAL CONTROL OF POST-HARVEST PATHOGENS OF POME FRUIT

by

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ABSTRACT

Blue mold (*Penicillium expansum*), grey mold (*Botrytis cinerea*) and mucor rot (*Mucor piriformis*) are important post-harvest diseases of pome fruit in British Columbia and throughout the world causing annual losses of 5 – 20%. Identification and quantification using novel DNA macroarray technology may assist in the development of prediction models and disease forecasting. Post-harvest pathogens were monitored and quantified throughout the growing season in four apple orchards in the Okanagan Valley, BC in 2007 and 2008. Their detection was variable due to field and year differences. The average percent detection of *P. expansum* (27.4%) and *M. piriformis* (19.2%) was higher than that of *B. cinerea* (6.2%). There was a positive correlation between total post-harvest pathogen detection in aerial samples just prior to harvest and after harvest in naturally infected fruit ($r = 0.74$; $p = 0.09$). *Pseudomonas fluorescens* (isolates 1-112, 2-28, 4-6) and *Serratia plymuthica* (isolate 6-25), isolated from the rhizosphere of legumes, were investigated for their biological control capabilities in semi-commercial storage conditions at 1°C in air and commercial storage conditions in controlled atmosphere and with 1-methylcyclopropene (1-MCP) application. Overall, isolate 6-25 provided control in the greatest number of treatments (51.7%) while isolate 1-112 provided the greatest level of control (75.8%) in treatments where significant control was exhibited. Isolate 4-6 was tagged with green fluorescent protein to gain insight into bacterial antagonist population and survival dynamics. Alone, its population increased 10 fold after 30 d in storage at 1°C and then decreased to concentrations similar to those at inoculation. In the presence of the pathogen, 4-6-*gfp* increased then decreased after 30 d in storage at 1°C to undetectable amounts. These data provide greater insight into the prediction, control and

population dynamics of pathogens and biological control agents as a means of preventing and controlling post-harvest storage diseases in pome fruit.

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* (one asterisk) – *p*value = 0.05 – 0.01
** (two asterisks) – *p*value = 0.01 – 0.001
*** (three asterisks) – *p*value = < 0.001
½ TSA – half-strength tryptic soy agar
½ TSB – half-strength tryptic soy broth
1-MCP – 1-methylcyclopropene
4-6-*gfp* – transformed *P. fluorescens* isolate 4-6 with GFP-containing plasmid pAG408
bc – biocontrol
BC – British Columbia
BCA – biological control agent
Bcin – *Botrytis cinerea*
βtub – β tubulin
CA – controlled atmosphere
CFU – colony forming units
CSLM – confocal scanning laser microscopy
gen - gentamicin
gfp – green fluorescent protein (gene)
GFP – green fluorescent protein (protein)
IS – infection severity
ISR – induced systemic resistance
kan - kanamycin
$\log_{10}$ – logarithmic to the base 10
LSD – least significant difference
MOA – mode of action
Mpir – *Mucor piriformis*
NDI – natural disease incidence
OD_{600} – optical density at 600 nm
PARC –Pacific Agri-Food Research Centre
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDA – potato dextrose agar
Pex – *Penicillium expansum*
pt – plant tissue
rDNA – ribosomal deoxyribonucleic acid
RFU – relative fluorescent units
TBZ – thiabendazole
TSA/B – trypic soy agar/broth

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CHAPTER 1: INTRODUCTION

According to the Food and Agriculture Organization of the United Nations, the world apple production in 2008 was greater than 69 million tonnes (FAOSTAT 2009). Apples (*Malus domestica*) are ranked fourth (by volume) in the world's production of fruit crops (BCMAFF 2004). In 2002, the British Columbia apple industry produced 136 000 tonnes or approximately 299 846 000 lbs of apples. Apples are produced in significantly higher volumes than any other tree fruit crop in BC (BCMAFF 2004). One major challenge to apple production is damage incurred by post-harvest diseases; annual losses can vary from 5 – 20% and up to 50% in developing countries (Janisiewicz and Korsten 2002). A variety of fungal pathogens is responsible for post-harvest disease; in BC, three such pathogens include *Penicillium expansum* (blue mold), *Botrytis cinerea* (grey mold) and *Mucor piriformis* (mucor rot) (Sholberg and Haag 1996).

A common method of disease prevention is through the use of fungicides. In Canada, two synthetic controls are registered for post-harvest use, Mertect® (thiabendazole) and Scholar® (fludioxonil) (PMRA 2007). However, due to growing health issues, the development of pathogen resistance and the demand for environmentally friendly sustainable practices, pesticides are now a major cause for concern (Errampalli et al. 2006; Michaildes and Spotts 1990; Sholberg and Haag 1996).

To reduce post-harvest rot, improved methods for pathogen identification prior to harvest and alternate technologies to chemical control are required to assist in disease prediction and control. Preliminary work has resulted in two novel approaches that address these demands: 1) a rapid DNA-based macroarray system that determines the presence of fungal pathogens responsible for post-harvest rot (Sholberg et al. 2005a) and

2) the identification of soil bacteria that can control fungal post-harvest pests (Hirkala et al. 2007; Hynes et al. 2008). This study will assist the project's long term goal to develop Canadian-based technologies that detect, predict and mitigate post-harvest disease in pome fruit and that are consistent with integrated pest management and sustainable production practices.

1.1 Post-harvest pathogens

Fruit infections can occur throughout the growing season, at harvest, during storage, transit and at the retail level (Jones and Aldwinckle 1990). Although global economic losses can be attributed to growing season diseases, post-harvest pathogens, despite modern storage facilities, can cause significant annual losses (Janisiewicz and Korsten 2002).

Post-harvest diseases, or latent infections, are asymptomatic throughout the growing season and disease is manifested only after periods of storage. As a result, these pathogens are often difficult to treat and prevent. Post-harvest fungal pathogens can be categorized in many ways, one of which describes the pathogenic point of entry. Rot characterization can include lenticel infections (pores that exist on the surface of the apple that facilitate gas exchange), core and eye rots and wound pathogens (Jijakli and Lepoivre 2004). Common lenticel infections include bitter and bull's eye rot and are commonly caused by *Colletotrichum gleosporioides* and *Cryptosporiopsis curvispora*, respectively (Jijakli and Lepoivre 2004). Core rots develop when an open sinus stems from the calyx end of the apple into the core (Miller 1959), which provides a point of entry for an invading pathogen. *Alternaria* spp. are the most commonly isolated fungi from core rots, but other fungal species such as *Cladosporium*, *Botrytis*, *Candida* and

Fusarium have also been identified (Ellis and Barrat 1983). Dry eye rots, characterized by a shallow, hard rot with a red border are predominantly caused by *Botrytis cinerea*.

Opportunistic wound pathogens invade apples as a result of fruit injury incurred by mishandling or harsh weather conditions. These types of infections are responsible for significant storage losses. Three important fungal pathogens that cause worldwide decay include *Penicillium expansum* (blue mold), *Botrytis cinerea* (grey mold) and to lesser extent, *Mucor piriformis* (mucor rot) (Michailides and Spotts 1990; Sanderson and Spotts 1995; Rosenberger 1990).

1.1.1 *Penicillium expansum*

Penicillium expansum Link, or apple blue mold, is a filamentous Ascomycete that causes one of the most important North American post-harvest storage diseases of pome fruit. Before the introduction of controlled atmosphere (CA) storage and fungicides, it accounted for 90% of post-harvest apple diseases (Rosenberger 1990). *P. expansum* produces the mycotoxin, patulin, which has been found in apple product derivatives such as apple juice, ciders, puree, vinegar, baby food and whole apples (Abramson et al. 2009; Doores 1983; Piemontese et al. 2005; Watanabe and Shimizu 2005). Its detection and control is, therefore, of utmost importance for food health and safety.

Although an opportunistic wound pathogen, *P. expansum* infection can originate from stem-end invasions, core rots and through lenticels. Infected fruit are characterized by light to dark brown, fleshy, circular lesions that surround wounds. Older lesions may produce a bluish spore mass. *P. expansum* can also form a dense powdery mass at the centre of the lesion (Rosenberger 1990) and can be characterized by an earthy, musty odour commonly used as a diagnostic tool (Vikram et al. 2004).

Spores of *P. expansum* are ubiquitous and can cause infection within orchards and packinghouses. This fungus can survive in organic debris on the orchard floor and within soil. Conidia also exist in the air and on apple surfaces (Lennox et al. 2003). Within packinghouses, spores have been isolated from fungicide-drench solutions, flume water, dump-tank water, air and storage room walls (Sholberg and Stokes 2006). Conidia can survive from season to season on contaminated bins, picking boxes and storage walls (Rosenberger 1990).

Sanitation, harvesting pre-senescent fruit and handling methods are the best strategy for minimizing disease incidence. This control can also be facilitated by fungicides applied as pre-harvest sprays or post-harvest dips (Eckert and Ogawa 1988). For example, pyrimethanil was used as a pre-harvest fungicide and applied twenty days prior to apple harvest. After apples were stored for six months, post-harvest blue mold was significantly reduced when compared to apples that were not treated with fungicides (Sholberg et al. 2005b). Mertect®, with an active ingredient of thiabendazole (TBZ), is an example of a post-harvest fungicide application. It was once highly effective; however, prolonged exposure to such agents has led to TBZ-resistant fungal strains (Sholberg and Haag 1996) that render chemical controls ineffective.

1.1.2 *Botrytis cinerea*

Botrytis cinerea Pers.:Fr is an Ascomycete and, like *P. expansum*, is an important post-harvest pathogen of pome fruit causing minor rot throughout the growing season and significant rot within packinghouses. It is the most important post-harvest pathogen of pears and is second to blue mold in importance to apples (Rosenberger 1990). *B. cinerea* infection may originate from wounds, stem punctures, or the stem or calyx portion of the

fruit. Upon infection, *B. cinerea* inflicts a light to dark brown, spongy decayed area around the damaged portion of the fruit. Under high humidity, fluffy white to grey mycelium and spore masses may appear on the decayed area. The optimal growth temperatures for this pathogen range from 20°C to 22°C however, conidia are capable of growth at as low as -2°C (Coley-Smith et al. 1980).

B. cinerea spores are ubiquitous, colonizing available organic matter within orchards. Throughout the growing season, this fungus can cause dry eye rot. *B. cinerea* also can be carried into packinghouses via contaminated storage bins. Once inside, conidia are predominantly spread by air currents and water splash. This pathogen is also known as a nest or cluster rot as secondary infection can occur through fruit to fruit contact; fungi on infected fruit can colonize healthy fruit and spread disease (Rosenberger 1990).

1.1.3 *Mucor piriformis*

Mucor piriformis belongs to the phylum Zygomycota and, until recently, was thought to be of minor importance as a post-harvest pathogen. However, *M. piriformis* is capable of causing major decay problems in fruit such as strawberries, pome and stone fruit. *M. piriformis* is typically saprotrophic in orchard soil and infects fruit through the stem or calyx end and puncture wounds (Michailides and Spotts 1990). Upon infection, the area surrounding the lesion becomes soft, watery, light brown and easily separable from the fruit tissue. Often, grey mycelium with dark sporangia appears upon the decayed area. Mucor rot has a distinct sweet smell with a clear, sticky exudate. This fungus sporulates from -1 to 24°C, with optimal growth at 21°C. Fungicides that are currently registered to control other post-harvest pome fruit pathogens are ineffective

against *M. piriformis* and other Zygomycetes (Michailides and Spotts 1990). Although Scholar^{TM}, with active ingredient fludioxonil, has been shown to be effective against *M. piriformis* (P. Sholberg, personal communication), sanitation methods are often the most effective method for preventing infection (Michailides and Spotts 1990).

1.2 Post-harvest factors that influence post-harvest pathology

Because of the ubiquitous nature of fungal spores, their presence throughout the apple growing season can influence post-harvest pathology. *P. expansum, B. cinerea* and *M. piriformis* can colonize organic matter on the orchard floor and within soil (Lennox et al. 2003; Michailides and Spotts 1990). *P. expansum* and *B. cinerea* conidia can additionally exist in the air and on fruit surfaces (Lennox et al. 2003). Low precipitation, physiological crop condition and the use of pre-harvest fungicides will have a reducing effect on decay levels after harvest (Sholberg and Conway 2004; Sholberg et al. 2003; Sholberg et al. 2005b).

However, post-harvest factors can also influence disease incidence. Within packinghouses, spores can survive in fungicide-drench solutions, flume water, dump-tank water and in the air and on walls of storage rooms (Lennox et al. 2003; Sholberg and Stokes 2006). Therefore, sanitation affects disease accumulation. The post-harvest system offers a unique, closed or semi-closed environment where manipulating temperature and atmosphere within the storage room and the use of chemical senescence inhibitors like 1-methylcyclopropene (1-MCP) are often easier to manage in comparison to pre-harvest factors that can contribute to disease.

1.2.1 Temperature

The first experimental cold storage facility in British Columbia was built in Summerland in 1929 (AAFC 2002). Optimal refrigeration temperatures range from -0.5°C for Braeburn apples in South Africa to as high as 5°C for Belle de Boskoop apples grown in the Netherlands (Kupferman 2003). Lowered temperatures slow the rate of apple respiration, thus retarding ripening (AAFC 2002). Temperature management is also critical to post-harvest disease control. Post-harvest fruit pathogens grow optimally between 20 to 25°C. Some fungi have minimum growth temperatures as low as -2°C and cannot be completely controlled without freezing the fruit. Their growth at these temperatures, however, is significantly reduced which leads to a reduction in post-harvest decay levels (Sholberg and Conway 2004).

1.2.2 Controlled atmosphere

"Normal air" refers to atmospheres that consist of 78 – 79% N_2, 20 – 21% O_2, ~ 0.03% CO_2 and trace amounts of other gases. Controlled atmosphere (CA) refers to atmospheres that differ from "normal air" and are under strict control (Yahia 2009). Here, optimal temperatures persist while oxygen and carbon dioxide concentrations are decreased and increased, respectively (Morales et al. 2007). The concept of CA relies on the fact that harvested fruits use oxygen and produce carbon dioxide. If the amount of oxygen is limited, fruits will not ripen or will ripen slowly (Yahia 2009). Apples are often stored under CA as this environment has been shown to be effective in delaying the onset of storage diseases (Smock 1979). Commercial CA storage can range from 1% – 3% O_2 and 0.4% – 4.5% CO_2 (Kupferman 2003). Additional studies revealed that as high as 13% CO_2 prevented close to 100% spore germination of *P. expansum* after

twenty days (Cossentine et al. 2004). However, CO_2 levels this high are not commercially acceptable as it may lead to fruit injury resulting in a decrease in fruit quality.

1.2.3 1-Methylcyclopropene

1-Methylcyclopropene (1-MCP) is a synthetic cyclopropene that blocks ethylene receptors and prevents apple ripening (Blankenship and Dole 2003). It has the ability to maintain post-harvest fruit and vegetable quality and its use provides insight into the role of ethylene in fruit senescence (Watkins 2006). Ripening physiology and quality of apples in response to 1-MCP application has been intensively studied (Watkins 2006). Less studied, are the effects of 1-MCP on disease incidence. It is thought that by preventing the ethylene-associated ripening process, apples will be better able to resist pathogens (Watkins 2006). However, prevention of decay in 1-MCP-treated apples has been inconsistent. For example, Golden Delicious apples treated with 1-MCP and *P. expansum* showed decreased disease incidence (Saftner et al. 2003). Alternatively, disease severity increased in Golden Delicious apples inoculated with *P. expansum* or *Colletotrichum acutatum* and treated with 1-MCP (Janisiewicz et al. 2003; Leverentz et al. 2003). Although initially successful, there is the potential of certain decay problems associated with 1-MCP use (P. Sholberg, personal communication).

1.3 Methods of detection and identification

Traditional methods for fungal identification were primarily based on morphology or phylogenetic characteristics. However, such techniques have limitations as morphological characterizations rely on fungi to be isolated or cultured, resulting in an

underestimation of the microbial community of interest (Bridge and Spooner 2001; Mazzola 2004). The advancement of molecular biology has led to more specific and sensitive fungal DNA-based detection methods that replaced assumptions made in previous studies. For example, Cruickshank and Pitt (1987) used enzyme gel electrophoresis, or a zymogram, to differentiate between *Penicillium* species. This study hypothesized that like species will display like zymograms. Results mostly confirmed taxonomy based on morphology; however, taxonomic differences were perceived.

Detection techniques based on morphology or electrophoresis are time consuming. A rapid method of detection is therefore required. Greater specificity, sensitivity and speed can be attributed to the advancement of the polymerase chain reaction (PCR), a rapid and sensitive primer-mediated enzymatic amplification of target DNA sequences (Saiki et al. 1985). Common PCR-based identification methods include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or terminal restriction length polymorphisms (T-RFLP) (Mazzola 2004). Although PCR-based identification methods provide a more accurate picture of a microbial community, these methods are often limited by gel resolution or, more importantly, the inability to detect a large number of different pathogens simultaneously (Sholberg et al 2005a).

The advent of DNA macroarray technology circumvents this problem. This system utilizes labelled PCR products hybridized to a nylon membrane that contains an assortment of anchored oligonucleotides or probes. Due to a chemiluminescent reaction, pathogen DNA, for example, is captured on X-ray film and is represented as a pattern of pre-determined dots. The presence of a signal indicates the presence of a pathogen that

can be identified and quantified. Levesque et al. (1998) were among the first to utilize macroarray technology for plant pathogen identification. This method was further developed for detection of economically important pre-harvest (Sholberg et al. 2005a) and post-harvest diseases of pome fruit (Hirkala et al 2007). These technologies have great potential as they can be used as a high-throughput detection and diagnostic technique that can identify numerous microorganisms across disciplines and environments.

1.4 Methods of control

1.4.1 Chemical control

Fungicides are used to prevent post-harvest decay. In Canada, two synthetic post-harvest controls are currently registered, Mertect® and Scholar® with active ingredients thiabendazole (TBZ) and fludioxonil, respectively (PMRA 2007). Although initially effective, fungicides are now a major cause for concern. Pathogens have developed resistance to chemical controls (Errampalli et al. 2006; Sholberg et al. 2005c) which negates their effectiveness. There are also growing health concerns that surround pesticide use (Hancock et al. 2008) and a demand for environmentally friendly sustainable post-harvest practices. An alternative is therefore of high priority. One possibility is the implementation of biological controls.

1.4.2 Biological control

Biological control can be defined as "the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (Cook and Baker 1983). As cited in Cook and Baker (1983),

biological control dates back to the early 1920s in which Hartley (1921) attempted to control damping off in pine seedlings with fungal antagonists. In 1927, Millard and Taylor tried to control common potato scab by the addition of *Streptomyces praecox* and grass clippings to autoclaved potted soil. Both studies noted that competition for nutrients may be a putative mechanism of action (Cook and Baker 1983).

Sixty years later, the first commercial bacterial biological control agent (BCA), *Agrobacterium radiobacter* strain K84, was registered with the United States Environmental Protection Agency in 1979. It controlled crown gall caused by *Agrobacterium radiobacter* pv. *tumifaciens*. Strain K84 lacked the tumor-inducing (Ti) plasmid present within the pathogenic strain and produced a bacteriocin, which inhibits the growth of certain tumorigenic-strains, and to which K84 was insensitive (Cook and Baker 1983).

Although the beginnings of biological control of plant pathogens date back to the early 1920s, practicing biological control within a post-harvest setting has been much less common. Currently, there are three BCAs registered for post-harvest use (Droby et al. 2009). The first and second, developed by JET Harvest Solutions (Longwood, FL), are Bio-Save 10LP and 11LP (Longwood, FL) with active ingredients of *Pseudomonas syringae* strain ESC-10 and ESC-11, respectively. Bio-Save 10LP targets post-harvest blue and grey mold and mucor rot in pome fruit while Bio-Save 11LP targets Rhizopus soft rot on sweet potatoes. The third, Shemer (*Metschnikowia fructicola*) is commercially used in Israel for prevention of sweet potato and carrot storage decay. Two yeast-based products, Aspire^{TM} and YieldPlus, are no longer available (Droby 2009).

Biological control of post-harvest pathogens of pome fruit using bacterial and

yeast antagonists has been studied by numerous research groups (Calvo et al. 2007; Etebarian et al. 2005; Janisiewicz 1988; Mikani et al. 2008; Morales et al. 2008; Nunes et al. 2002; Zhou et al. 2001). For example, *Candida sake* strain CPA-1 significantly decreased *P. expansum* lesion size by > 80% and reduced disease incidence by 50% (Teixidó et al. 1999). *Cryptococcus laurentii* and *Metschnikowia pulcherrima* also provided control against *P. expansum* (Conway et al. 2007). The epiphytic bacterium, *Rahnella aquatilis*, significantly inhibited *P. expansum* and *B. cinerea* on apples at 15°C and 4°C, respectively (Calvo et al. 2007). A saprophytic strain of *Pantoea agglomerans* also reduced *P. expansum* rot on Golden Delicious apples at 1°C in air and at 1°C in a low oxygen atmosphere by 81% and 100%, respectively (Nunes et al. 2002). A study conducted by Etebarian et al. (2005) showed that *Pseudomonas fluorescens* isolate 1100-6 significantly reduced the incidence of *P. expansum* and *P. solitium* on apples after 11 days at 20°C and 25 days at 5°C. In Canada, there are an increasing number of registrations and products under evaluation that are based upon microbially-active substances (Bailey et al. 2010). However, there are none registered for post-harvest use (Droby et al. 2009).

A demand therefore exists for the production and commercialization of a BCA for post-harvest use on pome fruit. A collection of rhizobacteria, isolated from the roots of legumes grown in Saskatchewan soils, were compiled and initially characterized for plant growth promoting traits such as the production of siderophores, ACC deaminase, root elongation and the suppression of legume fungal pathogens (Hynes et al. 2008). Fifteen of these isolates were further tested *in vitro* for suppression of *P. expansum*, *B. cinerea* and *M. piriformis* (Stokes et al. 2006). Nine isolates provided fungal control and were

then tested *in situ*. Four isolates provided consistent control at both 20ºC and 1ºC; isolates 1-112, 2-28, 4-6 and 6-25 (Hirkala, unpublished data) in combination with 1100-6 were selected for further study.

1.5 Mechanisms of action

The interaction between host, antagonist and pathogen is a complex, ecological relationship with high variability depending on the system of focus. The ability of an antagonist to suppress a pathogen may be due to more than one mechanism of action (MOA). A sound mechanistic understanding is essential for formulation development and biocontrol registration (Spadaro and Gullino 2004). Possible mechanisms include antibiosis (Janisiewicz et al. 1991; Kamesnky et al. 2003; Meziane et al. 2006), competition for nutrients (Elad 1996), parasitism (Frankowski et al. 2001; Watanabe et al. 2007) and induction of pathogen resistance in host tissue (Benhamou et al. 2000; Spadaro and Guillino 2004; Terry and Joyce 2004).

1.5.1 Antibiosis

One method of antagonistic action may be through the production of antibiotics. For example, pyrrolnitrin produced by strains of *Serratia plymuthica*, was associated with fungal suppression (Kamesnky et al. 2003; Meziane et al. 2006). To determine whether or not pyrrolnitrin assisted in antifungal activity, Meziane et al. (2006) created an *S. plymuthica* mutant that lacked the gene responsible for pyrrolnitrin production. The pyrrolnitrin-deficient mutant, IC1270-P1, lost its antifungal activity when tested against pathogens *Penicillium digitatum* and *Penicillium italicum*. In addition, purified pyrrolnitrin was effective in suppressing disease symptoms of *P. digitatum* and *P.*

italicum. Janisiewicz et al. (1991) also isolated pyrrolnitrin from *Pseudomonas cepacia* for control of *B. cinerea* and *P. expansum* on apples and pears. Pyrrolnitrin was found to provide control; however, the level of control varied due to wound type (cut, nail, bruise), pyrrolnitrin concentration and storage temperature (2ºC and 24ºC). A post-harvest dip solution of pyrrolnitrin may therefore be an effective solution against post-harvest pathogens. It should also be noted that the post-harvest fungicide, Scholar^{TM}, has an active ingredient of fludioxonil which belongs to the chemical class of phenylpyrroles. Phenylpyrroles are derived from pyrrolnitrin and are considered a reduced-risk chemical control agent (Errampalli 2004).

1.5.2 Competition for nutrients

Competition for nutrients has been widely studied due to nutritional demands of both antagonists and pathogens. *B. cinerea*, as with most necrotrophs, requires nutrients for germination and the initiation of the pathogenic process. In the absence of nutrients, *B. cinerea* becomes highly susceptible to degradation and control (Elad 1996; Janisiewicz et al. 2000). It is postulated that microorganisms capable of efficiently utilizing nutrients will make successful biological control agents (Elad 1996). Janisiewicz et al. (2000) proposed a simple way to study competition for nutrients that uses a 24-well tissue culture plate with cylindrical insets with 0.45 μm membranes attached to the bottom. Individual cylinders are placed in each of the 24-wells thereby permitting media nutrient and metabolite interchange while preventing pathogen and antagonist contact due to physical separation. In this system, competition for nutrients can be studied independently from competition for space. Bencheqroun et al. (2007) used this method to determine, *in vitro*, that the biocontrol antagonist, *Aureobasidium pullulans*, was

competing for nutrients, especially the amino acids glycine, glutamic acid and serine, with the pathogen, *Penicillium expansum*.

1.5.3 Parasitism

Antagonists can directly parasitize pathogens of post-harvest apples. One such method is through the production of lytic or cell wall degrading enzymes such as chitinase and β-1,3-glucanase. Bacterial strains that produce lytic enzymes will most likely have antifungal properties via cell wall hydrolysis. For example, *S. plymuthica* strain HRO-C48 was isolated from the rhizosphere of oilseed rape and shown to have antifungal properties associated with chitinase production. Frankowski et al. (2001) isolated and characterized two chitinolytic enzymes, one endochitinase (E.C. 3.2.1.14), CHIT60, and one *N*-acetyl-β-1,4-D-hexosaminidase (E.C. 3.2.1.52), CHIT100. *In vitro*, CHIT60 and CHIT100 showed direct inhibitory activity on spore germination and germ tube growth of *B. cinerea*. Alternate parasitic activity may involve direct attachment of antagonist to pathogenic fungal hyphae (Chan and Tian 2005; Watanabe et al. 2007).

1.5.4 Induced systemic resistance

Induced systemic resistance (ISR) is an observed phenomenon in which microorganisms activate host plant mechanisms that enhance their defensive capacity against potential pathogenic invasions. Protection can occur both locally and on areas of the plant that did not come into contact with inducing microorganisms (van Loon 2007). For example, Benhamou et al. (2000) determined that S. *plymuthica* strain R1G64 mediated induced systemic resistance in cucumber to protect against infection caused by *Pythium ultimum*. Upon pre-treatment with *S. plymuthica*, *P. ultimum* disease incidence

was reduced; cucumber root cells were structurally and biochemically modified with the formation of phenolic-enriched occluded depositions and structural barriers. These structural barriers were hypothesized to prevent pathogen movement towards the vascular stele. In ISR experiments, it is important to spatially separate inducing microorganisms and challenging pathogens (ie. root and leaf) to ensure that protection is plant- not microorganism-mediated (van Loon 2007).

1.6 Green fluorescent protein

Green fluorescent protein (GFP) is made up of 238 amino acids and exhibits green fluorescence at a peak emission of 509 nm by absorbing blue light maximally at 395 nm and minimally at 470 nm (Morin and Hastings 1971). In its native form, GFP was first discovered during the purification of the bioluminescent protein, aequorin, isolated from jellyfish (*Aequorea victoria*) (Shimomura et al. 1962). In *A. victoria*, green fluorescence occurs by an intermolecular energy transfer between aequorin and GFP; Ca^{2+} and aequorin interact and emit blue light, some of which is absorbed by GFP causing a color shift towards green (Morise et al. 1974). However, it wasn't until 1992 that the influence of GFP was realized. Prasher et al. (1992) paved the way for future GFP application by cloning and sequencing both cDNA and genomic clones from *Aequorea victoria*. In 1994, Chalfie et al. first expressed GFP within a living organism, highlighting sensory neurons in nematodes. These landmark studies influenced modern science as purified GFP has become one of the most important reporter genes in biology (Bloemberg 2007).

GFP is comprised of an eleven-stranded β barrel with a coaxial helix running through the centre. The chromophore is formed from the spontaneous cyclization of the

central helix consisting of the tripeptide, Ser65-Try66-Gly67 (Ormo et al. 1996). Introducing random amino acid substitutions into the twenty amino acids that flank the chromophore has led to altered forms of GFP some of which exhibit greater fluorescence. The first GFP modification was derived from a point mutation that altered Serine 65 to Threonine (S65T) (Heim et al.1995). This alteration shifted the excitation and emission maxima to 490 and 510 nm, respectively. Alterations of GFP not only affected intensity, but also increased color variety that is within the blue, cyan and yellow regions of the electromagnetic spectrum (Shaner et al. 2007).

Green fluorescent protein can be used as a reporter or tag to mark whole cells, study protein localization and monitor gene interactions and interactions between microorganisms. Its incorporation is non-invasive, it does not require an additional substrate for bioluminescence, nor does visualization necessitate fixation or staining protocols (Bloemberg 2007). The gene product of *gfp* can be expressed in both prokaryotes and eukaryotes (Chalfie et al. 1994) with no background gene expression (Tombolini et al. 1997). It is highly stable in the presence of denaturants and proteases and persists at high temperatures (65°C) and a wide range of pH values (6-12) (Bloemberg 2007; Chalfie et al. 1994; Ward et al. 1980).

Applications of GFP in biological control experiments can help elucidate mechanisms of action (MOA). Watanabe et al. (2007) transformed two fungal species with *gfp*, *Trichoderma asperellum* SKT-1, the antagonist, and *Gibberella fujikuroi* N-68, a pathogenic root fungus responsible for Bakanae disease of rice seedlings. The fluorescent protein was imaged by confocal scanning laser microscopy (CSLM) and mycoparasitism was suggested as the putative MOA. Scanning electron microscopy

(SEM) and CSLM showed a disappearance of GFP in *G. fujikuroi* upon contact with *T. asperellum* suggesting cell wall degradation.

In another example, Bolwerk et al. (2003) transformed red fluorescent protein (RFP) into two putative BCAs, *Pseudomonas fluorescens* WCS365 and *Pseudomonas chlororaphis* PCL1391, that have been shown to control *Fusarium oxysporum* f. sp. *radicis-lycopersici,* a causal agent of tomato foot and root rot (TFRR). Both *Pseudomonas* spp. contained RFP, whereas *Fusarium oxysporum* f. sp. *radicis-lycopersici* harboured GFP. CSLM revealed that both antagonists colonized the tomato root more quickly than the fungal pathogen and that the bacterial presence hindered root infection. A proposed MOA was that the presence of fungi initiated bacterial colonization of hyphae and subsequent production of fungal secondary metabolites.

1.7 Objectives

This thesis will build upon preliminary research and address the following three objectives: 1) validate the use of DNA macroarrays under field conditions; 2) assess bacterial antagonist performance under semi-commercial and commercial conditions and 3) determine how long and in what capacity a selected antagonist will colonize the fruit surface. I hypothesize that:

1) Macroarray technology will accurately identify and quantify post-harvest apple pathogens throughout the growing season and that pathogen prevalence will correlate with disease incidence post-harvest.
2) One or more of the bacterial antagonists will provide significant pathogen control in semi-commercial and commercial storage conditions.

3) The use of green fluorescent protein will facilitate visualization of bacterial colonization and survival on apple surfaces.

CHAPTER 2: MATERIALS AND METHODS

2.1 Antagonists

Four bacterial antagonists were obtained from Dr. L. Nelson and were identified by 16S rRNA sequence and fatty acid methyl ester analysis as *Pseudomonas fluorescens* (isolates 1-112, 2-28, 4-6) and *Serratia plymuthica* (isolate 6-25) (Hynes et al. 2008). Isolate 1100-6, *Pseudomonas fluorescens* (Etebarian et al. 2005), was provided by Dr. Peter Sholberg of the Pacific Agri-Food Research Centre (PARC) in Summerland, BC. The bacteria were grown in half-strength tryptic soy broth (½ TSB) (Becton, Dickinson and Company, Sparks, MD) at 28°C with shaking at 220 rpm. The final culture was centrifuged for 20 min at 3800 g and held at 20°C. Cell pellets were resuspended in sterile phosphate buffered saline solution (PBS) (1.2 g/L Na_2HPO_4, 0.18 g/L NaH_2PO_4, 8.5 g/L NaCl). The optical density (OD_{600}) was determined and the bacterial cells were diluted to 10^8 CFU/ml according to a standard curve relating OD_{600} to CFU/ml.

2.2 Pathogens

Penicillium expansum Link strain 1790, *Botrytis cinerea* Pers.:Fr strain B27 and *Mucor piriformis* Fischer strain 536 were provided by Dr. Peter Sholberg and grown on half-strength potato dextrose agar (½ PDA) (HiMedia Laboratories PVT. Ltd., India) for 7 days at 22°C. A spore suspension was created using sterile water and Tween 20 (MP Biomedicals, LLC, Solon, OH) and then enumerated using a Petroff-Hauser counter.

2.3 Orchard field sites

This study incorporated four conventional (non-organic) orchard field sites from which data were collected over two years. Two research orchards were located at PARC in Summerland, BC. Field 3 (49° 33' 58.49" N; 119° 38' 41.93" W) contained 166 Jonagold apple trees planted at high density. Field 12 (49° 33' 54.81" N; 119° 38' 56.73" W) contained 120 Gala apple trees planted at high density. The two commercial orchards were located in Kelowna, BC. The Kiran orchard (49° 50' 41.45" N; 119° 24' 54.24" W) grew Gala apples planted at high density. The Reekie orchard (49° 50' 58.78" N; 119° 23' 32.79" W) grew Red Delicious apples planted at medium density.

I-rods were used in this study to collect aerial spore samples. I-rods are clear polystyrene rods that rest within a sampling head of a rotating spore trap. When the motor spins, centrifugal force causes the I-rods coated in silicone grease to extend downwards at a 90^{o} angle and collect airborne particles (Aerobiology Research Laboratories, Nepean, ON). In the 2007 and 2008 growing seasons, Fields 3 and 12 contained one I-rod station per field. The Kiran orchard contained one I-rod station in both 2007 and 2008 and the Reekie orchard contained one I-rod station in 2008 only. Average daily temperatures (°C) and total daily precipitation (mm) from Environment Canada (2008) were plotted and used to compare with pathogen DNA detected throughout the apple growing seasons (Figure 1). Fungicide spray records for Fields 3 and 12 were provided. On May 15, 2007, the fungicide, Nova (myclobutanil) was applied to Field 12. On September 15, 2008, the fungicide Funginex (triforine) was applied to Field 3.

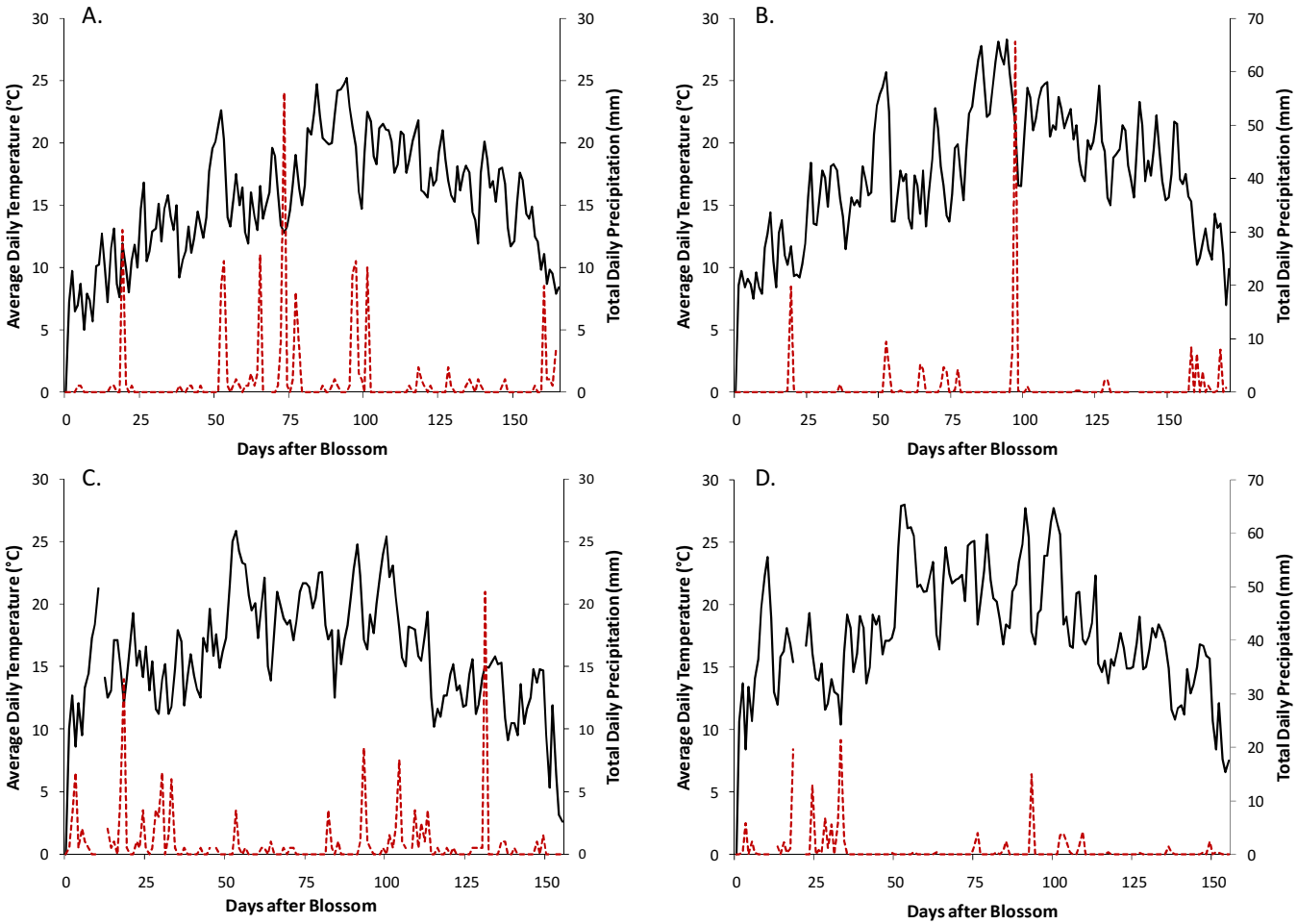

Figure 1. Average daily temperatures (°C) (solid line) and total daily precipitation (mm) (dashed line) at Kelowna, BC during the growing seasons of 2007 (A) and 2008 (B) and in Summerland, BC during the growing seasons of 2007 (C) and 2008 (D) as indicated by Environment Canada (2008).

2.4 pAG408

The *gfp*-containing plasmid, pAG408 (Suarez et al. 1997), was provided by Dr. Darren Korber, University of Saskatchewan, Saskatoon, Sask. pAG408 was maintained within the donor strain, *E. coli* S-17 λ pir, on Luria Burtani (LB) agar (10 g/ L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 50 µg/ml of kanamycin (kan) and 30 µg/ml of gentamicin (gen).

To test for antibiotic efficacy, pAG408 in *E. coli* S-17 λ pir was streaked onto three replicate plates each of LB agar, LB agar + 50 µg/ml kan, LB agar + 30 µg/ml gen and LB agar + 50 µg/ml of kan and 30 µg/ml of gen and grown at 28°C for 48 h. Furthermore, pAG408 plasmid confirmation was verified by extracting it from *E. coli* S-17 λ pir using the Wizard ® Plus Minipreps DNA Purification System (Promega, Madison, WI) as per manufacturer's instructions, and digesting 1 µg of DNA with 1 U *Hind*III (Invitrogen, Carlsbad, CA) for 1 h at 37°C.

2.5 Construction of the *gfp*-transconjugant

Bacterial isolate 4-6 was transformed with pAG408 via bacterial conjugation. Donor cells (*E. coli* S-17 λ pir containing pAG408) and recipient cells (4-6) were grown overnight at 28°C with shaking at 220 rpm. One millilitre of each culture was centrifuged at 10 000 x g for 20 min at 4°C. The supernatants were removed, the pellets washed twice with PBS then resuspended in 50 µl of PBS. Fifty microlitres each of the donors and recipients were combined and vortexed. The combined cultures were spotted on 0.22 µm sterile nylon membrane filters (Whatman, Maidstone, England) placed on LB agar plates supplemented with 50 µg/ml of kan and 30 µg/ml of gen. The plates were

incubated for 24 h at 28°C to allow for bacterial conjugation. The bacterial mixtures were then removed from the filters and plated on sodium citrate agar plates (0.2 g/L $MgS0_4$, 1.0 g/L $NH_4H_2PO_4$, 2.0 g/L sodium citrate, 5.0 g/L NaCl, 15 g/L agar) supplemented with 50 µg/ml of kan and 30 µg/ml of gen and then incubated for 48 h at 28°C. The combination of sodium citrate growth medium and antibiotics eliminated *E. coli* S-17 λ pir pAG408 cells and non-transformed bacterial cells; *E. coli* cannot use citrate as a carbon source and the wild-type biological control isolates cannot withstand the antibiotics. For a negative control, non-transformed *Pseudomonas fluorescens* isolate 1-112, *Serratia plymuthica* isolate 6-25 and *E. coli* S-17 λ pir containing pAG408 were spread on sodium citrate agar plates supplemented with 50 µg/ml of kan and 30 µg/ml of gen.

To confirm successful conjugation, 10 putative transconjugants were selected at random and their genomic DNA was extracted by boiling. One colony was placed in 100 µl of sterile water and the supernatant was used as the DNA template for the PCR that utilized *gfp*-specific primers. Three microlitres of DNA were used in the 25-µl reaction containing 1x PCR Buffer (TetraLink International, Buffalo, NY), 2.5 mM $MgCl_2$, 0.1 mM dNTP mix (Invitrogen, Carlsbad, CA) , 0.4 µM of each forward (*gfp* F: 5'-GAGTAAAGGAGAAGAACTTTTCA-3') and reverse primers (*gfp* R: 5'-TTATTTGTATAGTTCATCCATG-3') (Suarez et al. 1997) and 1 U of UltraTherm DNA polymerase (TetraLink International, Buffalo, NY). Amplification was performed on the Techne TC-3000 thermocycler (Barloworld Scientific, London, England) with the following conditions: 96°C for 5 min followed by 35 cycles of 95°C for 1 min, 54.8°C for 1 min and 72°C for 1 min with a final extension cycle at 72°C for 7 min.

2.6 Plasmid stability

Testing of the stability of exogenous DNA within bacterial isolate 4-6 was conducted according to Bloemberg et al. (1997) except that tryptic soy medium and the antibiotics, kanamycin and gentamicin, were used. Based on replicated stability results and the 2007 biocontrol experimental results, the promising *gfp*-tranconjgant biological control strain, *P. fluorescens* isolate 4-6, was selected as a suitable antagonist for bacterial survival and colonization studies.

2.7 *P. fluorescens* 4-6-*gfp* fitness analysis

Growth rates of 4-6-*gfp* and its wild-type counterpart (4-6 wild-type) were compared. Three replicates each of 4-6-*gfp* and 4-6 wild-type were grown in 10 ml of ½ TSB and incubated at 28°C with shaking at 220 rpm for 24 h. Cultures were spun for 20 min at 5000 x g and resuspended in 10 ml of phosphate buffered saline (PBS). Optical density (OD_{600}) was determined and cultures were diluted and standardized with PBS to within 0.100 of each other. One hundred microlitres of standardized culture were added to 10 ml of ½ TSB. OD_{600} readings were recorded at 0, 2, 4, 6, 8, 10, 12 and 24 h. The three replicates were averaged and represented graphically (time vs OD_{660}). Growth rates (μ) were calculated with the formula $\mu = (\log_{10} N - \log_{10} N_o) \times 2.303 / t - t_o)$ where N was a final cell number, N_o was an initial cell number, t was the time at N and t_o was the time at N_o.

Carbon utilization profiles of 4-6-wild-type and 4-6-*gfp* were also compared by determining carbon profiles using BioLog Phenotype MicroArrays for Microbial Cells

(Biolog, Inc., Hayward, CA) (microplate PM1 and PM2) as per the manufacturer's instructions

2.8 Spore, apple tissue collection and fruit washings

Apple leaves, blossoms and I-rods were collected at orchard field sites throughout the apple growing seasons (Table 1). In general, sampling was conducted three times per week at the beginning (early) and end (late) of the growing seasons in 2007 and 2008. Throughout the mid-growing seasons, samples were collected approximately once per week. At harvest, approximately 1250 apples were collected at random from each of Fields 3 and 12 and approximately 300 apples were collected at random from the Kiran and Reekie orchards.

Fruit surface washings were conducted on 20 apples each from Fields 3 and 12 in 2007 and 20 apples each from Fields 3, 12, the Kiran and the Reekie orchards in 2008. Twenty apples from each orchard were washed in a 600-ml beaker containing 200 ml of sterile distilled water and 200 µl of Tween 80. Each fruit was shaken for 5 min at 120 rpm and sonicated for 5 min (P. Sholberg, personal communication). After all fruit were washed, the solution was centrifuged at 13 400 g for 10 min. The supernatant was decanted and this process was repeated five times to a final volume of 10 ml that was centrifuged for 15 min at 7430 g. The supernatant was decanted and the remaining cells were transferred to a 2-ml tube and centrifuged at max (16 100 g) for 1 min. The supernatant was removed and 100 µl of water were added for storage at -20°C.

Table 1. Frequency of aerial and plant tissue sampling for Fields 3, 12 and the Kiran and Reekie orchards in the early, middle and late growing seasons of 2007 and 2008.

		Number of samples within sampling period (n)			
Field	**Sample type**	**Early**	**Mid**	**Late**	**Total**
2007 Growing Season					
Field 3	aerial	14	6	6	26
	plant tissue	2	3	2	7
Field 12	aerial	16	6	5	27
	plant tissue	2	3	2	7
Kiran	aerial	9	7	6	22
	plant tissue	4	7	5	16
Reekie	aerial	0	0	0	0
	plant tissue	6	8	4	18
2007 Total		**53**	**40**	**30**	**123**
2008 Growing Season					
Field 3	aerial	18	11	3	32
	plant tissue	4	0	2	6
Field 12	aerial	19	11	3	33
	plant tissue	4	0	2	6
Kiran	aerial	17	24	11	52
	plant tissue	18	24	10	52
Reekie	aerial	15	20	11	46
	plant tissue	16	24	11	51
2008 Total		**111**	**114**	**53**	**278**

2.9 Validation and field testing of DNA macroarrays

2.9.1 DNA extraction

DNA was extracted from I-rods, plant tissue samples and fruit washings using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. For DNA isolation, two I-rods were used per aerial

extraction and 0.25 g of plant tissue were used for tissue extractions. DNA was eluted in 100 µl of Buffer 5 and stored at -20°C until required for further use.

2.9.2 PCR amplification

PCR amplification was used to amplify universally conserved regions of the fungal genome, ribosomal DNA interspacer regions (rDNA ITS) and the β-tubulin gene. Approximately 10 – 20 ng of extracted DNA were used in 25-µl reactions containing 1x PCR Buffer (TetraLink International, Buffalo, NY), 2.5 mM $MgCl_2$, 0.1 mM digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany) (DIG)-dNTP mix , 0.4 µM of each forward and reverse primers and 1 U of UltraTherm DNA polymerase (TetraLink International, Buffalo, NY). Primers for rDNA were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3') (White et al. 1990) and β-tubulin primers were Bt-LEV-Lo1 (Fwd 5'-GTGAACTCCATCTCGTCCATA-3') and Bt-LEV-Up4 (5'-CAAGATCCGTGAGGAGT-3') (de Jong et al. 2001). Amplification was performed on the Techne TC-3000 thermocycler (Barloworld Scientific, London, England) with the following conditions: 96°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final extension cycle at 72°C for 7 min. DNA concentrations were estimated on 1% agarose gels using a Low Mass ladder (Invitrogen, Gaithersburg, MD). A minimum of 75 ng and a maximum of 100 ng of PCR-amplified DNA were required for hybridization to macroarrays.

2.9.3 DNA macroarray construction

Twenty-eight oligonucleotide macroarray probes were designed by Dr. Danielle Hirkala and constructed by Operon Technologies, Huntsville, AL (Table 2). The 5' end of the probe contained an amino C6 linker used to attach the oligonucleotide onto the Immunodyne ABC nylon membranes (PALL Europe Ltd., Portsmouth, England). The probes were resuspended in 0.5 M sodium bicarbonate buffer (pH 8.0) and diluted to a final concentration of 40 µm. In combination with 0.004% bromophenol blue, the resuspended probes were arranged according to a previously designed template (Figure 2). A 384-pin replicater (V&P Scientific, San Diego, CA) and pin guide (V&P Scientific, San Diego, CA) were used to "stamp" the probes onto the nylon membranes (Figure 2). The membranes were blocked for a minimum of 1 hour with hybridization buffer (6x SSC, 0.1% sarcosine, 0.02% SDS) and 1% skim milk at 55°C.

2.9.4 Hybridization and chemiluminescent detection

To denature the DIG-labelled PCR products, 75 – 100 ng of DNA were combined with 5 ml of hybridization buffer and placed in boiling water for 10 min. Membranes were added to the denatured probe solution for overnight hybridization at 55°C. Unbound probes were then washed from the membranes with pre-warmed 6x SSC (0.18% NaCl, 0.088% sodium citrate) and 1% SDS (sodium dodecyl sulphate) for 2 x 40 min at 55°C. Hybridized membranes were pooled and washed for 5 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl [pH 7.5], 0.3% Tween 20) and bound with a 1:25 000 dilution of anti-Digoxigenin-AP (alkaline phosphatase) Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) combined with washing buffer and 1% skim

Table 2. DNA macroarray probe sequences.

Name	Sequence 5' to 3'	Description
	rDNA-ITS	
UN-H1-up	[AminoC6]ACCTACGGAAACCTTGTTACGA	Universal to all fungi
Bot-all-H1b	[AminoC6]TTAGCTTGGTATTGAGTCTATGT	Universal to all *Botrytis* spp.
BC-H2d	[AminoC6]TATGCTCGCCAGAGAATACCAAA	*Botrytis cinerea*
BC-H3d	[AminoC6]GCTCGCCAGAGAATACCAAAAC	*Botrytis cinerea*
BT-H1d	[AminoC6]TATGCTCGCCAGAGAAAACCAAA	*Botrytis tulipae*
BT-H2d	[AminoC6]CTCGCCAGAGAAAACCAAAACT	*Botrytis tulipae*
PE-H2c	[AminoC6]CCCGAACTCTGCCTGAAGATT	*Penicillium expansum*
PE-H3u	[AminoC6]CAGACGACAATCTTCAGGCA	*Penicillium expansum*
Mpir-ITS-183H1	[AminoC6]TGGTGTCCTTAAAAATTATTATTAT	*Mucor piriformis*
Mpir-ITS-414H1	[AminoC6]AACACCCCACATCTTAAAAATC	*Mucor piriformis*
	β-tubulin	
UNbt-1	[AminoC6]CAAGAACATGATGGCTGCTTC	Universal to all fungi
UNbt-2	[AminoC6]CCAAGAACATGATGGCTGC	Universal to all fungi
UNbt-3	[AminoC6]TGTTCGACCCCAAGAACATG	Universal to all fungi
Pex-bt-84-H1-gag	[AminoC6]TCCGACGAGACTTTCTGTATC	*Penicillium expansum*
Pex-bt-84-H1-gcg	[AminoC6]TCCGACGCGACTTTCTGTATC	*Penicillium expansum* TBZ sensitive
Pex-bt-84-H1-gtg	[AminoC6]TCCGACGTGACTTTCTGTATC	*Penicillium expansum* TBZ resistant
Pcom-bt-1	[AminoC6]CCGTCAACATGGTCCCCTT	*Penicillium commune*
Psol-bt-1	[AminoC6]TCCCTCGTTTGCACTTCTT	*Penicillium solitum*
Psol-bt-2	[AminoC6]CCTTTCCGTCCGTCCACCAGCT	*Penicillium solitum*
Paur-bt-1	[AminoC6]CACACCTCTGATATCTTGCTAGG	*Penicillium aureum*
Paur-bt-2	[AminoC6]CGATGGACAGTAAGTTCTAATGG	*Penicillium aurantigriseum*
Bcin-133-H3	[AminoC6]TTACGATATTTGCATGAGAACCT	*Botrytis cinerea*
Bstok-144-H4	[AminoC6]GCATGAGAACCCTGAAGCTC	*Botrytis mali*
Bot-95-H1-GAG	[AminoC6]AACTCTGACGAGACCTTCTG	*Botrytis cinerea* TBZ sensitive
Bot-95-H1-GCG	[AminoC6]ACTCTGACGCGACCTTCTG	*Botrytis cinerea* TBZ resistant
MucorUN-bt-1	[AminoC6]ACATGGTTCCTTTCCCTCGT	Universal to all *Mucor* spp.
MucorUN-bt-2	[AminoC6]AAGGCTTTCTTGCATTGGTA	Universal to all *Mucor* spp.
MucorUN-bt-3	[AminoC6]GGTGCTGGTAACTCTTGGGC	Universal to all *Mucor* spp.

A.

Post-Harvest Macrorray Template								
	rDNA-ITS			β-tubulin				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	UN-H1-up	BT-H1d	Mpir-ITS-183H1	UNbt-1	Pex-bt-84-H1gag	Psol-bt-1	Bcin-133-H3	MucorUN-bt-1
2	Bot-all-H1-b	BT-H2d	Mpir-ITS-414H1	UNbt-2	Pex-bt-84-H1gcg	Psol-bt-2	Bstok-144-H4	MucorUN-bt-2
3	BC-H2d	PE-H2c		UNbt-3	Pex-bt-84-H1gtg	Paur-bt-1	Bot-95-H1-gag	MucorUN-bt-3
4	BC-H3d	PE-H3u			Pcom-bt-1	Paur-bt-2	Bot-95-H1-gcg	

B.

Post-Harvest Macrorray Template								
	rDNA-ITS			β-tubulin				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	● ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●
2	● ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●
3	● ●	● ●		● ●	● ●	● ●	● ●	● ●
4	● ●	● ●			● ●	● ●	● ●	

Figure 2. DNA macroarray pathogen template (A) and the corresponding oligonucleotide probe placements that are amine-linked to the nylon membrane (B). Columns A – C detect rDNA ITS PCR products; columns D – H detect β-tubulin PCR products. Each pathogen (A) corresponds with the dotted template (B) as it would appear on a developed macroarray. Each probe is blotted twice for positive confirmation. Grey boxes indicate no probe present.

milk powder for 30 min. Membranes were washed for 2 x 15 min then primed for 15 min in buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]).

For chemiluminescent detection, membranes were incubated for 5 min in buffer 3 plus 1:2000 dilution of chemiluminescent substrate, CDP-Star (Roche Diagnostics GmbH, Mannheim, Germany). Alkaline phosphatase enzymatically dephosphorylates CDP-Star, creating dioxetane phenolate anion, which decomposes and emits light maximally at 466 nm (Roche Diagnostics GmbH, Mannheim, Germany).

Membranes were bound in plastic wrap and immediately exposed to x-ray film (CL-X Posure ^{TM} Film, Thermo Scientific, Rockford, IL) for 2 h. After fixing and developing for 1 minute each (Kodak, Rochester, NY), the hybridized DIG-amplified PCR products were captured on x-ray film as a series of dots with varying grey intensities. The x-ray film was scanned and saved as a Jpeg picture file using a BenQ 4300u scanner (Taipei, Taiwan) for computer analysis. The hybridized DNA was quantified using ImageJ 1.30v software (National Institutes of Health, Bethesda, MD).

Each hybridization signal was assigned a relative greyscale value that ranged from 0 (completely back) to 255 (completely white). Greyscale values were in the form of two averaged measurements. The background was subtracted from each analyzed array using the following formula:

$$\textbf{Adjusted Greyscale Value} = \frac{127.5 \times \text{Original Greyscale Value}}{\text{Average Background}}$$

The adjusted greyscale values were transformed to DNA concentrations when compared to a standard curve produced from known DNA concentrations.

2.10 Biological control of post-harvest fungal pathogens

2.10.1 Inoculation of apples by drenching

Bacterial cultures were diluted in 5 L of water to a concentration of 10^8 CFU/ml and fungal cultures were diluted in 5 L of water to concentrations of 10^4 spores/ml for *P. expansum* and *B. cinerea* and 10^5 spores/ml for *M. piriformis.* Inoculation concentrations were previously determined by minimum infectious dose and minimum inhibitory concentration experiments (D. Hirkala, personal communication). Apples were surface sterilised with 90% ethanol and wounded in triplicate using a 3-mm diameter nail embedded in a cork. Wounded apples were placed in a plastic net bag and labelled by tagging. Each bag was placed in a 5-L bacterial suspension for 1 min then subsequently placed in a 5-L fungal suspension for 1 min. Following inoculation, apples were air dried on trays for 10 min then placed in mesh bags (10 apples / bag) and in crates (~100 apples / crate) for commercial storage or in covered plastic bins each with three 0.2-µm membrane filters attached to the lid for semi-commercial storage until lesions formed. In this study, commercial storage refers to controlled atmosphere (CA) storage conditions in a commercial packinghouse, whereas semi-commercial storage refers to storage in a research facility at 1°C in air.

2.10.2 Natural disease incidence

Following apple harvest in 2007, natural disease incidence (NDI) was determined by wounding 100 Jonagold apples from Field 3 and 100 Gala apples from Field 12. Fifty of the 100 apples were fumigated with 1-Methylcyclopropene (1-MCP) and 50 were not fumigated with 1-MCP. In 2008, NDI was determined by placing 260 apples, each from Fields 3 (Jonagold), 12, Kiran (Gala) and Reekie (Red Delicious) into controlled

atmosphere (CA) storage (1.5% O_2 and 1.5% CO_2) (n = 100 per orchard) in Naramata, BC or in air storage at 1°C (n = 40 per orchard) at PARC in Summerland, BC. Half of the apples were wounded in triplicate and the remaining half were not wounded. 1-MCP treatments were not used in 2008 harvest experiments. Disease incidence (% apples infected) and lesion diameters (mm) were measured after 3 and 6 months for the 2007 harvest, after 4.5 months of sealed CA storage and after 1, 2, 4 and 6 months at 1°C in air storage for apples harvested in 2008.

2.10.3 Natural disease incidence with bacterial antagonists

To measure bacterial antagonist efficacy against natural disease incidence for harvest 2007, 120 Jonagold apples from Field 3 and 120 Gala apples from Field 12 were wounded in triplicate and inoculated with one of five bacterial antagonists – isolates 1100-6, 1-112, 2-28, 4-6 or 6-25- and a non-inoculated control (10 apples / antagonist). Sixty of the 120 apples were fumigated with 1-MCP and the remaining 60 apples were not fumigated. Following harvest in 2008, 240 apples from each orchard, Field 3, 12, Kiran and Reekie, were inoculated as in section 2.10.1. One hundred and twenty apples from each orchard were stored in CA (60 wounded and 60 not wounded) and 120 apples were stored at 1°C in air (60 wounded and 60 not wounded). Disease incidence and lesion diameters were measured after 3 months in storage for apples harvested in 2007. For apples harvested in 2008, disease incidence and lesion diameters were measured after 4.5 months of sealed CA storage and after 1, 2, 4 and 6 months at 1°C in air storage. The efficacy of the biological control agents was compared to apples that had been wounded and not inoculated with bacteria.

2.10.4 Biological control of post-harvest pathogens

For apples harvested in 2007, disease inhibition was measured by inoculating 360 Jonagold apples from Field 3 and 360 Gala apples from Field 12 with one of three fungal pathogens, *P. expansum, B. cinerea* or *M. piriformis* and one of five bacterial control isolates, 1100-6, 1-112, 2-28, 4-6, 6-25 and a non-inoculated control. One hundred and eighty of the 360 apples were fumigated with 1-MCP. For apples harvested in 2008, 360 apples each from Fields 3 and 12 and the Kiran and Reekie orchards were inoculated as above. In 2008, 1-MCP was not used, but 180 apples per orchard were wounded and 180 apples per orchard were non-wounded. From each site, there were 10 apples / antagonist + 1 non-inoculated control / pathogen. Disease incidence and lesion diameters were measured after 3 months of storage for apples harvested in 2007 and after 1, 2, 4 and 6 months at 1°C in air storage for apples harvested in 2008. Pathogens were not permitted in commercial CA storage.

2.10.5 Bacterial survival on apple

Concentrations of 4-6-*gfp* were monitored by two experimental techniques- colony counts via dilution plating and direct scanning using the POLARstar Omega microplate reader (BMG Labtech, Germany) as in Etebarian et al. (2005). A standard curve was developed that described the relationship between colony forming units (CFU/ml) and relative fluorescent units (RFU) acquired by direct scanning.

The bacterial inoculum was prepared in ½ TSB with 50 µg/ml of kan and 30 µg/ml of gen and grown at 28°C for 24 h. *P. expansum* spores were harvested from 7-d-old ½ PDA plates. Final inoculation concentrations for 4-6-*gfp* and *P. expansum* were 10^8 CFU/ml and 10^4 spores/ml, respectively. Apples were prepared as in section 2.9.1

and inoculated by pipette (30 µl) with the following treatments: 1) non-inoculated control; 2) 4-6-*gfp* alone; 3) *P. expansum* alone and; 4) 4-6-*gfp* + *P. expansum*. Isolate 4-6-*gfp* was quantified by aseptically removing an apple core (5 mm in diameter x 5 mm deep) from two of the three apple wounds. Both cores were macerated with a sterile rod. One core, to be used for dilution plating, was placed in 1.1 ml of PBS and plated on Pseudomonas F agar (20 g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L anhydrous $MgSO_4$, 10 g/L agar [pH 7.2]) supplemented with 50 µg/ml kan and 30 µg/ml gen. The second core, to be used in direct scanning, was placed in 300 µl of PBS. Two hundred microlitres of the tissue slurry were added to a well in a black Nunc 96-well microplate (Thermo Fisher Scientific, Rochester, NY). Fluorescent readings were obtained using a 405 nm excitation filter and a 510 nm emission filter and a gain of 1200. The relative fluorescent units (RFU) of PBS and non-inoculated control were subtracted from the treatments to correct for background and apple fluorescence, respectively. Bacterial concentrations were determined approximately every 10 days for a total of 55 d.

2.11 Analysis

In all biocontrol experiments, disease was measured by disease incidence (percent apple infection) and disease severity (average lesion diameter in mm). From these two values, data were converted to infection severity (IS) (Spotts et al. 1999):

$$\textbf{Infection Severity (IS)} = \frac{\%\ \text{Apple Infection} * \text{Average Lesion Diameter in mm}}{100\%}$$

In order to correct for variance heterogeneity, infection severity values were $\log_{10}(n+0.5)$ transformed. Differences in means were analyzed by either a one-way or two-

way analysis of variance (ANOVA). *p*-values less than 0.05 were considered statistically significant. If the 2-way ANOVA interaction term was significant, the main effects were separated and a one-way ANOVA was used for subsequent analysis. Differences among means were determined by the least significant difference (LSD) test. The Pearson Product Moment Correlation Coefficient (*r*) was used to measure the linear association of two independent variables. Statistical analyses were conducted using CoStat Statistics Software (CoHort Software, Monterey, CA).

CHAPTER 3: RESULTS

3.1 Validation and field testing of DNA macroarrays

3.1.1 Quantification of post-harvest pathogens

Macroarray construction for the identification of post-harvest pathogens was completed by Dr. Danielle Hirkala and was based upon the prototype proposed in Sholberg et al. (2005a). The array displayed high specificity, no cross-reactivity and detected as little as 0.1 ng of pure culture fungal DNA extracted from *P. expansum* and *B. cinerea* and up to 0.01 ng of pure culture of *M. piriformis* (Hirkala, unpublished).

For the purpose of this study, three macroarray probes were selected for pathogen identification: PE-H3u for *P. expansum* detection, BC-H2d for *B. cinerea* detection and Mpir-ITS-414H1 for *M. piriformis* detection. Each of the three selected probes is comprised of sequences in the rDNA ITS region and is located on the left hand side of the macroarray template (Figure 2). These probes were selected because of their overall higher sensitivity compared to other probes. This decision was based upon all 2007 greyscale values obtained from environmental field data. Standard curves were determined for each of the three probes (Figures 3, 4 and 5).

To test the quantitative properties of the amino-linked oligonucleotides, 0 – 100 ng of pure culture fungal DNA was hybridized to the macroarray. Each detector oligonucleotide displayed varying levels of hybridization signal intensity; however, a linear relationship did exist between DNA concentration and hybridization signal strength. The linear range of the probe PE-H3u ((♦) r = -0.974; (●) r = -0.962) was between 0 and 3 ng (Figure 3), whereas the linear range for the probe BC-H2d

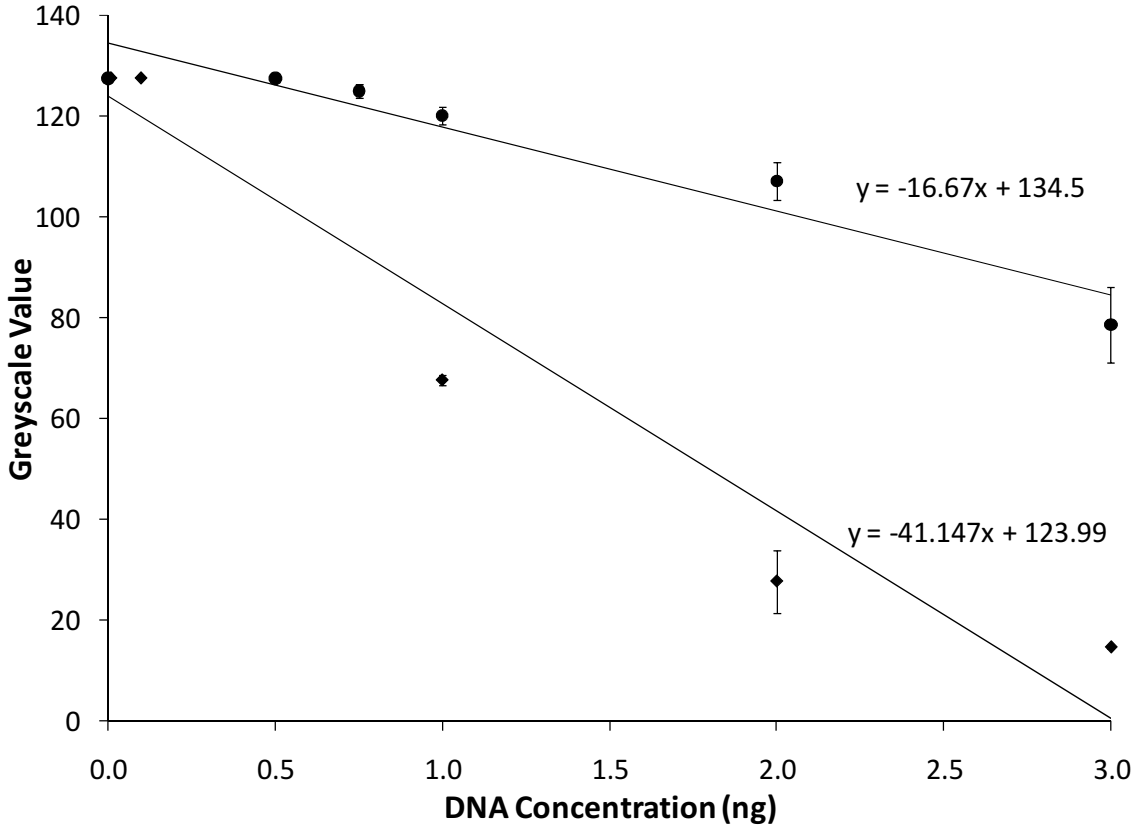

Figure 3. Quantitative assessment of *Penicillium expansum* probe PE-H3u illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.974); (●) Represents standard curve used for samples collected in 2008 (r = -0.962). Error bars represent standard error of the mean.

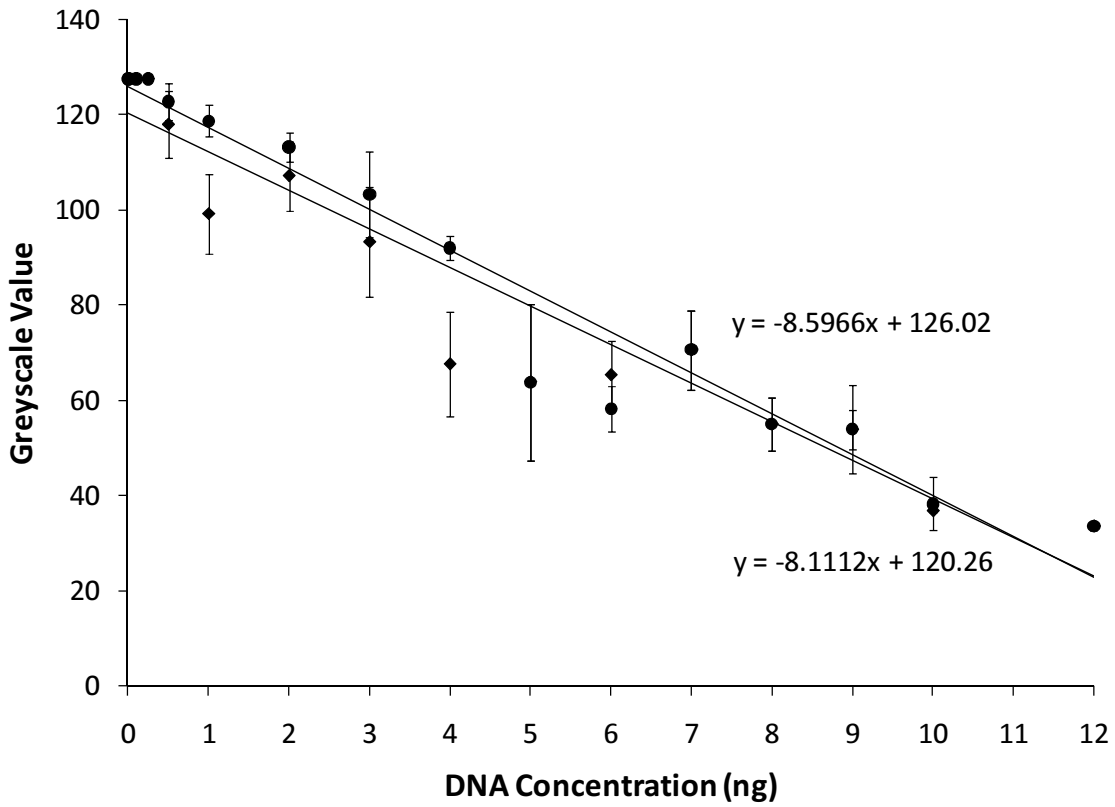

Figure 4. Quantitative assessment of *Botrytis cinerea* probe BC-H2d illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.960); (●) Represents standard curve used for samples collected in 2008 (r = -0.977). Error bars represent standard error of the mean.

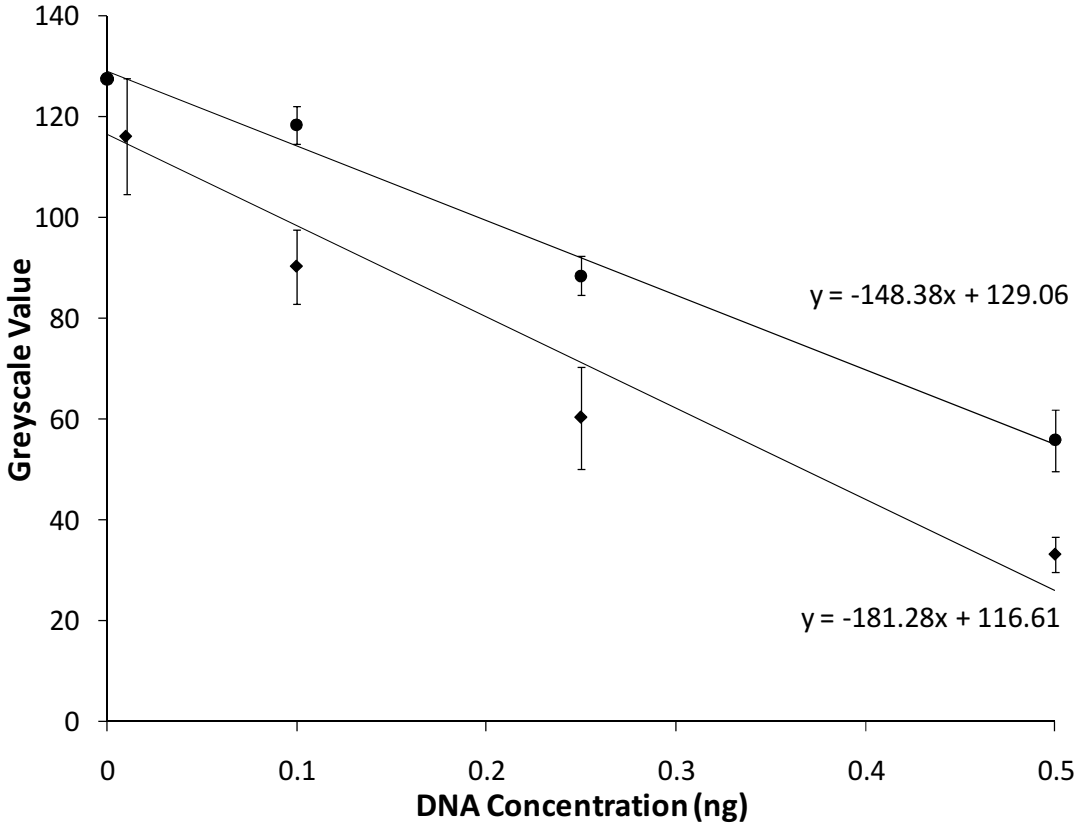

Figure 5. Quantitative assessment of *Mucor piriformis* probe Mpir-ITS-414H1 illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.970); (●) Represents standard curve used for samples collected in 2008 (r = -0.990). Error bars represent standard error of the mean.

((♦) r = -0.960; (●) r = -0.977) was between 0 and 12 ng (Figure 4) and the probe Mpir-ITS-414H1 ((♦) r = -0.970; (●) r = -0.990) was between 0 and 0.5 ng (Figure 5). Above these ranges, the curves deviated from linearity as the oligonucleotides reached their saturation level. Throughout the duration of this study, the intensity of the hybridization signals decreased over time which led to the construction of two separate quantification curves for each of the three pathogens (Figures 3, 4 and 5).

The two standard curves created for *P. expansum* DNA quantification (ng) were constructed in August 2008 (♦) and August 2009 (●) (Figure 3). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -41.147x + 123.99 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -16.67x + 134.5 (●).

The two standard curves created for *B. cinerea* DNA quantification (ng) were constructed in August 2008 (♦) and October 2009 (●) (Figure 4). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -8.1112x + 120.26 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -8.5966x + 126.02 (●).

The two standard curves created for *M. piriformis* DNA quantification (ng) were constructed in October 2008 (♦) and August 2009 (●) (Figure 5). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -181.28x + 116.61 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -148.38x + 129.06 (●).

3.1.2 Field application of macroarray technology

The DNA macroarray was used in two years of field monitoring studies to verify airborne and plant tissue pathogen spore loads in four orchards in the Okanagan Valley, BC: Field 3 in Summerland, BC (Figure 6); Field 12 in Summerland, BC (Figure 7); Kiran orchard in Kelowna, BC (Figure 8); Reekie orchard in Kelowna, BC (Figure 9).

All three probes had positive hybridization signals throughout the growing season with high variability between years and sample types. In Field 3 (Figure 6), all three pathogens, *P. expansum* (A. 2007, D. 2008), *B. cinerea* (B. 2007, E. 2008) and M. *piriformis* (C. 2007, F. 2008) were detected most frequently in the early and late growing seasons. In Field 12 (Figure 7), *P. expansum* was detected at the beginning and middle of the growing season in 2007 (A) and throughout the growing season in 2008 (D). Pathogen levels were much higher in 2008 (D) than in 2007 (A). *B. cinerea* was detected in the middle of the growing season in 2007 (B) and the beginning and the end of the growing season in 2008 (B). *B. cinerea* was not detected on plant tissue samples in either 2007 or 2008 (B, E). *M. piriformis* was detected at the beginning and middle of the growing season in 2007 (C) and at the beginning and the end of the growing season in 2008 (F). In the Kiran orchard (Figure 8), *P. expansum* (A. 2007, D. 2008), *B. cinerea* (B. 2007, E. 2008) and M. *piriformis* (C. 2007, F. 2008) were detected throughout the growing season in both 2007 and 2008. DNA concentrations for *P. expansum* and *B. cinerea* were higher in 2008 than in 2007. In the Reekie orchard (Figure 9), *P. expansum* was detected in the middle of the growing season in 2007 (A) and throughout the growing season in 2008 (D). *B. cinerea* was not detected in 2007 (B) and was detected throughout the growing season in 2008 (E). *M. piriformis* was detected in the middle of

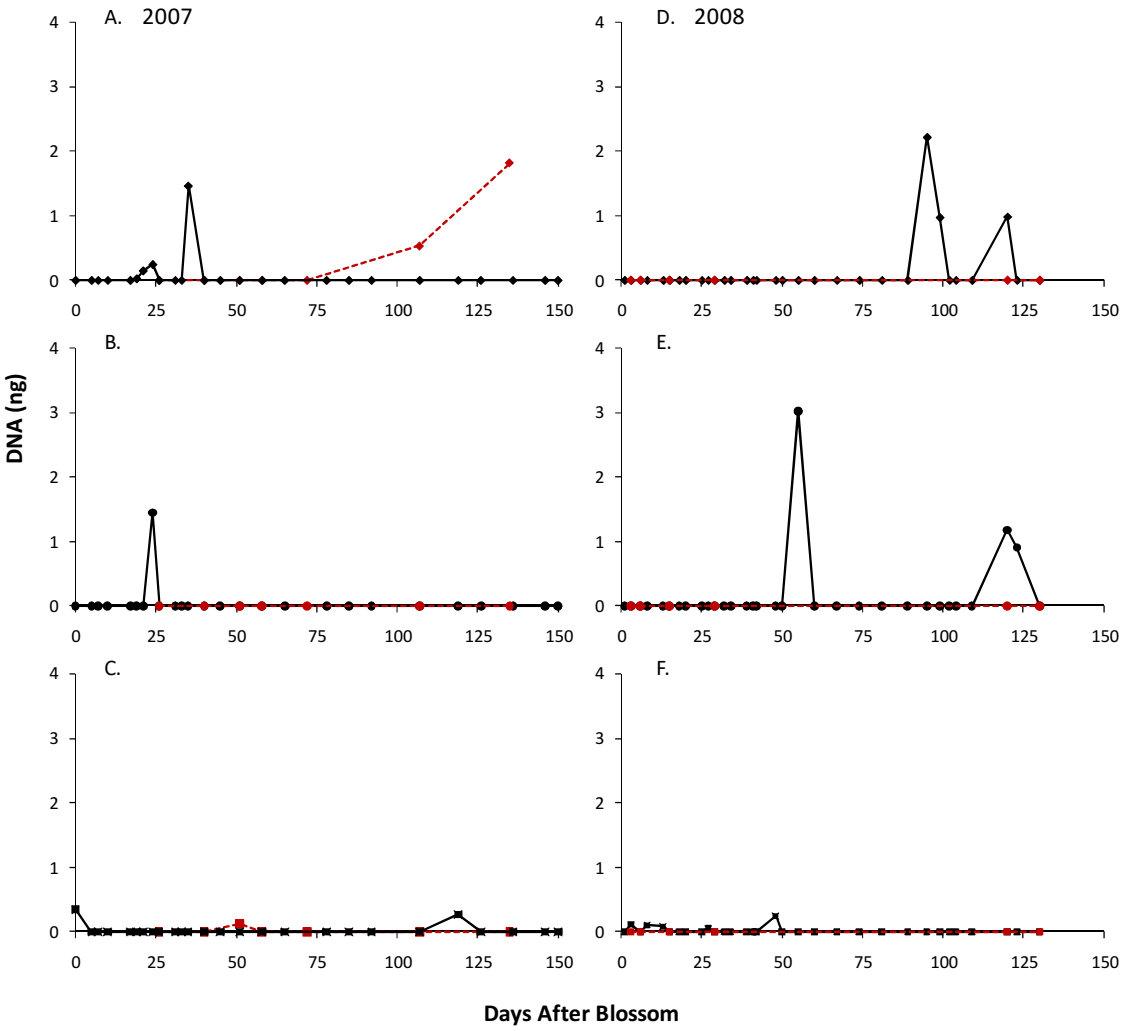

Figure 6. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from Field 3 (Summerland, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

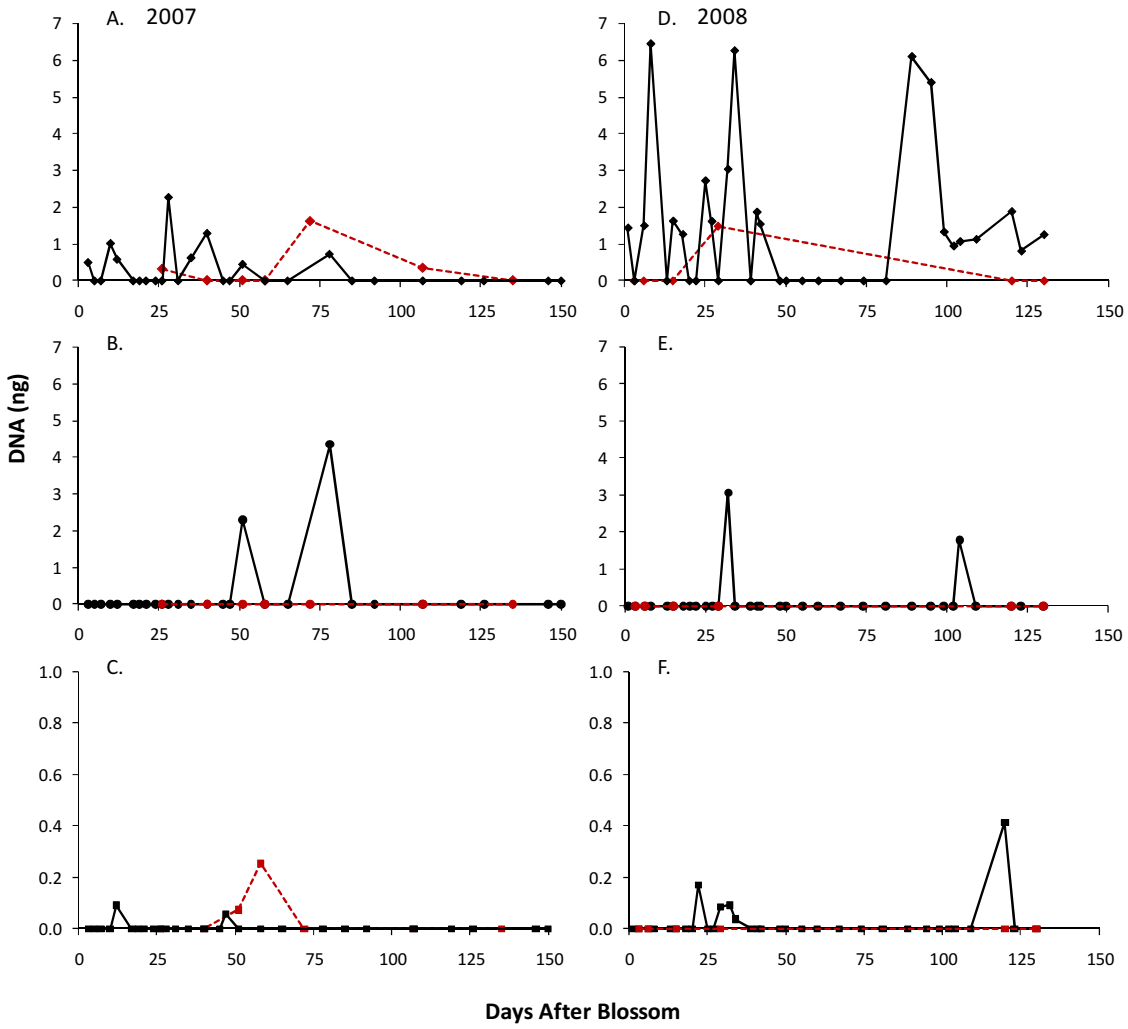

Figure 7. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from Field 12 (Summerland, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

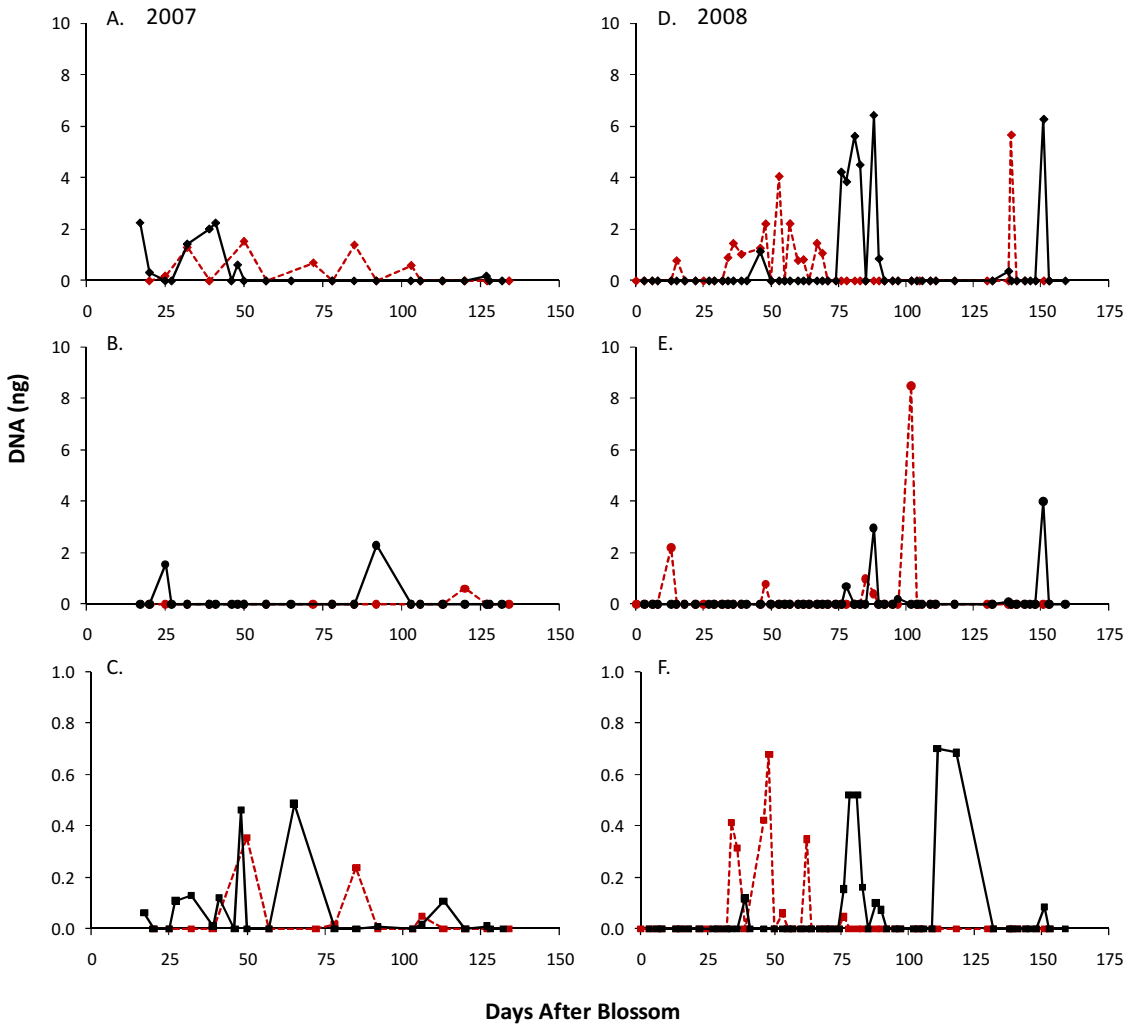

Figure 8. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from the Kiran orchard (Kelowna, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

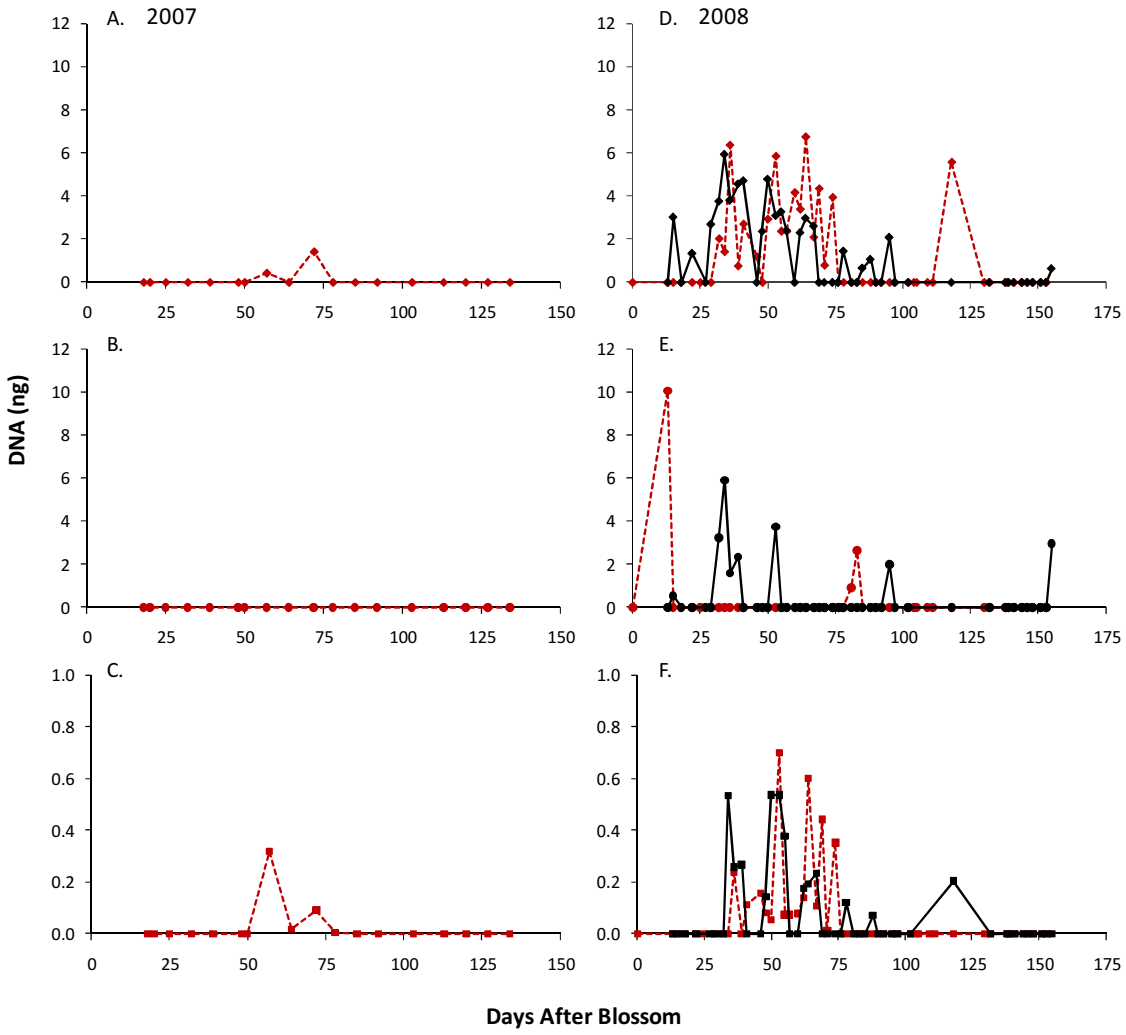

Figure 9. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from the Reekie orchard (Kelowna, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

the growing season in 2007 (C) and throughout the growing season in 2008 (F). All pathogen levels were higher in 2008 than in 2007.

Due to the observed variability, the data were arranged in an alternate way in order to determine if there were common patterns between pathogens and fields (Table 3). To determine what time in the growing season each of the three main post-harvest pathogens was primarily detected, the season was divided into three sections: 1) early (~1 – 50 days after blossom); 2) mid (~50 – 100 days after blossom) and; 3) late (~100 – 150+ days after blossom) growing season (Table 3). Aerial and plant tissue samples were analyzed separately and in combination by determining percent pathogen detection within either the early, mid or late growing seasons.

According to the tabulated data in Table 3, in 2007 and 2008, *P. expansum* was detected in each of the three growing season segments but differed by field and year. From DNA isolated from aerial samples collected in 2007, there was a significant effect of growing season segment on % *P. expansum* detected ($p = 0.06$) (Appendix, Table A1); the % *P. expansum* detected in the early season was significantly higher than that in the late growing season (Table 3). There was no effect of growing season segment on % *P. expansum* detected in aerial samples in 2008 (Table 3; Appendix, Table A4). From DNA isolated from plant tissue samples in 2007 there was no significant effect of the growing season segment on the % *P. expansum* detected (Table 3; Appendix, Table A2), but in 2008, there was a significant effect ($p = 0.053$) (Appendix, Table A5). The % *P. expansum* detected in the mid-growing season was significantly higher than that in the late-growing season (Table 3).

In 2007, there was no effect of the growing season segment on the % *B. cinerea* detected in DNA isolated from either aerial or plant tissue samples (Table 3; Appendix, Tables A1 and A2). In 2008, from DNA isolated from plant tissue samples, the % *B. cinerea* detected in the mid-growing season was significantly higher than that in the early and late-growing seasons (Table 3; Appendix, Table A5). Overall, *B. cinerea* was detected at much lower levels than either *P. expansum* or *M. piriformis*.

M. piriformis was detected in each of the three growing season segments but also differed by field and year (Table 3). From DNA isolated from plant tissue samples in 2007, the % *M. piriformis* detected was significantly higher in the mid-growing season than that in the early or late-growing seasons (Table 3; Appendix, Table A2). From DNA isolated from plant tissue samples in 2008, the % *M. piriformis* detected was significantly higher in the mid-growing season than that in the late-growing season ($p = 0.06$) (Table; Appendix, Table A5). In both 2007 and 2008, when aerial and plant tissue DNA samples were analyzed together, there was no effect of growing season segment (Table 3; Appendix, Tables A3 and A6).

In 2007 and 2008 combined, the selected probes detected *P. expansum* (27.4 ± 3.4%) and *M. piriformis* (19.2 ± 1.4%) most frequently, followed by *B. cinerea* (6.2 ± 1.4%) (Table 3; Appendix, Table A7). In addition, *P. expansum* was detected at the highest concentrations, followed by *B. cinerea* and then *M. piriformis*. Of the samples collected in 2007, the Kiran orchard had the highest percent detection (81.6%) followed by Field 12 (50%), the Reekie orchard (33.3%) and Field 3 (30.3%). In 2008, the Reekie orchard had the highest percent detection (80.4%) followed by Field 12 (71.8%), Kiran (46.2%) and Field 3 (28.9%) (Table 3).

Table 3. DNA macroarray detection (%) summary for *P. expansum*, *B. cinerea* and *M. piriformis* from four apple orchards throughout the 2007 and 2008 growing seasons. Growing season was divided into 3 sampling periods: 1) early-growing season (~ 1-50 days after blossom); 2) mid-growing season (~ 50-100 days after blossom) and; 3) late-growing season (~ 100-150+ days after blossom). Numbers in ( ) indicate standard error of the mean.

		% pathogen detection within sampling period[a]									
		P. expansum			***B. cinerea***			***M. piriformis***			**% detection per orchard**
Field	**Sample type**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	
2007 Growing Season											
Field 3	aerial	28.6	0.0	0.0	7.1	0.0	0.0	7.1	0.0	16.7	
	plant tissue	0.0	0.0	100.0	0.0	0.0	0.0	0.0	33.3	0.0	
Field ave		**25.0**[b]	**0.0**	**25.0**	**6.3**	**0.0**	**0.0**	**6.3**	**11.1**	**12.5**	**30.3**
Field 12	aerial	37.5	33.3	0.0	0.0	33.3	0.0	12.5	0.0	0.0	
	plant tissue	50.0	33.3	50.0	0.0	0.0	0.0	0.0	66.7	0.0	
Field ave		**38.9**	**33.3**	**14.3**	**0.0**	**22.2**	**0.0**	**11.1**	**22.2**	**0.0**	**50.0**
Kiran	aerial	66.7	0.0	16.7	11.1	14.3	0.0	66.7	28.6	50.0	
	plant tissue	50.0	57.1	0.0	0.0	0.0	20.0	0.0	42.9	20.0	
Field ave		**61.5**	**28.6**	**9.1**	**7.7**	**7.1**	**9.1**	**46.2**	**35.7**	**36.4**	**81.6**
Reekie	aerial	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	plant tissue	0.0	25.0	0.0	0.0	0.0	0.0	0.0	50.0	0.0	
Field ave		**0.0**	**25.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**50.0**	**0.0**	**33.3**
Ave % detection per sampling period (irod)[c]		**44.2a**[h] (11.5)	**11.1ab** (11.1)	**5.6b** (5.6)	**6.1a** (3.3)	**15.9a** (9.7)	**0.0a** (0.0)	**28.8a** (19.0)	**9.5a** (9.5)	**22.2a** (14.7)	
Ave % detection per sampling period (pt)[d]		**25.0a** (14.4)	**28.9a** (11.8)	**37.5a** (23.9)	**0.0a** (0.0)	**0.0a** (0.0)	**5.0a** (5.0)	**0.0b** (0.0)	**48.2a** (7.0)	**5.0b** (5.0)	
2007 ave % detection per sampling period (irod + pt)[e]		**31.4a** (12.9)	**21.7a** (7.4)	**12.1a** (5.2)	**3.5a** (2.0)	**7.3a** (5.2)	**2.3a** (2.3)	**15.9a** (10.3)	**29.8a** (8.4)	**12.2a** (8.6)	
2007 ave % detection per pathogen[f]				**24.0a** (5.6)			**4.2b** (1.7)			**20.6ab** (13.8)	

		% pathogen detection within sampling period									
		P. expansum			*B. cinerea*			*M. piriformis*			% detection per orchard
Field	**Sample type**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	
2008 Growing Season											
Field 3	aerial	0.0	18.2	33.3	0.0	9.1	66.7	27.8	0.0	0.0	
	plant tissue	0.0	ND	0.0	0.0	ND	0.0	0.0	ND	0.0	
Field ave		**0.0**	**18.2**	**20.0**	**0.0**	**9.1**	**40.0**	**22.7**	**0.0**	**0.0**	**28.9**
Field 12	aerial	57.9	54.5	100.0	5.3	9.1	0.0	21.1	0.0	33.3	
	plant tissue	25.0	ND	0.0	0.0	ND	0.0	0.0	ND	0.0	
Field ave		**52.2**	**54.5**	**60.0**	**4.3**	**9.1**	**0.0**	**17.4**	**0.0**	**20.0**	**71.8**
Kiran	aerial	5.9	25.0	18.2	0.0	12.5	18.2	5.9	29.2	18.2	
	plant tissue	38.9	20.8	10.0	5.6	12.5	0.0	27.8	8.3	0.0	
Field ave		**22.9**	**22.9**	**14.3**	**2.9**	**12.5**	**9.5**	**17.1**	**18.8**	**9.5**	**46.2**
Reekie	aerial	73.3	45.0	9.1	40.0	5.0	9.1	40.0	30.0	9.1	
	plant tissue	50.0	37.5	9.1	6.3	8.3	0.0	37.5	37.5	0.0	
Field ave		**61.3**	**40.9**	**9.1**	**22.6**	**6.8**	**4.5**	**38.7**	**34.1**	**4.5**	**80.4**
Ave % detection per sampling period (irod)[c]		**34.3a**[h] (18.4)	**35.7a** (8.5)	**40.2a** (20.6)	**11.3a** (9.6)	**8.9a** (1.5)	**23.5a** (14.9)	**23.7a** (7.1)	**14.8a** (8.5)	**15.2a** (7.1)	
Ave % detection per sampling period (pt)[d]		**28.5ab** (10.8)	**29.2a** (8.3)	**4.8b** (2.8)	**3.0b** (1.7)	**10.4a** (2.1)	**0.0b** (0.0)	**16.3ab** (9.6)	**22.2a** (14.6)	**0.0b** (0.0)	
2008 ave % detection per sampling period (irod + pt)[e]		**34.1a** (14.0)	**34.1a** (8.4)	**25.8a** (11.6)	**7.4a** (5.1)	**9.4a** (1.2)	**13.5a** (9.0)	**24.0a** (5.1)	**13.2a** (8.2)	**8.5a** (4.3)	
2008 ave % detection per pathogen[f]				**30.8a** (10.2)			**8.3b** (1.3)			**17.8ab** (3.8)	
2007/08 ave % detection per pathogen[g]				**27.4a** (3.4)			**6.2b** (1.4)			**19.2a** (1.4)	

a. % pathogen detection: $\frac{\text{\# of times a pathogen was detected within a select sampling period}}{\text{\# of samples taken within a select sampling period}} * 100\%$
b. Field averages were obtained by calculating the average % pathogen detection between both aerial and plant tissue samples
c. Ave % detection per sampling period (irod): irod = aerial sample; values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in aerial samples only
d. Ave % detection per sampling period (pt): pt = plant tissue sample; values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in plant tissue samples only
e. Ave % detection per sampling period: values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in aerial and plant tissue samples combined
f. Ave % detection per pathogen: values were determined by taking the average of the % pathogen detection values within all three sampling periods within each orchard with aerial and plant tissue samples combined
g. 2007/08 ave % detection per pathogen: values were determined by taking the average of the values obtained in Ave % detection per pathogen in both 2007 and 2008
h. Means followed by different letters within a pathogen and within a row are significantly different ($p \leq 0.06$) according to the least significant difference (LSD) test.

Fruit washings conducted after harvest in 2007 showed the presence of *B. cinerea* in Jonagold apples grown in Field 3 and the presence of *P. expansum, B. cinera* and *M. piriformis* in Gala apples grown in Field 12. Fruit washings conducted after harvest in 2008 showed the presence of *P. expansum* in Gala apples grown in the Kiran orchard (Table 4).

Table 4. Detection of *P. expansum*, *B. cinerea* and *M. piriformis* from fruit washings collected from Jonagold apples from Field 3, Gala apples from Field 12 and the Kiran orchard and Red Delicious apples from the Reekie orchard in 2007 and 2008 using DNA macroarrays.

	DNA detected (ng/20 apples)		
Field	***P. expansum***	***B. cinerea***	***M. piriformis***
Harvest 2007			
Field 3	ND[a]	0.48	ND
Field 12	0.78	0.66	0.05
Harvest 2008			
Field 3	ND	ND	ND
Field 12	ND	ND	ND
Kiran	1.41	ND	ND
Reekie	ND	ND	ND

a. ND = not detected

3.2 Natural disease incidence

3.2.1 Harvest 2007

The effect of incubation period (3 and 6 months) and 1-MCP use (1-MCP+/-) on infection severity (IS) was analyzed using a two-way ANOVA. The interaction terms for apples from both Field 3 and 12 were not significant (Appendix, Tables A8 and A9). In both Field 3 and 12, there was a greater IS at six months than at three months in 1°C storage. However, only in Field 3 was there a significant effect of 1-MCP application; 1-MCP reduced IS by 48.2% in comparison to the non-fumigated control (Tables 5 and 6).

Table 5. Natural disease incidence indicated by infection severity[a] in Jonagold apples grown in Field 3 (harvest 2007), treated or not treated with 1-MCP (1-MCP+/-) and stored for 3 or 6 months at 1°C in air.

	Field 3	**Field 3**	
Incubation Period	**3 months**	**6 months**	Overall Mean
1-MCP +	5.51	9.55	7.53 b[c]
1-MCP -	9.09	22.12	15.61 a
Overall Mean	7.30 b[b]	15.83 a	

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters are different at significance levels $p \leq 0.05$ according to least significant difference (LSD) test.
c. 1-MCP means with different letters are different at significance levels $p \leq 0.05$ LSD test.

Table 6. Natural disease incidence indicated by infection severity[a] in Gala apples grown in Field 12 (harvest 2007), treated or not treated with 1-MCP (1-MCP+/-) and stored for 3 or 6 months at 1°C in air

	Field 12	**Field 12**	
	3 months	**6 months**	Overall Mean
1-MCP +	7.64	30.28	18.95 a[c]
1-MCP -	11.78	22.29	16.56 a
Overall Mean	9.90 b[b]	26.28 a	

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters are different at significance levels $p \leq 0.05$ according to least significant difference (LSD) test.
c. 1-MCP means with different letters are different at significance levels $p \leq 0.05$ LSD test.

3.2.2 Harvest 2008

The effects of incubation period (1, 2, 4 and 6 months) on infection severity in each of the four orchards were analyzed using a one-way ANOVA. Results indicated that in all fields, there was a significant increase in IS from one to six months (Table 7; Appendix, Table A10). A one-way ANOVA also revealed that at two months incubation, Jonagold apples grown in Field 3 had significantly greater infection severities than in all

other orchards. At four and six months of incubation, Jonagold apples grown in Field 3 and Gala apples grown in Field 12 had significantly greater infection severities than in the commercial orchard apples, Gala grown in the Kiran orchard and Red Delicious grown in the Reekie orchard (Appendix, Table A11).

Table 7. Natural disease incidence indicated by infection severity[a] in Jonagold apples grown in Field 3, Gala apples grown in Field 12 and the Kiran orchard and Red Delicious apples grown in the Reekie orchard (harvest 2008) and stored for 1, 2, 4 or 6 months at 1°C in air.

Apple Variety	Field 3 Jonagold	Field 12 Gala	Kiran Gala	Reekie Red Delicious
1 month	0.00 dA	0.00 dA	0.36 bA	0.00 cA[c]
2 months	1.08 cA	0.54 cB	0.01 bB	0.00 cB
4 months	20.72 bA	19.95 bA	0.08 bB	2.28 bB
6 months	48.12 a[b]A	46.58 aA	2.06 aB	10.88 aB

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters within columns (lowercase) are different at significance levels at $p \leq 0.05$ according to least significant difference (LSD) test.
c. Incubation period means with different letters within rows (uppercase) are different at significance levels at $p \leq 0.05$ according to the LSD test.

In addition to storing apples at 1°C in air, fruit were also stored in controlled atmosphere (CA) for 4.5 months. Jonagold apples grown in Field 3 had the highest infection severity with significantly greater disease levels compared to Gala apples grown in Fields 12 and Kiran and Red Delicious apples grown in the Reekie orchard (Figure 10; Appendix, Table A12). The high infection severity found in Jonagold apples grown in Field 3 appeared to be due primarily to *P. expansum* (based on phenotypic observation).

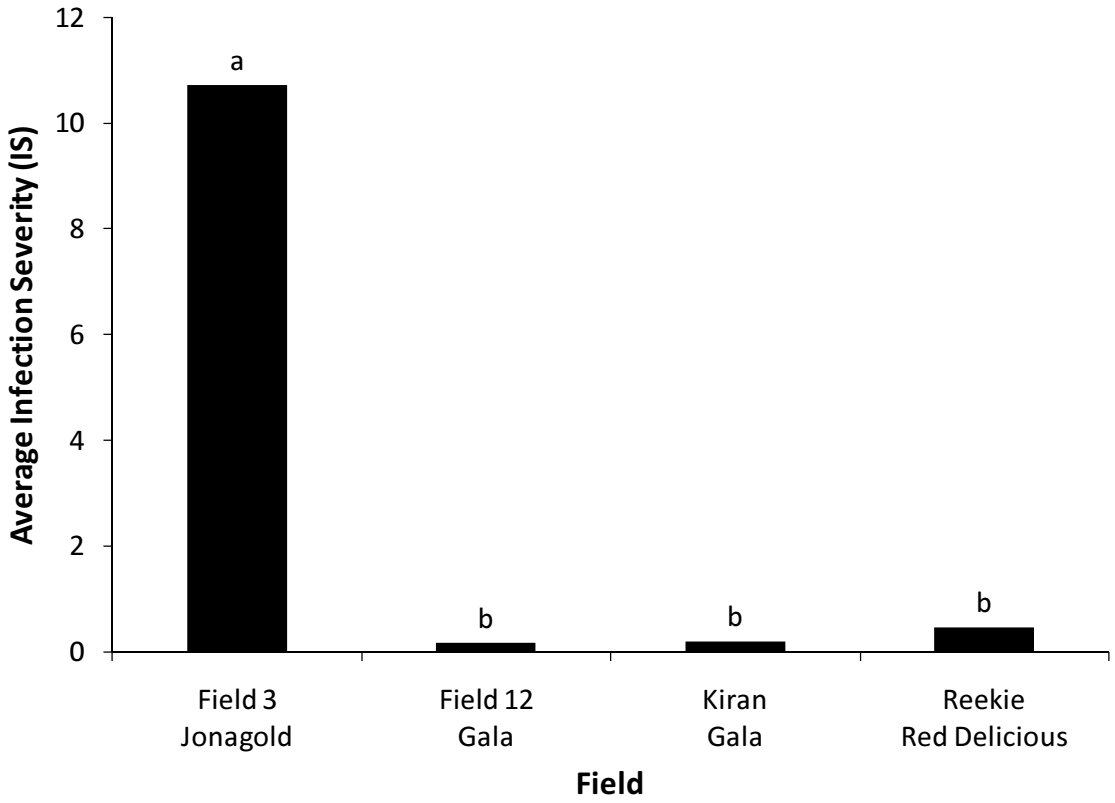

Figure 10. Natural disease incidence in Jonagold apples grown in Field 3, Gala apples grown in Field 12 and the Kiran Orchard and Red Delicious apples grown in the Reekie orchard and stored for 4.5 months in controlled atmosphere (CA) in Naramata, BC. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

3.3 Growing season detection and natural disease incidence

In this study, there was no correlation between % total pathogen detection (*P. expansum, B. cinerea* and *M. piriformis*) detected in the field throughout the entire growing season in both 2007 and 2008 combined (Table 3) and NDI after storage (r = -0.11; p = 0.8349). With respect to Fields 3 and 12 in 2007 and Fields 3 and 12 and the Kiran and Reekie orchards in 2008, there was no correlation between % pathogens detected in the early-growing season and their corresponding NDIs (r = -0.29; p = 0.5811) and % pathogens detected in the mid-growing season and their corresponding NDIs (r = -0.28; p = 0.5865). However, there was a positive correlation between % pathogens detected in the late-growing season and NDI (r = 0.79; p = 0.0612).

When the aerial samples were analyzed separately from the plant tissue samples, in the 2007 and 2008 data combined, there was no correlation between % pathogens detected in the early (r = -0.57; p = 0.2234) and mid-growing seasons and their corresponding NDIs (r = -0.36; p = 0.4838). However, there was a positive correlation between % pathogens detected in late-growing season and NDI (r = 0.74; p = 0.0903). When the plant tissue samples were separated from the aerial samples, in 2007 and 2008 combined, there was no correlation between % pathogens detected in either the mid (r = -0.22; p = 0.7810) or late-growing seasons (r = -0.21; p = 0.6826) and their corresponding NDIs. However, there was a negative correlation between % pathogens detected in the early-growing season and NDI (r = -0.79; p = 0.0623).

3.4 Antagonist efficacy, harvest 2007

3.4.1 Natural disease incidence and biocontrol inoculations

The effects of biological control treatment (no biocontrol, 1100-6, 1-112, 2-28, 4-6 or 6-25) and 1-MCP use (1-MCP+/-) were analyzed using a two-way ANOVA. For Jonagold apples grown in Field 3 and stored at 1°C in air for 3 months, there was no significant effect of the biological controls, 1-MCP use or their interaction (Appendix, Table A13). Results obtained with inoculated Gala apples from Field 12 indicated a significant interaction with both main effects, biological control treatment and 1-MCP use (Appendix, Table A14). Because the interaction term was significant, treatments were separated, followed by a one-way analysis of variance. 1-MCP significantly decreased IS in apples with no biological control treatment and apples that were treated with bacterial isolates 1100-6 and 2-28 (Figure 11; Appendix, Table A15). There were significant reductions in IS means in the absence of 1-MCP in apples treated with bacterial isolates 1-112, 4-6 and 6-25 in comparison to the non-inoculated control (Figure 11). In 1-MCP- treated apples, there was no significant effect of treatments compared to the non-inoculated control (Figure 11; Appendix, Table A16).

3.4.2 Post-harvest pathogen and biocontrol inoculations

Infection severity (IS) was determined with apples grown in Fields 3 and 12 and inoculated with one of three pathogens, *P. expansum*, *B. cinerea* or *M. piriformis* and one of five bacterial control isolates and the data were subjected to a two-way ANOVA. IS means for Jonagold apples from Field 3 and inoculated with *P. expansum*

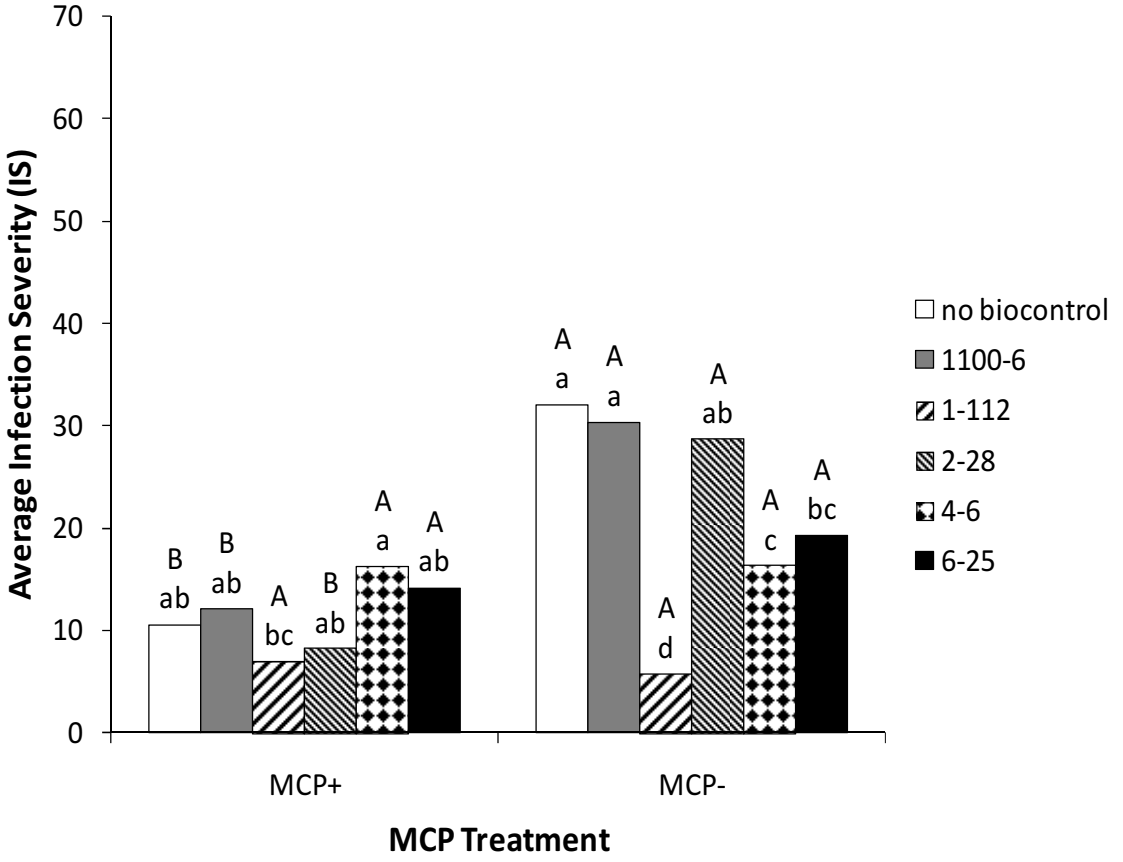

Figure 11. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on natural disease incidence in Gala apples grown in Field 12 (harvest 2007) and stored for 3 months in air at 1°C. IS means with different letters within treatments are different at significance levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

exhibited a significant effect of biological control treatment and of the interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A17). The resulting one-way ANOVA showed that the application of 1-MCP significantly decreased IS means in apples inoculated with isolate 1100-6 and increased IS in apples inoculated with isolate 6-25 (Figure 12a; Appendix, Table A18). In comparison to the non-inoculated control, isolates 1100-6, 1-112, 4-6 and 6-25 provided significant control of *P. expansum* in both the presence and absence of 1-MCP (Figure 12a; Appendix, Table A19).

For *B. cinerea*-treated apples from Field 3, the two-way ANOVA revealed a significant effect of biological control treatment and 1-MCP and of their interaction (Appendix, Table A20). The one-way ANOVAs revealed a significant increase in IS in 1-MCP-treated apples that were not inoculated with a biocontrol and apples inoculated with isolates 2-28 and 4-6 (Figure 12b; Appendix, Table A21). In comparison to a non-inoculated control, isolates 1100-6, 1-112 and 6-25 provided significant control of *B. cinerea* in the presence of 1-MCP. There was no significant reduction in IS in apples in the absence of 1-MCP (Figure 12b; Appendix, Table A22).

For *M. piriformis*-treated apples grown in Field 3, there was a significant effect of the biological control treatment and of the interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A23). The one-way ANOVAs revealed a significant increase in IS in non-1-MCP treated apples inoculated with isolate 4-6 (Figure 12c; Appendix, Table A24). There was no effect of inoculation with any of the biological control isolates in the presence of 1-MCP; however, there was a significant increase in IS in non-1-MCP-treated apples inoculated with isolate 4-6 when

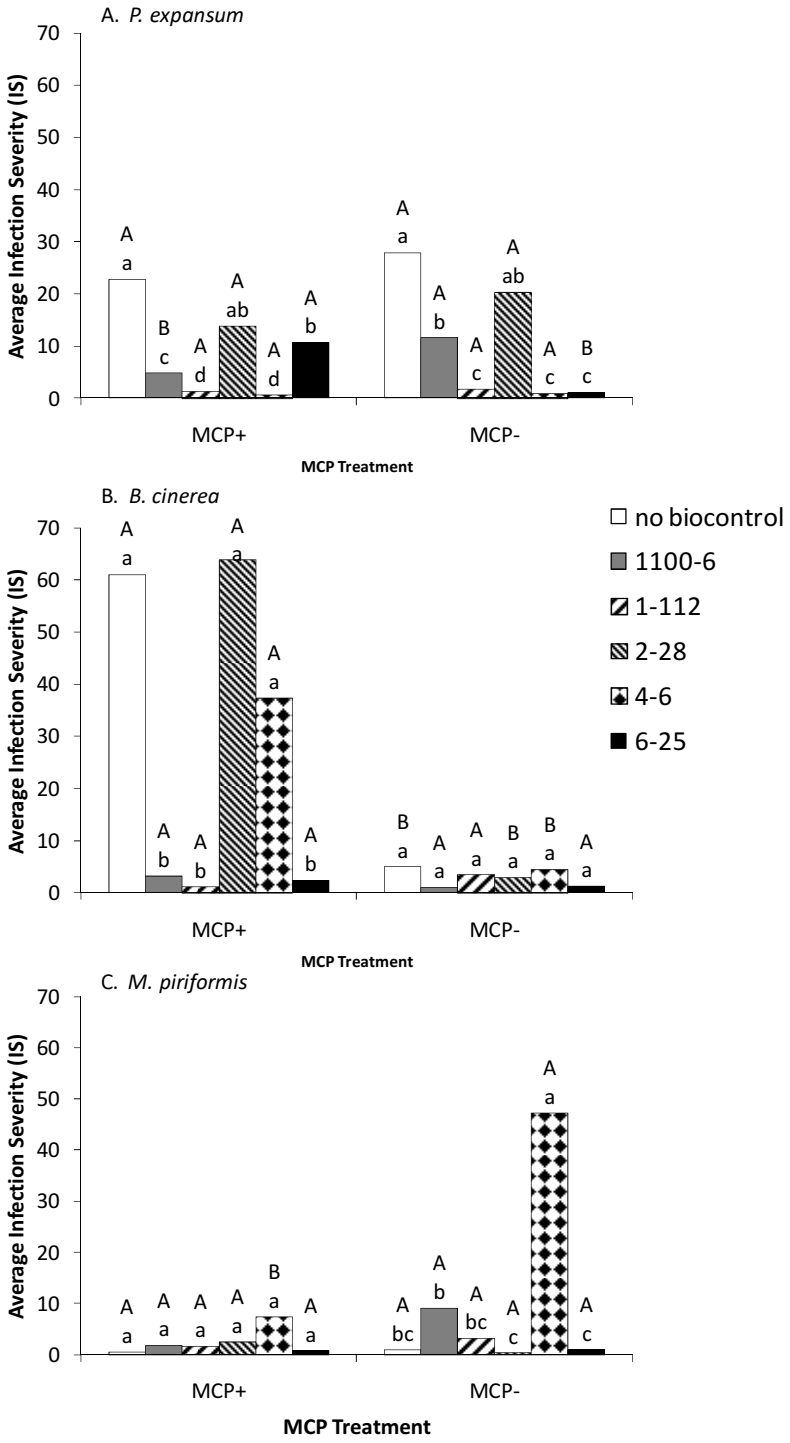

Figure 12. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on infection severity in Jonagold apples from Field 3 (harvest 2007), inoculated with *P. expansum* (A), *B. cinerea* (B) and *M. piriformis* (C) and stored for 3 months at 1°C in air. IS means with different letters within treatments are different at significance levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

compared to 1-MCP-treated apples (Figure 12c; Appendix, Table A25).

For Gala apples from Field 12 treated with *P. expansum* and each of five biological control isolates, there was a significant effect of 1-MCP use and no significant interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A26). 1-MCP significantly increased the mean IS (48.16 ± 0.50) compared to that obtained in the absence of 1-MCP (45.69 ± 0.68). There was no overall significant effect of the biological control isolates (Appendix, Table A26). There were no data for Gala apples from Field 12 inoculated with *B. cinerea* and the five biological control isolates as there was complete apple decay after 3 months of storage at 1°C.

For Gala apples from Field 12 treated with *M. piriformis* and each of the five biological controls, there was a significant interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A27). The one-way ANOVAs revealed a significant decrease in IS in 1-MCP-treated apples inoculated with isolate 1-112 compared to non-1-MCP-treated apples and a significant increase in IS in non-1-MCP-treated apples inoculated with isolate 2-28 when compared to 1-MCP-treated apples (Figure 13; Appendix, Table A28). In the presence of 1-MCP, isolate 2-28 exhibited significant biological control activity compared to the non-inoculated control. There was no significant reduction in IS in non-1-MCP treated apples when compared to a non-inoculated control (Figure 13; Appendix, Table A29).

3.5 Antagonist efficacy, harvest 2008

For all 2008 biological control experiments, apples were wounded and non-wounded. Non-wounded apples had a very low frequency of infection with low

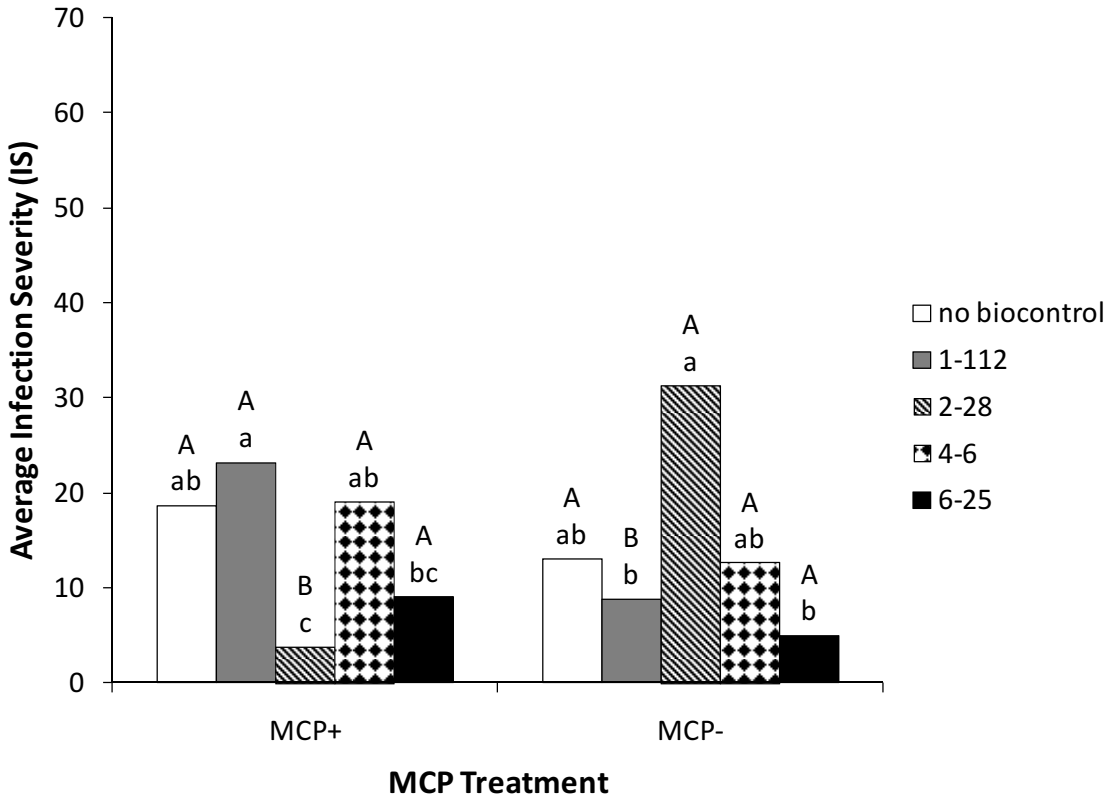

Figure 13. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on infection severity in Gala apples from Field 12 (harvest 2007), inoculated with *M. piriformis* and stored for 3 months at 1°C in air. IS means with different letters within treatments are different at significant levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

variation. As a result, the variable was eliminated from 2008 biological control experimental analysis as it explained little about the corresponding dependent and independent variables (Figure 14).

3.5.1 Natural disease incidence and biocontrol inoculations

The effects of biological control treatment (no biocontrol, 1100-6, 1-112, 2-28, 4-6 or 6-25) and incubation period (1, 2, 4 and 6 months) were analyzed using a two-way ANOVA. For Jonagold and Gala apples grown in Fields 3 and 12, respectively, then stored at 1°C in air, there was a significant effect of incubation period and of the interaction between the main effects, biological control treatment and incubation period (Appendix, Tables A30 and A31). For Jonagold apples grown in Field 3, there was no significant reduction in IS in apples treated with the five biological controls after 2 and 4 months in storage (Figure 15a and 15b). However, after six months in storage, isolates 2-28 and 6-25 significantly reduced IS compared to the non- inoculated control (Figure 15c; Appendix, Table A32). For Gala apples grown in Field 12, isolates 1100-6, 1-112, 4-6 and 6-25 significantly reduced IS compared to the non-inoculated control after two months in storage (Figure 15d; Appendix, Table A33). There was no significant reduction in IS after four and six months storage compared to the non-inoculated control (Figure 15e and 15f).

Jonagold apples grown in Field 3, Gala apples grown in Fields 12 and the Kiran orchard and Red Delicious apples grown in the Reekie orchard were also stored for 4.5 months in CA storage. A two-way ANOVA with replication indicated there was a significant effect of biological control treatment and location and of the interaction between biological control treatment and location (Appendix, Table A34). The one-way

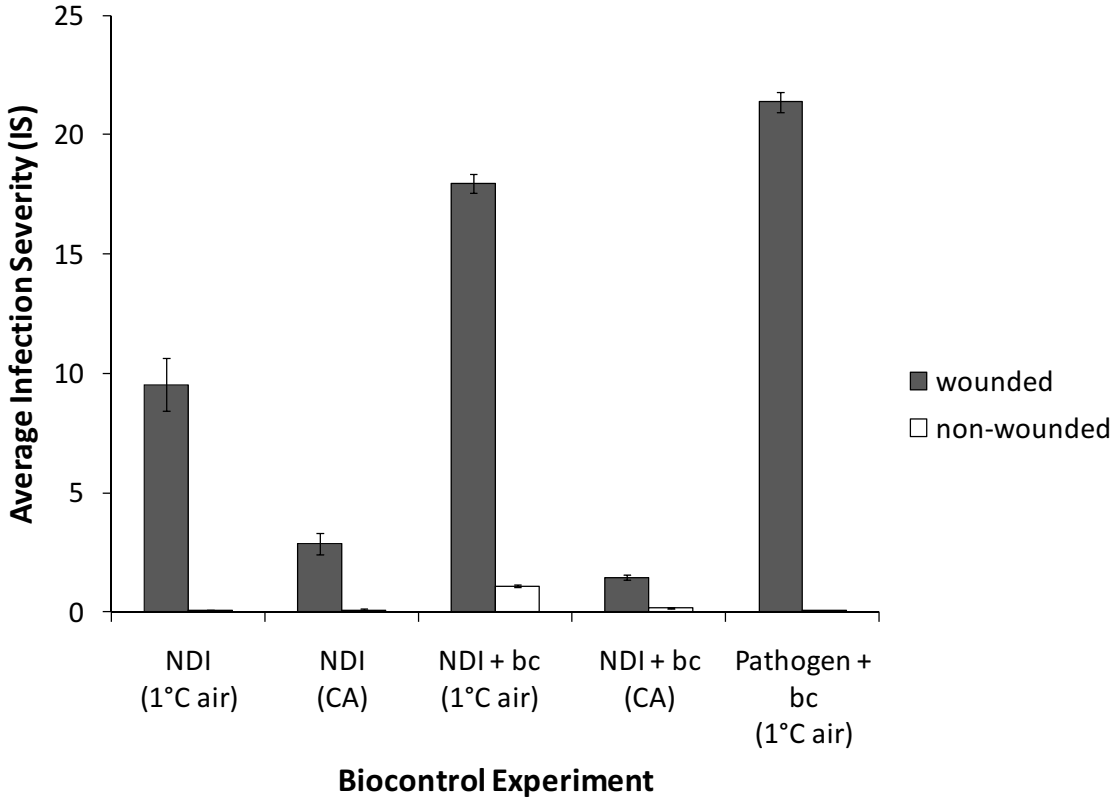

Figure 14. Average infection severity for all wounded and non-wounded apples from individual biological control treatments conducted in 2008. Experiments were as follows: 1) Natural disease incidence (NDI) at 1°C in air; 2) Natural disease incidence (NDI) in controlled atmosphere (CA); 3) Natural disease incidence (NDI) in combination with biocontrols (bc) at 1°C in air; 4) Natural disease incidence (NDI) in combination with biocontrols (bc) in CA; 5) Pathogen in combination with biocontrols at 1°C in air. Error bars represent standard error of the mean.

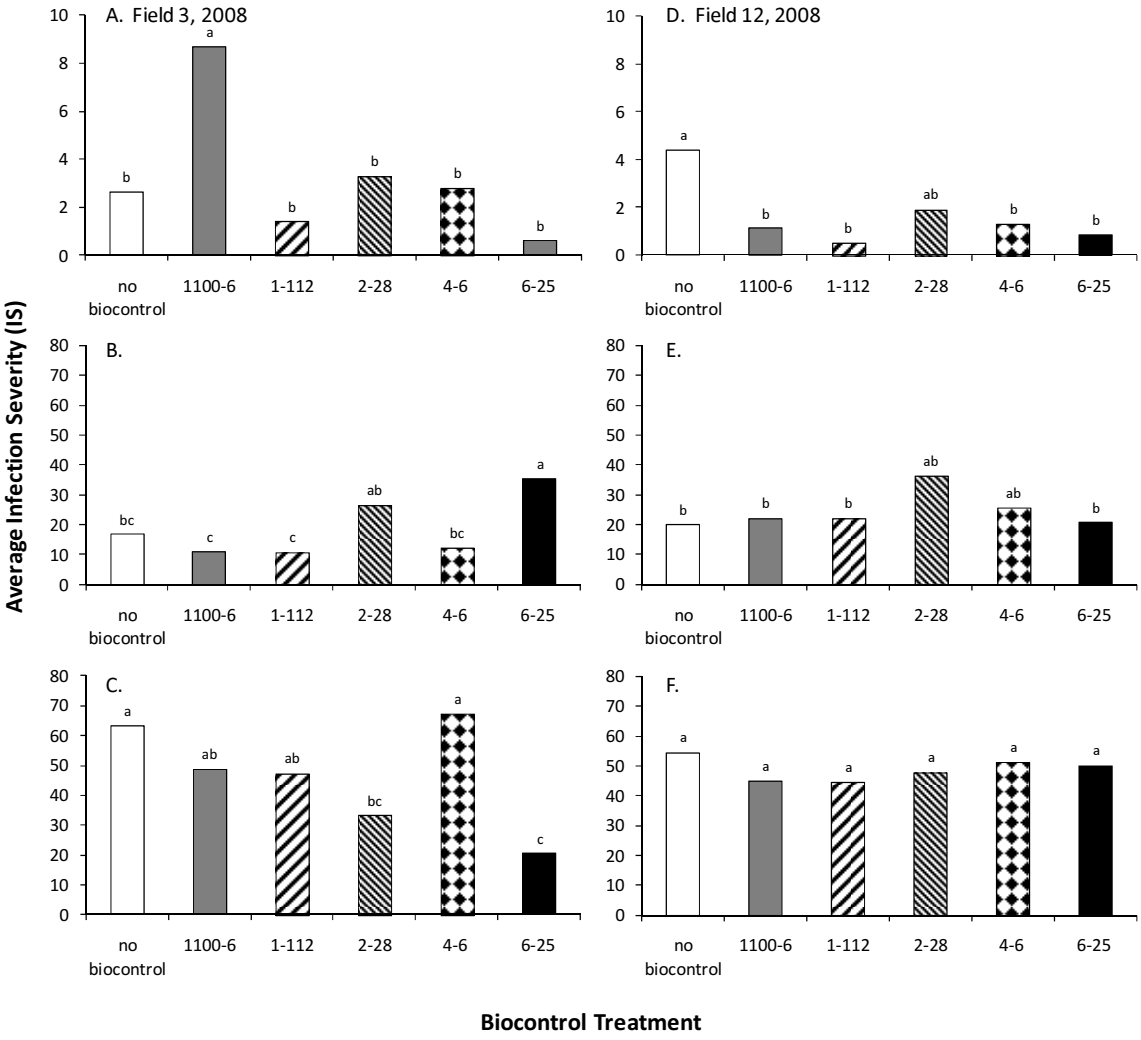

Figure 15. Effect of biological control isolates on natural disease incidence in Jonagold apples grown in Field 3 (A – C) and Gala apples grown in Field 12 (D – F) (harvest 2008) and stored for 2 (A&D), 4 (B&E) or 6 (C&F) months at 1°C in air. IS means with different letters within figures are different at significance levels $p \leq 0.05$ according to LSD test.

ANOVAs revealed a significant decrease in IS in Jonagold apples grown in Field 3 and inoculated with biological control isolates 1100-6, 1-112, 2-28, 4-6 and 6-25 (Figure 16; Appendix, Table A35). There was no significant reduction in IS in Gala apples grown in Field 12 and inoculated with one of five biological controls (data not shown; Appendix, Table A35). In Gala apples grown in the Kiran orchard, there was little overall disease. However, isolates 1100-6, 2-28, 4-6 and 6-25 significantly reduced IS compared to the non-inoculated control (Figure 17; Appendix, Table A35). For Red Delicious apples grown in the Reekie orchard, there was no significant effect on IS when inoculated with the biological control isolates (data not shown; Appendix, Table A35).

3.5.2 Post-harvest pathogen and biocontrol inoculations

Infection severity (IS) was determined with apples grown in Fields 3 and 12 and inoculated with one of three pathogens, *P. expansum*, *B. cinerea* or *M. piriformis* and one of five bacterial control isolates. One way ANOVAs were computed to determine if there were significant effects of the biological control agents on infection severities within each field.

For Jonagold apples grown in Field 3 and inoculated with *P. expansum* there was a significant reduction in IS in apples treated with isolates 4-6 and 6-25 compared to the non-inoculated control (Figure 18a; Appendix, Table A36). For Gala apples grown in Field 12 and inoculated with *P. expansum*, there was a significant reduction in IS in apples treated with isolates 1100-6, 2-28 and 6-25 compared to the non-inoculated control (Figure 19, Appendix, Table A36). In Jonagold apples grown in Field 3 and Gala

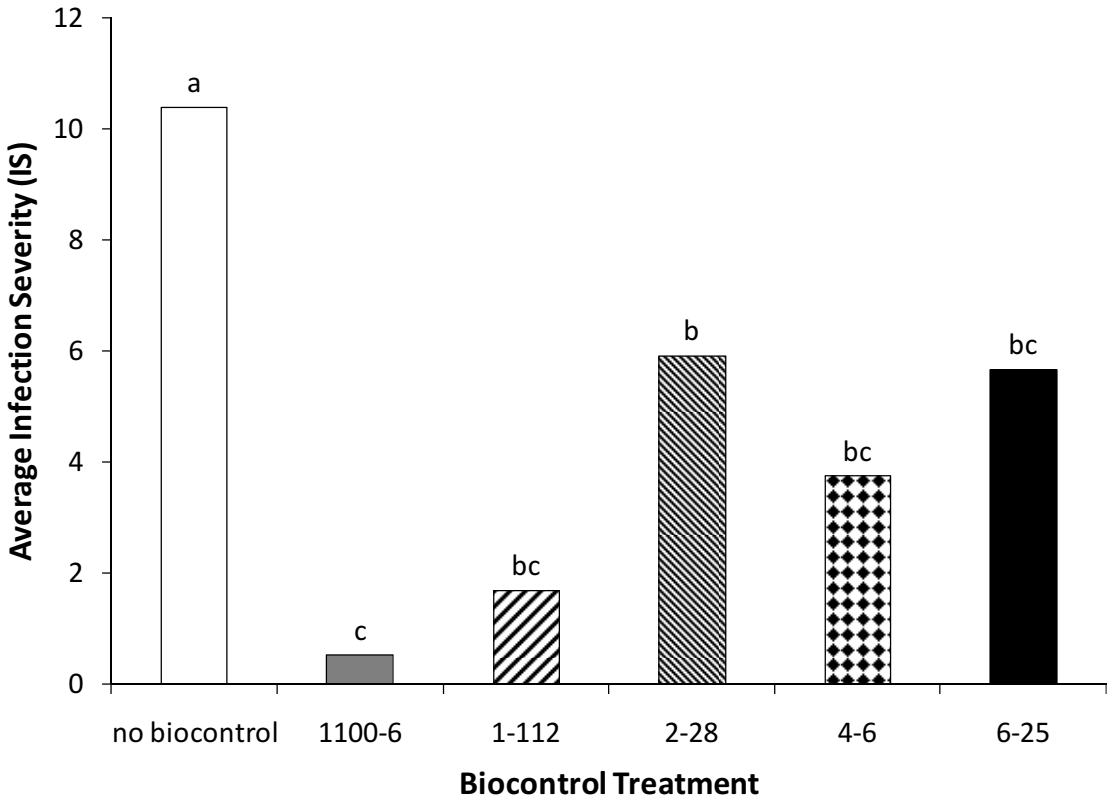

Figure 16. Effect of biological control isolates on natural disease incidence in Jonagold apples grown in Field 3 (harvest 2008) and stored for 4.5 months in CA. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

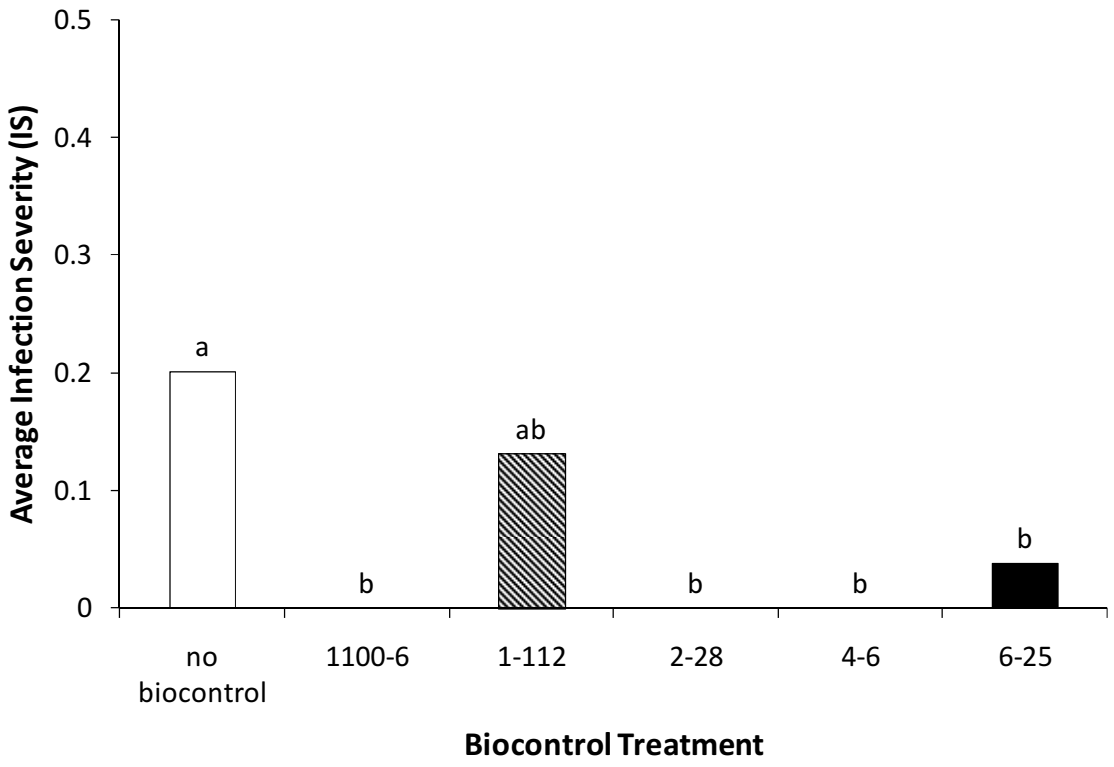

Figure 17. Effect of biological control isolates on natural disease incidence in Gala apples grown in the Kiran orchard (harvest 2008) and stored for 4.5 months in CA. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

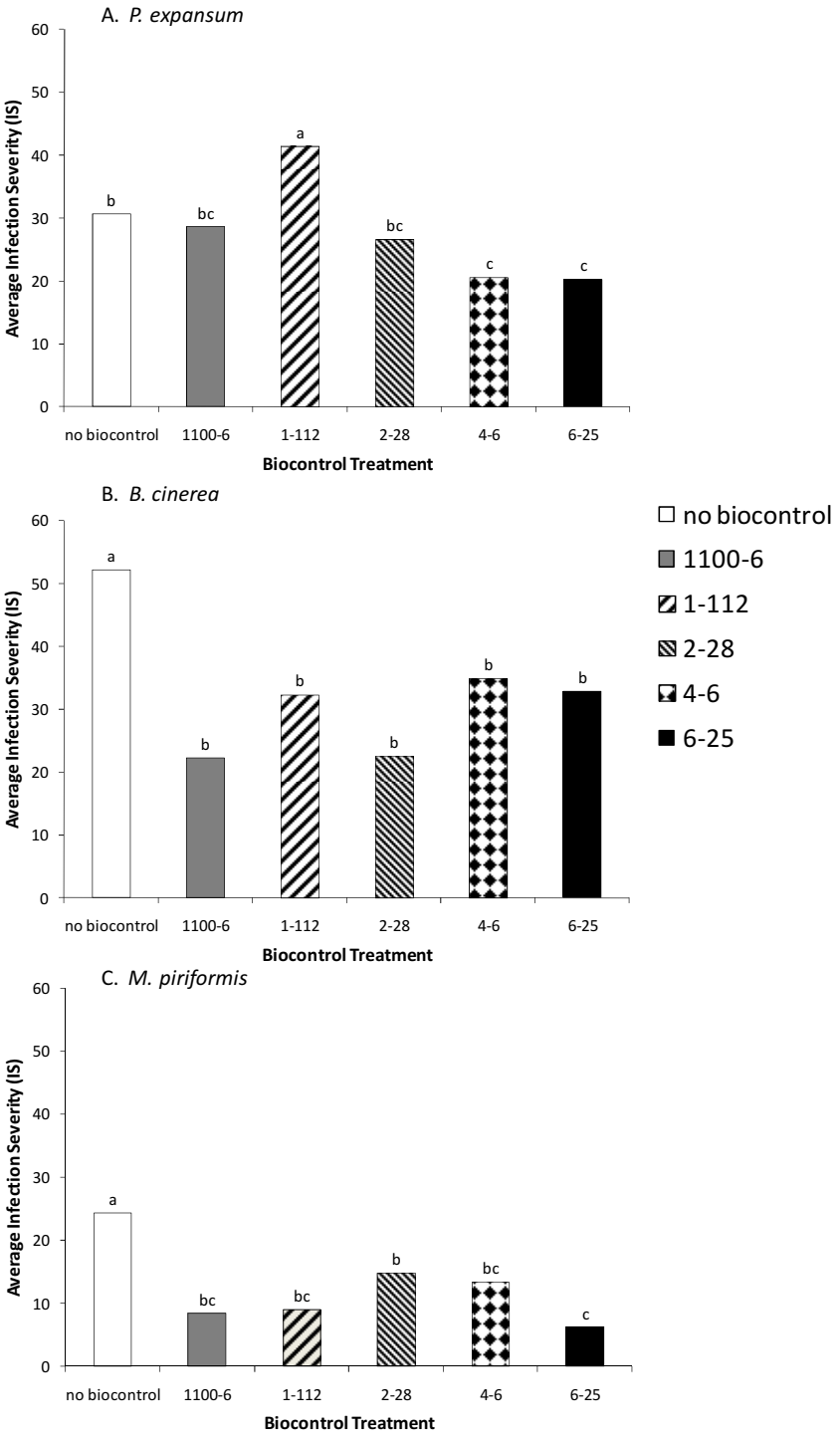

Figure 18. Effect of biological control isolates on infection severity in Jonagold apples grown in Field 3 (harvest 2008), inoculated with *P. expansum* (A) and *B. cinerea* (B) and stored for 2 months at 1°C in air and inoculated with *M. piriformis* (C) and stored for 1 month at 1°C in air . IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

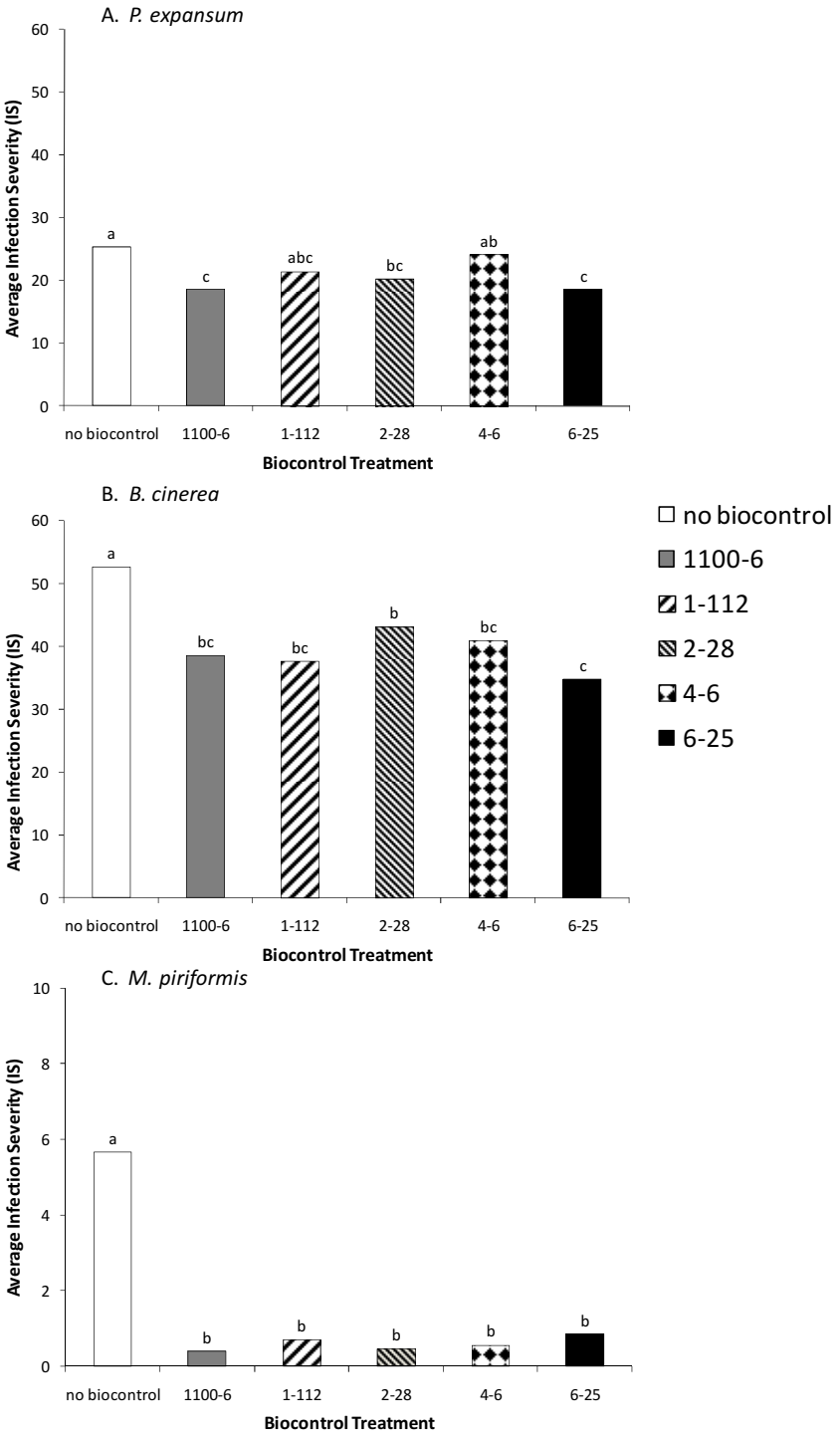

Figure 19. Effect of biological control isolates on infection severity in Gala apples grown in Field 12 (harvest 2008), inoculated with *P. expansum* (A) and *B. cinerea* (B) and stored for 2 months at 1°C in air and inoculated with *M. piriformis* (C) and stored for 1 month at 1°C in air. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

apples grown in Field 12 and inoculated with either *B. cinerea* (Figures 18b and 19b; Appendix, Table A37) or *M. piriformis* (Figures 18c and 19c; Appendix, Table A38) there was a significant reduction in IS in apples treated with all isolates, 1100-6, 1-112, 2-28, 4-6 and 6-25.

Table 8 summarizes all biological control treatments in 2007 and 2008 and indicates the percent decrease (or increase) of all BCAs. Overall, results obtained from two years of experimental trials indicated that the bacterial isolate that provided significant control in the greatest number of treatments was *Serratia plymuthica* isolate 6-25 (51.7%) followed by *P. fluorescens* isolates 4-6 (41.4%), 1100-6 (37.9%), 1-112 (34.5%) and 2-28 (31%). The bacterial isolate that provided the greatest reduction in infection severity, in cases where significant control was exhibited, was isolate 1-112 followed by isolates 1100-6, 4-6, 6-25 and 2-28. Their respective average reduction in IS was 75.8%, 61%, 59%, 57.6% and 56.4%. In 2007, isolates 1100-6, 1-112 and 6-25 provided significant control of both *P. expansum* and *B. cinerea*. Isolate 4-6 provided significant control of *P. expansum*, only. Isolate 2-28 was the only bacterial strain to provide significant control of *M. piriformis*. In 2008, isolates 1100-6, 2-28, 4-6 and 6-25 provided significant control of all pathogens, *P. expansum*, *B. cinerea* and *M. piriformis*. Isolate 1-112 provided significant control of both *P. expansum* and *B. cinerea* (Table 8). All bacterial antagonists provided greater than 90% control in at least two experiments (Table 8).

Table 8. Summary of antagonist efficacy experiments for harvest 2007 and 2008.

						Percent disease decrease or (increase)[a]				
Experiment[b]	**Storage Type[c]**	**Field**	**Apple Variety**	**Storage Time (months)**	**1-MCP+/-**	**1100-6**	**1-112**	**2-28**	**4-6**	**6-25**
Harvest 2007										
NDI + bc	1C air	3	Jonagold	3	MCP +	NS	NS	NS	NS	NS
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	NS	NS
NDI + bc	1C air	12	Gala	3	MCP +	NS	NS	NS	NS	NS
	1C air	12	Gala	3	MCP -	NS	82.1	NS	49.2	39.9
Pex + bc	1C air	3	Jonagold	3	MCP +	78.7	94.3	NS	97.3	53.2
	1C air	3	Jonagold	3	MCP -	58.6	94.1	NS	96.9	96.2
	1C air	12	Gala	3	MCP+&-	NS	NS	NS	7.8	5.7
Bcin + bc	1C air	3	Jonagold	3	MCP +	94.7	98.1	NS	NS	96.1
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	NS	NS
Mpir + bc	1C air	3	Jonagold	3	MCP +	NS	NS	NS	NS	NS
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	(98.1)	NS
	1C air	12	Gala	3	MCP +	NS	NS	91.6	NS	NS
	1C air	12	Gala	3	MCP -	NS	NS	NS	NS	NS
Harvest 2008										
NDI + bc	1C air	3	Jonagold	2	N/A	(69.6)	NS	NS	NS	NS
	1C air	3	Jonagold	4	N/A	NS	NS	NS	NS	(52.9)
	1C air	3	Jonagold	6	N/A	NS	NS	47.4	NS	67.5
	1C air	12	Gala	2	N/A	74.7	88.3	NS	70.4	80.8
	1C air	12	Gala	4	N/A	NS	NS	NS	NS	NS
	1C air	12	Gala	6	N/A	NS	NS	NS	NS	NS

						Percent disease decrease or (increase)[a]				
Experiment[b]	**Storage Type[c]**	**Field**	**Apple Variety**	**Storage Time (months)**	**1-MCP+/-**	**1100-6**	**1-112**	**2-28**	**4-6**	**6-25**
Harvest 2008										
NDI + bc	CA	3	Jonagold	4.5	N/A	94.9	83.8	43.0	63.9	45.5
	CA	12	Gala	4.5	N/A	NS	NS	NS	NS	NS
	CA	Kiran	Gala	4.5	N/A	100	NS	100	100	81.1
	CA	Reekie	Red Delicious	4.5	N/A	NS	NS	NS	NS	NS
Pex + bc	1C air	3	Jonagold	2	N/A	NS	(26.0)	NS	32.8	33.9
	1C air	12	Gala	2	N/A	26.7	NS	20.0	NS	26.7
Bcin + bc	1C air	3	Jonagold	2	N/A	57.4	38.1	56.8	33.1	37.1
	1C air	12	Gala	2	N/A	26.9	28.6	18.3	22.3	33.9
Mpir + bc	1C air	3	Jonagold	1	N/A	65.4	62.8	39.0	45.0	74.6
	1C air	12	Gala	1	N/A	93.0	87.6	91.8	90.0	85.0

a. Percentages indicate a decrease in IS; percentages in brackets indicate an increase in IS; NS = not significant
b. NDI = natural disease incidence; bc = biocontrol; Pex = *P. expansum*; Bcin = *B. cinerea*; Mpir = *M. piriformis*
c. CA = controlled atmosphere

3.6 Bacterial survival on apple using GFP

3.6.1 Plasmid confirmation

E. coli S-17 λ pir containing the *gfp*-plasmid, pAG408, grew on LB agar without antibiotics and LB agar supplemented with either 50 µg/ml kan or 30 µg/ml gen or both antibiotics combined. Alternatively, the bacterial isolates 1100-6, 1-112, 2-28, 4-6 and 6-25 only grew on agar plates that were not supplemented with antibiotics.

When pAG408 was digested with *Hind*III, the plasmid was cut into two fragments which measured approximately 2.1 and 3.6 kb (Figure 20). These fragments approximated the total plasmid size of 5.7 kb (Suarez et al. 1997). The undigested plasmid migrated to approximately 10 kb due to its supercoiled conformation

3.6.2 Confirmation of *gfp* transformation

After transformation of genetic material from the host (E. coli S-17 λ pir) into the donor (isolate 4-6), transconjugants were amplified by PCR using gfp-specific primers. Successful gfp amplification was observed when a band was present around 714 bp (Figure 21). The absence of banding suggested an unsuccessful transformation. Confirmation of *gfp* transformation was additionally confirmed by fluorescence microscopy *in vitro* (Figure 22) and *in vivo* (Figure 23).

3.6.3 Plasmid stability

After four successive days of sub-culturing and growth in the absence of antibiotic selection, 80.3 ± 6.8% of the transformed *P. fluorescens* isolate 4-6 (4-6-*gfp*) retained kanamycin and gentamicin resistance. These results, in combination with prior biological control experimental results, indicated that 4-6-*gfp* was an appropriate tool for

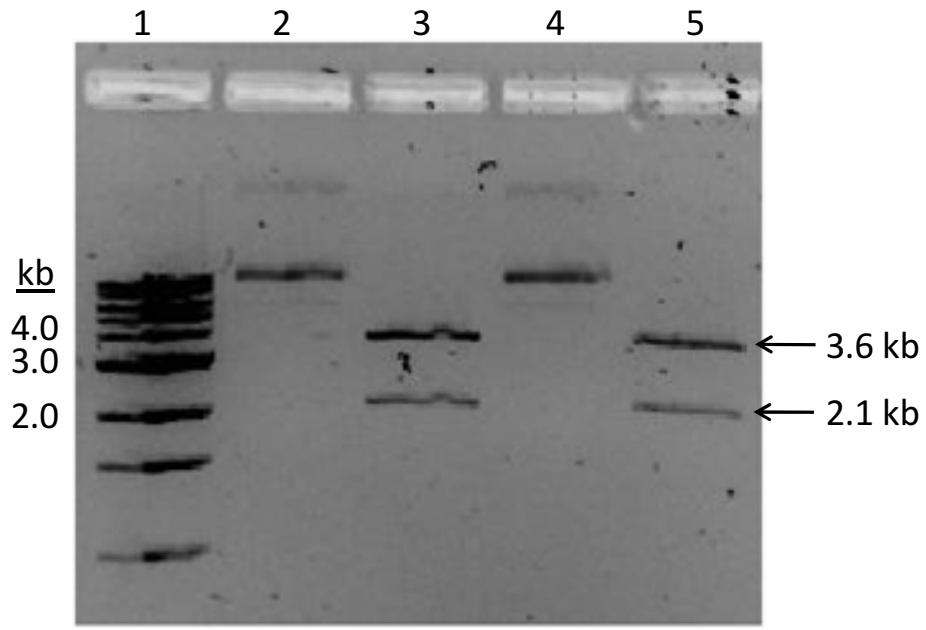

Figure 20. *Hind*III restriction digest of *gfp*-containing plasmid, pAG408. Lane 1: 1 kb ladder (New England Biolabs, Ipswich, MA); Lanes 2 and 4: undigested pAG408; Lanes 3 and 5: digested pAG408.

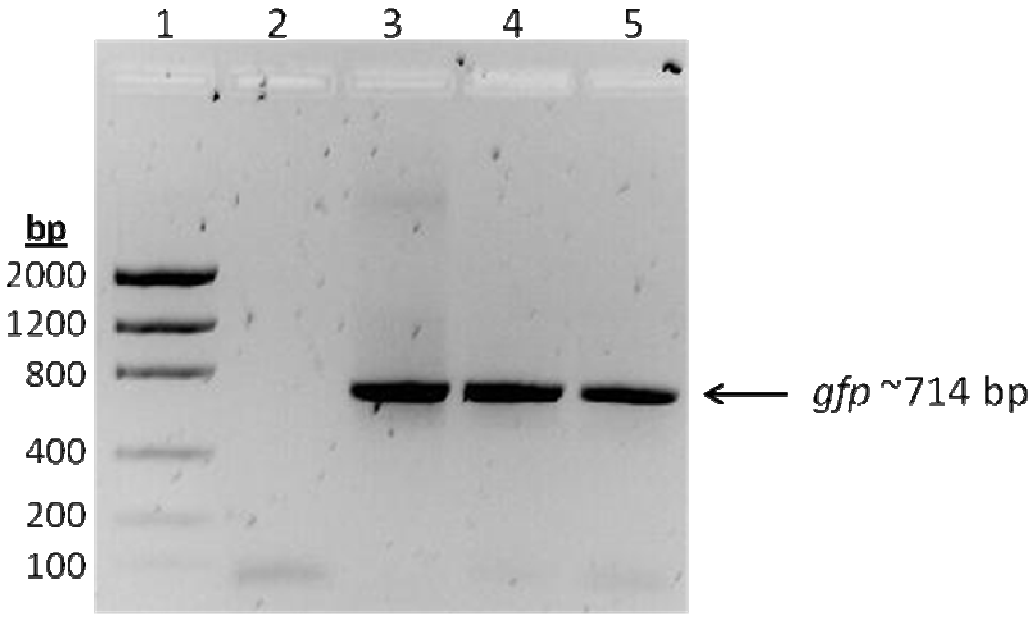

Figure 21. PCR amplification of *gfp* in *P. fluorescens* isolate 4-6 transformed with pAG408 using *gfp*-specific primers. Sample were loaded on a 1% TBE agarose gel and stained with Sybr Safe (Invitrogen, Carlsbad, CA). Lane 1) Low DNA Mass Ladder (Invitrogen, Carlsbad, CA); 2) negative control (ddH_20); 3) extracted plasmid, pAG408; 4 and 5) *gfp* transconjugant of bacterial isolate 4-6.

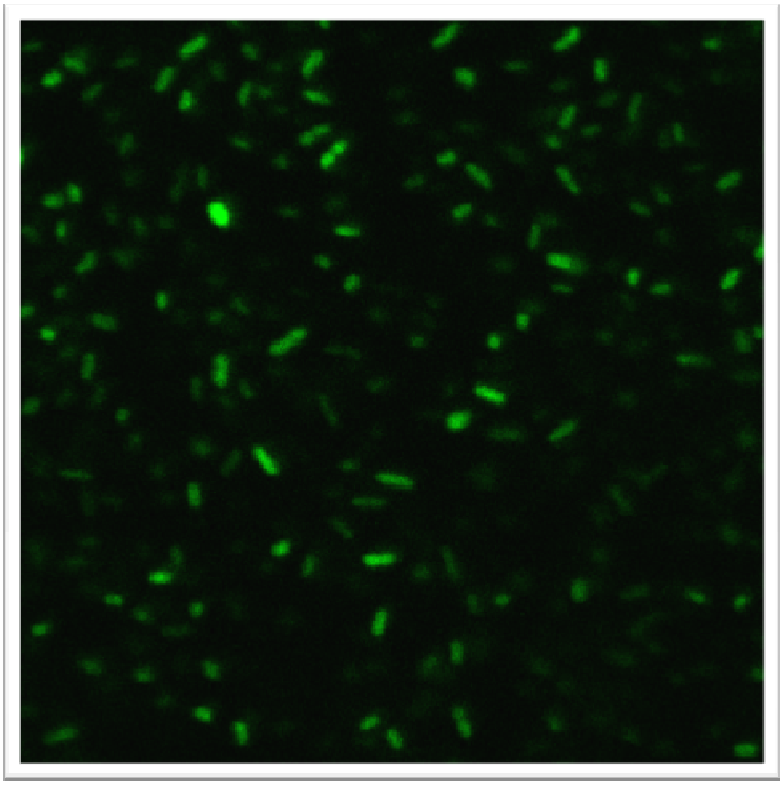

Figure 22. Fluorescence microscopy of *P. fluorescens* isolate 4-6 transformed with pAG408 exhibiting green fluorescence in ½ TSB supplemented with 50 µg/ml kan and 30µg/ml gen.

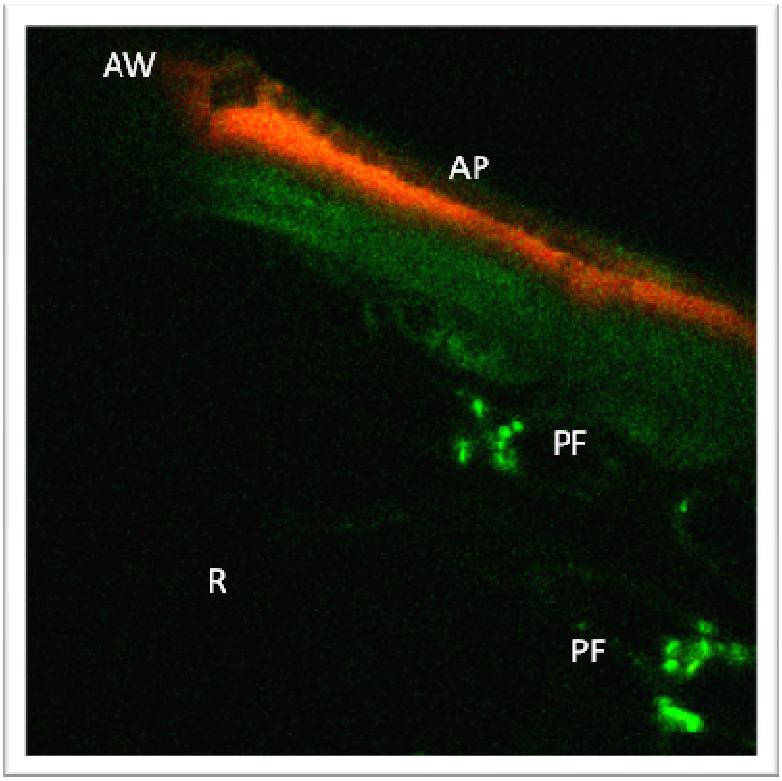

Figure 23. Fluorescence microscopy of an apple wound inoculated with *P. fluorescens* isolate 4-6-*gfp*. AP = apple peel; AW = apple wound; PF = *P. fluorescens* isolate 4-6-*gfp*; R = receptacle or fruit flesh.

studying bacterial survival on apples in the absence of antibiotic selection.

3.6.4 *P. fluorescens* 4-6-*gfp* fitness analysis

There was no significant difference between the growth rates (μ) (Figure 24; Appendix, Table A39) of *P. fluorescens* isolate 4-6-wild-type (0.71 ± 0.04/h) and 4-6-*gfp* (0.75 ± 0.06/h) and sole source carbon utilization patterns (Table 9).

Table 9. Sole carbon source utilization by *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* as indicated by Biolog Phenotye MicroArrays™ (Biolog, Inc., Hayward, CA) for sugars and organic acids commonly found in apple juice (Eisele and Drake 2005).

Apple Carbon Sources	**Bacterial Isolate**	
	4-6-wild-type	**4-6-*gfp***
Fructose	+	+
Sucrose	+	+
Glucose	+	+
Sorbitol	+	+
L-Malic Acid	+	+
Quinic Acid	+	+
Citric Acid	+	+
Fumaric Acid	+	+

3.6.5 Bacterial survival on apple

For standard bacterial suspensions of 4-6-*gfp*, there was a strong linear relationship between CFU/ml and relative fluorescent units (RFU) (r = 0.999) (Figure 25). The fluorescent values obtained by direct scanning of inoculated apple samples (Figure 26) were too low to extrapolate to cell densities from the formula y = 1.8041x + 6.5652 (Figure 25). However, trends could be discerned based on RFUs (Figure 26).

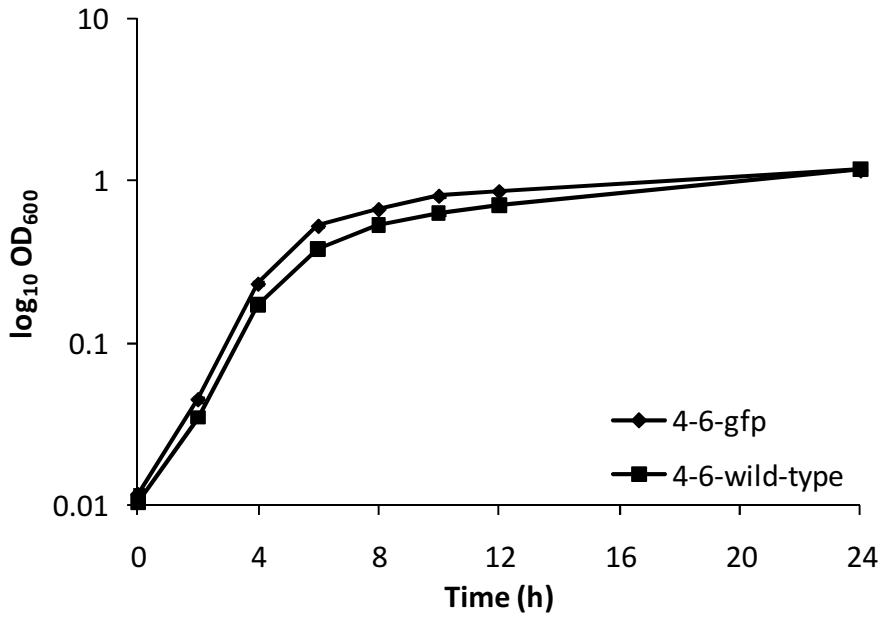

Figure 24. Growth characteristics of *P. fluorescens* 4-6-wild-type and *P. fluorescens* 4-6-*gfp* in half-strength TSB at 28°C for 24 h. Error bars represent standard error of the mean (n = 3).

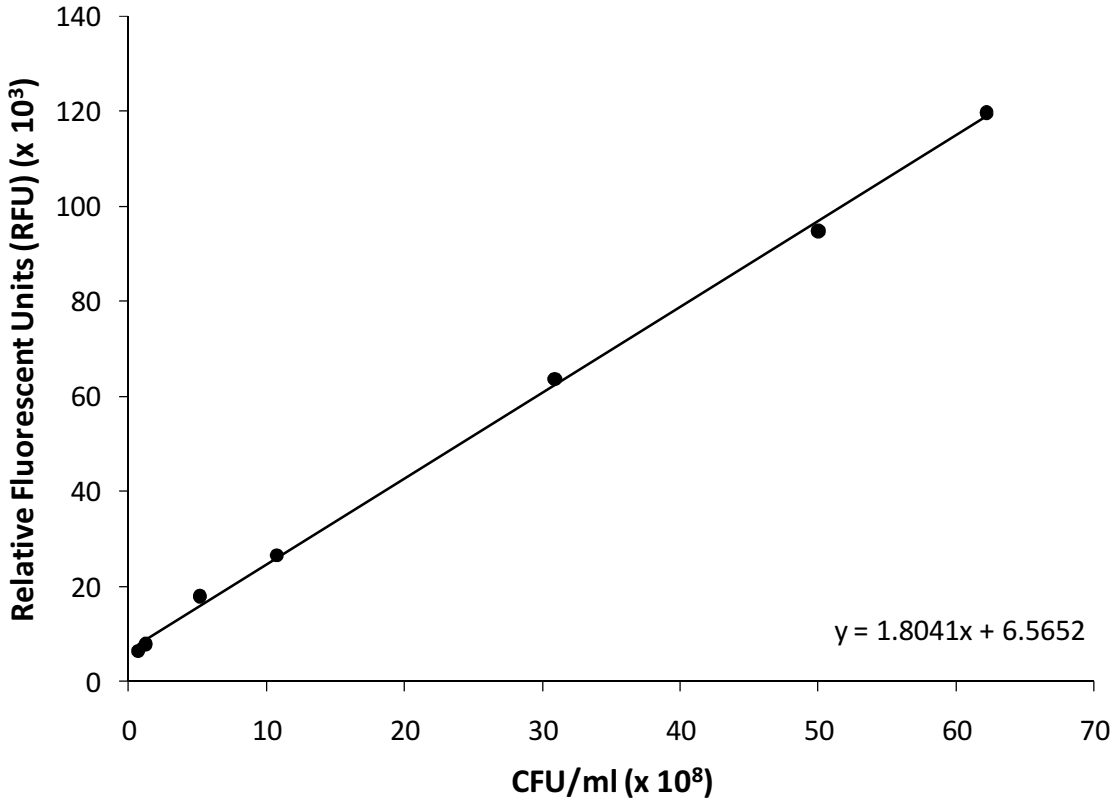

Figure 25. Relationship between *P. fluorescens* isolate 4-6-*gfp* cell density (CFU/ml) and relative fluorescent units (RFU) as determined by dilution plating and direct scanning (r = 0.999). Values are an average of three replicates.

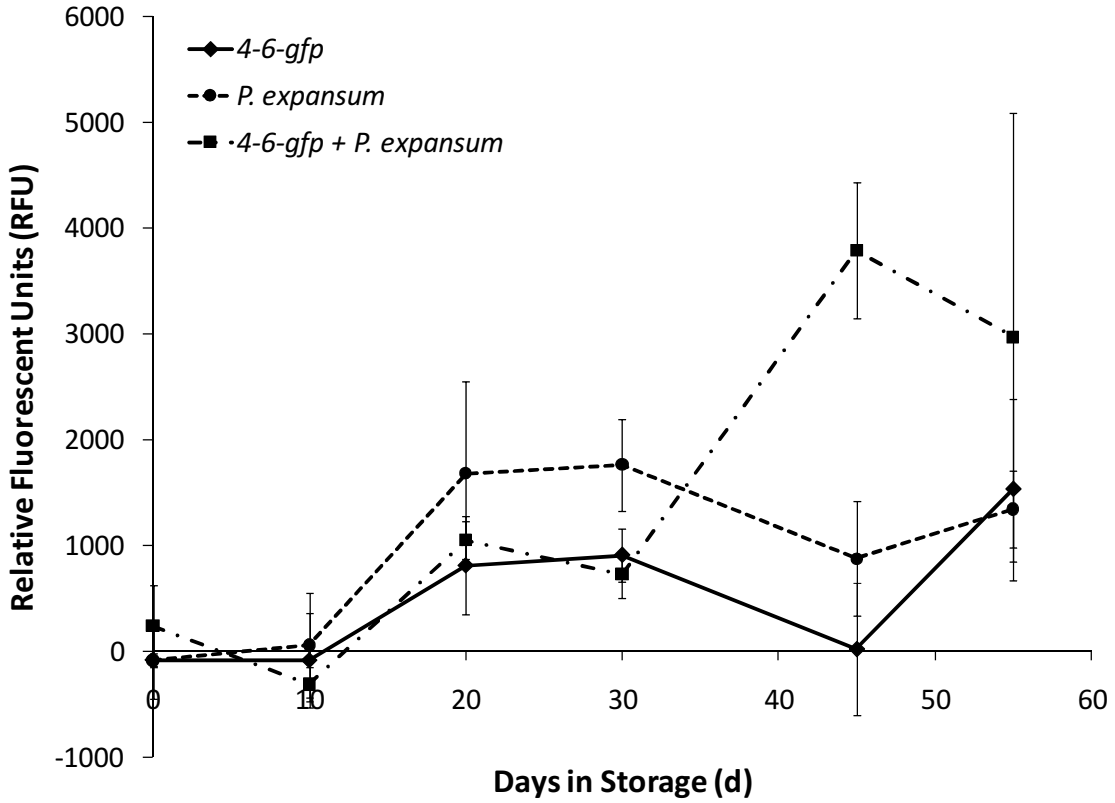

Figure 26. Bacterial survival of 4-6-*gfp* (♦), *P. expansum* (●) and *P. expansum* + 4-6-*gfp* (■) on Gala apples stored for 55 d at 1°C in air using the direct scanning method. Error bars represent standard error of the mean (n = 4).

Isolate 4-6-*gfp* alone increased during 20 d in storage then decreased after 30 d in storage, followed by an increase at 55 d in storage. *P. expansum* alone followed the same trend; however, the overall RFUs were higher. The RFUs obtained for 4-6-*gfp* in the presence of *P. expansum* initially decreased in the first 10 d of storage and increased until 45 d in storage, followed by a decrease at 55 d in storage.

The selective growth medium, PF agar supplemented with 50 µg/ml kan and 30 µg/ml gen, was effective in isolating 4-6-*gfp* and no other resident bacteria on the apple surface were detected. Over 55 d of dilutions and platings, there was no bacterial growth on the plates with extracted material from the untreated and *P. expansum* only-inoculated apples.

A two-way ANOVA indicated a significant interaction between bacterial strain, 4-6-wild-type and 4-6-*gfp*, and time (Appendix, Table A40). One-way ANOVAs revealed no significant differences in cell densities between apples inoculated with 4-6-*gfp* + *P. expansum* and apples inoculated solely with 4-6-*gfp* up to 20 d in 4°C storage (Figure 27; Appendix, Table A41). From 30 to 55 d of storage, populations of 4-6-*gfp* in the presence of *P. expansum* differed significantly from populations of 4-6-*gfp* alone. When challenged with the pathogen, bacterial populations remained constant until lesions developed after 30 d, after which populations rapidly declined. When 4-6-*gfp* was unchallenged with the pathogen, its population increased from 20 – 30 d in storage then declined to slightly greater than its population at time 0. From 0 to 40 d in storage, 4-6-*gfp* significantly reduced IS compared to the control *(P. expansum* alone) (Table 10; Appendix, Table A42). There was no significant reduction in IS of apples treated with 4-

6-*gfp* in the presence of *P. expansum* after 40 d in storage (Table 10; Appendix, Table A42).

Table 10. Effect of biological control isolate 4-6-*gfp* on infection severity in Gala apples inoculated with and without *P. expansum* and stored for 55 days at 1°C in air.
IS means with different letters within columns are different at significant levels $p \leq 0.05$ according to the least significant difference (LSD) test.

Treatments	**Days in storage**					
	10	**20**	**30**	**40**	**45**	**55**
Untreated	0.0	0.0	0.0	0.0	0.0	0.0
4-6-*gfp* alone	0.0	0.0	0.0	0.0	0.0	0.0
P. expansum alone	0.0	0.0	2.7 a	7.8 a	11.5 a	19.6 a
4-6-*gfp* + *P. expansum*	0.0	0.0	0.0 b	3.0 b	8.4 a	16.9 a

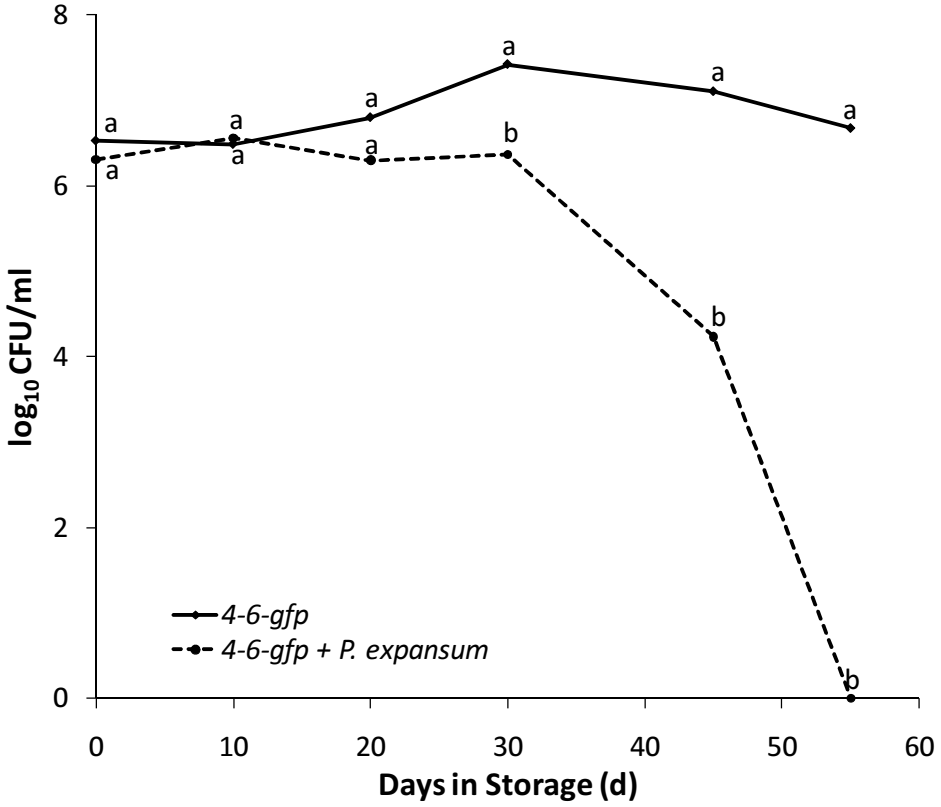

Figure 27. Bacterial survival of 4-6-*gfp* (♦) and 4-6-*gfp* + *P. expansum* (●) on Gala apples stored for 55 d at 1°C in air using the dilution plating method CFU/ml means with different letters within sampling times are different at significance levels $p \leq 0.05$ according to LSD test (n = 4).

CHAPTER 4: DISCUSSION

4.1 DNA macroarray for post-harvest apple pathogens

4.1.1 DNA macroarray quantification

In the current study, it was hypothesized that macroarray technology would accurately identify and quantify post-harvest apple pathogens throughout the growing season and that the frequency of pathogen detection would correlate with disease incidence post-harvest. This study reports that post-harvest pathogen quantification can be accomplished using DNA macroarray technology and that apple post-harvest pathogens can be detected throughout the apple growing season. Although many studies have used macroarray technology to detect and identify various plant pathogens and biological control microorganisms (Fessehaie et al. 2003; Izzo and Mazzola 2009; Le Floch et al. 2007; Levesque et al. 1998; Lievens et al. 2003; Robideau et al. 2008; Sholberg et al. 2005a; Tambong et al. 2006), few have quantified macroarray hybridization signal intensity (Lievens et al. 2005; Lievens et al. 2007; Sholberg et al. 2006) as we have done in this study.

Pathogen DNA was quantified by hybridizing 0 – 100 ng of pure culture DNA extracts of *P. expansum*, *B. cinerea* and *M. piriformis* to the macroarray. The detector probes or oligonucleotide sequences had varying levels of hybridization signal intensity. As found by Sholberg et al. (2006) who correlated greyscale value and cell density, a linear relationship was found between greyscale value and DNA concentration. However, in this study, the hybridization signals became saturated outside of the linear range at >3 ng for *P. expansum*, >12 ng for *B. cinerea* and >0.5 ng for *M. piriformis* (data not shown). Lievens et al. (2005), who quantified DNA concentrations of

phytopathogenic fungi, *Verticillium albo-atrum*, *Verticillium dahlia*, *Fusarium oxysporum*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*, also reported a linear relationship between signal intensity and DNA concentration. Linearity was established when 0.5 fmol of detector probe was spotted on a membrane and template concentrations ranged from 0.25 pg to 2.5 ng which was in the same range or somewhat lower than the linear range in the present study. Below and above 0.5 fmol, the curves deviated from linearity. Lievens et al. (2007) also reported that hybridization signals between 0.25 to 25 pg (0.00025 to 0.025 ng), that detect *Trichoderma* spp. and *Trichoderma hamatum*, a disease suppressive organism, resulted in a logarithmic relationship between signal intensity and template DNA concentration. This was lower than the linear range reported in the present study. Above 25 pg, the standard curves deviated from linearity.

Although a range of detector probe concentrations were not tested within this study, Lievens et al. (2005) noted that altering the amount of oligonucleotide spotted on the membrane can affect the range of DNA concentrations detected by a specific macroarray probe. Once a greater knowledge base exists about the level of post-harvest pathogenic DNA found throughout a crop's growing season and its correlation with disease manifestation, the DNA oligonucleotide detector probe concentrations can be altered to detect ranges typically found in field conditions. This will allow for more accurate DNA quantification and disease management. For enhanced and more precise quantification, it would be advantageous to simultaneously employ hybridization signal analysis with real-time PCR. The ability to accurately quantify a pathogen is useful in

plant disease management as information can be used to assess potential risk and disease development (Lievens and Thomma 2005).

4.1.2 Field monitoring of apple post-harvest pathogens

The current study was the first of its kind to monitor post-harvest pathogen presence via molecular technologies prior to commercial storage and disease development. Of the samples collected in each of the three growing season sampling periods, there was a tendency to detect total populations of all three post-harvest fungi more frequently in the early and mid-growing seasons of 2007 (56.6% - early; 60% - mid; 33.3% - late) and 2008 (67.6% - early; 63.2% - mid; 34% - late). This tendency to detect lower pathogen levels from air and plant tissue samples in the late growing season corresponds with the lower pathogen levels detected on whole apples collected at harvest (Table 4). In addition, the increase in early season fungal detection corresponds with results obtained by Teixidó et al. (1999) who observed total bacterial, yeast and filamentous fungal populations on Golden Delicious apples. However, they found an even greater increase in microorganisms at the apple bud stage followed by a decrease in populations at blossom. It was suggested that as buds form blossoms, there is a loss of external structure due to blossom drop, which accounts for the reported decrease in microorganisms after the apple budding stage. In the current investigation, samples were not collected at the apple bud stage. The rise in total fungal propagules may therefore correspond with precipitation events in the mid-growing season of 2007 and the early and mid-growing season of 2008 (Figure 1).

Of the three major post-harvest pathogens, *P. expansum*, on average, was detected most frequently (27.4. ± 3.4%) and was found throughout the growing season. In 2008 in

Fields 3 and 12 there was an increased detection frequency at the end of the growing season (Table 3). The abundance of *P. expansum* found within this study contradicts results of Amiri and Bompeix (2005) and Teixidó et al. (1999) who reported low levels of *Penicillium* inoculum within the orchard atmosphere. However, the aerial sampling period of Amiri and Bompeix (2005) was between July and September which excludes the early growing season.

In contrast to *P. expansum, B. cinerea* was detected the least number of times (6.2 ± 1.4%) and found throughout the growing season of 2007 and 2008. The most important infective units of *B. cinerea* are the conidia produced in late winter and early spring from over-wintering mycelia and/or sclerotia on host tissue or soil surfaces (Elad et al. 2007); grey mold is rarely seen in the field (Rosenberger 1990). The low levels of *B. cinerea* as indicated by the macroarray correspond with these results.

M. piriformis was detected the second most frequently (19.2 ± 1.4%) and found most frequently in the mid-growing season of 2007 and the early to mid-growing season of 2008 in DNA isolated from plant tissue samples. These findings support Guo et al. (1999) who found an increase in *M. piriformis* populations within orchard soil from December to March and April, followed by a decrease afterwards. It was suggested that the increase in *M. piriformis* corresponds with apples left on the orchard floor and cool weather. Alternatively, these results contradict those found by Dobson and Spotts (1988) who recovered no *M. piriformis* from orchard air but found an abundance of *B. cinerea* and *P. expansum*. Sporangiospores of *M. piriformis* are soilborne (Michailides and Spotts 1986); greater than 75% are found in the top 2 cm of the soil profile and are dispersed primarily by rain splash and insects (Dobson and Spotts 1988). The detection

of an abundance of *M. piriformis* in this study may correspond to precipitation bursts found in both 2007 and 2008 (Figure 9). Sporangiospores may be aerosolized by the falling rain and then trapped by the aerial spore sampler.

Earlier studies, as mentioned above, that detected fungal abundance throughout the orchard growing season utilized culturing methods for fungal detection and identification (Amiri and Bompeix 2005; Dobson and Spotts 1988; Teixidó et al. 1999). In the case of *P. expansum* and *M. piriformis*, the macroarray detected the presence of pathogens not commonly found within the orchard. Culturing methodologies for detection and relative quantification may underestimate fungal abundance, thus showing the power of macroarray technology.

Data obtained using the macroarray may be useful in developing a disease forecasting model such as that presented by Spotts et al. (2009). For future experiments, it would be beneficial to correlate pathogen findings throughout the growing season with species-specific identification of natural disease incidence found within the packinghouse. In this study, a positive correlation was only evident between natural disease incidence and percent pathogens detected in the late-growing season for aerial and plant tissue samples analyzed together ($r = 0.79$) and for aerial samples ($r = 0.74$) when analysed separately from plant tissue samples. This finding corresponds with Lennox et al. (2003) who observed a significant correlation between the density of *B. cinerea* conidia on the pear fruit surface and grey mold disease incidence in cold storage. In addition, Walter et al. (1997) found a strong correlation between *B. cinerea* berry fruit contamination and latent boysenberry infection in New Zealand. Given the cost and time involved in collecting and analyzing DNA for macroarray identification and

quantification, the majority of samples could be collected, for example, near the end of the growing season. It should be noted, however, that Spotts et al. (2009) found that in creating an at-harvest prediction model for grey mold risk in pears, surface DNA was the least important predictor in the model as opposed to orchard rating and fungicide applications. Also of note, within this study, there was a negative correlation between percent pathogens detected in the early-growing season and natural disease incidence when plant tissue samples were analysed separately from aerial samples (r = -0.79). This data suggests that perhaps plant tissue samples collected in the early-growing season are not optimal predictors for natural disease incidence, thereby supporting the correlations mentioned above. More work is required in order to determine the relatedness between growing season pathogen levels and post-harvest disease prevalence.

The variability in the results of this study suggests that a multitude of factors contribute to disease incidence found post-harvest. Coley-Smith et al. (1980) proposed that disease outbursts may be a result of complex interactions between the production and dispersal of various inocula, infection pathogenesis, pathogen survival along with temperature, rainfall, humidity and crop protection, nutrition and phenology. Variability may also be explained by the application of pre-harvest fungicides and their effect on resident microflora. According to Sholberg and Boulé (2008), the application of Nova®, a fungicide used to control powdery mildew, reduced apple leaf and fruit microflora in two years of study. In the current study, Nova® was applied to Field 12 on May 15, 2007 which may help to explain the reduced pathogen loads when compared to the 2008 results (Figure 6). It should not be forgotten that the majority of post-harvest diseases are

a result of pathogens entering the fruit via wounds, all of which should be considered in accurate disease forecasting models.

4.2 Biological control of post-harvest pathogens

Five putative bacterial control agents were applied to apples harvested from commercial and experimental orchards in the Okanagan Valley, BC. It was hypothesized that one or more of the bacterial antagonists would provide significant pathogen control in semi-commercial and commercial storage conditions. This study showed that the four *P. fluorescens* isolates and one *S. plymuthica* isolate exhibited some control and antagonistic efficacy against post-harvest apple pathogens (Table 8). As indicated, *Serratia plymuthica* isolate 6-25 provided significant control in the greatest number of treatments (57.6%) (Table 8). These results suggest that this bacterium may provide control against a variety of post-harvest diseases and storage conditions and its use may be applied to other crops. Using *S. plymuthica* as a BCA, however, is a challenging endeavour as concerns surround its biosafety, a major consideration when selecting an antagonist (Droby et al. 2009). *Serratia plymuthica* belongs to the family Enterobacteriaceae. Members of this family, such as *Escherichia coli* strain O157:H7, have been known to cause human infections (Health Protection Agency 2007). Although opportunistic pathogenic strains of *S. plymuthica* have been isolated (Carrero et al. 1995; Clark and Janda 1985; Domingo et al. 1994) infection is rare compared to other members of the genus, *Serratia*, or of the family, Enterobacteriaceae.

Despite concerns surrounding its use as a biological control agent, *S. plymuthica* isolate 6-25 provided significant control in this study, against apple post-harvest pathogens, *P. expansum*, *B. cinerea* and *M. piriformis*. *S. plymuthica* biological control

efficacy has also been demonstrated by other research groups, although not on post-harvest apple pathogens. *S. plymuthica* strain HRO-C48 has been studied to control *Verticillium dahliae,* the causal agent of Verticillum wilt in oil-seed rape (Müller and Berg 2008). HRO-C48 also suppressed growth of *V. dahliae* and *Phytophthora cactorum* in strawberries (Kurze et al. 2001) and a commercial product based on strain HRO-C48 has been developed, RhizoStar® (e-nema GmbH, Raisdorf, Germany) (Müller and Berg 2008). *S. plymuthica* strain 5-6 also showed significant control of dry rot of potato caused by *Fusarium sambucinum* (Gould et al. 2008). The post-harvest biological control provided by *S. plymuthica* isolate 6-25 of this study should not be disregarded as the potential exists, as evident by other studies, for commercial application.

This study also showed that pseudomonads, specifically *P. fluorescens*, are also capable of providing control against post-harvest pathogens. According to Stockwell and Stack (2007), *Pseudomonas* spp., have been studied for decades as model organisms for biological control of plant diseases. In this study the greatest reduction in IS, in cases where significant control was exhibited, was with *P. fluorescens* isolate 1-112 followed by 1100-6 (Table 8). Other pseudomonads are also being studied for their efficacy against post-harvest pathogens. Mikani et al. (2008) reported significant control of *Botrytis mali*, formerly thought of as *B. cinerea* and recently revived by O'Gorman et al. (2008), using 10 strains of *P. fluorescens* isolated from leaf surfaces and apple fruit. Similar to the level of control found in this study, the results of Mikani et al. (2008), reported approximately 34.9% to 95.9% *B. mali* inhibition on Golden Delicious apples after 25 d at 5°C. In addition, Zhou et al. (2001) studied four strains of *Pseudomonas syringae* that were isolated from the phyllosphere of apple trees and controlled blue

mold, following inoculation by pipette, by 64 – 70% at 4°C after 28 d. The bacterial isolates also controlled grey mold, but to a lesser extent. Similar to methods used in this study, Zhou et al. (2001) also inoculated apples by dipping and found that a *P. syringae* isolate controlled blue mold by 93% after 133 d at 1°C in air, with the overall incidence of blue mold at 30%. In many cases, (Figure 12a) the bacterial isolates used in the present study exhibited similar and greater levels of control than those reported by Mikani et al. (2008) and Zhou et al. (2001). The effectiveness of these isolates may be due to the unique environment in which they were isolated. Many BCAs are found on the fruit surface or directly within a wound. The microbial agents used in this study, however, were isolated from the rhizosphere of legumes grown in cold Saskatchewan soils and may be more adapted to cold storage conditions. Droby et al. (2009) outlined the importance of antagonist selection and the use of a variety of screening procedures in finding effective BCAs. An assortment of selection techniques would increase biocontrol species diversity and therefore the potential for a successful product. These experiments illustrate the potential of *S. plymuthica* and *P. fluorescens* isolates for biological control application and commercialization.

4.2.1 Bacterial antagonists and CA storage

One type of commercial storage application used in this study was controlled atmosphere (CA). This research confirmed the hypothesis that one or more antagonists would provide control in commercial conditions. In all experiments, the lowest disease levels were found in apples stored in CA storage for 4.5 months following harvest in 2008. Many suggestions have been made regarding the selection criteria for potential BCAs (Wilson and Wisniewski 1989). One such criterion is the ability of the antagonist

to be compatible with current post-harvest commercial processing procedures such as CA (Janisiewicz and Korsten 2002; Wilson and Wisniewski 1989). This current study indicated that the selected antagonists were compatible with CA storage practices and may actually extend apple storage time due to enhanced pathogen suppression when compared to storage at 1°C. For example, the highest disease incidence recorded in CA was in Jonagold apples which reached an average IS of 10.39 ± 1.6 after 4.5 months in storage (Figure 10). The biological antagonists provided significant control against naturally occurring diseases in all orchards but Field 12 and the Reekie orchard (data not shown). In comparison, for Jonagold and Gala apples grown in Fields 3 and 12 then stored at 1°C in air for 4 months, non-inoculated controls had IS levels of 15.65 ± 5.0 (Figure 16b) and 19.90 ± 4.8 (Figure 16e), respectively. These values were almost double the infection severities found in CA. In both Jonagold and Gala apples harvested from Fields 3 and 12, respectively, the bacterial antagonists did not provide a significant reduction in IS after 4 months in storage (Figure 16b and e). However, isolate 6-25 reduced IS in Jonagold apples grown in Field 3 after 6 months in storage (Figure 16c). The decrease in apple disease supports the findings of Smock (1979) as CA effectively delayed the onset of storage decay.

In the CA-stored apples, natural disease incidence was very low even after 4.5 months of storage, except in the case of Jonagold apples from Field 3 which appeared to be due primarily to *P. expansum* (based on phenotypic observation). The gas composition of a packinghouse can directly or indirectly suppress fungal decay. Direct inhibition affects mycelium development and spore germination of resident pathogens. Alternatively, storage conditions also affect senescence and ripening of hosts, which, in

turn, affects their ability to resist infections, thereby indirectly controlling fungal decay (Barkai-Golan 2001). The implementation of CA is a balance between O_2 and CO_2 concentrations. General recommendations include 1 – 2 % O_2 and 0.5 – 2% CO_2. Concentrations outside of these ranges may lead to fruit injury which can affect fruit flavour and appearance (Kupferman 2003). However, post-harvest pathogens are only strikingly suppressed after O_2 concentrations have been lowered to less than 1% and CO_2 concentrations increased to greater than 10% (El-Goorani and Sommer 1981). The CA storage conditions in this study were approximately 1.5% O_2 and 1.5% CO_2, a range in which, if the conditions were right, may lead to the fungal outbreak that was observed in the Jonagold apples.

In the current study, the antagonistic effects of the bacterial isolates were difficult to observe because there was little natural disease present; if little disease was present, then there was little disease to control. However, the outbreak in Jonagold apples from Field 3 demonstrated the antagonistic potential of the bacteria because significant disease was present. While all of the antagonists demonstrated significant control, isolate 1100-6 and 1-112 were particularly effective, providing 94.9% and 83.8% reduction in disease symptoms in Jonagold apples from Field 3 (Figure 17). Although not directly inoculated with a pathogen, these BCAs performed better than the commercially registered BCA, Bio-Save 10 LP™, in studies conducted by Janisiewicz and Jeffers (1997) and Zhou et al. (2001). Using Bio-Save™ during 133 and 123 days in CA storage, Zhou et al. (2001) reported a 57% and a 73% reduction of blue mold in Empire and Delicious apples, respectively. It would be beneficial to test the efficacy of the bacterial antagonists against artificially inoculated pathogens under CA conditions as in Conway et al. (2007),

Janisiewicz et al. (2008) and Tian et al. (2002). However, during the present study, permission for pathogen inoculation within a commercial storage facility was not given.

4.2.2 Bacterial antagonists and 1-MCP

Another type of commercial storage application tested in this study was the use of 1-MCP. This research confirmed the hypothesis that one or more antagonists would provide control in commercial conditions, although to a lesser extent. 1-MCP was applied to Jonagold apples grown in Field 3 and Gala apples grown in Field 12 in the biological control experiments using apples from the 2007 harvest. It was postulated that the application of 1-MCP would result in a decrease in disease incidence and severity of non-inoculated wounded apples or apples inoculated with putative biological control agents and/or post-harvest pathogens. However, when the overall effect of 1-MCP was examined, a reduction in infection severity occurred in only 14% of the experimental treatments. For example, 1-MCP application reduced IS caused by natural disease incidence in Jonagold apples from Field 3 (Table 5) by 51.8% and in Gala apples from Field 12 (Figure 11) by 67.1%. Variable levels of control confirm findings of Saftner et al. (2003) who studied the effects of pre-storage heat, CA storage and pre- and post-application of MCP on inhibition of fungal decay and maintenance of Golden Delicious fruit quality. Pre-storage treatment of MCP reduced lesion size (mm) of fruit inoculated with *B. cinerea* and *P. expansum* by 34% and 17.9%, respectively. Post-storage application of MCP had no effect on reducing lesion diameter. Variable responses to 1-MCP application are not uncommon. Watkins (2008) indicated that the apple is a fruit with many cultivars that differ in their ripening rates, harvest criteria, post-harvest handling practices and storage periods in air and CA. In addition, time between harvest

and 1-MCP treatment and repeated application have variable effects in disease resistance responses. In the current study, when a decrease in infection severity was observed, it appeared to be primarily in experiments where there was no artificial inoculation of *P. expansum*, *B. cinerea* or *M. piriformis*; the only pathogens present were those that occurred naturally.

Contradictory to the intended outcome of 1-MCP application, some treatments in the present study resulted in an increase in infection severity of apples treated with 1-MCP, especially those apples inoculated with *B. cinerea* (Figure 12b). An increase in decay after 1-MCP application was also observed by Janisiewicz et al. (2003) and Leverentz et al. (2003) on Golden Delicious apple fruit and by Bedford et al. (2002) who reported an increase in *B. cinerea* decay when apples were fumigated with 1-MCP. Commercial application of 1-MCP as SmartFresh® is thought to delay apple ripening by preventing ethylene production via preferential binding to the ethylene receptor site and extending the action of natural defence mechanisms due to delayed ripening (Blankenship and Dole 2003). However, the reverse was observed in some cases as 1-MCP appeared to hinder plant defence mechanisms. According to Marcos et al. (2005), endogenous ethylene may be an important component in plant resistance and defence gene regulation; the application of 1-MCP may therefore compromise a plant's defence response system (Jiang et al. 2001).

Despite the controversy surrounding chemical control agents, fungicides still remain the preferred choice for the prevention of post-harvest diseases. Although fungicide-resistant pathogens have increased within the population, chemical control provides a consistent and effective reduction in disease. Within the agricultural

community, there is a relatively high level of scepticism surrounding the use of biological control products (Droby et al. 2009). Commercially acceptable decay levels are generally below 5% (Janisiewicz and Korsten 2002) and because of the inconsistency and variability of biofungicides, the packinghouse industry will often choose the most economically viable option that consistently reduces rot.

Disease control was exhibited by the bacterial antagonists in this study, but efficacy was highly variable, as observed by other researchers. The tritrophic interaction between host, pathogen and antagonist is a highly complex one that demands a more sound and fundamental understanding. In addition to exploring the mechanism(s) of action for fungal suppression, combination and alternative treatments for enhanced efficacy should be explored. Such alternate biocontrol treatments, outlined by Sharma et al. (2009) include, for example, the addition of low doses of fungicides, salt additives, or nutrients and plant products, as well as the use of mixed cultures or manipulation of the physical environment. Rapid antagonist colonization of fruit wounds is critical to controlling decay; therefore, enhancements leading to improved wound colonization should be further explored (Janisiewicz and Korsten 2002).

4.3 Bacterial survival on apple

Acquiring basic knowledge about potential antagonists will provide a greater understanding into the mechanisms of actions, which will, in turn, offer insight into means for achieving more consistent control. Disease prevention relies on a quantitative relationship between antagonist and pathogen (Janisiewicz 1988) and knowledge of antagonist cell number during time in storage will assist in enhanced disease suppression (Etebarian and Sholberg 2005). In the present study, *Pseudomonas fluorescens* isolate 4-

6 was tagged with GFP to determine the survival characteristics of this antagonist alone and when challenged with *P. expansum*. It was hypothesized that green fluorescent protein would facilitate visualization of bacterial colonization and survival on apple surfaces. In this study, *gfp* did facilitate bacterial visualization, but indirectly as pAG408 provided antibiotic resistance of isolate 4-6 which permitted selective plating that excluded other organisms during survival analysis.

Bacterial concentrations were determined using direct scanning and colony counts. There was a strong linear relationship between fluorescent intensity and cell density (Figure 26). These results corresponded with those found by Etebarian and Sholberg (2005) who studied population levels of *P. fluorescens* isolate 1100-6 labelled with GFP using direct scanning, cell counts and microscopy. This and the current study illustrated that measuring fluorescent intensity as a means of estimating cell concentration was an effective method of population assessment. The initial inoculation concentration of 4-6-*gfp* (10^8 CFU/ml), however, combined with the method of recovery, resulted in fluorescent readings that were not high enough to quantify using the linear equation $y = 1.8041x + 6.5652$ (Figure 26). Using a Fast Prep machine for tissue maceration as in Etebarian and Sholberg (2005) or in Etebarian et al. (2005) may overcome issues incurred by extraction.

Despite low values, the raw microplate data were compared to plate counting data. Relative fluorescent units (RFUs) (Figure 27) and cell densities (CFU/ml) (Figure 28) of isolate 4-6-*gfp* followed similar patterns; when cell counts increased, so did RFUs. However, there was an inverse relationship between RFUs and CFU/ml for 4-6-*gfp* inoculated in the presence of *P. expansum*; RFUs increased while cell counts decreased.

Two possible explanations are that *P. expansum* auto-fluoresces or that the decayed apple tissue auto-fluoresces. The latter explanation corresponds with the formation of lesions at 30 d (Table 10). Increased antagonist populations after 30 d of storage were also found by Janisiewicz and Marchi (1992) who reported a 10 – 100 fold increase in populations of *Pseudomonas syringae* inoculated on pears stored at 1°C. Etebarian and Sholberg (2005) also found an increase in *P. fluorescens* isolate 1100-6 GFP on apples after 35 days in 5°C storage. However, unlike the survival characteristics of 4-6-*gfp* + *P. expansum* in this study, 1100-6 GFP + *P. expansum* maintained as high or higher cell numbers than 1100-6 GFP alone. Low cell counts after lesion development may be attributed to difficulties in extracting apple cores from completely decayed tissue. Even so, after 30 d at 1°C in air storage, 4-6-*gfp* appeared to lose some of its antagonistic capabilities (Table 10). Information such as this should be considered when implementing control strategies for optimal post-harvest disease management.

CHAPTER 5: CONCLUSIONS

This thesis built upon preliminary research and addressed the following three objectives: 1) to validate the performance of DNA macroarrays under field conditions; 2) to assess bacterial antagonist performance under semi-commercial and commercial conditions and; 3) to determine how long and in what capacity a selected antagonist will colonize the fruit surface.

The first hypothesis addressed the first objective and stated that macroarray technology would accurately identify and quantify post-harvest apple pathogens throughout the growing season and that disease prevalence would correlate with disease incidence post-harvest. Results confirmed that in two years of field trials, the DNA macroarray is a fast and easy technique capable of detecting apple post-harvest pathogens throughout the apple growing season (Table 3). Commercial application of this diagnostic and quantitative technique would provide accurate information to assist growers and field personnel in crop management. A positive correlation existed between pathogen detection at the end of the growing season and natural disease incidence found post-harvest. More work is required to obtain information about the importance of monitoring pre-harvest pathogen loads and their contribution to post-harvest disease.

The second hypothesis addressed the second objective and stated that one or more of the bacterial antagonists would provide significant pathogen control in semi-commercial and commercial storage conditions. Results indicated that despite variability between years, variety and biological control efficacy, the potential BCAs demonstrated control at 1°C in air and particularly in CA storage (Table 8). Biocontrol efficacy in combination with 1-MCP was less effective and the application of 1-MCP greatly

increased disease levels with *B. cinerea* artificial inoculation (Figure 12b). Combining some or all of the bacterial control agents used in this study in conjunction with other organic amendments such as salts (ie. sodium bicarbonate) or manipulating the physical environment (ie. heat treatment) may provide consistency in control which the industry demands.

The third hypothesis addressed the third objective and stated that the use of green fluorescent protein would facilitate visualization of bacterial colonization and survival on apple surfaces. Results indicated that green fluorescent protein was an effective technique to monitor population levels as the *gfp*-marked strain behaved similarly to the wild-type strain (Figure 25; Table 9). The fluorescence levels obtained by direct scanning, however, were not sufficient to distinguish 4-6-*gfp* from background auto-fluorescence. The presence of the *gfp*-containing plasmid within *P. fluorescens* isolate 4-6 therefore provided an indirect method of monitoring population levels as pAG408 contained antibiotic resistant genes that allowed for strain-specific isolation from an inoculated apple wound. Bacterial populations were found to initially increase then decrease within an apple wound during 55 d in storage at 1°C (Figure 28). In the presence of *P. expansum*, 4-6-*gfp* populations declined, which coincided with *P. expansum* lesion development. The information obtained within this study will assist the fruit industry in detecting, quantifying and biologically controlling post-harvest pathogens of pome fruit.

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APPENDIX

Appendix A

Table A1. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	2628.336	4.585	0.0619
B. cinerea	2	384.596	1.857	0.2357
M. piriformis	2	573.896	0.429	0.6697

Table A2. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	327.860	0.134	0.8766
B. cinerea	2	66.667	1.0	0.4053
M. piriformis	2	5625.402	28.266	0.0001***

Table A3. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	741.125	1.119	0.3681
B. cinerea	2	55.512	0.567	0.5863
M. piriformis	2	683.272	1.018	0.3994

Table A4. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	75.335	0.034	0.9668
B. cinerea	2	488.562	0.579	0.5800
M. piriformis	2	203.247	0.438	0.6583

Table A5. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	1702.334	4.590	0.0533
B. cinerea	2	148.328	12.683	0.0047**
M. piriformis	2	1336.256	4.316	0.0601

Table A6. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	182.052	0.171	0.8461
B. cinerea	2	76.432	0.262	0.7753
M. piriformis	2	503.152	1.681	0.2398

Table A7. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in all growing season sections for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Detected pathogens (*P. expansum*, *B. cinerea* or *M. piriformis*)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2007 growing season	2	896.063	4.116	0.0538
2008 growing season	2	1022.252	3.222	0.0880
2007/08 growing season	2	454.769	19.248	0.0194*

Table A8. Key data extracted from the two-way ANOVA table for infection severity data collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Incubation Period	1	3639.591	13.081	0.0004***
1-MCP+/-	1	3260.433	11.718	0.0008***
Interaction				
Incubation period x MCP+/-	1	1008.275	3.624	0.0584

Table A9. Key data extracted from the two-way ANOVA table for infection severity data collected from Gala apples grown in Field 12 analyzed for natural disease incidence after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Incubation Period	1	13.767	36.073	0.0000***
1-MCP+/-	1	0.175	0.458	0.4995
Interaction				
Incubation period x MCP+/-	1	0.286	0.750	0.3876

Table A10. The effect of incubation period (1, 2, 4 and 6 months) on natural disease incidence and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3, Gala apples grown in Field 12 and Kiran and Red Delicious apples grown in Reekie and analyzed for natural disease incidence from harvest after 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control Treatment

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3	3	61.3201	74.6611	0.0000***
Field 12	3	79.6754	276.1476	0.0000***
Kiran	3	0.8366	3.2372	0.0268*
Reekie	3	11.1988	17.9952	0.0000***

Table A11. Key data extracted from the one-way ANOVA table constructed from infection severity data collected from Fields 3, 12, Kiran and Reekie analyzed for natural disease incidence for apples stored in CA storage for 4.5 months from harvest 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1 month storage	3	0.9484	1.5636	0.1195
2 months storage	3	10.0842	7.1456	0.0001***
4 months storage	3	8863.9197	27.1892	0.0000***
6 months storage	3	40909.7401	48.0806	0.0000***

Table A12. Key data extracted from the one-way ANOVA table constructed from infection severity data collected from Fields 3, 12, Kiran and Reekie analyzed for natural disease incidence for apples stored in CA storage for 4.5 months from harvest 2008.

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field	3	29.2208	55.0013	0.0000***

Table A13. Key data extracted from the two-way ANOVA table for infection severity data collected from Jonagold apples grown in Field 3 analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2007..

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	1	0.900	1.192	0.3182
1-MCP+/-	1	0.342	2.262	0.1355
Interaction				
Biological Control x MCP+/-	5	0.332	0.439	0.8202

Table A14. Key data extracted from the two-way ANOVA table for infection severity data collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2007..

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	1	3039.868	5.773	0.0001***
1-MCP+/-	1	3435.342	32.620	0.0000***
Interaction				
Biological Control x MCP+/-	5	2779.181	0.439	0.0002***

Table A15. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates from harvest 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	2310.748	18.214	0.0005***
1100-6	1	1674.926	11.450	0.0033**
1-112	1	7.358	0.243	0.6279
2-28	1	1542.633	26.057	0.0001***
4-6	1	0.020	1.49e-4	0.9904
6-25	1	130.302	0.966	0.3387

Table A16. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are constructed from IS results collected from Field 12 Gala apples and analyzed for natural disease incidence in combination with bacterial isolates from harvest 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	624.112	1.329	0.2659
1-MCP-	5	5194.937	8.902	0.0000***

Table A17. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and inoculated with *P. expansum* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	32.862	61.071	0.0000***
1-MCP+/-	1	0.015	0.413	0.7077
Interaction				
Biological Control x MCP+/-	5	5.626	1.125	0.0000***

Table A18. The effect of 1-MCP in six separate biological control categories and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0420	1.775	0.1994
1100-6	1	1.4724	7.422	0.0139*
1-112	1	0.0425	0.215	0.6482
2-28	1	0.0872	2.057	0.1687
4-6	1	0.0329	0.302	0.5893
6-25	1	3.9641	52.958	0.0000***

Table A19. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are constructed from infection severity results collected from Jonagold grown in Field 3 analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates from harvest in 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	16.7121	31.798	0.0000***
1-MCP-	5	21.7763	39.548	0.0000***

Table A20. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and inoculated with *B. cinerea* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	21.222	20.760	0.0000***
1-MCP+/-	1	15.998	78.249	0.0000***
Interaction				
Biological Control x MCP+/-	5	13.841	13.534	0.0000***

Table A21. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 20077. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	9.706	45.191	0.0000***
1100-6	1	0.3931	1.793	0.1973
1-112	1	0.2607	1.060	0.3169
2-28	1	13.3139	80.502	0.0000***
4-6	1	5.9336	29.726	0.0000***
6-25	1	0.2310	1.271	0.2743

Table A22. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 2007.

Main Effect Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	33.7372	53.215	0.0000
1-MCP-	5	1.3253	0.940	0.4630

Table A23. Key data extracted from the two-way ANOVA table constructed from infection severity data collected from Jonogold apples grown in Field 3 and inoculated with *M. piriformis* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	14.346	10.660	0.0000***
1-MCP+/-	1	0.784	2.913	0.0908
Interaction				
Biological Control x MCP+/-	5	7.164	5.323	0.0002***

Table A24. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data is are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect 1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0020	0.0181	0.8943
1100-6	1	0.6318	1.3963	0.2527
1-112	1	0.0194	0.0729	0.7902
2-28	1	0.6870	4.3055	0.0526
4-6	1	6.5402	13.1253	0.0019**
6-25	1	0.0677	0.5298	0.4761

Table A25. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	1.1507	0.9683	0.4455
1-MCP-	5	20.3591	13.5439	0.0000

Table A26. Key data extracted from the two-way ANOVA table constructed from infection severity data collected from Gala apples from Field 12 and inoculated with *P. expansum* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	192.271	1.940	0.0936
1-MCP+/-	1	182.887	9.226	0.0030**
Interaction				
Biological Control x MCP+/-	5	164.951	1.664	0.1494

Table A27. Key data extracted from the two-way ANOVA table from infection severity data collected from Gala apples grown in Field 12 and inoculated with *M. piriformis* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	4	3.015	1.729	0.1505
1-MCP+/-	1	6.578	1.508e-5	0.9969
Interaction				
Biological Control x MCP+/-	4	8.469	4.856	0.0014***

Table A28. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect 1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0242	0.0487	0.8278
1-112	1	1.9035	4.7987	0.0419*
2-28	1	6.0306	14.5425	0.0013**
4-6	1	0.4423	1.1814	0.2914
6-25	1	0.0683	0.1370	0.7156

Table A29. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007.

Main Effect Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	7.0326	3.9878	0.0075**
1-MCP-	5	4.4516	2.5814	0.0498*

Table A30. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2008.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	1.2214	0.8840	0.4926
Incubation Period	3	105.9743	127.8366	0.0000***
Interaction				
Biological Control x Incubation Period	15	14.3647	3.4656	0.0000***

Table A31. Key data extracted from the two-way ANOVA table from infection severity data collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2008.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	5	0.5763	0.8804	0.4950
Incubation Period	3	149.6138	380.9182	0.0000***
Interaction				
Biological Control x Incubation Period	15	3.6138	1.8400	0.0309*

Table A32. The effect of the incubation period on biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2 months	5	5.5753	4.3605	0.0021**
4 months	5	5067.4972	3.9609	0.0039**
6 months	5	15646.1017	5.2559	0.0005***

Table A33. The effect of the incubation period on biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2 months	5	2.1850	2.8870	0.0221*
4 months	5	1876.4999	2.5715	0.0370*
6 months	5	743.3109	0.7762	0.5782

Table A34. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold (Field 3), Gala (Fields 12 and Kiran) and Red Delicious (Reekie) apples after 4.5 months in CA storage and analyzed for natural disease incidence following inoculation with bacterial isolates after harvest in 2008.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	5	3.3247	5.4749	0.0001***
Field	3	12.3636	33.9323	0.0000***
Interaction				
Biological Control x Field	15	5.8261	3.1980	0.0000***

Table A35. The effect of the biological control treatments on infection severity and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3, Gala apples grown in Field 12 and Kiran and Red Delicious apples grown in Reekie after 4.5 months in CA stroage and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control Treatment

Category	DF	Type III SS	*F*-value	*p*-value
Field 3	5	7.7543	4.8907	0.0009***
Field 12	5	0.1933	1.1827	0.3298
Kiran	5	0.1136	2.2402	0.0633
Reekie	5	1.0897	1.7312	0.1432

Table A36. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control

Category	DF	Type III SS	*F*-value	*p*-value
Field 3, Jonagold	5	3041.4892	6.8050	0.0001***
Field 12, Gala	5	401.2654	3.1239	0.0151*

Table A37. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3, Jonagold	5	5981.4974	4.9960	0.0008***
Field 12, Gala	5	1962.7067	6.4391	0.0001***

Table A38. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3, Jonagold	5	2115.5372	4.7271	0.0012**
Field 12, Gala	5	3.0721	4.1326	0.0030**

Table A39. Growth rate (μ) comparisons of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* and the resulting key data extracted from the one-way ANOVA table.

Main Effect				
Bacterial Isolate 4-6-wt vs 4-6-*gfp*				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Growth Rate (μ)	1	0.0022	0.6492	0.4656

Table A40. Key data extracted from the two-way ANOVA table constructed from cell densities (CFU/ml) of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* stored for 55 d at 1°C in air.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Bacterial strain	1	50.5367	670.6401	0.0000***
Time	5	72.3075	191.9023	0.0000***
Interaction				
Bacterial strain x Time	5	72.7250	171.9775	0.0000***

Table A41. Cell density comparisons determined by dilution plating method of *P. fluorescens* isolates 4-6-*gfp* and 4-6-*gfp* + *P. expansum* stored for 55 d at 1°C in air and the resulting key data extracted from the one-way ANOVA table. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Bacterial Isolate 4-6-*gfp* vs 4-6-*gfp* + *P. expansum*

Category	DF	Type III SS	*F*-value	*p*-value
0 d storage	1	0.1061	2.3329	0.1610
10 d storage	1	8.4137e-4	0.0157	0.9024
20 d storage	1	0.5121	4.0261	0.0758
30 d storage	1	2.3762	36.5429	0.0005***
45 d storage	1	16.6408	120.1450	0.0000***
55 d storage	1	4.418e13	8.8646	0.0206*

Table A42. Infection severity comparisons of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* stored for 55 d at 1°C in air and the resulting key data extracted from the one-way ANOVA table. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Bacterial Isolate 4-6-*gfp* vs 4-6-*gfp* + *P. expansum*

Category	DF	Type III SS	*F*-value	*p*-value
30 d storage	1	21.6680	7.6629	0.0040**
40 d storage	1	46.8838	14.1739	0.0093**
45 d storage	1	18.9113	1.3841	0.2840
55 d storage	1	13.9568	0.5615	0.4820

DETECTION, QUANTIFICATION AND BIOLOGICAL CONTROL OF POST-HARVEST PATHOGENS OF POME FRUIT

by

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ABSTRACT

Blue mold (*Penicillium expansum*), grey mold (*Botrytis cinerea*) and mucor rot (*Mucor piriformis*) are important post-harvest diseases of pome fruit in British Columbia and throughout the world causing annual losses of 5 – 20%. Identification and quantification using novel DNA macroarray technology may assist in the development of prediction models and disease forecasting. Post-harvest pathogens were monitored and quantified throughout the growing season in four apple orchards in the Okanagan Valley, BC in 2007 and 2008. Their detection was variable due to field and year differences. The average percent detection of *P. expansum* (27.4%) and *M. piriformis* (19.2%) was higher than that of *B. cinerea* (6.2%). There was a positive correlation between total post-harvest pathogen detection in aerial samples just prior to harvest and after harvest in naturally infected fruit ($r = 0.74$; $p = 0.09$). *Pseudomonas fluorescens* (isolates 1-112, 2-28, 4-6) and *Serratia plymuthica* (isolate 6-25), isolated from the rhizosphere of legumes, were investigated for their biological control capabilities in semi-commercial storage conditions at 1°C in air and commercial storage conditions in controlled atmosphere and with 1-methylcyclopropene (1-MCP) application. Overall, isolate 6-25 provided control in the greatest number of treatments (51.7%) while isolate 1-112 provided the greatest level of control (75.8%) in treatments where significant control was exhibited. Isolate 4-6 was tagged with green fluorescent protein to gain insight into bacterial antagonist population and survival dynamics. Alone, its population increased 10 fold after 30 d in storage at 1°C and then decreased to concentrations similar to those at inoculation. In the presence of the pathogen, 4-6-*gfp* increased then decreased after 30 d in storage at 1°C to undetectable amounts. These data provide greater insight into the prediction, control and

population dynamics of pathogens and biological control agents as a means of preventing and controlling post-harvest storage diseases in pome fruit.

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* (one asterisk) – *p*value = 0.05 – 0.01
** (two asterisks) – *p*value = 0.01 – 0.001
*** (three asterisks) – *p*value = < 0.001
½ TSA – half-strength tryptic soy agar
½ TSB – half-strength tryptic soy broth
1-MCP – 1-methylcyclopropene
4-6-*gfp* – transformed *P. fluorescens* isolate 4-6 with GFP-containing plasmid pAG408
bc – biocontrol
BC – British Columbia
BCA – biological control agent
Bcin – *Botrytis cinerea*
βtub – β tubulin
CA – controlled atmosphere
CFU – colony forming units
CSLM – confocal scanning laser microscopy
gen - gentamicin
gfp – green fluorescent protein (gene)
GFP – green fluorescent protein (protein)
IS – infection severity
ISR – induced systemic resistance
kan - kanamycin
$\log_{10}$ – logarithmic to the base 10
LSD – least significant difference
MOA – mode of action
Mpir – *Mucor piriformis*
NDI – natural disease incidence
OD_{600} – optical density at 600 nm
PARC –Pacific Agri-Food Research Centre
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDA – potato dextrose agar
Pex – *Penicillium expansum*
pt – plant tissue
rDNA – ribosomal deoxyribonucleic acid
RFU – relative fluorescent units
TBZ – thiabendazole
TSA/B – trypic soy agar/broth

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CHAPTER 1: INTRODUCTION

According to the Food and Agriculture Organization of the United Nations, the world apple production in 2008 was greater than 69 million tonnes (FAOSTAT 2009). Apples (*Malus domestica*) are ranked fourth (by volume) in the world's production of fruit crops (BCMAFF 2004). In 2002, the British Columbia apple industry produced 136 000 tonnes or approximately 299 846 000 lbs of apples. Apples are produced in significantly higher volumes than any other tree fruit crop in BC (BCMAFF 2004). One major challenge to apple production is damage incurred by post-harvest diseases; annual losses can vary from 5 – 20% and up to 50% in developing countries (Janisiewicz and Korsten 2002). A variety of fungal pathogens is responsible for post-harvest disease; in BC, three such pathogens include *Penicillium expansum* (blue mold), *Botrytis cinerea* (grey mold) and *Mucor piriformis* (mucor rot) (Sholberg and Haag 1996).

A common method of disease prevention is through the use of fungicides. In Canada, two synthetic controls are registered for post-harvest use, Mertect® (thiabendazole) and Scholar® (fludioxonil) (PMRA 2007). However, due to growing health issues, the development of pathogen resistance and the demand for environmentally friendly sustainable practices, pesticides are now a major cause for concern (Errampalli et al. 2006; Michaildes and Spotts 1990; Sholberg and Haag 1996).

To reduce post-harvest rot, improved methods for pathogen identification prior to harvest and alternate technologies to chemical control are required to assist in disease prediction and control. Preliminary work has resulted in two novel approaches that address these demands: 1) a rapid DNA-based macroarray system that determines the presence of fungal pathogens responsible for post-harvest rot (Sholberg et al. 2005a) and

2) the identification of soil bacteria that can control fungal post-harvest pests (Hirkala et al. 2007; Hynes et al. 2008). This study will assist the project's long term goal to develop Canadian-based technologies that detect, predict and mitigate post-harvest disease in pome fruit and that are consistent with integrated pest management and sustainable production practices.

1.1 Post-harvest pathogens

Fruit infections can occur throughout the growing season, at harvest, during storage, transit and at the retail level (Jones and Aldwinckle 1990). Although global economic losses can be attributed to growing season diseases, post-harvest pathogens, despite modern storage facilities, can cause significant annual losses (Janisiewicz and Korsten 2002).

Post-harvest diseases, or latent infections, are asymptomatic throughout the growing season and disease is manifested only after periods of storage. As a result, these pathogens are often difficult to treat and prevent. Post-harvest fungal pathogens can be categorized in many ways, one of which describes the pathogenic point of entry. Rot characterization can include lenticel infections (pores that exist on the surface of the apple that facilitate gas exchange), core and eye rots and wound pathogens (Jijakli and Lepoivre 2004). Common lenticel infections include bitter and bull's eye rot and are commonly caused by *Colletotrichum gleosporioides* and *Cryptosporiopsis curvispora*, respectively (Jijakli and Lepoivre 2004). Core rots develop when an open sinus stems from the calyx end of the apple into the core (Miller 1959), which provides a point of entry for an invading pathogen. *Alternaria* spp. are the most commonly isolated fungi from core rots, but other fungal species such as *Cladosporium*, *Botrytis*, *Candida* and

Fusarium have also been identified (Ellis and Barrat 1983). Dry eye rots, characterized by a shallow, hard rot with a red border are predominantly caused by *Botrytis cinerea*.

Opportunistic wound pathogens invade apples as a result of fruit injury incurred by mishandling or harsh weather conditions. These types of infections are responsible for significant storage losses. Three important fungal pathogens that cause worldwide decay include *Penicillium expansum* (blue mold), *Botrytis cinerea* (grey mold) and to lesser extent, *Mucor piriformis* (mucor rot) (Michailides and Spotts 1990; Sanderson and Spotts 1995; Rosenberger 1990).

1.1.1 *Penicillium expansum*

Penicillium expansum Link, or apple blue mold, is a filamentous Ascomycete that causes one of the most important North American post-harvest storage diseases of pome fruit. Before the introduction of controlled atmosphere (CA) storage and fungicides, it accounted for 90% of post-harvest apple diseases (Rosenberger 1990). *P. expansum* produces the mycotoxin, patulin, which has been found in apple product derivatives such as apple juice, ciders, puree, vinegar, baby food and whole apples (Abramson et al. 2009; Doores 1983; Piemontese et al. 2005; Watanabe and Shimizu 2005). Its detection and control is, therefore, of utmost importance for food health and safety.

Although an opportunistic wound pathogen, *P. expansum* infection can originate from stem-end invasions, core rots and through lenticels. Infected fruit are characterized by light to dark brown, fleshy, circular lesions that surround wounds. Older lesions may produce a bluish spore mass. *P. expansum* can also form a dense powdery mass at the centre of the lesion (Rosenberger 1990) and can be characterized by an earthy, musty odour commonly used as a diagnostic tool (Vikram et al. 2004).

Spores of *P. expansum* are ubiquitous and can cause infection within orchards and packinghouses. This fungus can survive in organic debris on the orchard floor and within soil. Conidia also exist in the air and on apple surfaces (Lennox et al. 2003). Within packinghouses, spores have been isolated from fungicide-drench solutions, flume water, dump-tank water, air and storage room walls (Sholberg and Stokes 2006). Conidia can survive from season to season on contaminated bins, picking boxes and storage walls (Rosenberger 1990).

Sanitation, harvesting pre-senescent fruit and handling methods are the best strategy for minimizing disease incidence. This control can also be facilitated by fungicides applied as pre-harvest sprays or post-harvest dips (Eckert and Ogawa 1988). For example, pyrimethanil was used as a pre-harvest fungicide and applied twenty days prior to apple harvest. After apples were stored for six months, post-harvest blue mold was significantly reduced when compared to apples that were not treated with fungicides (Sholberg et al. 2005b). Mertect®, with an active ingredient of thiabendazole (TBZ), is an example of a post-harvest fungicide application. It was once highly effective; however, prolonged exposure to such agents has led to TBZ-resistant fungal strains (Sholberg and Haag 1996) that render chemical controls ineffective.

1.1.2 *Botrytis cinerea*

Botrytis cinerea Pers.:Fr is an Ascomycete and, like *P. expansum*, is an important post-harvest pathogen of pome fruit causing minor rot throughout the growing season and significant rot within packinghouses. It is the most important post-harvest pathogen of pears and is second to blue mold in importance to apples (Rosenberger 1990). *B. cinerea* infection may originate from wounds, stem punctures, or the stem or calyx portion of the

fruit. Upon infection, *B. cinerea* inflicts a light to dark brown, spongy decayed area around the damaged portion of the fruit. Under high humidity, fluffy white to grey mycelium and spore masses may appear on the decayed area. The optimal growth temperatures for this pathogen range from 20°C to 22°C however, conidia are capable of growth at as low as -2°C (Coley-Smith et al. 1980).

B. cinerea spores are ubiquitous, colonizing available organic matter within orchards. Throughout the growing season, this fungus can cause dry eye rot. *B. cinerea* also can be carried into packinghouses via contaminated storage bins. Once inside, conidia are predominantly spread by air currents and water splash. This pathogen is also known as a nest or cluster rot as secondary infection can occur through fruit to fruit contact; fungi on infected fruit can colonize healthy fruit and spread disease (Rosenberger 1990).

1.1.3 *Mucor piriformis*

Mucor piriformis belongs to the phylum Zygomycota and, until recently, was thought to be of minor importance as a post-harvest pathogen. However, *M. piriformis* is capable of causing major decay problems in fruit such as strawberries, pome and stone fruit. *M. piriformis* is typically saprotrophic in orchard soil and infects fruit through the stem or calyx end and puncture wounds (Michailides and Spotts 1990). Upon infection, the area surrounding the lesion becomes soft, watery, light brown and easily separable from the fruit tissue. Often, grey mycelium with dark sporangia appears upon the decayed area. Mucor rot has a distinct sweet smell with a clear, sticky exudate. This fungus sporulates from -1 to 24°C, with optimal growth at 21°C. Fungicides that are currently registered to control other post-harvest pome fruit pathogens are ineffective

against *M. piriformis* and other Zygomycetes (Michailides and Spotts 1990). Although Scholar^{TM}, with active ingredient fludioxonil, has been shown to be effective against *M. piriformis* (P. Sholberg, personal communication), sanitation methods are often the most effective method for preventing infection (Michailides and Spotts 1990).

1.2 Post-harvest factors that influence post-harvest pathology

Because of the ubiquitous nature of fungal spores, their presence throughout the apple growing season can influence post-harvest pathology. *P. expansum, B. cinerea* and *M. piriformis* can colonize organic matter on the orchard floor and within soil (Lennox et al. 2003; Michailides and Spotts 1990). *P. expansum* and *B. cinerea* conidia can additionally exist in the air and on fruit surfaces (Lennox et al. 2003). Low precipitation, physiological crop condition and the use of pre-harvest fungicides will have a reducing effect on decay levels after harvest (Sholberg and Conway 2004; Sholberg et al. 2003; Sholberg et al. 2005b).

However, post-harvest factors can also influence disease incidence. Within packinghouses, spores can survive in fungicide-drench solutions, flume water, dump-tank water and in the air and on walls of storage rooms (Lennox et al. 2003; Sholberg and Stokes 2006). Therefore, sanitation affects disease accumulation. The post-harvest system offers a unique, closed or semi-closed environment where manipulating temperature and atmosphere within the storage room and the use of chemical senescence inhibitors like 1-methylcyclopropene (1-MCP) are often easier to manage in comparison to pre-harvest factors that can contribute to disease.

1.2.1 Temperature

The first experimental cold storage facility in British Columbia was built in Summerland in 1929 (AAFC 2002). Optimal refrigeration temperatures range from -0.5°C for Braeburn apples in South Africa to as high as 5°C for Belle de Boskoop apples grown in the Netherlands (Kupferman 2003). Lowered temperatures slow the rate of apple respiration, thus retarding ripening (AAFC 2002). Temperature management is also critical to post-harvest disease control. Post-harvest fruit pathogens grow optimally between 20 to 25°C. Some fungi have minimum growth temperatures as low as -2°C and cannot be completely controlled without freezing the fruit. Their growth at these temperatures, however, is significantly reduced which leads to a reduction in post-harvest decay levels (Sholberg and Conway 2004).

1.2.2 Controlled atmosphere

"Normal air" refers to atmospheres that consist of 78 – 79% N_2, 20 – 21% O_2, ~ 0.03% CO_2 and trace amounts of other gases. Controlled atmosphere (CA) refers to atmospheres that differ from "normal air" and are under strict control (Yahia 2009). Here, optimal temperatures persist while oxygen and carbon dioxide concentrations are decreased and increased, respectively (Morales et al. 2007). The concept of CA relies on the fact that harvested fruits use oxygen and produce carbon dioxide. If the amount of oxygen is limited, fruits will not ripen or will ripen slowly (Yahia 2009). Apples are often stored under CA as this environment has been shown to be effective in delaying the onset of storage diseases (Smock 1979). Commercial CA storage can range from 1% – 3% O_2 and 0.4% – 4.5% CO_2 (Kupferman 2003). Additional studies revealed that as high as 13% CO_2 prevented close to 100% spore germination of *P. expansum* after

twenty days (Cossentine et al. 2004). However, CO_2 levels this high are not commercially acceptable as it may lead to fruit injury resulting in a decrease in fruit quality.

1.2.3 1-Methylcyclopropene

1-Methylcyclopropene (1-MCP) is a synthetic cyclopropene that blocks ethylene receptors and prevents apple ripening (Blankenship and Dole 2003). It has the ability to maintain post-harvest fruit and vegetable quality and its use provides insight into the role of ethylene in fruit senescence (Watkins 2006). Ripening physiology and quality of apples in response to 1-MCP application has been intensively studied (Watkins 2006). Less studied, are the effects of 1-MCP on disease incidence. It is thought that by preventing the ethylene-associated ripening process, apples will be better able to resist pathogens (Watkins 2006). However, prevention of decay in 1-MCP-treated apples has been inconsistent. For example, Golden Delicious apples treated with 1-MCP and *P. expansum* showed decreased disease incidence (Saftner et al. 2003). Alternatively, disease severity increased in Golden Delicious apples inoculated with *P. expansum* or *Colletotrichum acutatum* and treated with 1-MCP (Janisiewicz et al. 2003; Leverentz et al. 2003). Although initially successful, there is the potential of certain decay problems associated with 1-MCP use (P. Sholberg, personal communication).

1.3 Methods of detection and identification

Traditional methods for fungal identification were primarily based on morphology or phylogenetic characteristics. However, such techniques have limitations as morphological characterizations rely on fungi to be isolated or cultured, resulting in an

underestimation of the microbial community of interest (Bridge and Spooner 2001; Mazzola 2004). The advancement of molecular biology has led to more specific and sensitive fungal DNA-based detection methods that replaced assumptions made in previous studies. For example, Cruickshank and Pitt (1987) used enzyme gel electrophoresis, or a zymogram, to differentiate between *Penicillium* species. This study hypothesized that like species will display like zymograms. Results mostly confirmed taxonomy based on morphology; however, taxonomic differences were perceived.

Detection techniques based on morphology or electrophoresis are time consuming. A rapid method of detection is therefore required. Greater specificity, sensitivity and speed can be attributed to the advancement of the polymerase chain reaction (PCR), a rapid and sensitive primer-mediated enzymatic amplification of target DNA sequences (Saiki et al. 1985). Common PCR-based identification methods include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or terminal restriction length polymorphisms (T-RFLP) (Mazzola 2004). Although PCR-based identification methods provide a more accurate picture of a microbial community, these methods are often limited by gel resolution or, more importantly, the inability to detect a large number of different pathogens simultaneously (Sholberg et al 2005a).

The advent of DNA macroarray technology circumvents this problem. This system utilizes labelled PCR products hybridized to a nylon membrane that contains an assortment of anchored oligonucleotides or probes. Due to a chemiluminescent reaction, pathogen DNA, for example, is captured on X-ray film and is represented as a pattern of pre-determined dots. The presence of a signal indicates the presence of a pathogen that

can be identified and quantified. Levesque et al. (1998) were among the first to utilize macroarray technology for plant pathogen identification. This method was further developed for detection of economically important pre-harvest (Sholberg et al. 2005a) and post-harvest diseases of pome fruit (Hirkala et al 2007). These technologies have great potential as they can be used as a high-throughput detection and diagnostic technique that can identify numerous microorganisms across disciplines and environments.

1.4 Methods of control

1.4.1 Chemical control

Fungicides are used to prevent post-harvest decay. In Canada, two synthetic post-harvest controls are currently registered, Mertect® and Scholar® with active ingredients thiabendazole (TBZ) and fludioxonil, respectively (PMRA 2007). Although initially effective, fungicides are now a major cause for concern. Pathogens have developed resistance to chemical controls (Errampalli et al. 2006; Sholberg et al. 2005c) which negates their effectiveness. There are also growing health concerns that surround pesticide use (Hancock et al. 2008) and a demand for environmentally friendly sustainable post-harvest practices. An alternative is therefore of high priority. One possibility is the implementation of biological controls.

1.4.2 Biological control

Biological control can be defined as "the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (Cook and Baker 1983). As cited in Cook and Baker (1983),

biological control dates back to the early 1920s in which Hartley (1921) attempted to control damping off in pine seedlings with fungal antagonists. In 1927, Millard and Taylor tried to control common potato scab by the addition of *Streptomyces praecox* and grass clippings to autoclaved potted soil. Both studies noted that competition for nutrients may be a putative mechanism of action (Cook and Baker 1983).

Sixty years later, the first commercial bacterial biological control agent (BCA), *Agrobacterium radiobacter* strain K84, was registered with the United States Environmental Protection Agency in 1979. It controlled crown gall caused by *Agrobacterium radiobacter* pv. *tumifaciens*. Strain K84 lacked the tumor-inducing (Ti) plasmid present within the pathogenic strain and produced a bacteriocin, which inhibits the growth of certain tumorigenic-strains, and to which K84 was insensitive (Cook and Baker 1983).

Although the beginnings of biological control of plant pathogens date back to the early 1920s, practicing biological control within a post-harvest setting has been much less common. Currently, there are three BCAs registered for post-harvest use (Droby et al. 2009). The first and second, developed by JET Harvest Solutions (Longwood, FL), are Bio-Save 10LP and 11LP (Longwood, FL) with active ingredients of *Pseudomonas syringae* strain ESC-10 and ESC-11, respectively. Bio-Save 10LP targets post-harvest blue and grey mold and mucor rot in pome fruit while Bio-Save 11LP targets Rhizopus soft rot on sweet potatoes. The third, Shemer (*Metschnikowia fructicola*) is commercially used in Israel for prevention of sweet potato and carrot storage decay. Two yeast-based products, Aspire^{TM} and YieldPlus, are no longer available (Droby 2009).

Biological control of post-harvest pathogens of pome fruit using bacterial and

yeast antagonists has been studied by numerous research groups (Calvo et al. 2007; Etebarian et al. 2005; Janisiewicz 1988; Mikani et al. 2008; Morales et al. 2008; Nunes et al. 2002; Zhou et al. 2001). For example, *Candida sake* strain CPA-1 significantly decreased *P. expansum* lesion size by > 80% and reduced disease incidence by 50% (Teixidó et al. 1999). *Cryptococcus laurentii* and *Metschnikowia pulcherrima* also provided control against *P. expansum* (Conway et al. 2007). The epiphytic bacterium, *Rahnella aquatilis*, significantly inhibited *P. expansum* and *B. cinerea* on apples at 15°C and 4°C, respectively (Calvo et al. 2007). A saprophytic strain of *Pantoea agglomerans* also reduced *P. expansum* rot on Golden Delicious apples at 1°C in air and at 1°C in a low oxygen atmosphere by 81% and 100%, respectively (Nunes et al. 2002). A study conducted by Etebarian et al. (2005) showed that *Pseudomonas fluorescens* isolate 1100-6 significantly reduced the incidence of *P. expansum* and *P. solitium* on apples after 11 days at 20°C and 25 days at 5°C. In Canada, there are an increasing number of registrations and products under evaluation that are based upon microbially-active substances (Bailey et al. 2010). However, there are none registered for post-harvest use (Droby et al. 2009).

A demand therefore exists for the production and commercialization of a BCA for post-harvest use on pome fruit. A collection of rhizobacteria, isolated from the roots of legumes grown in Saskatchewan soils, were compiled and initially characterized for plant growth promoting traits such as the production of siderophores, ACC deaminase, root elongation and the suppression of legume fungal pathogens (Hynes et al. 2008). Fifteen of these isolates were further tested *in vitro* for suppression of *P. expansum*, *B. cinerea* and *M. piriformis* (Stokes et al. 2006). Nine isolates provided fungal control and were

then tested *in situ*. Four isolates provided consistent control at both 20ºC and 1ºC; isolates 1-112, 2-28, 4-6 and 6-25 (Hirkala, unpublished data) in combination with 1100-6 were selected for further study.

1.5 Mechanisms of action

The interaction between host, antagonist and pathogen is a complex, ecological relationship with high variability depending on the system of focus. The ability of an antagonist to suppress a pathogen may be due to more than one mechanism of action (MOA). A sound mechanistic understanding is essential for formulation development and biocontrol registration (Spadaro and Gullino 2004). Possible mechanisms include antibiosis (Janisiewicz et al. 1991; Kamesnky et al. 2003; Meziane et al. 2006), competition for nutrients (Elad 1996), parasitism (Frankowski et al. 2001; Watanabe et al. 2007) and induction of pathogen resistance in host tissue (Benhamou et al. 2000; Spadaro and Guillino 2004; Terry and Joyce 2004).

1.5.1 Antibiosis

One method of antagonistic action may be through the production of antibiotics. For example, pyrrolnitrin produced by strains of *Serratia plymuthica*, was associated with fungal suppression (Kamesnky et al. 2003; Meziane et al. 2006). To determine whether or not pyrrolnitrin assisted in antifungal activity, Meziane et al. (2006) created an *S. plymuthica* mutant that lacked the gene responsible for pyrrolnitrin production. The pyrrolnitrin-deficient mutant, IC1270-P1, lost its antifungal activity when tested against pathogens *Penicillium digitatum* and *Penicillium italicum*. In addition, purified pyrrolnitrin was effective in suppressing disease symptoms of *P. digitatum* and *P.*

italicum. Janisiewicz et al. (1991) also isolated pyrrolnitrin from *Pseudomonas cepacia* for control of *B. cinerea* and *P. expansum* on apples and pears. Pyrrolnitrin was found to provide control; however, the level of control varied due to wound type (cut, nail, bruise), pyrrolnitrin concentration and storage temperature (2ºC and 24ºC). A post-harvest dip solution of pyrrolnitrin may therefore be an effective solution against post-harvest pathogens. It should also be noted that the post-harvest fungicide, Scholar^{TM}, has an active ingredient of fludioxonil which belongs to the chemical class of phenylpyrroles. Phenylpyrroles are derived from pyrrolnitrin and are considered a reduced-risk chemical control agent (Errampalli 2004).

1.5.2 Competition for nutrients

Competition for nutrients has been widely studied due to nutritional demands of both antagonists and pathogens. *B. cinerea*, as with most necrotrophs, requires nutrients for germination and the initiation of the pathogenic process. In the absence of nutrients, *B. cinerea* becomes highly susceptible to degradation and control (Elad 1996; Janisiewicz et al. 2000). It is postulated that microorganisms capable of efficiently utilizing nutrients will make successful biological control agents (Elad 1996). Janisiewicz et al. (2000) proposed a simple way to study competition for nutrients that uses a 24-well tissue culture plate with cylindrical insets with 0.45 μm membranes attached to the bottom. Individual cylinders are placed in each of the 24-wells thereby permitting media nutrient and metabolite interchange while preventing pathogen and antagonist contact due to physical separation. In this system, competition for nutrients can be studied independently from competition for space. Bencheqroun et al. (2007) used this method to determine, *in vitro*, that the biocontrol antagonist, *Aureobasidium pullulans*, was

competing for nutrients, especially the amino acids glycine, glutamic acid and serine, with the pathogen, *Penicillium expansum*.

1.5.3 Parasitism

Antagonists can directly parasitize pathogens of post-harvest apples. One such method is through the production of lytic or cell wall degrading enzymes such as chitinase and β-1,3-glucanase. Bacterial strains that produce lytic enzymes will most likely have antifungal properties via cell wall hydrolysis. For example, *S. plymuthica* strain HRO-C48 was isolated from the rhizosphere of oilseed rape and shown to have antifungal properties associated with chitinase production. Frankowski et al. (2001) isolated and characterized two chitinolytic enzymes, one endochitinase (E.C. 3.2.1.14), CHIT60, and one *N*-acetyl-β-1,4-D-hexosaminidase (E.C. 3.2.1.52), CHIT100. *In vitro*, CHIT60 and CHIT100 showed direct inhibitory activity on spore germination and germ tube growth of *B. cinerea*. Alternate parasitic activity may involve direct attachment of antagonist to pathogenic fungal hyphae (Chan and Tian 2005; Watanabe et al. 2007).

1.5.4 Induced systemic resistance

Induced systemic resistance (ISR) is an observed phenomenon in which microorganisms activate host plant mechanisms that enhance their defensive capacity against potential pathogenic invasions. Protection can occur both locally and on areas of the plant that did not come into contact with inducing microorganisms (van Loon 2007). For example, Benhamou et al. (2000) determined that S. *plymuthica* strain R1G64 mediated induced systemic resistance in cucumber to protect against infection caused by *Pythium ultimum*. Upon pre-treatment with *S. plymuthica*, *P. ultimum* disease incidence

was reduced; cucumber root cells were structurally and biochemically modified with the formation of phenolic-enriched occluded depositions and structural barriers. These structural barriers were hypothesized to prevent pathogen movement towards the vascular stele. In ISR experiments, it is important to spatially separate inducing microorganisms and challenging pathogens (ie. root and leaf) to ensure that protection is plant- not microorganism-mediated (van Loon 2007).

1.6 Green fluorescent protein

Green fluorescent protein (GFP) is made up of 238 amino acids and exhibits green fluorescence at a peak emission of 509 nm by absorbing blue light maximally at 395 nm and minimally at 470 nm (Morin and Hastings 1971). In its native form, GFP was first discovered during the purification of the bioluminescent protein, aequorin, isolated from jellyfish (*Aequorea victoria*) (Shimomura et al. 1962). In *A. victoria*, green fluorescence occurs by an intermolecular energy transfer between aequorin and GFP; Ca^{2+} and aequorin interact and emit blue light, some of which is absorbed by GFP causing a color shift towards green (Morise et al. 1974). However, it wasn't until 1992 that the influence of GFP was realized. Prasher et al. (1992) paved the way for future GFP application by cloning and sequencing both cDNA and genomic clones from *Aequorea victoria*. In 1994, Chalfie et al. first expressed GFP within a living organism, highlighting sensory neurons in nematodes. These landmark studies influenced modern science as purified GFP has become one of the most important reporter genes in biology (Bloemberg 2007).

GFP is comprised of an eleven-stranded β barrel with a coaxial helix running through the centre. The chromophore is formed from the spontaneous cyclization of the

central helix consisting of the tripeptide, Ser65-Try66-Gly67 (Ormo et al. 1996). Introducing random amino acid substitutions into the twenty amino acids that flank the chromophore has led to altered forms of GFP some of which exhibit greater fluorescence. The first GFP modification was derived from a point mutation that altered Serine 65 to Threonine (S65T) (Heim et al.1995). This alteration shifted the excitation and emission maxima to 490 and 510 nm, respectively. Alterations of GFP not only affected intensity, but also increased color variety that is within the blue, cyan and yellow regions of the electromagnetic spectrum (Shaner et al. 2007).

Green fluorescent protein can be used as a reporter or tag to mark whole cells, study protein localization and monitor gene interactions and interactions between microorganisms. Its incorporation is non-invasive, it does not require an additional substrate for bioluminescence, nor does visualization necessitate fixation or staining protocols (Bloemberg 2007). The gene product of *gfp* can be expressed in both prokaryotes and eukaryotes (Chalfie et al. 1994) with no background gene expression (Tombolini et al. 1997). It is highly stable in the presence of denaturants and proteases and persists at high temperatures (65°C) and a wide range of pH values (6-12) (Bloemberg 2007; Chalfie et al. 1994; Ward et al. 1980).

Applications of GFP in biological control experiments can help elucidate mechanisms of action (MOA). Watanabe et al. (2007) transformed two fungal species with *gfp*, *Trichoderma asperellum* SKT-1, the antagonist, and *Gibberella fujikuroi* N-68, a pathogenic root fungus responsible for Bakanae disease of rice seedlings. The fluorescent protein was imaged by confocal scanning laser microscopy (CSLM) and mycoparasitism was suggested as the putative MOA. Scanning electron microscopy

(SEM) and CSLM showed a disappearance of GFP in *G. fujikuroi* upon contact with *T. asperellum* suggesting cell wall degradation.

In another example, Bolwerk et al. (2003) transformed red fluorescent protein (RFP) into two putative BCAs, *Pseudomonas fluorescens* WCS365 and *Pseudomonas chlororaphis* PCL1391, that have been shown to control *Fusarium oxysporum* f. sp. *radicis-lycopersici,* a causal agent of tomato foot and root rot (TFRR). Both *Pseudomonas* spp. contained RFP, whereas *Fusarium oxysporum* f. sp. *radicis-lycopersici* harboured GFP. CSLM revealed that both antagonists colonized the tomato root more quickly than the fungal pathogen and that the bacterial presence hindered root infection. A proposed MOA was that the presence of fungi initiated bacterial colonization of hyphae and subsequent production of fungal secondary metabolites.

1.7 Objectives

This thesis will build upon preliminary research and address the following three objectives: 1) validate the use of DNA macroarrays under field conditions; 2) assess bacterial antagonist performance under semi-commercial and commercial conditions and 3) determine how long and in what capacity a selected antagonist will colonize the fruit surface. I hypothesize that:

1) Macroarray technology will accurately identify and quantify post-harvest apple pathogens throughout the growing season and that pathogen prevalence will correlate with disease incidence post-harvest.
2) One or more of the bacterial antagonists will provide significant pathogen control in semi-commercial and commercial storage conditions.

3) The use of green fluorescent protein will facilitate visualization of bacterial colonization and survival on apple surfaces.

CHAPTER 2: MATERIALS AND METHODS

2.1 Antagonists

Four bacterial antagonists were obtained from Dr. L. Nelson and were identified by 16S rRNA sequence and fatty acid methyl ester analysis as *Pseudomonas fluorescens* (isolates 1-112, 2-28, 4-6) and *Serratia plymuthica* (isolate 6-25) (Hynes et al. 2008). Isolate 1100-6, *Pseudomonas fluorescens* (Etebarian et al. 2005), was provided by Dr. Peter Sholberg of the Pacific Agri-Food Research Centre (PARC) in Summerland, BC. The bacteria were grown in half-strength tryptic soy broth (½ TSB) (Becton, Dickinson and Company, Sparks, MD) at 28°C with shaking at 220 rpm. The final culture was centrifuged for 20 min at 3800 g and held at 20°C. Cell pellets were resuspended in sterile phosphate buffered saline solution (PBS) (1.2 g/L Na_2HPO_4, 0.18 g/L NaH_2PO_4, 8.5 g/L NaCl). The optical density (OD_{600}) was determined and the bacterial cells were diluted to 10^8 CFU/ml according to a standard curve relating OD_{600} to CFU/ml.

2.2 Pathogens

Penicillium expansum Link strain 1790, *Botrytis cinerea* Pers.:Fr strain B27 and *Mucor piriformis* Fischer strain 536 were provided by Dr. Peter Sholberg and grown on half-strength potato dextrose agar (½ PDA) (HiMedia Laboratories PVT. Ltd., India) for 7 days at 22°C. A spore suspension was created using sterile water and Tween 20 (MP Biomedicals, LLC, Solon, OH) and then enumerated using a Petroff-Hauser counter.

2.3 Orchard field sites

This study incorporated four conventional (non-organic) orchard field sites from which data were collected over two years. Two research orchards were located at PARC in Summerland, BC. Field 3 (49° 33' 58.49" N; 119° 38' 41.93" W) contained 166 Jonagold apple trees planted at high density. Field 12 (49° 33' 54.81" N; 119° 38' 56.73" W) contained 120 Gala apple trees planted at high density. The two commercial orchards were located in Kelowna, BC. The Kiran orchard (49° 50' 41.45" N; 119° 24' 54.24" W) grew Gala apples planted at high density. The Reekie orchard (49° 50' 58.78" N; 119° 23' 32.79" W) grew Red Delicious apples planted at medium density.

I-rods were used in this study to collect aerial spore samples. I-rods are clear polystyrene rods that rest within a sampling head of a rotating spore trap. When the motor spins, centrifugal force causes the I-rods coated in silicone grease to extend downwards at a 90^{o} angle and collect airborne particles (Aerobiology Research Laboratories, Nepean, ON). In the 2007 and 2008 growing seasons, Fields 3 and 12 contained one I-rod station per field. The Kiran orchard contained one I-rod station in both 2007 and 2008 and the Reekie orchard contained one I-rod station in 2008 only. Average daily temperatures (°C) and total daily precipitation (mm) from Environment Canada (2008) were plotted and used to compare with pathogen DNA detected throughout the apple growing seasons (Figure 1). Fungicide spray records for Fields 3 and 12 were provided. On May 15, 2007, the fungicide, Nova (myclobutanil) was applied to Field 12. On September 15, 2008, the fungicide Funginex (triforine) was applied to Field 3.

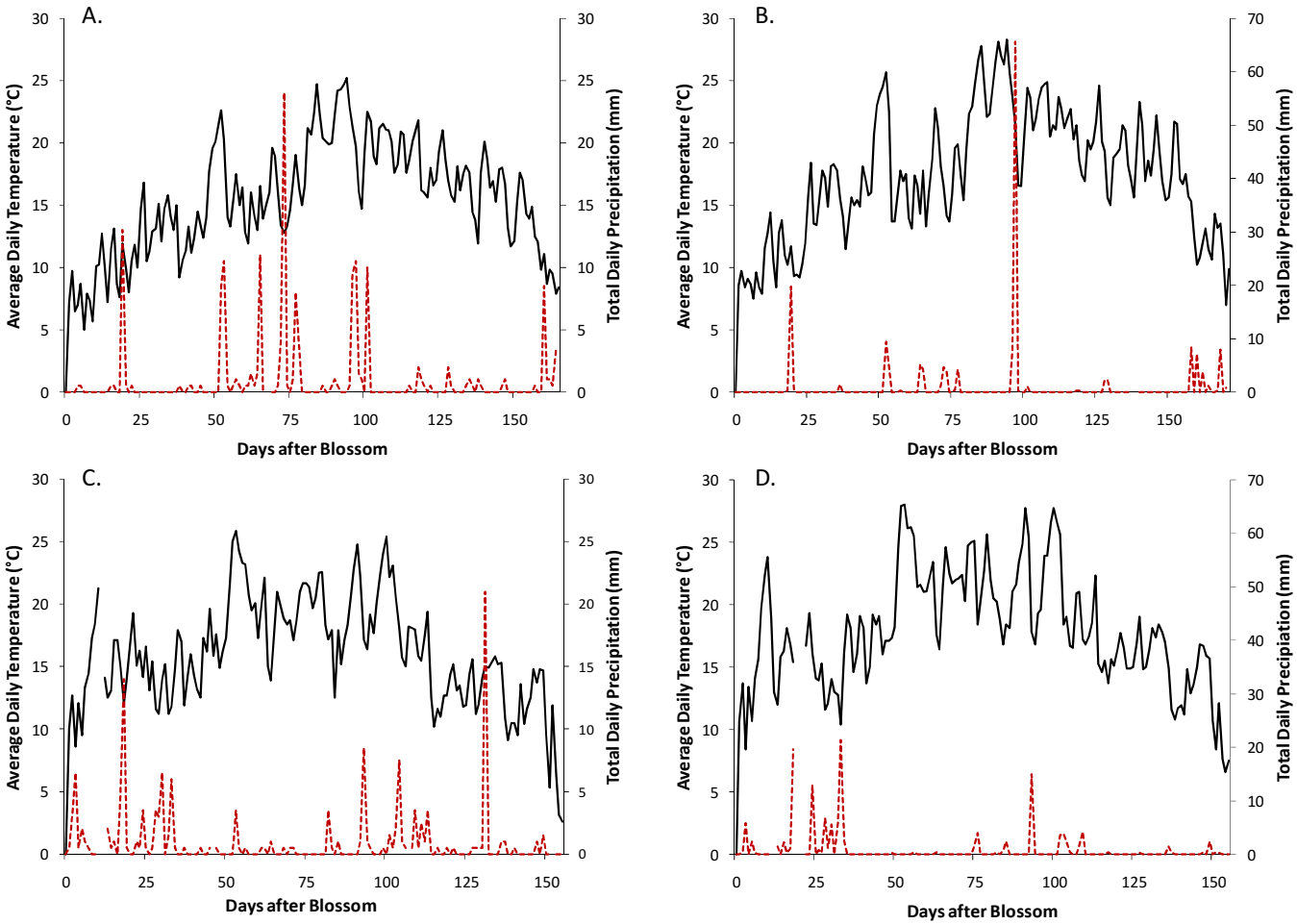

Figure 1. Average daily temperatures (°C) (solid line) and total daily precipitation (mm) (dashed line) at Kelowna, BC during the growing seasons of 2007 (A) and 2008 (B) and in Summerland, BC during the growing seasons of 2007 (C) and 2008 (D) as indicated by Environment Canada (2008).

2.4 pAG408

The *gfp*-containing plasmid, pAG408 (Suarez et al. 1997), was provided by Dr. Darren Korber, University of Saskatchewan, Saskatoon, Sask. pAG408 was maintained within the donor strain, *E. coli* S-17 λ pir, on Luria Burtani (LB) agar (10 g/ L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 50 µg/ml of kanamycin (kan) and 30 µg/ml of gentamicin (gen).

To test for antibiotic efficacy, pAG408 in *E. coli* S-17 λ pir was streaked onto three replicate plates each of LB agar, LB agar + 50 µg/ml kan, LB agar + 30 µg/ml gen and LB agar + 50 µg/ml of kan and 30 µg/ml of gen and grown at 28°C for 48 h. Furthermore, pAG408 plasmid confirmation was verified by extracting it from *E. coli* S-17 λ pir using the Wizard ® Plus Minipreps DNA Purification System (Promega, Madison, WI) as per manufacturer's instructions, and digesting 1 µg of DNA with 1 U *Hind*III (Invitrogen, Carlsbad, CA) for 1 h at 37°C.

2.5 Construction of the *gfp*-transconjugant

Bacterial isolate 4-6 was transformed with pAG408 via bacterial conjugation. Donor cells (*E. coli* S-17 λ pir containing pAG408) and recipient cells (4-6) were grown overnight at 28°C with shaking at 220 rpm. One millilitre of each culture was centrifuged at 10 000 x g for 20 min at 4°C. The supernatants were removed, the pellets washed twice with PBS then resuspended in 50 µl of PBS. Fifty microlitres each of the donors and recipients were combined and vortexed. The combined cultures were spotted on 0.22 µm sterile nylon membrane filters (Whatman, Maidstone, England) placed on LB agar plates supplemented with 50 µg/ml of kan and 30 µg/ml of gen. The plates were

incubated for 24 h at 28°C to allow for bacterial conjugation. The bacterial mixtures were then removed from the filters and plated on sodium citrate agar plates (0.2 g/L $MgS0_4$, 1.0 g/L $NH_4H_2PO_4$, 2.0 g/L sodium citrate, 5.0 g/L NaCl, 15 g/L agar) supplemented with 50 µg/ml of kan and 30 µg/ml of gen and then incubated for 48 h at 28°C. The combination of sodium citrate growth medium and antibiotics eliminated *E. coli* S-17 λ pir pAG408 cells and non-transformed bacterial cells; *E. coli* cannot use citrate as a carbon source and the wild-type biological control isolates cannot withstand the antibiotics. For a negative control, non-transformed *Pseudomonas fluorescens* isolate 1-112, *Serratia plymuthica* isolate 6-25 and *E. coli* S-17 λ pir containing pAG408 were spread on sodium citrate agar plates supplemented with 50 µg/ml of kan and 30 µg/ml of gen.

To confirm successful conjugation, 10 putative transconjugants were selected at random and their genomic DNA was extracted by boiling. One colony was placed in 100 µl of sterile water and the supernatant was used as the DNA template for the PCR that utilized *gfp*-specific primers. Three microlitres of DNA were used in the 25-µl reaction containing 1x PCR Buffer (TetraLink International, Buffalo, NY), 2.5 mM $MgCl_2$, 0.1 mM dNTP mix (Invitrogen, Carlsbad, CA) , 0.4 µM of each forward (*gfp* F: 5'-GAGTAAAGGAGAAGAACTTTTCA-3') and reverse primers (*gfp* R: 5'-TTATTTGTATAGTTCATCCATG-3') (Suarez et al. 1997) and 1 U of UltraTherm DNA polymerase (TetraLink International, Buffalo, NY). Amplification was performed on the Techne TC-3000 thermocycler (Barloworld Scientific, London, England) with the following conditions: 96°C for 5 min followed by 35 cycles of 95°C for 1 min, 54.8°C for 1 min and 72°C for 1 min with a final extension cycle at 72°C for 7 min.

2.6 Plasmid stability

Testing of the stability of exogenous DNA within bacterial isolate 4-6 was conducted according to Bloemberg et al. (1997) except that tryptic soy medium and the antibiotics, kanamycin and gentamicin, were used. Based on replicated stability results and the 2007 biocontrol experimental results, the promising *gfp*-tranconjgant biological control strain, *P. fluorescens* isolate 4-6, was selected as a suitable antagonist for bacterial survival and colonization studies.

2.7 *P. fluorescens* 4-6-*gfp* fitness analysis

Growth rates of 4-6-*gfp* and its wild-type counterpart (4-6 wild-type) were compared. Three replicates each of 4-6-*gfp* and 4-6 wild-type were grown in 10 ml of ½ TSB and incubated at 28°C with shaking at 220 rpm for 24 h. Cultures were spun for 20 min at 5000 x g and resuspended in 10 ml of phosphate buffered saline (PBS). Optical density (OD_{600}) was determined and cultures were diluted and standardized with PBS to within 0.100 of each other. One hundred microlitres of standardized culture were added to 10 ml of ½ TSB. OD_{600} readings were recorded at 0, 2, 4, 6, 8, 10, 12 and 24 h. The three replicates were averaged and represented graphically (time vs OD_{660}). Growth rates (μ) were calculated with the formula $\mu = (\log_{10} N - \log_{10} N_o) \times 2.303 / t - t_o)$ where N was a final cell number, N_o was an initial cell number, t was the time at N and t_o was the time at N_o.

Carbon utilization profiles of 4-6-wild-type and 4-6-*gfp* were also compared by determining carbon profiles using BioLog Phenotype MicroArrays for Microbial Cells

(Biolog, Inc., Hayward, CA) (microplate PM1 and PM2) as per the manufacturer's instructions

2.8 Spore, apple tissue collection and fruit washings

Apple leaves, blossoms and I-rods were collected at orchard field sites throughout the apple growing seasons (Table 1). In general, sampling was conducted three times per week at the beginning (early) and end (late) of the growing seasons in 2007 and 2008. Throughout the mid-growing seasons, samples were collected approximately once per week. At harvest, approximately 1250 apples were collected at random from each of Fields 3 and 12 and approximately 300 apples were collected at random from the Kiran and Reekie orchards.

Fruit surface washings were conducted on 20 apples each from Fields 3 and 12 in 2007 and 20 apples each from Fields 3, 12, the Kiran and the Reekie orchards in 2008. Twenty apples from each orchard were washed in a 600-ml beaker containing 200 ml of sterile distilled water and 200 µl of Tween 80. Each fruit was shaken for 5 min at 120 rpm and sonicated for 5 min (P. Sholberg, personal communication). After all fruit were washed, the solution was centrifuged at 13 400 g for 10 min. The supernatant was decanted and this process was repeated five times to a final volume of 10 ml that was centrifuged for 15 min at 7430 g. The supernatant was decanted and the remaining cells were transferred to a 2-ml tube and centrifuged at max (16 100 g) for 1 min. The supernatant was removed and 100 µl of water were added for storage at -20°C.

Table 1. Frequency of aerial and plant tissue sampling for Fields 3, 12 and the Kiran and Reekie orchards in the early, middle and late growing seasons of 2007 and 2008.

		Number of samples within sampling period (n)			
Field	**Sample type**	**Early**	**Mid**	**Late**	**Total**
2007 Growing Season					
Field 3	aerial	14	6	6	26
	plant tissue	2	3	2	7
Field 12	aerial	16	6	5	27
	plant tissue	2	3	2	7
Kiran	aerial	9	7	6	22
	plant tissue	4	7	5	16
Reekie	aerial	0	0	0	0
	plant tissue	6	8	4	18
2007 Total		**53**	**40**	**30**	**123**
2008 Growing Season					
Field 3	aerial	18	11	3	32
	plant tissue	4	0	2	6
Field 12	aerial	19	11	3	33
	plant tissue	4	0	2	6
Kiran	aerial	17	24	11	52
	plant tissue	18	24	10	52
Reekie	aerial	15	20	11	46
	plant tissue	16	24	11	51
2008 Total		**111**	**114**	**53**	**278**

2.9 Validation and field testing of DNA macroarrays

2.9.1 DNA extraction

DNA was extracted from I-rods, plant tissue samples and fruit washings using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. For DNA isolation, two I-rods were used per aerial

extraction and 0.25 g of plant tissue were used for tissue extractions. DNA was eluted in 100 µl of Buffer 5 and stored at -20°C until required for further use.

2.9.2 PCR amplification

PCR amplification was used to amplify universally conserved regions of the fungal genome, ribosomal DNA interspacer regions (rDNA ITS) and the β-tubulin gene. Approximately 10 – 20 ng of extracted DNA were used in 25-µl reactions containing 1x PCR Buffer (TetraLink International, Buffalo, NY), 2.5 mM $MgCl_2$, 0.1 mM digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany) (DIG)-dNTP mix , 0.4 µM of each forward and reverse primers and 1 U of UltraTherm DNA polymerase (TetraLink International, Buffalo, NY). Primers for rDNA were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3') (White et al. 1990) and β-tubulin primers were Bt-LEV-Lo1 (Fwd 5'-GTGAACTCCATCTCGTCCATA-3') and Bt-LEV-Up4 (5'-CAAGATCCGTGAGGAGT-3') (de Jong et al. 2001). Amplification was performed on the Techne TC-3000 thermocycler (Barloworld Scientific, London, England) with the following conditions: 96°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final extension cycle at 72°C for 7 min. DNA concentrations were estimated on 1% agarose gels using a Low Mass ladder (Invitrogen, Gaithersburg, MD). A minimum of 75 ng and a maximum of 100 ng of PCR-amplified DNA were required for hybridization to macroarrays.

2.9.3 DNA macroarray construction

Twenty-eight oligonucleotide macroarray probes were designed by Dr. Danielle Hirkala and constructed by Operon Technologies, Huntsville, AL (Table 2). The 5' end of the probe contained an amino C6 linker used to attach the oligonucleotide onto the Immunodyne ABC nylon membranes (PALL Europe Ltd., Portsmouth, England). The probes were resuspended in 0.5 M sodium bicarbonate buffer (pH 8.0) and diluted to a final concentration of 40 µm. In combination with 0.004% bromophenol blue, the resuspended probes were arranged according to a previously designed template (Figure 2). A 384-pin replicater (V&P Scientific, San Diego, CA) and pin guide (V&P Scientific, San Diego, CA) were used to "stamp" the probes onto the nylon membranes (Figure 2). The membranes were blocked for a minimum of 1 hour with hybridization buffer (6x SSC, 0.1% sarcosine, 0.02% SDS) and 1% skim milk at 55°C.

2.9.4 Hybridization and chemiluminescent detection

To denature the DIG-labelled PCR products, 75 – 100 ng of DNA were combined with 5 ml of hybridization buffer and placed in boiling water for 10 min. Membranes were added to the denatured probe solution for overnight hybridization at 55°C. Unbound probes were then washed from the membranes with pre-warmed 6x SSC (0.18% NaCl, 0.088% sodium citrate) and 1% SDS (sodium dodecyl sulphate) for 2 x 40 min at 55°C. Hybridized membranes were pooled and washed for 5 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl [pH 7.5], 0.3% Tween 20) and bound with a 1:25 000 dilution of anti-Digoxigenin-AP (alkaline phosphatase) Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) combined with washing buffer and 1% skim

Table 2. DNA macroarray probe sequences.

Name	Sequence 5' to 3'	Description
	rDNA-ITS	
UN-H1-up	[AminoC6]ACCTACGGAAACCTTGTTACGA	Universal to all fungi
Bot-all-H1b	[AminoC6]TTAGCTTGGTATTGAGTCTATGT	Universal to all *Botrytis* spp.
BC-H2d	[AminoC6]TATGCTCGCCAGAGAATACCAAA	*Botrytis cinerea*
BC-H3d	[AminoC6]GCTCGCCAGAGAATACCAAAAC	*Botrytis cinerea*
BT-H1d	[AminoC6]TATGCTCGCCAGAGAAAACCAAA	*Botrytis tulipae*
BT-H2d	[AminoC6]CTCGCCAGAGAAAACCAAAACT	*Botrytis tulipae*
PE-H2c	[AminoC6]CCCGAACTCTGCCTGAAGATT	*Penicillium expansum*
PE-H3u	[AminoC6]CAGACGACAATCTTCAGGCA	*Penicillium expansum*
Mpir-ITS-183H1	[AminoC6]TGGTGTCCTTAAAAATTATTATTAT	*Mucor piriformis*
Mpir-ITS-414H1	[AminoC6]AACACCCCACATCTTAAAAATC	*Mucor piriformis*
	β-tubulin	
UNbt-1	[AminoC6]CAAGAACATGATGGCTGCTTC	Universal to all fungi
UNbt-2	[AminoC6]CCAAGAACATGATGGCTGC	Universal to all fungi
UNbt-3	[AminoC6]TGTTCGACCCCAAGAACATG	Universal to all fungi
Pex-bt-84-H1-gag	[AminoC6]TCCGACGAGACTTTCTGTATC	*Penicillium expansum*
Pex-bt-84-H1-gcg	[AminoC6]TCCGACGCGACTTTCTGTATC	*Penicillium expansum* TBZ sensitive
Pex-bt-84-H1-gtg	[AminoC6]TCCGACGTGACTTTCTGTATC	*Penicillium expansum* TBZ resistant
Pcom-bt-1	[AminoC6]CCGTCAACATGGTCCCCTT	*Penicillium commune*
Psol-bt-1	[AminoC6]TCCCTCGTTTGCACTTCTT	*Penicillium solitum*
Psol-bt-2	[AminoC6]CCTTTCCGTCCGTCCACCAGCT	*Penicillium solitum*
Paur-bt-1	[AminoC6]CACACCTCTGATATCTTGCTAGG	*Penicillium aureum*
Paur-bt-2	[AminoC6]CGATGGACAGTAAGTTCTAATGG	*Penicillium aurantigriseum*
Bcin-133-H3	[AminoC6]TTACGATATTTGCATGAGAACCT	*Botrytis cinerea*
Bstok-144-H4	[AminoC6]GCATGAGAACCCTGAAGCTC	*Botrytis mali*
Bot-95-H1-GAG	[AminoC6]AACTCTGACGAGACCTTCTG	*Botrytis cinerea* TBZ sensitive
Bot-95-H1-GCG	[AminoC6]ACTCTGACGCGACCTTCTG	*Botrytis cinerea* TBZ resistant
MucorUN-bt-1	[AminoC6]ACATGGTTCCTTTCCCTCGT	Universal to all *Mucor* spp.
MucorUN-bt-2	[AminoC6]AAGGCTTTCTTGCATTGGTA	Universal to all *Mucor* spp.
MucorUN-bt-3	[AminoC6]GGTGCTGGTAACTCTTGGGC	Universal to all *Mucor* spp.

A.

Post-Harvest Macrorray Template								
	rDNA-ITS			β-tubulin				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	UN-H1-up	BT-H1d	Mpir-ITS-183H1	UNbt-1	Pex-bt-84-H1gag	Psol-bt-1	Bcin-133-H3	MucorUN-bt-1
2	Bot-all-H1-b	BT-H2d	Mpir-ITS-414H1	UNbt-2	Pex-bt-84-H1gcg	Psol-bt-2	Bstok-144-H4	MucorUN-bt-2
3	BC-H2d	PE-H2c		UNbt-3	Pex-bt-84-H1gtg	Paur-bt-1	Bot-95-H1-gag	MucorUN-bt-3
4	BC-H3d	PE-H3u			Pcom-bt-1	Paur-bt-2	Bot-95-H1-gcg	

B.

Post-Harvest Macrorray Template								
	rDNA-ITS			β-tubulin				
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	● ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●
2	● ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●
3	● ●	● ●		● ●	● ●	● ●	● ●	● ●
4	● ●	● ●			● ●	● ●	● ●	

Figure 2. DNA macroarray pathogen template (A) and the corresponding oligonucleotide probe placements that are amine-linked to the nylon membrane (B). Columns A – C detect rDNA ITS PCR products; columns D – H detect β-tubulin PCR products. Each pathogen (A) corresponds with the dotted template (B) as it would appear on a developed macroarray. Each probe is blotted twice for positive confirmation. Grey boxes indicate no probe present.

milk powder for 30 min. Membranes were washed for 2 x 15 min then primed for 15 min in buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]).

For chemiluminescent detection, membranes were incubated for 5 min in buffer 3 plus 1:2000 dilution of chemiluminescent substrate, CDP-Star (Roche Diagnostics GmbH, Mannheim, Germany). Alkaline phosphatase enzymatically dephosphorylates CDP-Star, creating dioxetane phenolate anion, which decomposes and emits light maximally at 466 nm (Roche Diagnostics GmbH, Mannheim, Germany).

Membranes were bound in plastic wrap and immediately exposed to x-ray film (CL-X Posure ^{TM} Film, Thermo Scientific, Rockford, IL) for 2 h. After fixing and developing for 1 minute each (Kodak, Rochester, NY), the hybridized DIG-amplified PCR products were captured on x-ray film as a series of dots with varying grey intensities. The x-ray film was scanned and saved as a Jpeg picture file using a BenQ 4300u scanner (Taipei, Taiwan) for computer analysis. The hybridized DNA was quantified using ImageJ 1.30v software (National Institutes of Health, Bethesda, MD).

Each hybridization signal was assigned a relative greyscale value that ranged from 0 (completely back) to 255 (completely white). Greyscale values were in the form of two averaged measurements. The background was subtracted from each analyzed array using the following formula:

$$\textbf{Adjusted Greyscale Value} = \frac{127.5 \times \text{Original Greyscale Value}}{\text{Average Background}}$$

The adjusted greyscale values were transformed to DNA concentrations when compared to a standard curve produced from known DNA concentrations.

2.10 Biological control of post-harvest fungal pathogens

2.10.1 Inoculation of apples by drenching

Bacterial cultures were diluted in 5 L of water to a concentration of 10^8 CFU/ml and fungal cultures were diluted in 5 L of water to concentrations of 10^4 spores/ml for *P. expansum* and *B. cinerea* and 10^5 spores/ml for *M. piriformis.* Inoculation concentrations were previously determined by minimum infectious dose and minimum inhibitory concentration experiments (D. Hirkala, personal communication). Apples were surface sterilised with 90% ethanol and wounded in triplicate using a 3-mm diameter nail embedded in a cork. Wounded apples were placed in a plastic net bag and labelled by tagging. Each bag was placed in a 5-L bacterial suspension for 1 min then subsequently placed in a 5-L fungal suspension for 1 min. Following inoculation, apples were air dried on trays for 10 min then placed in mesh bags (10 apples / bag) and in crates (~100 apples / crate) for commercial storage or in covered plastic bins each with three 0.2-µm membrane filters attached to the lid for semi-commercial storage until lesions formed. In this study, commercial storage refers to controlled atmosphere (CA) storage conditions in a commercial packinghouse, whereas semi-commercial storage refers to storage in a research facility at 1°C in air.

2.10.2 Natural disease incidence

Following apple harvest in 2007, natural disease incidence (NDI) was determined by wounding 100 Jonagold apples from Field 3 and 100 Gala apples from Field 12. Fifty of the 100 apples were fumigated with 1-Methylcyclopropene (1-MCP) and 50 were not fumigated with 1-MCP. In 2008, NDI was determined by placing 260 apples, each from Fields 3 (Jonagold), 12, Kiran (Gala) and Reekie (Red Delicious) into controlled

atmosphere (CA) storage (1.5% O_2 and 1.5% CO_2) (n = 100 per orchard) in Naramata, BC or in air storage at 1°C (n = 40 per orchard) at PARC in Summerland, BC. Half of the apples were wounded in triplicate and the remaining half were not wounded. 1-MCP treatments were not used in 2008 harvest experiments. Disease incidence (% apples infected) and lesion diameters (mm) were measured after 3 and 6 months for the 2007 harvest, after 4.5 months of sealed CA storage and after 1, 2, 4 and 6 months at 1°C in air storage for apples harvested in 2008.

2.10.3 Natural disease incidence with bacterial antagonists

To measure bacterial antagonist efficacy against natural disease incidence for harvest 2007, 120 Jonagold apples from Field 3 and 120 Gala apples from Field 12 were wounded in triplicate and inoculated with one of five bacterial antagonists – isolates 1100-6, 1-112, 2-28, 4-6 or 6-25- and a non-inoculated control (10 apples / antagonist). Sixty of the 120 apples were fumigated with 1-MCP and the remaining 60 apples were not fumigated. Following harvest in 2008, 240 apples from each orchard, Field 3, 12, Kiran and Reekie, were inoculated as in section 2.10.1. One hundred and twenty apples from each orchard were stored in CA (60 wounded and 60 not wounded) and 120 apples were stored at 1°C in air (60 wounded and 60 not wounded). Disease incidence and lesion diameters were measured after 3 months in storage for apples harvested in 2007. For apples harvested in 2008, disease incidence and lesion diameters were measured after 4.5 months of sealed CA storage and after 1, 2, 4 and 6 months at 1°C in air storage. The efficacy of the biological control agents was compared to apples that had been wounded and not inoculated with bacteria.

2.10.4 Biological control of post-harvest pathogens

For apples harvested in 2007, disease inhibition was measured by inoculating 360 Jonagold apples from Field 3 and 360 Gala apples from Field 12 with one of three fungal pathogens, *P. expansum, B. cinerea* or *M. piriformis* and one of five bacterial control isolates, 1100-6, 1-112, 2-28, 4-6, 6-25 and a non-inoculated control. One hundred and eighty of the 360 apples were fumigated with 1-MCP. For apples harvested in 2008, 360 apples each from Fields 3 and 12 and the Kiran and Reekie orchards were inoculated as above. In 2008, 1-MCP was not used, but 180 apples per orchard were wounded and 180 apples per orchard were non-wounded. From each site, there were 10 apples / antagonist + 1 non-inoculated control / pathogen. Disease incidence and lesion diameters were measured after 3 months of storage for apples harvested in 2007 and after 1, 2, 4 and 6 months at 1°C in air storage for apples harvested in 2008. Pathogens were not permitted in commercial CA storage.

2.10.5 Bacterial survival on apple

Concentrations of 4-6-*gfp* were monitored by two experimental techniques- colony counts via dilution plating and direct scanning using the POLARstar Omega microplate reader (BMG Labtech, Germany) as in Etebarian et al. (2005). A standard curve was developed that described the relationship between colony forming units (CFU/ml) and relative fluorescent units (RFU) acquired by direct scanning.

The bacterial inoculum was prepared in ½ TSB with 50 µg/ml of kan and 30 µg/ml of gen and grown at 28°C for 24 h. *P. expansum* spores were harvested from 7-d-old ½ PDA plates. Final inoculation concentrations for 4-6-*gfp* and *P. expansum* were 10^8 CFU/ml and 10^4 spores/ml, respectively. Apples were prepared as in section 2.9.1

and inoculated by pipette (30 µl) with the following treatments: 1) non-inoculated control; 2) 4-6-*gfp* alone; 3) *P. expansum* alone and; 4) 4-6-*gfp* + *P. expansum*. Isolate 4-6-*gfp* was quantified by aseptically removing an apple core (5 mm in diameter x 5 mm deep) from two of the three apple wounds. Both cores were macerated with a sterile rod. One core, to be used for dilution plating, was placed in 1.1 ml of PBS and plated on Pseudomonas F agar (20 g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L anhydrous $MgSO_4$, 10 g/L agar [pH 7.2]) supplemented with 50 µg/ml kan and 30 µg/ml gen. The second core, to be used in direct scanning, was placed in 300 µl of PBS. Two hundred microlitres of the tissue slurry were added to a well in a black Nunc 96-well microplate (Thermo Fisher Scientific, Rochester, NY). Fluorescent readings were obtained using a 405 nm excitation filter and a 510 nm emission filter and a gain of 1200. The relative fluorescent units (RFU) of PBS and non-inoculated control were subtracted from the treatments to correct for background and apple fluorescence, respectively. Bacterial concentrations were determined approximately every 10 days for a total of 55 d.

2.11 Analysis

In all biocontrol experiments, disease was measured by disease incidence (percent apple infection) and disease severity (average lesion diameter in mm). From these two values, data were converted to infection severity (IS) (Spotts et al. 1999):

$$\textbf{Infection Severity (IS)} = \frac{\%\ \text{Apple Infection} * \text{Average Lesion Diameter in mm}}{100\%}$$

In order to correct for variance heterogeneity, infection severity values were $\log_{10}(n+0.5)$ transformed. Differences in means were analyzed by either a one-way or two-

way analysis of variance (ANOVA). *p*-values less than 0.05 were considered statistically significant. If the 2-way ANOVA interaction term was significant, the main effects were separated and a one-way ANOVA was used for subsequent analysis. Differences among means were determined by the least significant difference (LSD) test. The Pearson Product Moment Correlation Coefficient (*r*) was used to measure the linear association of two independent variables. Statistical analyses were conducted using CoStat Statistics Software (CoHort Software, Monterey, CA).

CHAPTER 3: RESULTS

3.1 Validation and field testing of DNA macroarrays

3.1.1 Quantification of post-harvest pathogens

Macroarray construction for the identification of post-harvest pathogens was completed by Dr. Danielle Hirkala and was based upon the prototype proposed in Sholberg et al. (2005a). The array displayed high specificity, no cross-reactivity and detected as little as 0.1 ng of pure culture fungal DNA extracted from *P. expansum* and *B. cinerea* and up to 0.01 ng of pure culture of *M. piriformis* (Hirkala, unpublished).

For the purpose of this study, three macroarray probes were selected for pathogen identification: PE-H3u for *P. expansum* detection, BC-H2d for *B. cinerea* detection and Mpir-ITS-414H1 for *M. piriformis* detection. Each of the three selected probes is comprised of sequences in the rDNA ITS region and is located on the left hand side of the macroarray template (Figure 2). These probes were selected because of their overall higher sensitivity compared to other probes. This decision was based upon all 2007 greyscale values obtained from environmental field data. Standard curves were determined for each of the three probes (Figures 3, 4 and 5).

To test the quantitative properties of the amino-linked oligonucleotides, 0 – 100 ng of pure culture fungal DNA was hybridized to the macroarray. Each detector oligonucleotide displayed varying levels of hybridization signal intensity; however, a linear relationship did exist between DNA concentration and hybridization signal strength. The linear range of the probe PE-H3u ((♦) r = -0.974; (●) r = -0.962) was between 0 and 3 ng (Figure 3), whereas the linear range for the probe BC-H2d

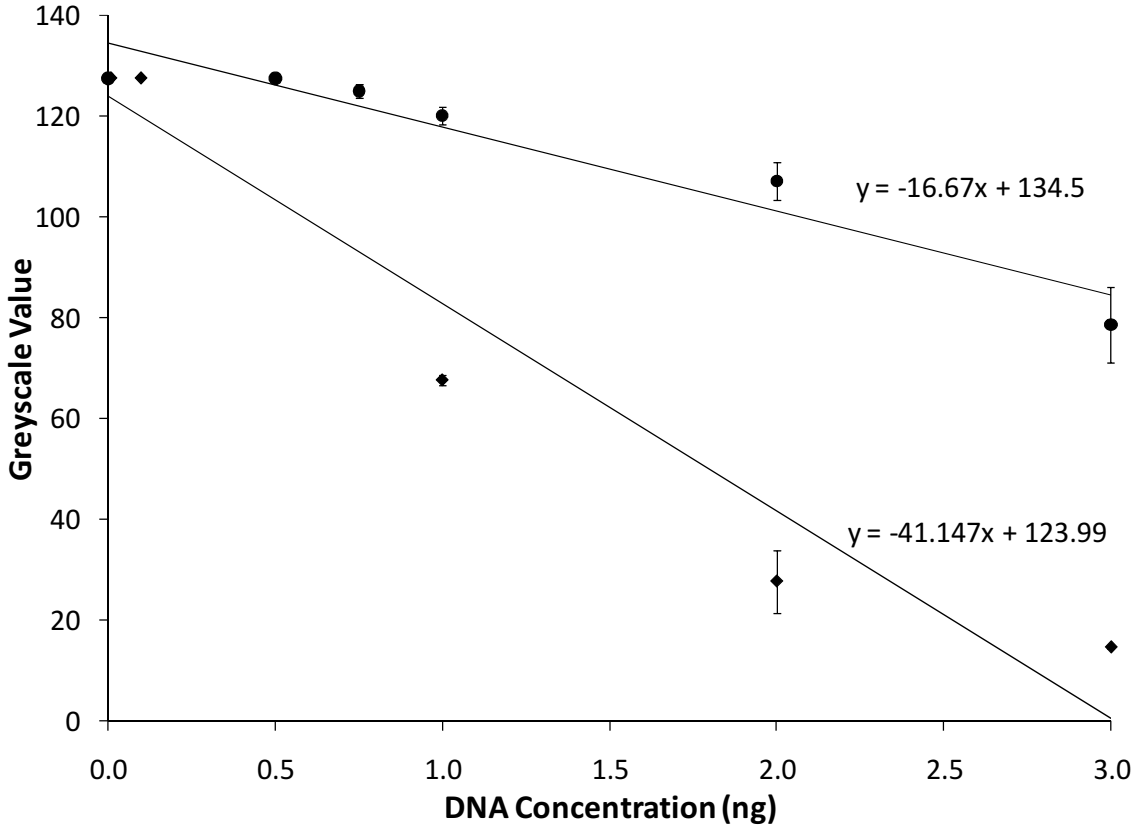

Figure 3. Quantitative assessment of *Penicillium expansum* probe PE-H3u illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.974); (●) Represents standard curve used for samples collected in 2008 (r = -0.962). Error bars represent standard error of the mean.

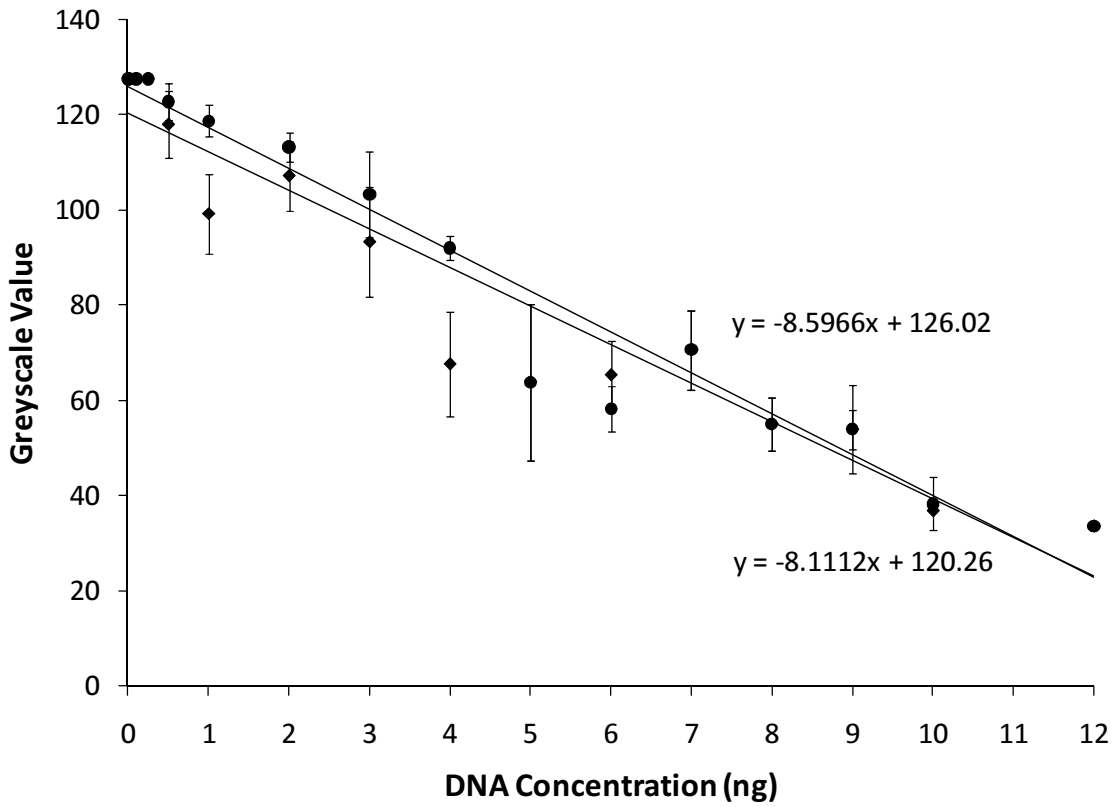

Figure 4. Quantitative assessment of *Botrytis cinerea* probe BC-H2d illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.960); (●) Represents standard curve used for samples collected in 2008 (r = -0.977). Error bars represent standard error of the mean.

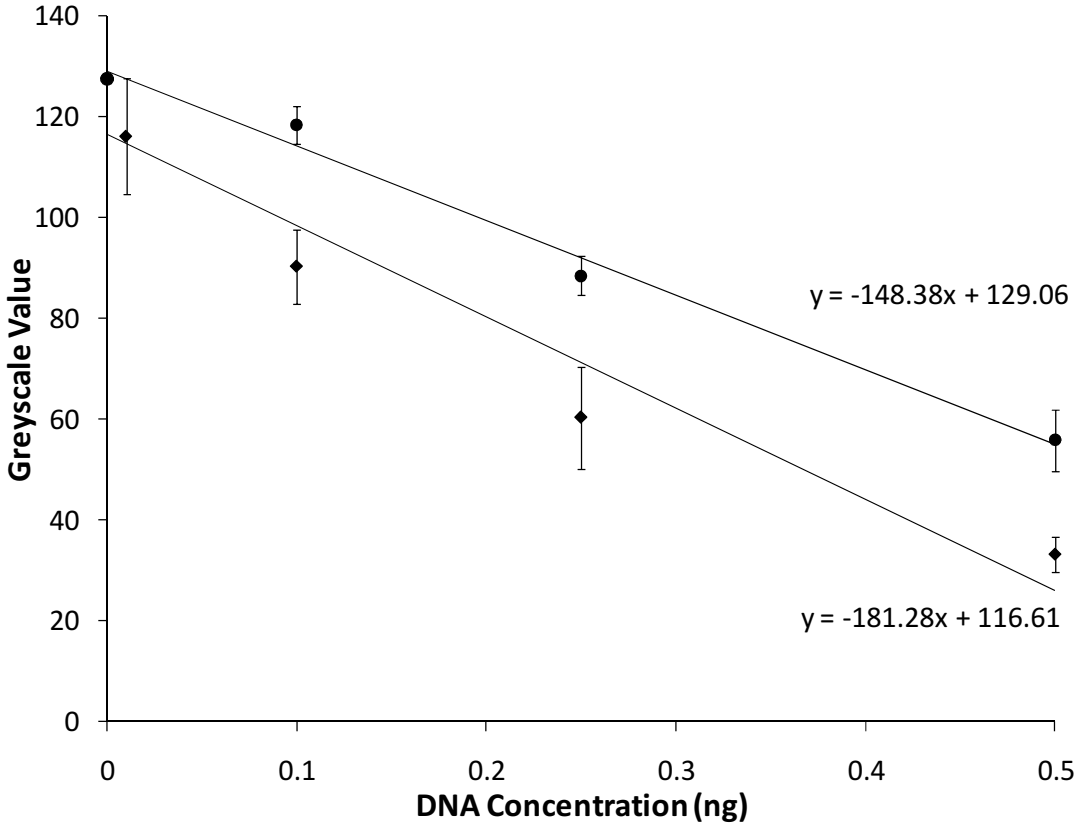

Figure 5. Quantitative assessment of *Mucor piriformis* probe Mpir-ITS-414H1 illustrating the relationship between greyscale value and DNA concentration (ng). (♦) Represents standard curve used for samples collected in 2007 (r = -0.970); (●) Represents standard curve used for samples collected in 2008 (r = -0.990). Error bars represent standard error of the mean.

((♦) r = -0.960; (●) r = -0.977) was between 0 and 12 ng (Figure 4) and the probe Mpir-ITS-414H1 ((♦) r = -0.970; (●) r = -0.990) was between 0 and 0.5 ng (Figure 5). Above these ranges, the curves deviated from linearity as the oligonucleotides reached their saturation level. Throughout the duration of this study, the intensity of the hybridization signals decreased over time which led to the construction of two separate quantification curves for each of the three pathogens (Figures 3, 4 and 5).

The two standard curves created for *P. expansum* DNA quantification (ng) were constructed in August 2008 (♦) and August 2009 (●) (Figure 3). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -41.147x + 123.99 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -16.67x + 134.5 (●).

The two standard curves created for *B. cinerea* DNA quantification (ng) were constructed in August 2008 (♦) and October 2009 (●) (Figure 4). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -8.1112x + 120.26 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -8.5966x + 126.02 (●).

The two standard curves created for *M. piriformis* DNA quantification (ng) were constructed in October 2008 (♦) and August 2009 (●) (Figure 5). Positive hybridization signals that were present in samples assessed in 2008 were quantified using the formula y = -181.28x + 116.61 (♦). Positive hybridization signals that were present in samples assessed in 2009 were quantified using the formula y = -148.38x + 129.06 (●).

3.1.2 Field application of macroarray technology

The DNA macroarray was used in two years of field monitoring studies to verify airborne and plant tissue pathogen spore loads in four orchards in the Okanagan Valley, BC: Field 3 in Summerland, BC (Figure 6); Field 12 in Summerland, BC (Figure 7); Kiran orchard in Kelowna, BC (Figure 8); Reekie orchard in Kelowna, BC (Figure 9).

All three probes had positive hybridization signals throughout the growing season with high variability between years and sample types. In Field 3 (Figure 6), all three pathogens, *P. expansum* (A. 2007, D. 2008), *B. cinerea* (B. 2007, E. 2008) and M. *piriformis* (C. 2007, F. 2008) were detected most frequently in the early and late growing seasons. In Field 12 (Figure 7), *P. expansum* was detected at the beginning and middle of the growing season in 2007 (A) and throughout the growing season in 2008 (D). Pathogen levels were much higher in 2008 (D) than in 2007 (A). *B. cinerea* was detected in the middle of the growing season in 2007 (B) and the beginning and the end of the growing season in 2008 (B). *B. cinerea* was not detected on plant tissue samples in either 2007 or 2008 (B, E). *M. piriformis* was detected at the beginning and middle of the growing season in 2007 (C) and at the beginning and the end of the growing season in 2008 (F). In the Kiran orchard (Figure 8), *P. expansum* (A. 2007, D. 2008), *B. cinerea* (B. 2007, E. 2008) and M. *piriformis* (C. 2007, F. 2008) were detected throughout the growing season in both 2007 and 2008. DNA concentrations for *P. expansum* and *B. cinerea* were higher in 2008 than in 2007. In the Reekie orchard (Figure 9), *P. expansum* was detected in the middle of the growing season in 2007 (A) and throughout the growing season in 2008 (D). *B. cinerea* was not detected in 2007 (B) and was detected throughout the growing season in 2008 (E). *M. piriformis* was detected in the middle of

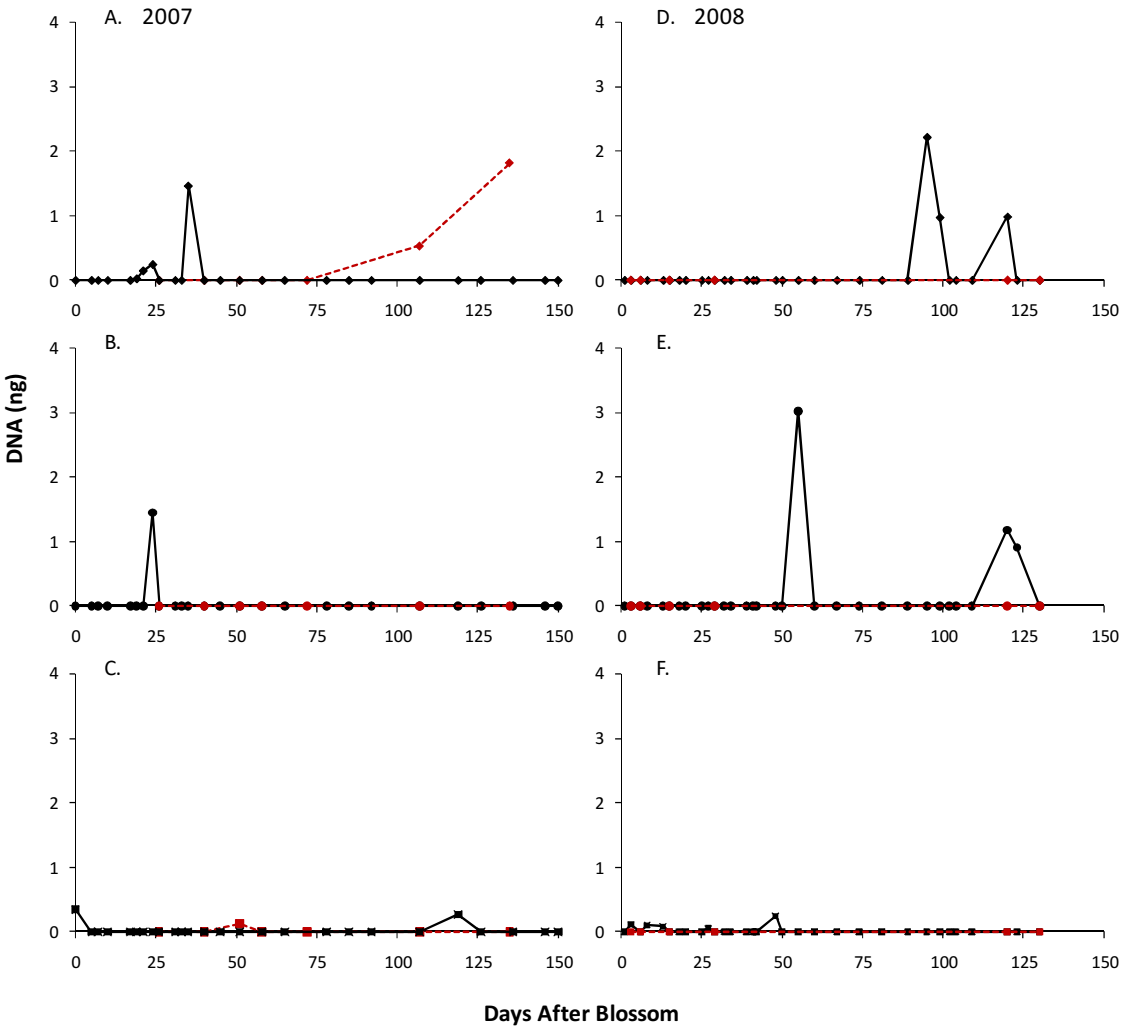

Figure 6. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from Field 3 (Summerland, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

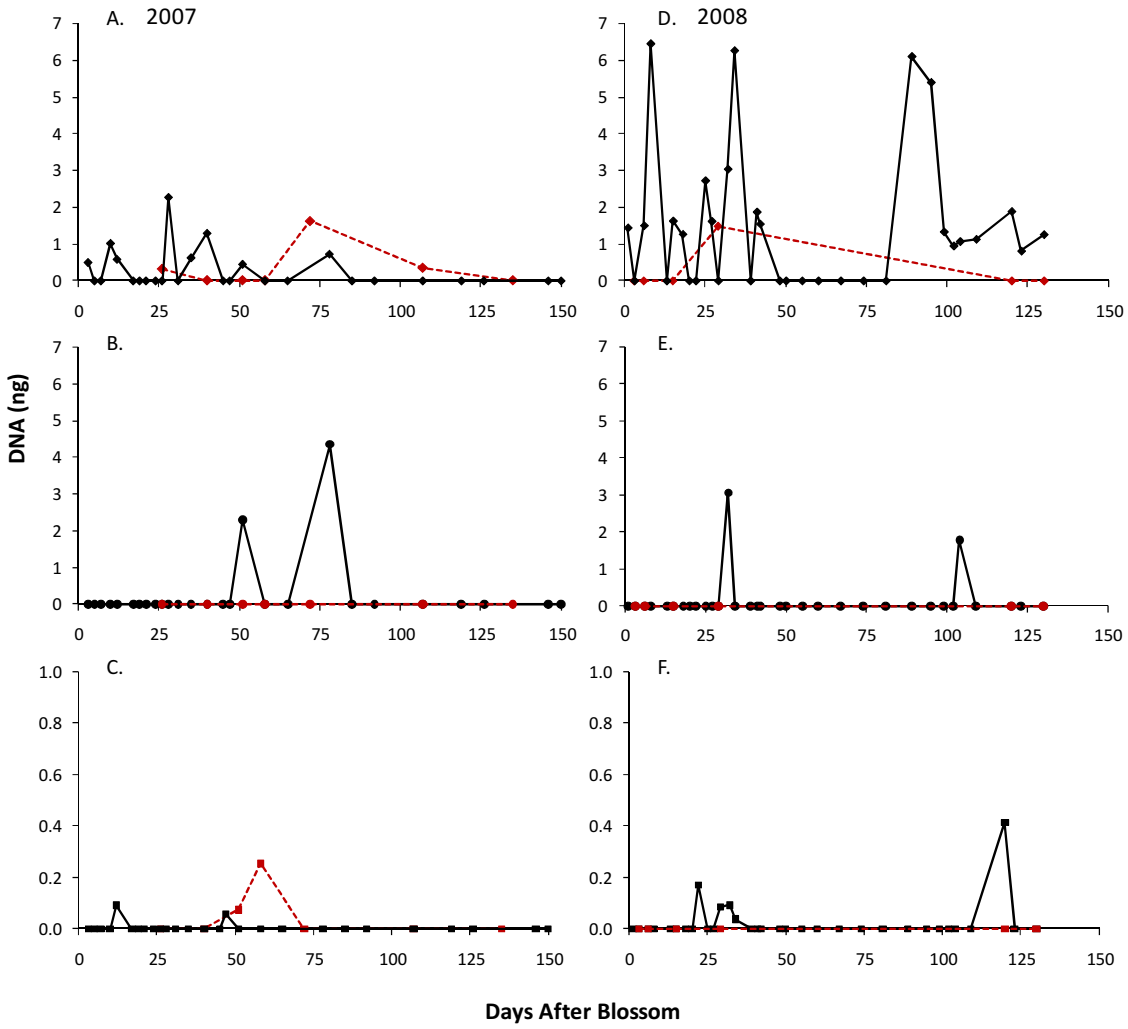

Figure 7. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from Field 12 (Summerland, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

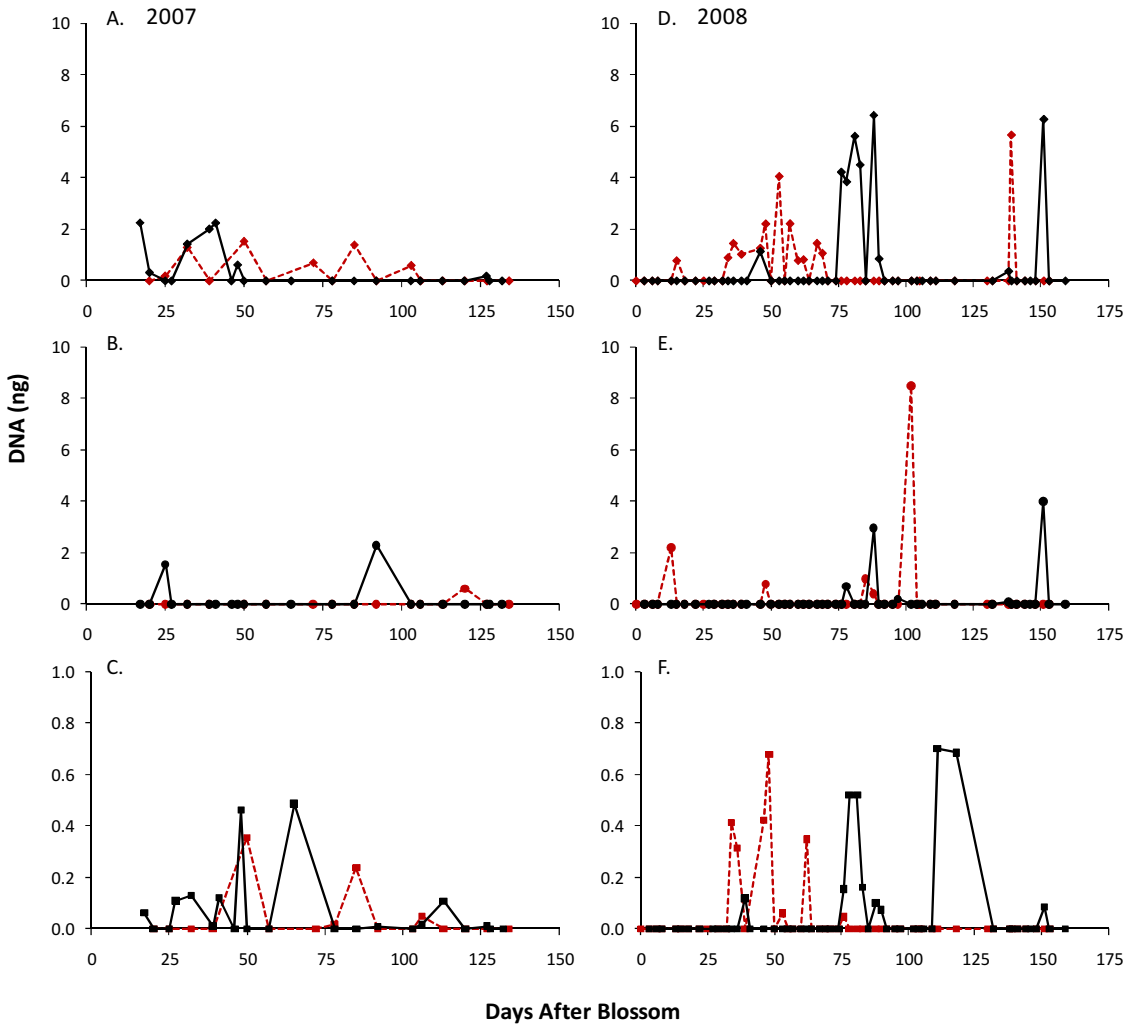

Figure 8. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from the Kiran orchard (Kelowna, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

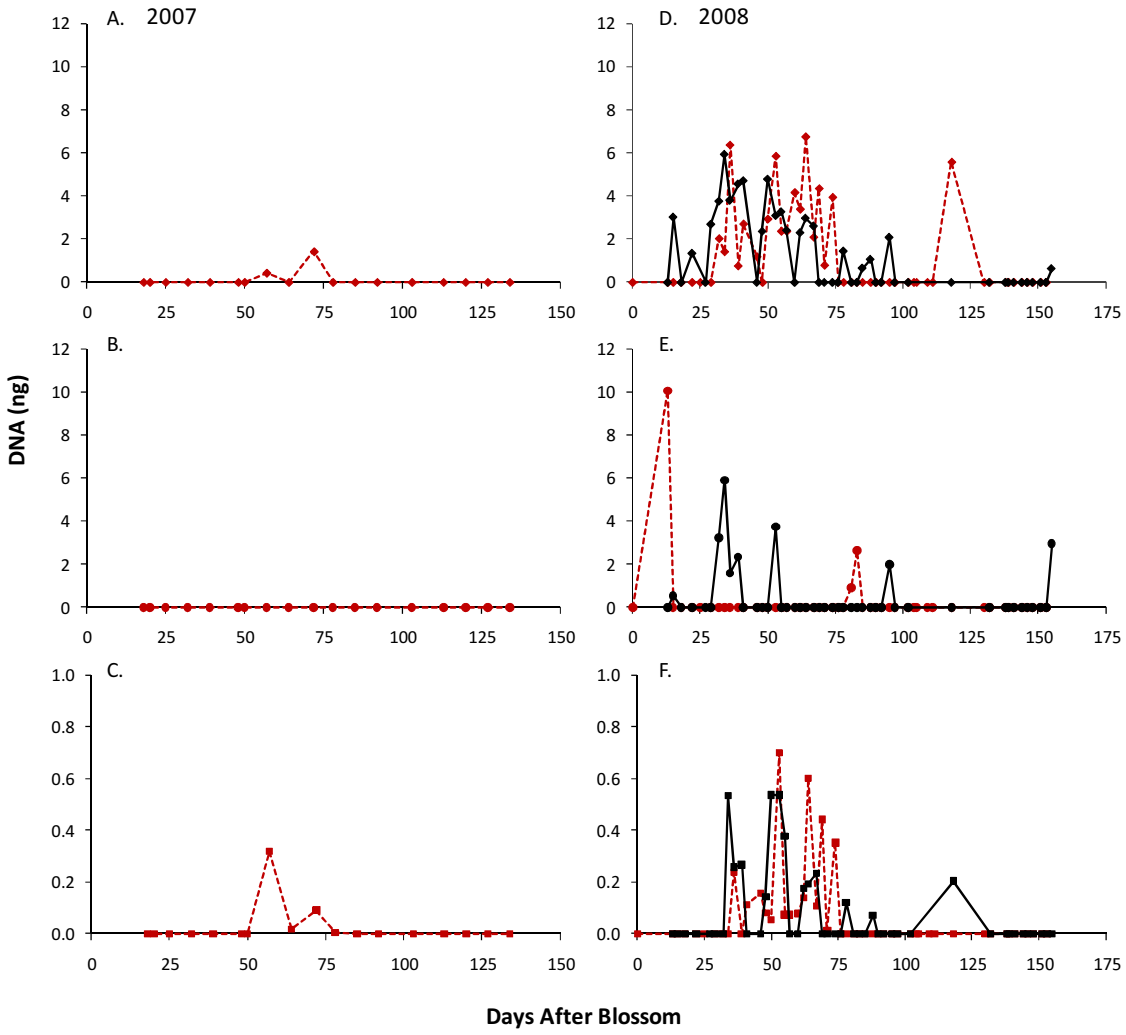

Figure 9. Concentrations of *P. expansum* (A,D), *B. cinerea* (B,E) and *M. piriformis* (C,F) from aerial (solid line) and plant tissue samples (dashed line) collected from the Reekie orchard (Kelowna, BC) in 2007 (A – C) and 2008 (D – F) using DNA macroarrays and identified by post-harvest pathogen rDNA ITS macroarray probes, PE-H3u (♦), BC-H2d (●) and Mpir-ITS-414H1 (■).

the growing season in 2007 (C) and throughout the growing season in 2008 (F). All pathogen levels were higher in 2008 than in 2007.

Due to the observed variability, the data were arranged in an alternate way in order to determine if there were common patterns between pathogens and fields (Table 3). To determine what time in the growing season each of the three main post-harvest pathogens was primarily detected, the season was divided into three sections: 1) early (~1 – 50 days after blossom); 2) mid (~50 – 100 days after blossom) and; 3) late (~100 – 150+ days after blossom) growing season (Table 3). Aerial and plant tissue samples were analyzed separately and in combination by determining percent pathogen detection within either the early, mid or late growing seasons.

According to the tabulated data in Table 3, in 2007 and 2008, *P. expansum* was detected in each of the three growing season segments but differed by field and year. From DNA isolated from aerial samples collected in 2007, there was a significant effect of growing season segment on % *P. expansum* detected ($p = 0.06$) (Appendix, Table A1); the % *P. expansum* detected in the early season was significantly higher than that in the late growing season (Table 3). There was no effect of growing season segment on % *P. expansum* detected in aerial samples in 2008 (Table 3; Appendix, Table A4). From DNA isolated from plant tissue samples in 2007 there was no significant effect of the growing season segment on the % *P. expansum* detected (Table 3; Appendix, Table A2), but in 2008, there was a significant effect ($p = 0.053$) (Appendix, Table A5). The % *P. expansum* detected in the mid-growing season was significantly higher than that in the late-growing season (Table 3).

In 2007, there was no effect of the growing season segment on the % *B. cinerea* detected in DNA isolated from either aerial or plant tissue samples (Table 3; Appendix, Tables A1 and A2). In 2008, from DNA isolated from plant tissue samples, the % *B. cinerea* detected in the mid-growing season was significantly higher than that in the early and late-growing seasons (Table 3; Appendix, Table A5). Overall, *B. cinerea* was detected at much lower levels than either *P. expansum* or *M. piriformis*.

M. piriformis was detected in each of the three growing season segments but also differed by field and year (Table 3). From DNA isolated from plant tissue samples in 2007, the % *M. piriformis* detected was significantly higher in the mid-growing season than that in the early or late-growing seasons (Table 3; Appendix, Table A2). From DNA isolated from plant tissue samples in 2008, the % *M. piriformis* detected was significantly higher in the mid-growing season than that in the late-growing season ($p = 0.06$) (Table; Appendix, Table A5). In both 2007 and 2008, when aerial and plant tissue DNA samples were analyzed together, there was no effect of growing season segment (Table 3; Appendix, Tables A3 and A6).

In 2007 and 2008 combined, the selected probes detected *P. expansum* (27.4 ± 3.4%) and *M. piriformis* (19.2 ± 1.4%) most frequently, followed by *B. cinerea* (6.2 ± 1.4%) (Table 3; Appendix, Table A7). In addition, *P. expansum* was detected at the highest concentrations, followed by *B. cinerea* and then *M. piriformis*. Of the samples collected in 2007, the Kiran orchard had the highest percent detection (81.6%) followed by Field 12 (50%), the Reekie orchard (33.3%) and Field 3 (30.3%). In 2008, the Reekie orchard had the highest percent detection (80.4%) followed by Field 12 (71.8%), Kiran (46.2%) and Field 3 (28.9%) (Table 3).

Table 3. DNA macroarray detection (%) summary for *P. expansum*, *B. cinerea* and *M. piriformis* from four apple orchards throughout the 2007 and 2008 growing seasons. Growing season was divided into 3 sampling periods: 1) early-growing season (~ 1-50 days after blossom); 2) mid-growing season (~ 50-100 days after blossom) and; 3) late-growing season (~ 100-150+ days after blossom). Numbers in ( ) indicate standard error of the mean.

		% pathogen detection within sampling period[a]									
		P. expansum			***B. cinerea***			***M. piriformis***			**% detection per orchard**
Field	**Sample type**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	
2007 Growing Season											
Field 3	aerial	28.6	0.0	0.0	7.1	0.0	0.0	7.1	0.0	16.7	
	plant tissue	0.0	0.0	100.0	0.0	0.0	0.0	0.0	33.3	0.0	
Field ave		**25.0**[b]	**0.0**	**25.0**	**6.3**	**0.0**	**0.0**	**6.3**	**11.1**	**12.5**	**30.3**
Field 12	aerial	37.5	33.3	0.0	0.0	33.3	0.0	12.5	0.0	0.0	
	plant tissue	50.0	33.3	50.0	0.0	0.0	0.0	0.0	66.7	0.0	
Field ave		**38.9**	**33.3**	**14.3**	**0.0**	**22.2**	**0.0**	**11.1**	**22.2**	**0.0**	**50.0**
Kiran	aerial	66.7	0.0	16.7	11.1	14.3	0.0	66.7	28.6	50.0	
	plant tissue	50.0	57.1	0.0	0.0	0.0	20.0	0.0	42.9	20.0	
Field ave		**61.5**	**28.6**	**9.1**	**7.7**	**7.1**	**9.1**	**46.2**	**35.7**	**36.4**	**81.6**
Reekie	aerial	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	plant tissue	0.0	25.0	0.0	0.0	0.0	0.0	0.0	50.0	0.0	
Field ave		**0.0**	**25.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**50.0**	**0.0**	**33.3**
Ave % detection per sampling period (irod)[c]		**44.2a**[h] (11.5)	**11.1ab** (11.1)	**5.6b** (5.6)	**6.1a** (3.3)	**15.9a** (9.7)	**0.0a** (0.0)	**28.8a** (19.0)	**9.5a** (9.5)	**22.2a** (14.7)	
Ave % detection per sampling period (pt)[d]		**25.0a** (14.4)	**28.9a** (11.8)	**37.5a** (23.9)	**0.0a** (0.0)	**0.0a** (0.0)	**5.0a** (5.0)	**0.0b** (0.0)	**48.2a** (7.0)	**5.0b** (5.0)	
2007 ave % detection per sampling period (irod + pt)[e]		**31.4a** (12.9)	**21.7a** (7.4)	**12.1a** (5.2)	**3.5a** (2.0)	**7.3a** (5.2)	**2.3a** (2.3)	**15.9a** (10.3)	**29.8a** (8.4)	**12.2a** (8.6)	
2007 ave % detection per pathogen[f]				**24.0a** (5.6)			**4.2b** (1.7)			**20.6ab** (13.8)	

		% pathogen detection within sampling period									
		P. expansum			*B. cinerea*			*M. piriformis*			% detection per orchard
Field	**Sample type**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	
2008 Growing Season											
Field 3	aerial	0.0	18.2	33.3	0.0	9.1	66.7	27.8	0.0	0.0	
	plant tissue	0.0	ND	0.0	0.0	ND	0.0	0.0	ND	0.0	
Field ave		**0.0**	**18.2**	**20.0**	**0.0**	**9.1**	**40.0**	**22.7**	**0.0**	**0.0**	**28.9**
Field 12	aerial	57.9	54.5	100.0	5.3	9.1	0.0	21.1	0.0	33.3	
	plant tissue	25.0	ND	0.0	0.0	ND	0.0	0.0	ND	0.0	
Field ave		**52.2**	**54.5**	**60.0**	**4.3**	**9.1**	**0.0**	**17.4**	**0.0**	**20.0**	**71.8**
Kiran	aerial	5.9	25.0	18.2	0.0	12.5	18.2	5.9	29.2	18.2	
	plant tissue	38.9	20.8	10.0	5.6	12.5	0.0	27.8	8.3	0.0	
Field ave		**22.9**	**22.9**	**14.3**	**2.9**	**12.5**	**9.5**	**17.1**	**18.8**	**9.5**	**46.2**
Reekie	aerial	73.3	45.0	9.1	40.0	5.0	9.1	40.0	30.0	9.1	
	plant tissue	50.0	37.5	9.1	6.3	8.3	0.0	37.5	37.5	0.0	
Field ave		**61.3**	**40.9**	**9.1**	**22.6**	**6.8**	**4.5**	**38.7**	**34.1**	**4.5**	**80.4**
Ave % detection per sampling period (irod)[c]		**34.3a**[h] (18.4)	**35.7a** (8.5)	**40.2a** (20.6)	**11.3a** (9.6)	**8.9a** (1.5)	**23.5a** (14.9)	**23.7a** (7.1)	**14.8a** (8.5)	**15.2a** (7.1)	
Ave % detection per sampling period (pt)[d]		**28.5ab** (10.8)	**29.2a** (8.3)	**4.8b** (2.8)	**3.0b** (1.7)	**10.4a** (2.1)	**0.0b** (0.0)	**16.3ab** (9.6)	**22.2a** (14.6)	**0.0b** (0.0)	
2008 ave % detection per sampling period (irod + pt)[e]		**34.1a** (14.0)	**34.1a** (8.4)	**25.8a** (11.6)	**7.4a** (5.1)	**9.4a** (1.2)	**13.5a** (9.0)	**24.0a** (5.1)	**13.2a** (8.2)	**8.5a** (4.3)	
2008 ave % detection per pathogen[f]				**30.8a** (10.2)			**8.3b** (1.3)			**17.8ab** (3.8)	
2007/08 ave % detection per pathogen[g]				**27.4a** (3.4)			**6.2b** (1.4)			**19.2a** (1.4)	

a. % pathogen detection: $\frac{\text{\# of times a pathogen was detected within a select sampling period}}{\text{\# of samples taken within a select sampling period}} * 100\%$
b. Field averages were obtained by calculating the average % pathogen detection between both aerial and plant tissue samples
c. Ave % detection per sampling period (irod): irod = aerial sample; values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in aerial samples only
d. Ave % detection per sampling period (pt): pt = plant tissue sample; values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in plant tissue samples only
e. Ave % detection per sampling period: values were determined by taking the average of the % detection values per sampling period per pathogen within each orchard in aerial and plant tissue samples combined
f. Ave % detection per pathogen: values were determined by taking the average of the % pathogen detection values within all three sampling periods within each orchard with aerial and plant tissue samples combined
g. 2007/08 ave % detection per pathogen: values were determined by taking the average of the values obtained in Ave % detection per pathogen in both 2007 and 2008
h. Means followed by different letters within a pathogen and within a row are significantly different ($p \leq 0.06$) according to the least significant difference (LSD) test.

Fruit washings conducted after harvest in 2007 showed the presence of *B. cinerea* in Jonagold apples grown in Field 3 and the presence of *P. expansum, B. cinera* and *M. piriformis* in Gala apples grown in Field 12. Fruit washings conducted after harvest in 2008 showed the presence of *P. expansum* in Gala apples grown in the Kiran orchard (Table 4).

Table 4. Detection of *P. expansum*, *B. cinerea* and *M. piriformis* from fruit washings collected from Jonagold apples from Field 3, Gala apples from Field 12 and the Kiran orchard and Red Delicious apples from the Reekie orchard in 2007 and 2008 using DNA macroarrays.

	DNA detected (ng/20 apples)		
Field	***P. expansum***	***B. cinerea***	***M. piriformis***
Harvest 2007			
Field 3	ND[a]	0.48	ND
Field 12	0.78	0.66	0.05
Harvest 2008			
Field 3	ND	ND	ND
Field 12	ND	ND	ND
Kiran	1.41	ND	ND
Reekie	ND	ND	ND

a. ND = not detected

3.2 Natural disease incidence

3.2.1 Harvest 2007

The effect of incubation period (3 and 6 months) and 1-MCP use (1-MCP+/-) on infection severity (IS) was analyzed using a two-way ANOVA. The interaction terms for apples from both Field 3 and 12 were not significant (Appendix, Tables A8 and A9). In both Field 3 and 12, there was a greater IS at six months than at three months in 1°C storage. However, only in Field 3 was there a significant effect of 1-MCP application; 1-MCP reduced IS by 48.2% in comparison to the non-fumigated control (Tables 5 and 6).

Table 5. Natural disease incidence indicated by infection severity[a] in Jonagold apples grown in Field 3 (harvest 2007), treated or not treated with 1-MCP (1-MCP+/-) and stored for 3 or 6 months at 1°C in air.

	Field 3	**Field 3**	
Incubation Period	**3 months**	**6 months**	Overall Mean
1-MCP +	5.51	9.55	7.53 b[c]
1-MCP -	9.09	22.12	15.61 a
Overall Mean	7.30 b[b]	15.83 a	

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters are different at significance levels $p \leq 0.05$ according to least significant difference (LSD) test.
c. 1-MCP means with different letters are different at significance levels $p \leq 0.05$ LSD test.

Table 6. Natural disease incidence indicated by infection severity[a] in Gala apples grown in Field 12 (harvest 2007), treated or not treated with 1-MCP (1-MCP+/-) and stored for 3 or 6 months at 1°C in air

	Field 12	**Field 12**	
	3 months	**6 months**	Overall Mean
1-MCP +	7.64	30.28	18.95 a[c]
1-MCP -	11.78	22.29	16.56 a
Overall Mean	9.90 b[b]	26.28 a	

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters are different at significance levels $p \leq 0.05$ according to least significant difference (LSD) test.
c. 1-MCP means with different letters are different at significance levels $p \leq 0.05$ LSD test.

3.2.2 Harvest 2008

The effects of incubation period (1, 2, 4 and 6 months) on infection severity in each of the four orchards were analyzed using a one-way ANOVA. Results indicated that in all fields, there was a significant increase in IS from one to six months (Table 7; Appendix, Table A10). A one-way ANOVA also revealed that at two months incubation, Jonagold apples grown in Field 3 had significantly greater infection severities than in all

other orchards. At four and six months of incubation, Jonagold apples grown in Field 3 and Gala apples grown in Field 12 had significantly greater infection severities than in the commercial orchard apples, Gala grown in the Kiran orchard and Red Delicious grown in the Reekie orchard (Appendix, Table A11).

Table 7. Natural disease incidence indicated by infection severity[a] in Jonagold apples grown in Field 3, Gala apples grown in Field 12 and the Kiran orchard and Red Delicious apples grown in the Reekie orchard (harvest 2008) and stored for 1, 2, 4 or 6 months at 1°C in air.

Apple Variety	Field 3 Jonagold	Field 12 Gala	Kiran Gala	Reekie Red Delicious
1 month	0.00 dA	0.00 dA	0.36 bA	0.00 cA[c]
2 months	1.08 cA	0.54 cB	0.01 bB	0.00 cB
4 months	20.72 bA	19.95 bA	0.08 bB	2.28 bB
6 months	48.12 a[b]A	46.58 aA	2.06 aB	10.88 aB

a. Infection severity (IS) = percent apple infection x average lesion diameter (mm)/100 (Spotts et al. 1999).
b. Incubation period means with different letters within columns (lowercase) are different at significance levels at $p \leq 0.05$ according to least significant difference (LSD) test.
c. Incubation period means with different letters within rows (uppercase) are different at significance levels at $p \leq 0.05$ according to the LSD test.

In addition to storing apples at 1°C in air, fruit were also stored in controlled atmosphere (CA) for 4.5 months. Jonagold apples grown in Field 3 had the highest infection severity with significantly greater disease levels compared to Gala apples grown in Fields 12 and Kiran and Red Delicious apples grown in the Reekie orchard (Figure 10; Appendix, Table A12). The high infection severity found in Jonagold apples grown in Field 3 appeared to be due primarily to *P. expansum* (based on phenotypic observation).

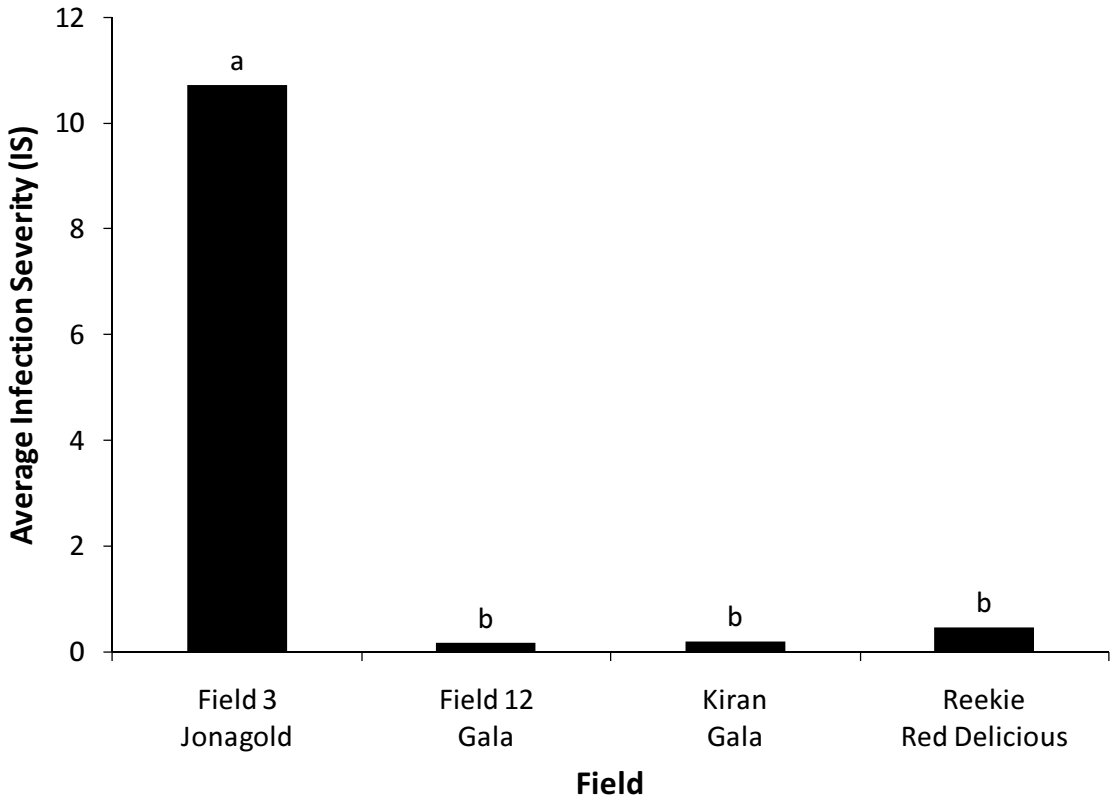

Figure 10. Natural disease incidence in Jonagold apples grown in Field 3, Gala apples grown in Field 12 and the Kiran Orchard and Red Delicious apples grown in the Reekie orchard and stored for 4.5 months in controlled atmosphere (CA) in Naramata, BC. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

3.3 Growing season detection and natural disease incidence

In this study, there was no correlation between % total pathogen detection (*P. expansum, B. cinerea* and *M. piriformis*) detected in the field throughout the entire growing season in both 2007 and 2008 combined (Table 3) and NDI after storage (r = -0.11; p = 0.8349). With respect to Fields 3 and 12 in 2007 and Fields 3 and 12 and the Kiran and Reekie orchards in 2008, there was no correlation between % pathogens detected in the early-growing season and their corresponding NDIs (r = -0.29; p = 0.5811) and % pathogens detected in the mid-growing season and their corresponding NDIs (r = -0.28; p = 0.5865). However, there was a positive correlation between % pathogens detected in the late-growing season and NDI (r = 0.79; p = 0.0612).

When the aerial samples were analyzed separately from the plant tissue samples, in the 2007 and 2008 data combined, there was no correlation between % pathogens detected in the early (r = -0.57; p = 0.2234) and mid-growing seasons and their corresponding NDIs (r = -0.36; p = 0.4838). However, there was a positive correlation between % pathogens detected in late-growing season and NDI (r = 0.74; p = 0.0903). When the plant tissue samples were separated from the aerial samples, in 2007 and 2008 combined, there was no correlation between % pathogens detected in either the mid (r = -0.22; p = 0.7810) or late-growing seasons (r = -0.21; p = 0.6826) and their corresponding NDIs. However, there was a negative correlation between % pathogens detected in the early-growing season and NDI (r = -0.79; p = 0.0623).

3.4 Antagonist efficacy, harvest 2007

3.4.1 Natural disease incidence and biocontrol inoculations

The effects of biological control treatment (no biocontrol, 1100-6, 1-112, 2-28, 4-6 or 6-25) and 1-MCP use (1-MCP+/-) were analyzed using a two-way ANOVA. For Jonagold apples grown in Field 3 and stored at 1°C in air for 3 months, there was no significant effect of the biological controls, 1-MCP use or their interaction (Appendix, Table A13). Results obtained with inoculated Gala apples from Field 12 indicated a significant interaction with both main effects, biological control treatment and 1-MCP use (Appendix, Table A14). Because the interaction term was significant, treatments were separated, followed by a one-way analysis of variance. 1-MCP significantly decreased IS in apples with no biological control treatment and apples that were treated with bacterial isolates 1100-6 and 2-28 (Figure 11; Appendix, Table A15). There were significant reductions in IS means in the absence of 1-MCP in apples treated with bacterial isolates 1-112, 4-6 and 6-25 in comparison to the non-inoculated control (Figure 11). In 1-MCP- treated apples, there was no significant effect of treatments compared to the non-inoculated control (Figure 11; Appendix, Table A16).

3.4.2 Post-harvest pathogen and biocontrol inoculations

Infection severity (IS) was determined with apples grown in Fields 3 and 12 and inoculated with one of three pathogens, *P. expansum*, *B. cinerea* or *M. piriformis* and one of five bacterial control isolates and the data were subjected to a two-way ANOVA. IS means for Jonagold apples from Field 3 and inoculated with *P. expansum*

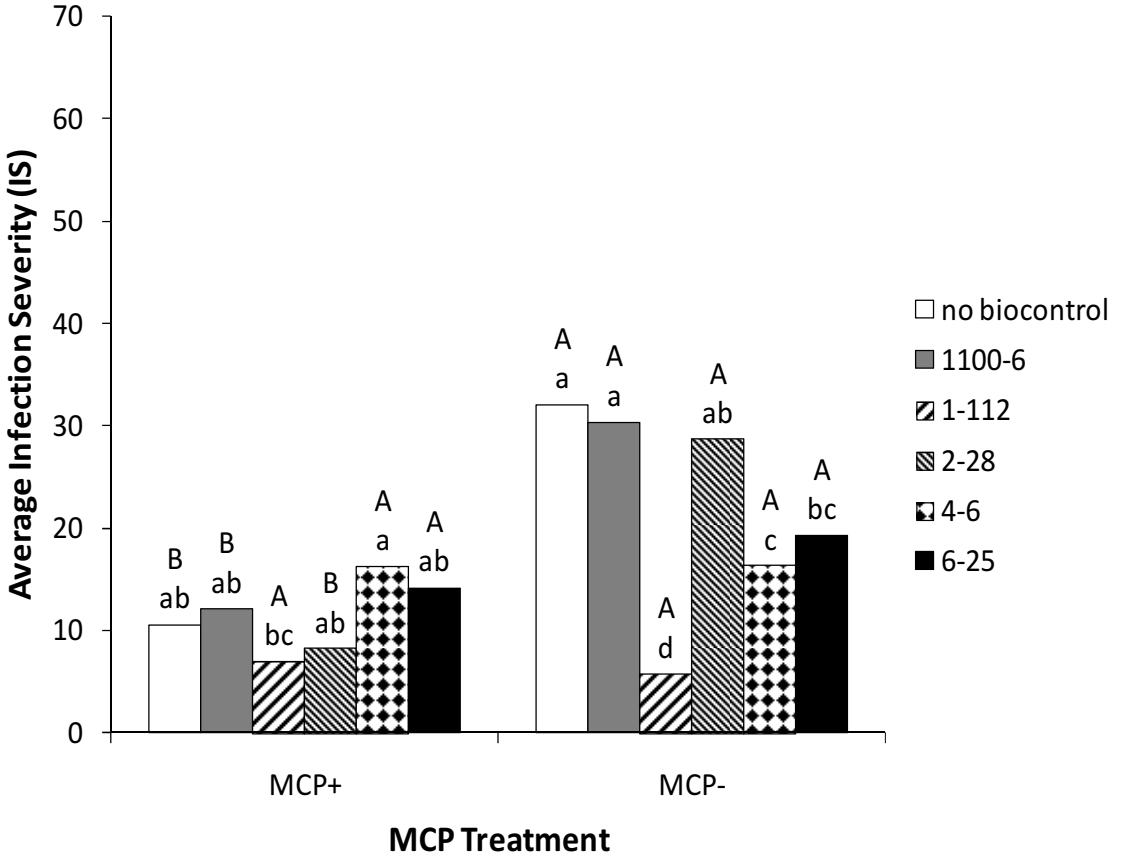

Figure 11. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on natural disease incidence in Gala apples grown in Field 12 (harvest 2007) and stored for 3 months in air at 1°C. IS means with different letters within treatments are different at significance levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

exhibited a significant effect of biological control treatment and of the interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A17). The resulting one-way ANOVA showed that the application of 1-MCP significantly decreased IS means in apples inoculated with isolate 1100-6 and increased IS in apples inoculated with isolate 6-25 (Figure 12a; Appendix, Table A18). In comparison to the non-inoculated control, isolates 1100-6, 1-112, 4-6 and 6-25 provided significant control of *P. expansum* in both the presence and absence of 1-MCP (Figure 12a; Appendix, Table A19).

For *B. cinerea*-treated apples from Field 3, the two-way ANOVA revealed a significant effect of biological control treatment and 1-MCP and of their interaction (Appendix, Table A20). The one-way ANOVAs revealed a significant increase in IS in 1-MCP-treated apples that were not inoculated with a biocontrol and apples inoculated with isolates 2-28 and 4-6 (Figure 12b; Appendix, Table A21). In comparison to a non-inoculated control, isolates 1100-6, 1-112 and 6-25 provided significant control of *B. cinerea* in the presence of 1-MCP. There was no significant reduction in IS in apples in the absence of 1-MCP (Figure 12b; Appendix, Table A22).

For *M. piriformis*-treated apples grown in Field 3, there was a significant effect of the biological control treatment and of the interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A23). The one-way ANOVAs revealed a significant increase in IS in non-1-MCP treated apples inoculated with isolate 4-6 (Figure 12c; Appendix, Table A24). There was no effect of inoculation with any of the biological control isolates in the presence of 1-MCP; however, there was a significant increase in IS in non-1-MCP-treated apples inoculated with isolate 4-6 when

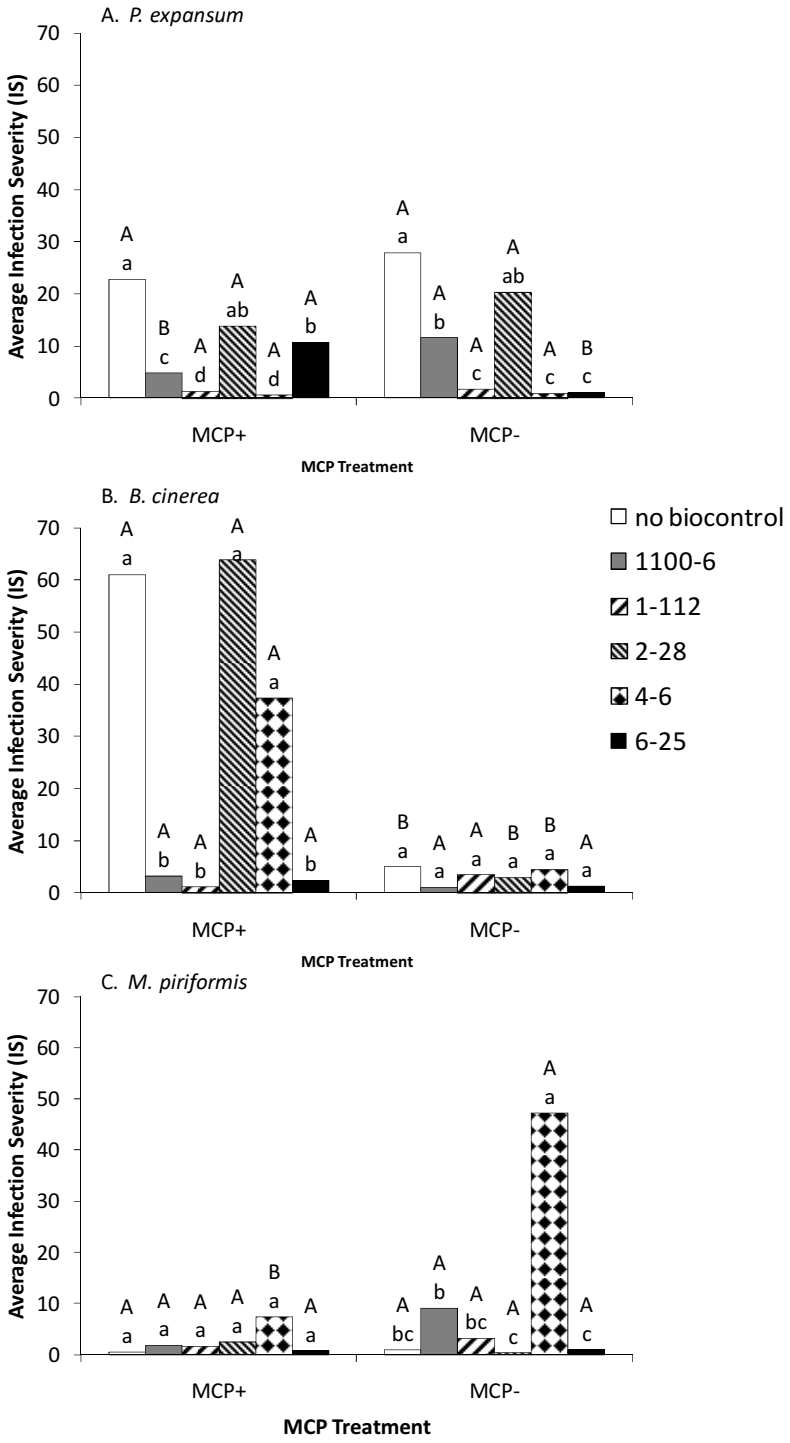

Figure 12. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on infection severity in Jonagold apples from Field 3 (harvest 2007), inoculated with *P. expansum* (A), *B. cinerea* (B) and *M. piriformis* (C) and stored for 3 months at 1°C in air. IS means with different letters within treatments are different at significance levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

compared to 1-MCP-treated apples (Figure 12c; Appendix, Table A25).

For Gala apples from Field 12 treated with *P. expansum* and each of five biological control isolates, there was a significant effect of 1-MCP use and no significant interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A26). 1-MCP significantly increased the mean IS (48.16 ± 0.50) compared to that obtained in the absence of 1-MCP (45.69 ± 0.68). There was no overall significant effect of the biological control isolates (Appendix, Table A26). There were no data for Gala apples from Field 12 inoculated with *B. cinerea* and the five biological control isolates as there was complete apple decay after 3 months of storage at 1°C.

For Gala apples from Field 12 treated with *M. piriformis* and each of the five biological controls, there was a significant interaction between the main effects, biological control treatment and 1-MCP use (Appendix, Table A27). The one-way ANOVAs revealed a significant decrease in IS in 1-MCP-treated apples inoculated with isolate 1-112 compared to non-1-MCP-treated apples and a significant increase in IS in non-1-MCP-treated apples inoculated with isolate 2-28 when compared to 1-MCP-treated apples (Figure 13; Appendix, Table A28). In the presence of 1-MCP, isolate 2-28 exhibited significant biological control activity compared to the non-inoculated control. There was no significant reduction in IS in non-1-MCP treated apples when compared to a non-inoculated control (Figure 13; Appendix, Table A29).

3.5 Antagonist efficacy, harvest 2008

For all 2008 biological control experiments, apples were wounded and non-wounded. Non-wounded apples had a very low frequency of infection with low

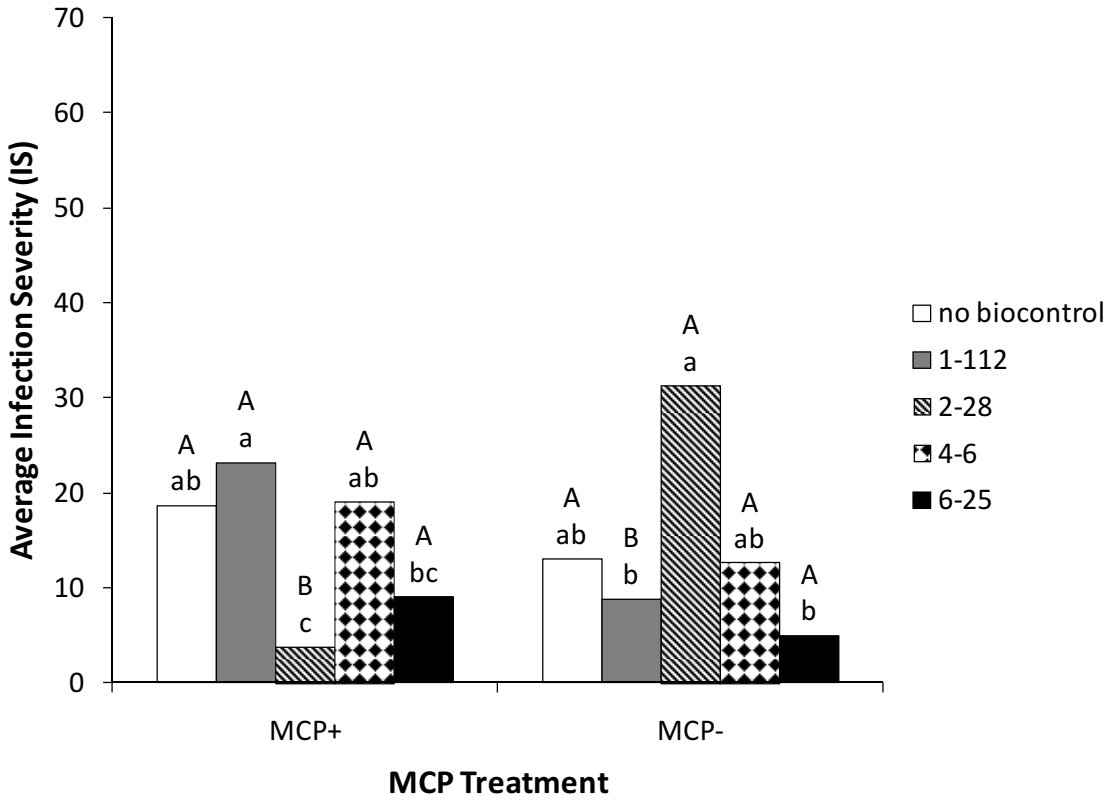

Figure 13. Effect of 1-MCP (capital letters) and biological control isolates (lowercase letters) on infection severity in Gala apples from Field 12 (harvest 2007), inoculated with *M. piriformis* and stored for 3 months at 1°C in air. IS means with different letters within treatments are different at significant levels $p \leq 0.05$ according to LSD test. 1-MCP effect comparisons (capital letters) can be made between 1-MCP treatment groups. Biological control activity comparisons (lowercase letters) can be made within 1-MCP treatment groups.

variation. As a result, the variable was eliminated from 2008 biological control experimental analysis as it explained little about the corresponding dependent and independent variables (Figure 14).

3.5.1 Natural disease incidence and biocontrol inoculations

The effects of biological control treatment (no biocontrol, 1100-6, 1-112, 2-28, 4-6 or 6-25) and incubation period (1, 2, 4 and 6 months) were analyzed using a two-way ANOVA. For Jonagold and Gala apples grown in Fields 3 and 12, respectively, then stored at 1°C in air, there was a significant effect of incubation period and of the interaction between the main effects, biological control treatment and incubation period (Appendix, Tables A30 and A31). For Jonagold apples grown in Field 3, there was no significant reduction in IS in apples treated with the five biological controls after 2 and 4 months in storage (Figure 15a and 15b). However, after six months in storage, isolates 2-28 and 6-25 significantly reduced IS compared to the non- inoculated control (Figure 15c; Appendix, Table A32). For Gala apples grown in Field 12, isolates 1100-6, 1-112, 4-6 and 6-25 significantly reduced IS compared to the non-inoculated control after two months in storage (Figure 15d; Appendix, Table A33). There was no significant reduction in IS after four and six months storage compared to the non-inoculated control (Figure 15e and 15f).

Jonagold apples grown in Field 3, Gala apples grown in Fields 12 and the Kiran orchard and Red Delicious apples grown in the Reekie orchard were also stored for 4.5 months in CA storage. A two-way ANOVA with replication indicated there was a significant effect of biological control treatment and location and of the interaction between biological control treatment and location (Appendix, Table A34). The one-way

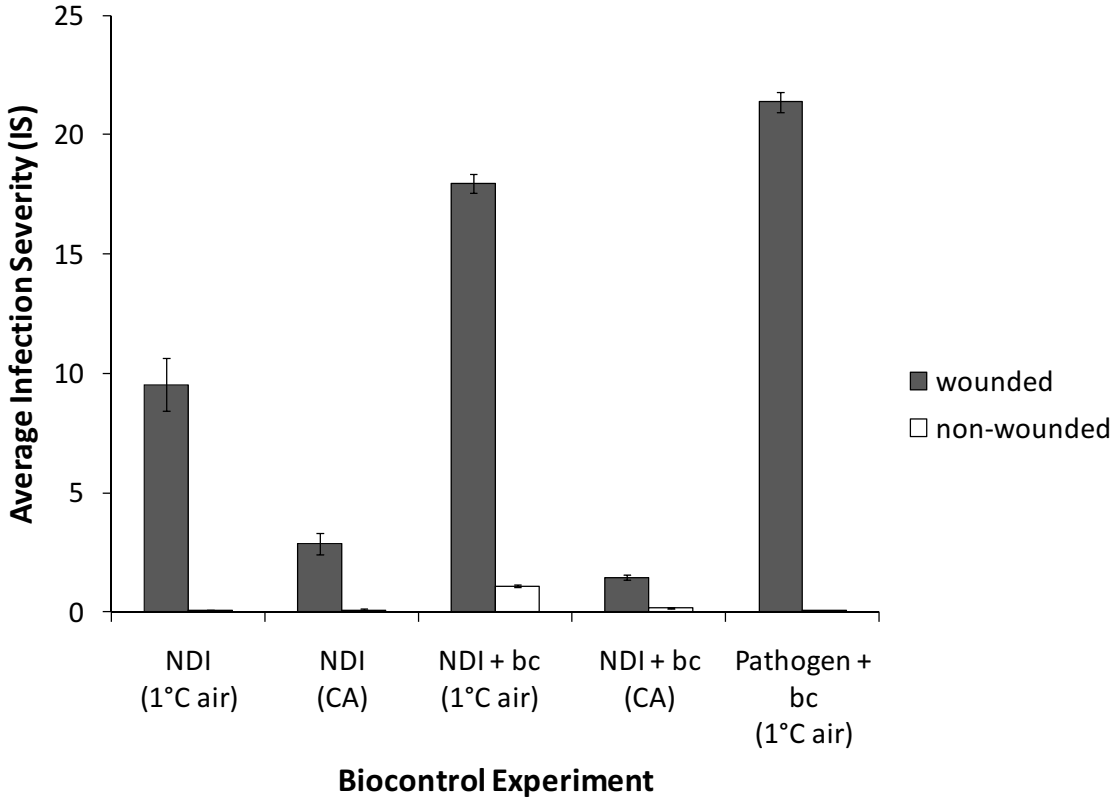

Figure 14. Average infection severity for all wounded and non-wounded apples from individual biological control treatments conducted in 2008. Experiments were as follows: 1) Natural disease incidence (NDI) at 1°C in air; 2) Natural disease incidence (NDI) in controlled atmosphere (CA); 3) Natural disease incidence (NDI) in combination with biocontrols (bc) at 1°C in air; 4) Natural disease incidence (NDI) in combination with biocontrols (bc) in CA; 5) Pathogen in combination with biocontrols at 1°C in air. Error bars represent standard error of the mean.

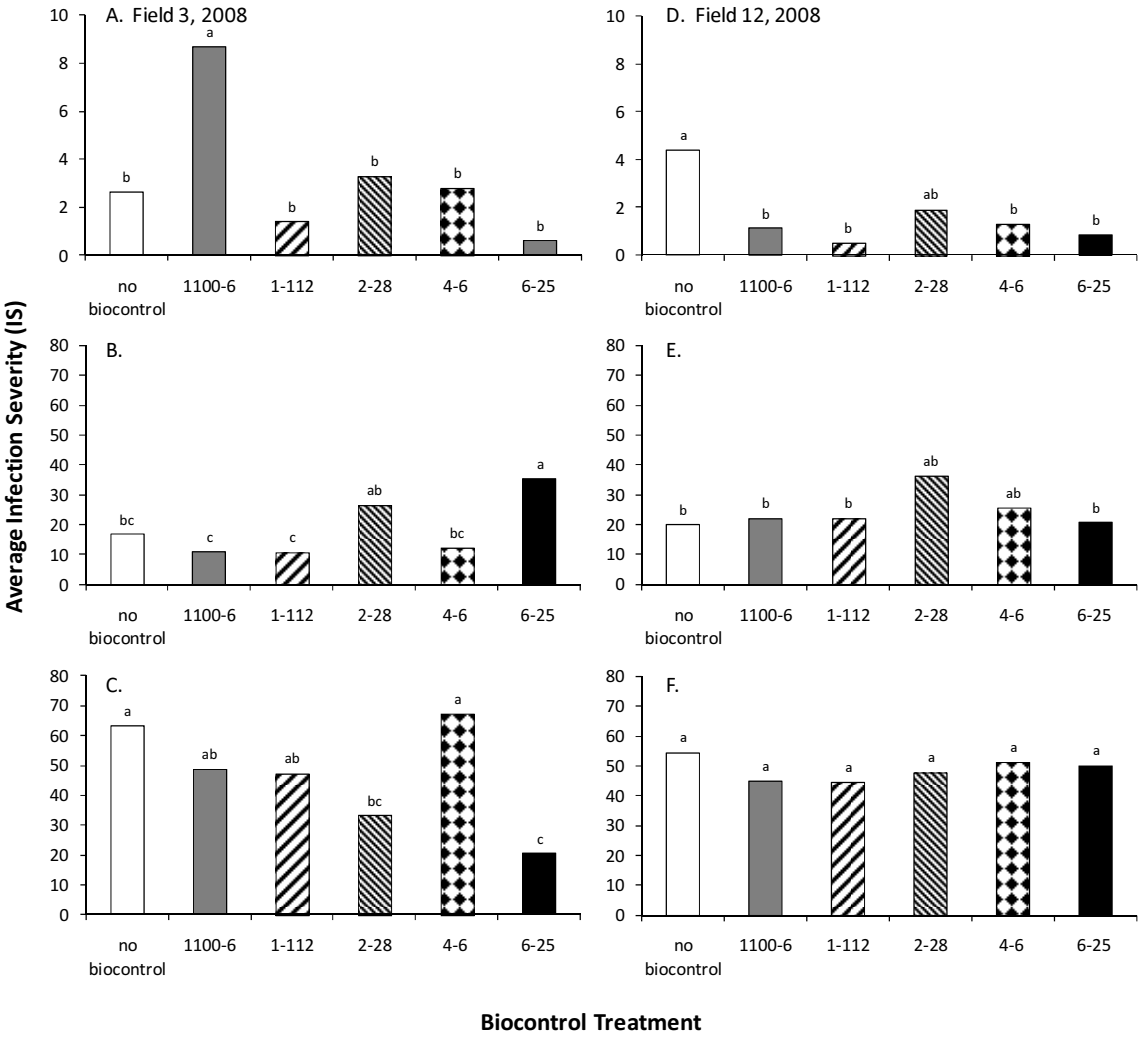

Figure 15. Effect of biological control isolates on natural disease incidence in Jonagold apples grown in Field 3 (A – C) and Gala apples grown in Field 12 (D – F) (harvest 2008) and stored for 2 (A&D), 4 (B&E) or 6 (C&F) months at 1°C in air. IS means with different letters within figures are different at significance levels $p \leq 0.05$ according to LSD test.

ANOVAs revealed a significant decrease in IS in Jonagold apples grown in Field 3 and inoculated with biological control isolates 1100-6, 1-112, 2-28, 4-6 and 6-25 (Figure 16; Appendix, Table A35). There was no significant reduction in IS in Gala apples grown in Field 12 and inoculated with one of five biological controls (data not shown; Appendix, Table A35). In Gala apples grown in the Kiran orchard, there was little overall disease. However, isolates 1100-6, 2-28, 4-6 and 6-25 significantly reduced IS compared to the non-inoculated control (Figure 17; Appendix, Table A35). For Red Delicious apples grown in the Reekie orchard, there was no significant effect on IS when inoculated with the biological control isolates (data not shown; Appendix, Table A35).

3.5.2 Post-harvest pathogen and biocontrol inoculations

Infection severity (IS) was determined with apples grown in Fields 3 and 12 and inoculated with one of three pathogens, *P. expansum*, *B. cinerea* or *M. piriformis* and one of five bacterial control isolates. One way ANOVAs were computed to determine if there were significant effects of the biological control agents on infection severities within each field.

For Jonagold apples grown in Field 3 and inoculated with *P. expansum* there was a significant reduction in IS in apples treated with isolates 4-6 and 6-25 compared to the non-inoculated control (Figure 18a; Appendix, Table A36). For Gala apples grown in Field 12 and inoculated with *P. expansum*, there was a significant reduction in IS in apples treated with isolates 1100-6, 2-28 and 6-25 compared to the non-inoculated control (Figure 19, Appendix, Table A36). In Jonagold apples grown in Field 3 and Gala

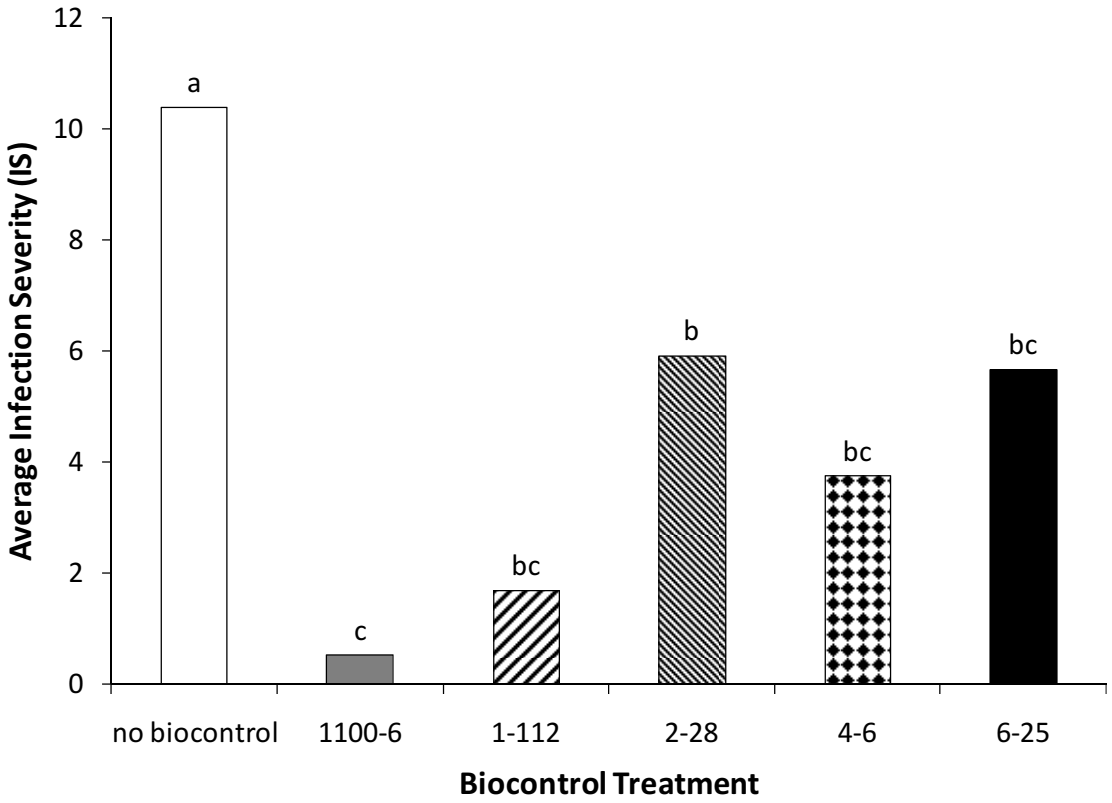

Figure 16. Effect of biological control isolates on natural disease incidence in Jonagold apples grown in Field 3 (harvest 2008) and stored for 4.5 months in CA. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

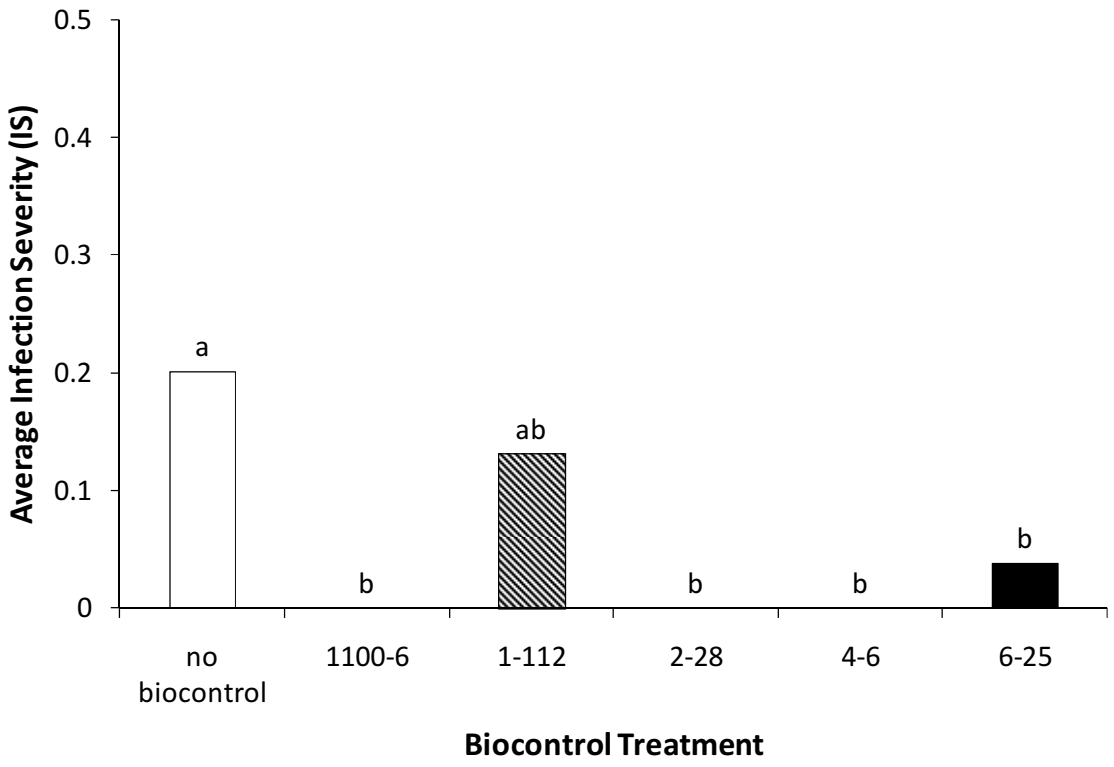

Figure 17. Effect of biological control isolates on natural disease incidence in Gala apples grown in the Kiran orchard (harvest 2008) and stored for 4.5 months in CA. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

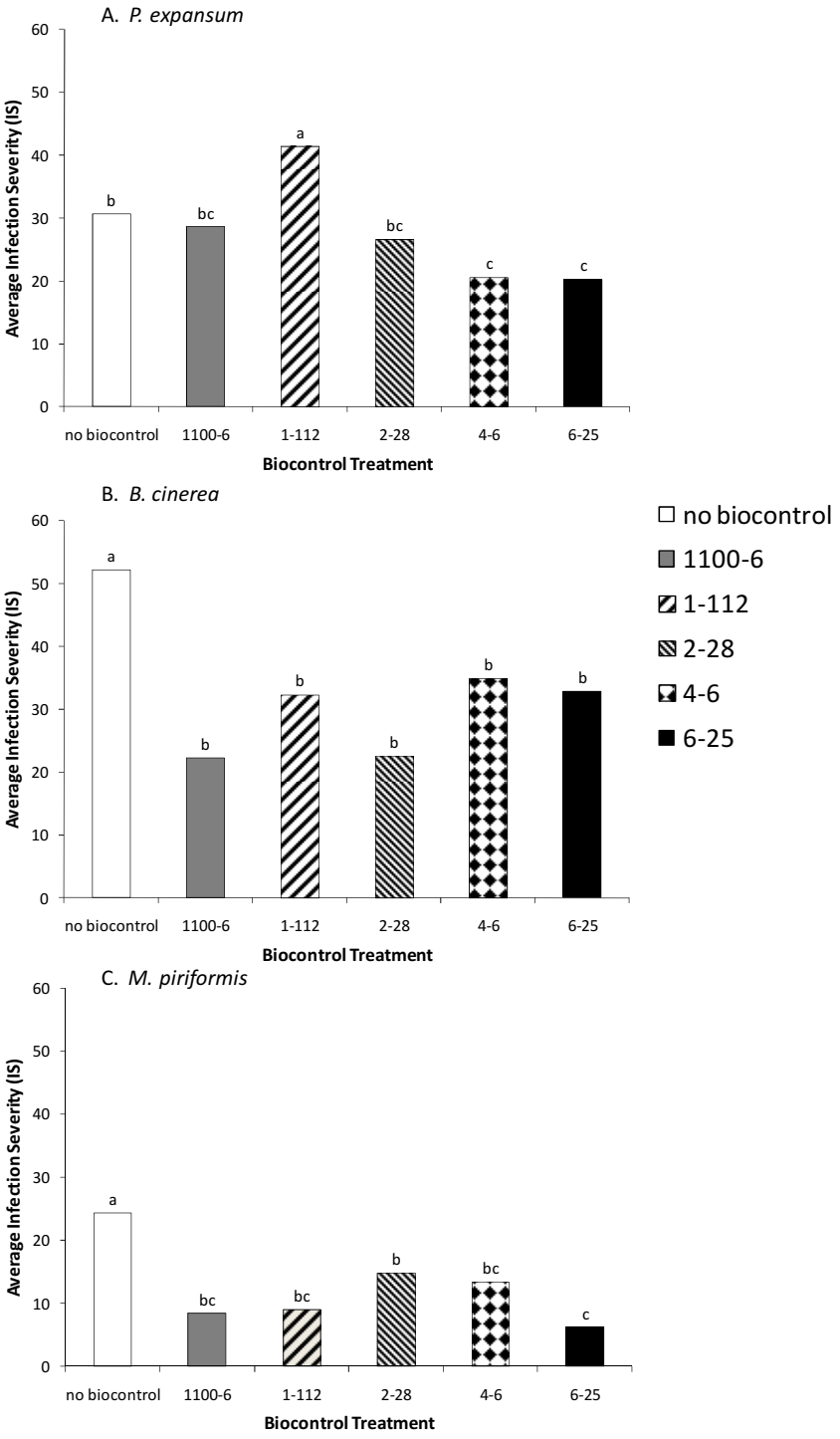

Figure 18. Effect of biological control isolates on infection severity in Jonagold apples grown in Field 3 (harvest 2008), inoculated with *P. expansum* (A) and *B. cinerea* (B) and stored for 2 months at 1°C in air and inoculated with *M. piriformis* (C) and stored for 1 month at 1°C in air . IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

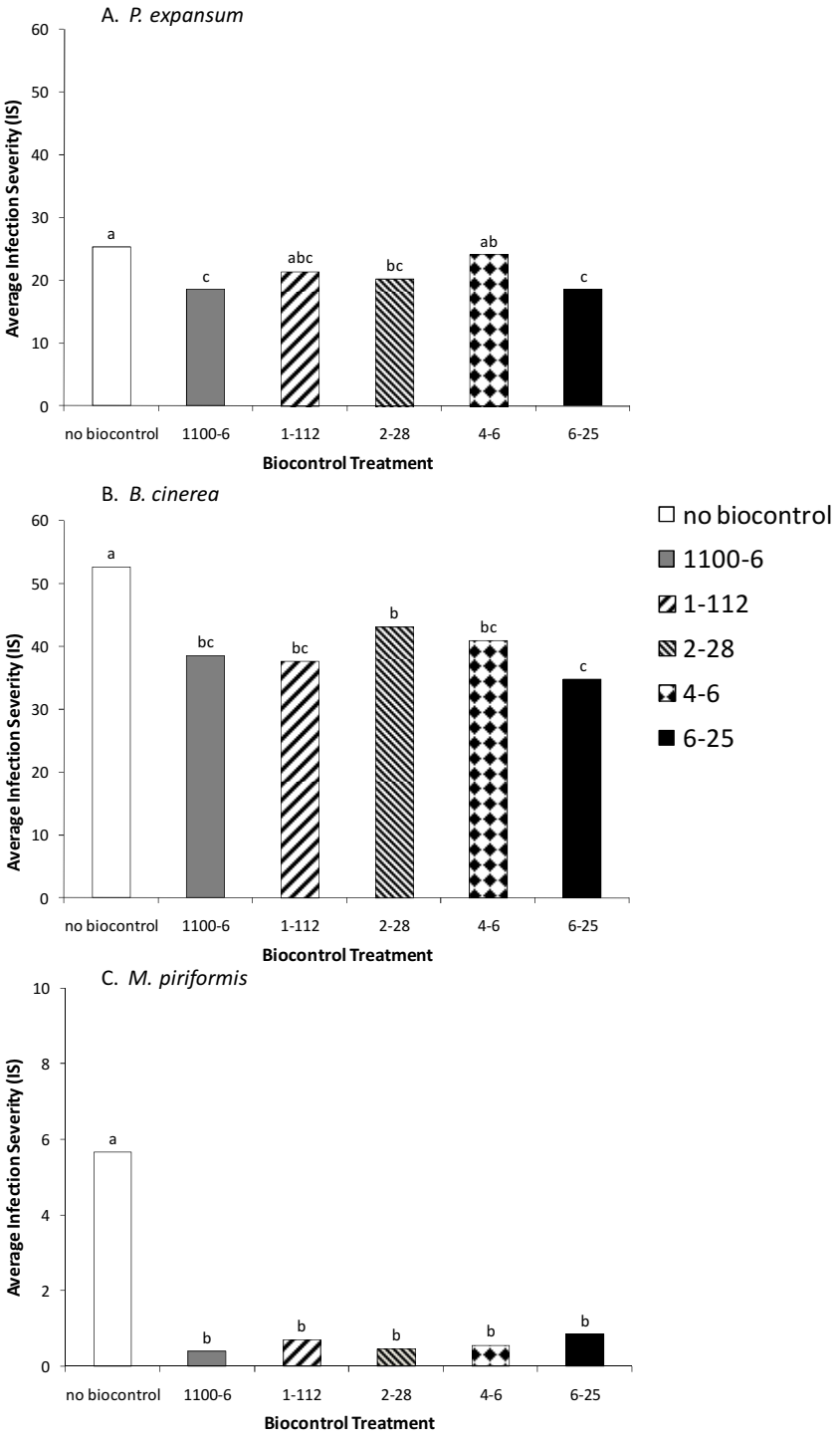

Figure 19. Effect of biological control isolates on infection severity in Gala apples grown in Field 12 (harvest 2008), inoculated with *P. expansum* (A) and *B. cinerea* (B) and stored for 2 months at 1°C in air and inoculated with *M. piriformis* (C) and stored for 1 month at 1°C in air. IS means with different letters are different at significance levels $p \leq 0.05$ according to LSD test.

apples grown in Field 12 and inoculated with either *B. cinerea* (Figures 18b and 19b; Appendix, Table A37) or *M. piriformis* (Figures 18c and 19c; Appendix, Table A38) there was a significant reduction in IS in apples treated with all isolates, 1100-6, 1-112, 2-28, 4-6 and 6-25.

Table 8 summarizes all biological control treatments in 2007 and 2008 and indicates the percent decrease (or increase) of all BCAs. Overall, results obtained from two years of experimental trials indicated that the bacterial isolate that provided significant control in the greatest number of treatments was *Serratia plymuthica* isolate 6-25 (51.7%) followed by *P. fluorescens* isolates 4-6 (41.4%), 1100-6 (37.9%), 1-112 (34.5%) and 2-28 (31%). The bacterial isolate that provided the greatest reduction in infection severity, in cases where significant control was exhibited, was isolate 1-112 followed by isolates 1100-6, 4-6, 6-25 and 2-28. Their respective average reduction in IS was 75.8%, 61%, 59%, 57.6% and 56.4%. In 2007, isolates 1100-6, 1-112 and 6-25 provided significant control of both *P. expansum* and *B. cinerea*. Isolate 4-6 provided significant control of *P. expansum*, only. Isolate 2-28 was the only bacterial strain to provide significant control of *M. piriformis*. In 2008, isolates 1100-6, 2-28, 4-6 and 6-25 provided significant control of all pathogens, *P. expansum*, *B. cinerea* and *M. piriformis*. Isolate 1-112 provided significant control of both *P. expansum* and *B. cinerea* (Table 8). All bacterial antagonists provided greater than 90% control in at least two experiments (Table 8).

Table 8. Summary of antagonist efficacy experiments for harvest 2007 and 2008.

						Percent disease decrease or (increase)[a]				
Experiment[b]	**Storage Type[c]**	**Field**	**Apple Variety**	**Storage Time (months)**	**1-MCP+/-**	**1100-6**	**1-112**	**2-28**	**4-6**	**6-25**
Harvest 2007										
NDI + bc	1C air	3	Jonagold	3	MCP +	NS	NS	NS	NS	NS
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	NS	NS
NDI + bc	1C air	12	Gala	3	MCP +	NS	NS	NS	NS	NS
	1C air	12	Gala	3	MCP -	NS	82.1	NS	49.2	39.9
Pex + bc	1C air	3	Jonagold	3	MCP +	78.7	94.3	NS	97.3	53.2
	1C air	3	Jonagold	3	MCP -	58.6	94.1	NS	96.9	96.2
	1C air	12	Gala	3	MCP+&-	NS	NS	NS	7.8	5.7
Bcin + bc	1C air	3	Jonagold	3	MCP +	94.7	98.1	NS	NS	96.1
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	NS	NS
Mpir + bc	1C air	3	Jonagold	3	MCP +	NS	NS	NS	NS	NS
	1C air	3	Jonagold	3	MCP -	NS	NS	NS	(98.1)	NS
	1C air	12	Gala	3	MCP +	NS	NS	91.6	NS	NS
	1C air	12	Gala	3	MCP -	NS	NS	NS	NS	NS
Harvest 2008										
NDI + bc	1C air	3	Jonagold	2	N/A	(69.6)	NS	NS	NS	NS
	1C air	3	Jonagold	4	N/A	NS	NS	NS	NS	(52.9)
	1C air	3	Jonagold	6	N/A	NS	NS	47.4	NS	67.5
	1C air	12	Gala	2	N/A	74.7	88.3	NS	70.4	80.8
	1C air	12	Gala	4	N/A	NS	NS	NS	NS	NS
	1C air	12	Gala	6	N/A	NS	NS	NS	NS	NS

						Percent disease decrease or (increase)[a]				
Experiment[b]	**Storage Type[c]**	**Field**	**Apple Variety**	**Storage Time (months)**	**1-MCP+/-**	**1100-6**	**1-112**	**2-28**	**4-6**	**6-25**
Harvest 2008										
NDI + bc	CA	3	Jonagold	4.5	N/A	94.9	83.8	43.0	63.9	45.5
	CA	12	Gala	4.5	N/A	NS	NS	NS	NS	NS
	CA	Kiran	Gala	4.5	N/A	100	NS	100	100	81.1
	CA	Reekie	Red Delicious	4.5	N/A	NS	NS	NS	NS	NS
Pex + bc	1C air	3	Jonagold	2	N/A	NS	(26.0)	NS	32.8	33.9
	1C air	12	Gala	2	N/A	26.7	NS	20.0	NS	26.7
Bcin + bc	1C air	3	Jonagold	2	N/A	57.4	38.1	56.8	33.1	37.1
	1C air	12	Gala	2	N/A	26.9	28.6	18.3	22.3	33.9
Mpir + bc	1C air	3	Jonagold	1	N/A	65.4	62.8	39.0	45.0	74.6
	1C air	12	Gala	1	N/A	93.0	87.6	91.8	90.0	85.0

a. Percentages indicate a decrease in IS; percentages in brackets indicate an increase in IS; NS = not significant
b. NDI = natural disease incidence; bc = biocontrol; Pex = *P. expansum*; Bcin = *B. cinerea*; Mpir = *M. piriformis*
c. CA = controlled atmosphere

3.6 Bacterial survival on apple using GFP

3.6.1 Plasmid confirmation

E. coli S-17 λ pir containing the *gfp*-plasmid, pAG408, grew on LB agar without antibiotics and LB agar supplemented with either 50 µg/ml kan or 30 µg/ml gen or both antibiotics combined. Alternatively, the bacterial isolates 1100-6, 1-112, 2-28, 4-6 and 6-25 only grew on agar plates that were not supplemented with antibiotics.

When pAG408 was digested with *Hind*III, the plasmid was cut into two fragments which measured approximately 2.1 and 3.6 kb (Figure 20). These fragments approximated the total plasmid size of 5.7 kb (Suarez et al. 1997). The undigested plasmid migrated to approximately 10 kb due to its supercoiled conformation

3.6.2 Confirmation of *gfp* transformation

After transformation of genetic material from the host (E. coli S-17 λ pir) into the donor (isolate 4-6), transconjugants were amplified by PCR using gfp-specific primers. Successful gfp amplification was observed when a band was present around 714 bp (Figure 21). The absence of banding suggested an unsuccessful transformation. Confirmation of *gfp* transformation was additionally confirmed by fluorescence microscopy *in vitro* (Figure 22) and *in vivo* (Figure 23).

3.6.3 Plasmid stability

After four successive days of sub-culturing and growth in the absence of antibiotic selection, 80.3 ± 6.8% of the transformed *P. fluorescens* isolate 4-6 (4-6-*gfp*) retained kanamycin and gentamicin resistance. These results, in combination with prior biological control experimental results, indicated that 4-6-*gfp* was an appropriate tool for

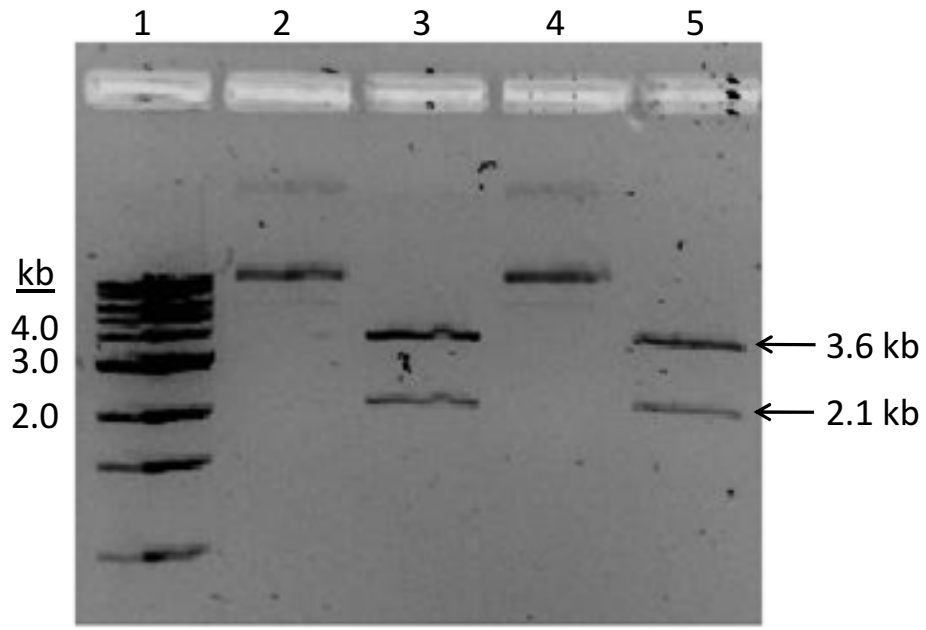

Figure 20. *Hind*III restriction digest of *gfp*-containing plasmid, pAG408. Lane 1: 1 kb ladder (New England Biolabs, Ipswich, MA); Lanes 2 and 4: undigested pAG408; Lanes 3 and 5: digested pAG408.

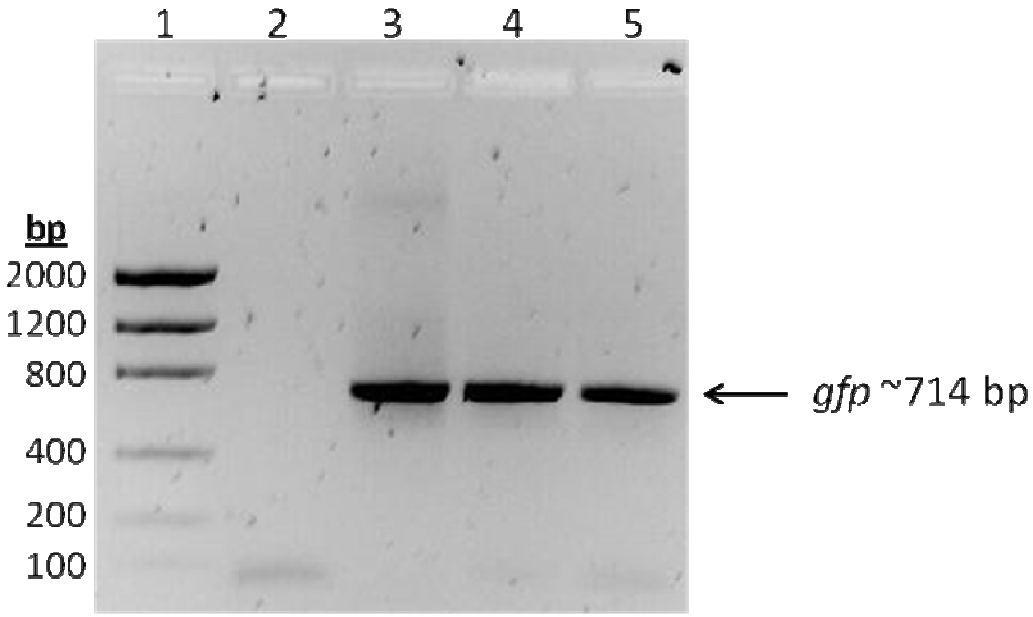

Figure 21. PCR amplification of *gfp* in *P. fluorescens* isolate 4-6 transformed with pAG408 using *gfp*-specific primers. Sample were loaded on a 1% TBE agarose gel and stained with Sybr Safe (Invitrogen, Carlsbad, CA). Lane 1) Low DNA Mass Ladder (Invitrogen, Carlsbad, CA); 2) negative control (ddH_20); 3) extracted plasmid, pAG408; 4 and 5) *gfp* transconjugant of bacterial isolate 4-6.

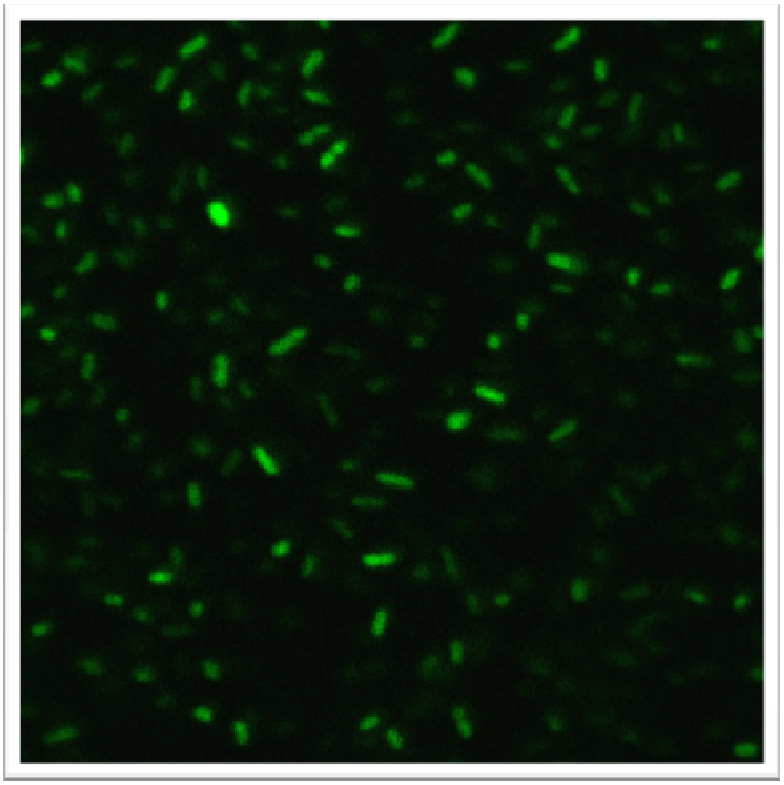

Figure 22. Fluorescence microscopy of *P. fluorescens* isolate 4-6 transformed with pAG408 exhibiting green fluorescence in ½ TSB supplemented with 50 µg/ml kan and 30µg/ml gen.

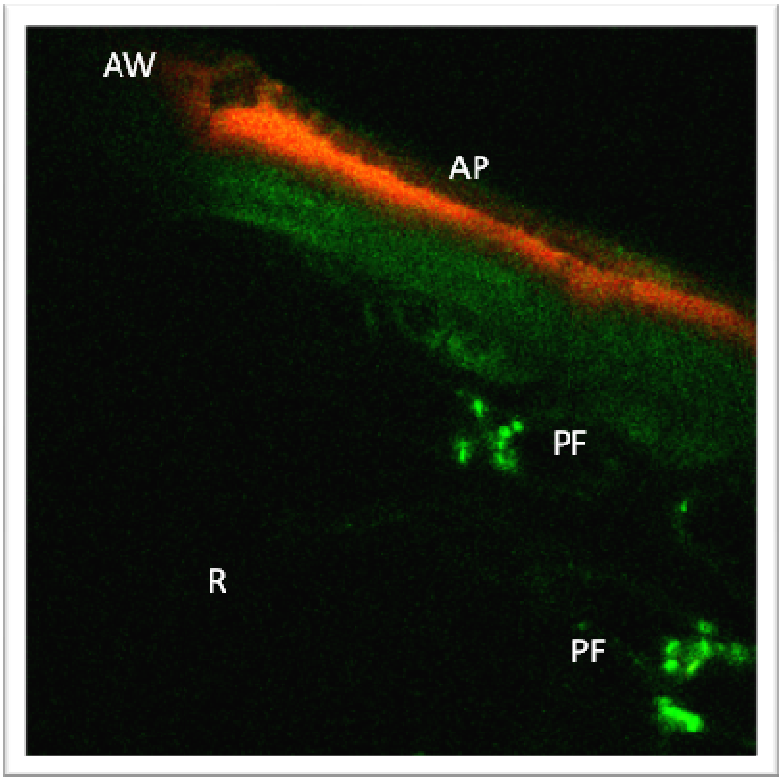

Figure 23. Fluorescence microscopy of an apple wound inoculated with *P. fluorescens* isolate 4-6-*gfp*. AP = apple peel; AW = apple wound; PF = *P. fluorescens* isolate 4-6-*gfp*; R = receptacle or fruit flesh.

studying bacterial survival on apples in the absence of antibiotic selection.

3.6.4 *P. fluorescens* 4-6-*gfp* fitness analysis

There was no significant difference between the growth rates (μ) (Figure 24; Appendix, Table A39) of *P. fluorescens* isolate 4-6-wild-type (0.71 ± 0.04/h) and 4-6-*gfp* (0.75 ± 0.06/h) and sole source carbon utilization patterns (Table 9).

Table 9. Sole carbon source utilization by *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* as indicated by Biolog Phenotye MicroArrays™ (Biolog, Inc., Hayward, CA) for sugars and organic acids commonly found in apple juice (Eisele and Drake 2005).

Apple Carbon Sources	**Bacterial Isolate**	
	4-6-wild-type	**4-6-*gfp***
Fructose	+	+
Sucrose	+	+
Glucose	+	+
Sorbitol	+	+
L-Malic Acid	+	+
Quinic Acid	+	+
Citric Acid	+	+
Fumaric Acid	+	+

3.6.5 Bacterial survival on apple

For standard bacterial suspensions of 4-6-*gfp*, there was a strong linear relationship between CFU/ml and relative fluorescent units (RFU) (r = 0.999) (Figure 25). The fluorescent values obtained by direct scanning of inoculated apple samples (Figure 26) were too low to extrapolate to cell densities from the formula y = 1.8041x + 6.5652 (Figure 25). However, trends could be discerned based on RFUs (Figure 26).

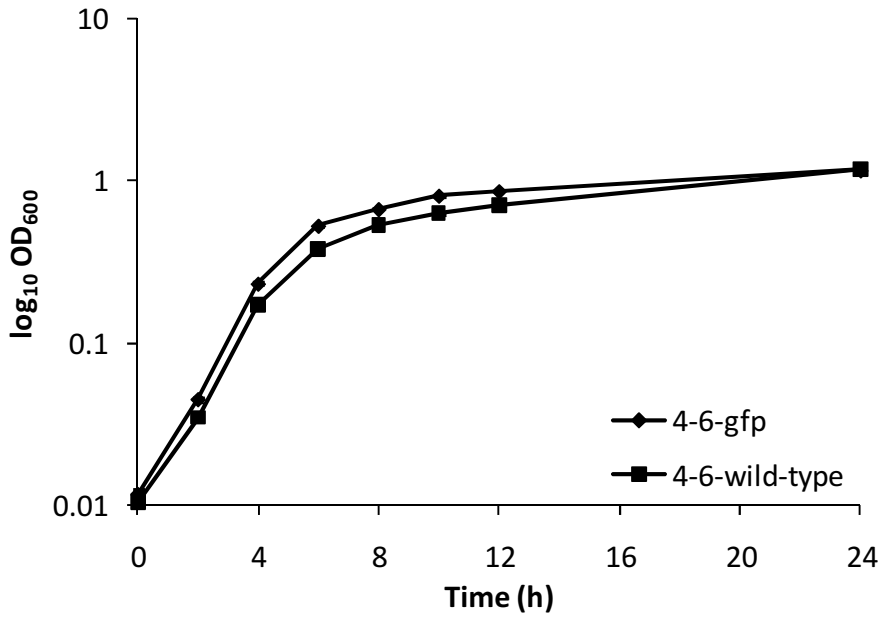

Figure 24. Growth characteristics of *P. fluorescens* 4-6-wild-type and *P. fluorescens* 4-6-*gfp* in half-strength TSB at 28°C for 24 h. Error bars represent standard error of the mean (n = 3).

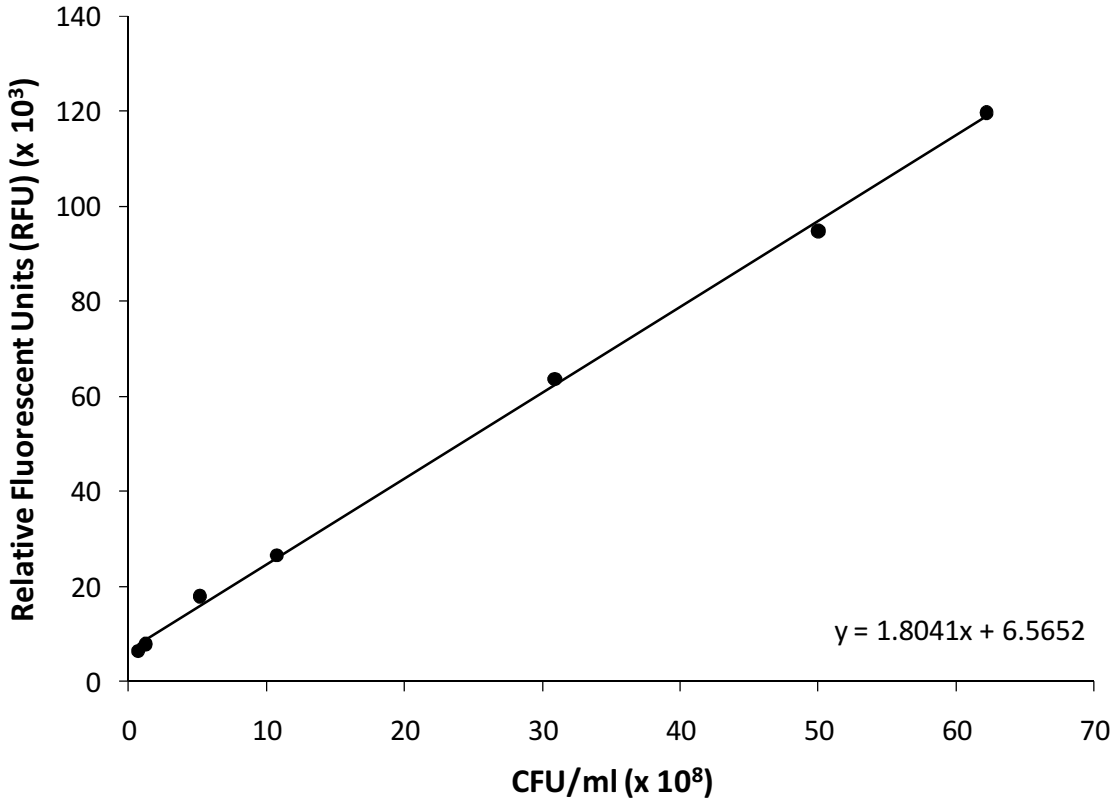

Figure 25. Relationship between *P. fluorescens* isolate 4-6-*gfp* cell density (CFU/ml) and relative fluorescent units (RFU) as determined by dilution plating and direct scanning (r = 0.999). Values are an average of three replicates.

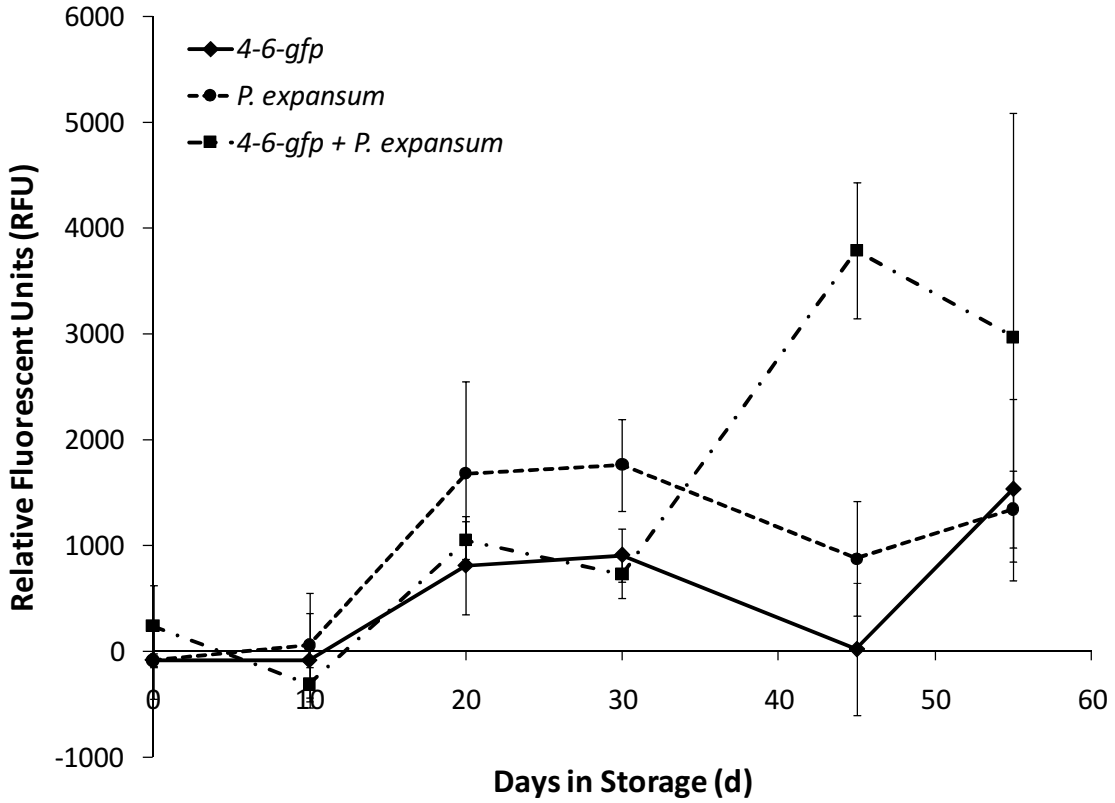

Figure 26. Bacterial survival of 4-6-*gfp* (♦), *P. expansum* (●) and *P. expansum* + 4-6-*gfp* (■) on Gala apples stored for 55 d at 1°C in air using the direct scanning method. Error bars represent standard error of the mean (n = 4).

Isolate 4-6-*gfp* alone increased during 20 d in storage then decreased after 30 d in storage, followed by an increase at 55 d in storage. *P. expansum* alone followed the same trend; however, the overall RFUs were higher. The RFUs obtained for 4-6-*gfp* in the presence of *P. expansum* initially decreased in the first 10 d of storage and increased until 45 d in storage, followed by a decrease at 55 d in storage.

The selective growth medium, PF agar supplemented with 50 µg/ml kan and 30 µg/ml gen, was effective in isolating 4-6-*gfp* and no other resident bacteria on the apple surface were detected. Over 55 d of dilutions and platings, there was no bacterial growth on the plates with extracted material from the untreated and *P. expansum* only-inoculated apples.

A two-way ANOVA indicated a significant interaction between bacterial strain, 4-6-wild-type and 4-6-*gfp*, and time (Appendix, Table A40). One-way ANOVAs revealed no significant differences in cell densities between apples inoculated with 4-6-*gfp* + *P. expansum* and apples inoculated solely with 4-6-*gfp* up to 20 d in 4°C storage (Figure 27; Appendix, Table A41). From 30 to 55 d of storage, populations of 4-6-*gfp* in the presence of *P. expansum* differed significantly from populations of 4-6-*gfp* alone. When challenged with the pathogen, bacterial populations remained constant until lesions developed after 30 d, after which populations rapidly declined. When 4-6-*gfp* was unchallenged with the pathogen, its population increased from 20 – 30 d in storage then declined to slightly greater than its population at time 0. From 0 to 40 d in storage, 4-6-*gfp* significantly reduced IS compared to the control *(P. expansum* alone) (Table 10; Appendix, Table A42). There was no significant reduction in IS of apples treated with 4-

6-*gfp* in the presence of *P. expansum* after 40 d in storage (Table 10; Appendix, Table A42).

Table 10. Effect of biological control isolate 4-6-*gfp* on infection severity in Gala apples inoculated with and without *P. expansum* and stored for 55 days at 1°C in air.
IS means with different letters within columns are different at significant levels $p \leq 0.05$ according to the least significant difference (LSD) test.

Treatments	**Days in storage**					
	10	**20**	**30**	**40**	**45**	**55**
Untreated	0.0	0.0	0.0	0.0	0.0	0.0
4-6-*gfp* alone	0.0	0.0	0.0	0.0	0.0	0.0
P. expansum alone	0.0	0.0	2.7 a	7.8 a	11.5 a	19.6 a
4-6-*gfp* + *P. expansum*	0.0	0.0	0.0 b	3.0 b	8.4 a	16.9 a

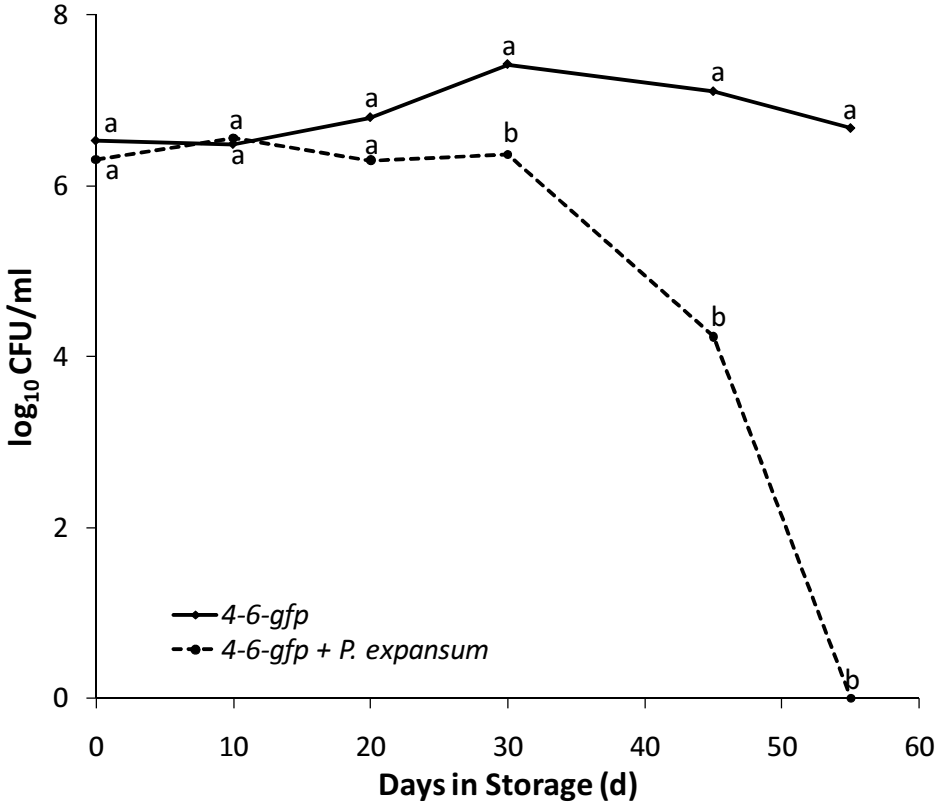

Figure 27. Bacterial survival of 4-6-*gfp* (♦) and 4-6-*gfp* + *P. expansum* (●) on Gala apples stored for 55 d at 1°C in air using the dilution plating method CFU/ml means with different letters within sampling times are different at significance levels $p \leq 0.05$ according to LSD test (n = 4).

CHAPTER 4: DISCUSSION

4.1 DNA macroarray for post-harvest apple pathogens

4.1.1 DNA macroarray quantification

In the current study, it was hypothesized that macroarray technology would accurately identify and quantify post-harvest apple pathogens throughout the growing season and that the frequency of pathogen detection would correlate with disease incidence post-harvest. This study reports that post-harvest pathogen quantification can be accomplished using DNA macroarray technology and that apple post-harvest pathogens can be detected throughout the apple growing season. Although many studies have used macroarray technology to detect and identify various plant pathogens and biological control microorganisms (Fessehaie et al. 2003; Izzo and Mazzola 2009; Le Floch et al. 2007; Levesque et al. 1998; Lievens et al. 2003; Robideau et al. 2008; Sholberg et al. 2005a; Tambong et al. 2006), few have quantified macroarray hybridization signal intensity (Lievens et al. 2005; Lievens et al. 2007; Sholberg et al. 2006) as we have done in this study.

Pathogen DNA was quantified by hybridizing 0 – 100 ng of pure culture DNA extracts of *P. expansum*, *B. cinerea* and *M. piriformis* to the macroarray. The detector probes or oligonucleotide sequences had varying levels of hybridization signal intensity. As found by Sholberg et al. (2006) who correlated greyscale value and cell density, a linear relationship was found between greyscale value and DNA concentration. However, in this study, the hybridization signals became saturated outside of the linear range at >3 ng for *P. expansum*, >12 ng for *B. cinerea* and >0.5 ng for *M. piriformis* (data not shown). Lievens et al. (2005), who quantified DNA concentrations of

phytopathogenic fungi, *Verticillium albo-atrum*, *Verticillium dahlia*, *Fusarium oxysporum*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*, also reported a linear relationship between signal intensity and DNA concentration. Linearity was established when 0.5 fmol of detector probe was spotted on a membrane and template concentrations ranged from 0.25 pg to 2.5 ng which was in the same range or somewhat lower than the linear range in the present study. Below and above 0.5 fmol, the curves deviated from linearity. Lievens et al. (2007) also reported that hybridization signals between 0.25 to 25 pg (0.00025 to 0.025 ng), that detect *Trichoderma* spp. and *Trichoderma hamatum*, a disease suppressive organism, resulted in a logarithmic relationship between signal intensity and template DNA concentration. This was lower than the linear range reported in the present study. Above 25 pg, the standard curves deviated from linearity.

Although a range of detector probe concentrations were not tested within this study, Lievens et al. (2005) noted that altering the amount of oligonucleotide spotted on the membrane can affect the range of DNA concentrations detected by a specific macroarray probe. Once a greater knowledge base exists about the level of post-harvest pathogenic DNA found throughout a crop's growing season and its correlation with disease manifestation, the DNA oligonucleotide detector probe concentrations can be altered to detect ranges typically found in field conditions. This will allow for more accurate DNA quantification and disease management. For enhanced and more precise quantification, it would be advantageous to simultaneously employ hybridization signal analysis with real-time PCR. The ability to accurately quantify a pathogen is useful in

plant disease management as information can be used to assess potential risk and disease development (Lievens and Thomma 2005).

4.1.2 Field monitoring of apple post-harvest pathogens

The current study was the first of its kind to monitor post-harvest pathogen presence via molecular technologies prior to commercial storage and disease development. Of the samples collected in each of the three growing season sampling periods, there was a tendency to detect total populations of all three post-harvest fungi more frequently in the early and mid-growing seasons of 2007 (56.6% - early; 60% - mid; 33.3% - late) and 2008 (67.6% - early; 63.2% - mid; 34% - late). This tendency to detect lower pathogen levels from air and plant tissue samples in the late growing season corresponds with the lower pathogen levels detected on whole apples collected at harvest (Table 4). In addition, the increase in early season fungal detection corresponds with results obtained by Teixidó et al. (1999) who observed total bacterial, yeast and filamentous fungal populations on Golden Delicious apples. However, they found an even greater increase in microorganisms at the apple bud stage followed by a decrease in populations at blossom. It was suggested that as buds form blossoms, there is a loss of external structure due to blossom drop, which accounts for the reported decrease in microorganisms after the apple budding stage. In the current investigation, samples were not collected at the apple bud stage. The rise in total fungal propagules may therefore correspond with precipitation events in the mid-growing season of 2007 and the early and mid-growing season of 2008 (Figure 1).

Of the three major post-harvest pathogens, *P. expansum*, on average, was detected most frequently (27.4. ± 3.4%) and was found throughout the growing season. In 2008 in

Fields 3 and 12 there was an increased detection frequency at the end of the growing season (Table 3). The abundance of *P. expansum* found within this study contradicts results of Amiri and Bompeix (2005) and Teixidó et al. (1999) who reported low levels of *Penicillium* inoculum within the orchard atmosphere. However, the aerial sampling period of Amiri and Bompeix (2005) was between July and September which excludes the early growing season.

In contrast to *P. expansum, B. cinerea* was detected the least number of times (6.2 ± 1.4%) and found throughout the growing season of 2007 and 2008. The most important infective units of *B. cinerea* are the conidia produced in late winter and early spring from over-wintering mycelia and/or sclerotia on host tissue or soil surfaces (Elad et al. 2007); grey mold is rarely seen in the field (Rosenberger 1990). The low levels of *B. cinerea* as indicated by the macroarray correspond with these results.

M. piriformis was detected the second most frequently (19.2 ± 1.4%) and found most frequently in the mid-growing season of 2007 and the early to mid-growing season of 2008 in DNA isolated from plant tissue samples. These findings support Guo et al. (1999) who found an increase in *M. piriformis* populations within orchard soil from December to March and April, followed by a decrease afterwards. It was suggested that the increase in *M. piriformis* corresponds with apples left on the orchard floor and cool weather. Alternatively, these results contradict those found by Dobson and Spotts (1988) who recovered no *M. piriformis* from orchard air but found an abundance of *B. cinerea* and *P. expansum*. Sporangiospores of *M. piriformis* are soilborne (Michailides and Spotts 1986); greater than 75% are found in the top 2 cm of the soil profile and are dispersed primarily by rain splash and insects (Dobson and Spotts 1988). The detection

of an abundance of *M. piriformis* in this study may correspond to precipitation bursts found in both 2007 and 2008 (Figure 9). Sporangiospores may be aerosolized by the falling rain and then trapped by the aerial spore sampler.

Earlier studies, as mentioned above, that detected fungal abundance throughout the orchard growing season utilized culturing methods for fungal detection and identification (Amiri and Bompeix 2005; Dobson and Spotts 1988; Teixidó et al. 1999). In the case of *P. expansum* and *M. piriformis*, the macroarray detected the presence of pathogens not commonly found within the orchard. Culturing methodologies for detection and relative quantification may underestimate fungal abundance, thus showing the power of macroarray technology.

Data obtained using the macroarray may be useful in developing a disease forecasting model such as that presented by Spotts et al. (2009). For future experiments, it would be beneficial to correlate pathogen findings throughout the growing season with species-specific identification of natural disease incidence found within the packinghouse. In this study, a positive correlation was only evident between natural disease incidence and percent pathogens detected in the late-growing season for aerial and plant tissue samples analyzed together ($r = 0.79$) and for aerial samples ($r = 0.74$) when analysed separately from plant tissue samples. This finding corresponds with Lennox et al. (2003) who observed a significant correlation between the density of *B. cinerea* conidia on the pear fruit surface and grey mold disease incidence in cold storage. In addition, Walter et al. (1997) found a strong correlation between *B. cinerea* berry fruit contamination and latent boysenberry infection in New Zealand. Given the cost and time involved in collecting and analyzing DNA for macroarray identification and

quantification, the majority of samples could be collected, for example, near the end of the growing season. It should be noted, however, that Spotts et al. (2009) found that in creating an at-harvest prediction model for grey mold risk in pears, surface DNA was the least important predictor in the model as opposed to orchard rating and fungicide applications. Also of note, within this study, there was a negative correlation between percent pathogens detected in the early-growing season and natural disease incidence when plant tissue samples were analysed separately from aerial samples (r = -0.79). This data suggests that perhaps plant tissue samples collected in the early-growing season are not optimal predictors for natural disease incidence, thereby supporting the correlations mentioned above. More work is required in order to determine the relatedness between growing season pathogen levels and post-harvest disease prevalence.

The variability in the results of this study suggests that a multitude of factors contribute to disease incidence found post-harvest. Coley-Smith et al. (1980) proposed that disease outbursts may be a result of complex interactions between the production and dispersal of various inocula, infection pathogenesis, pathogen survival along with temperature, rainfall, humidity and crop protection, nutrition and phenology. Variability may also be explained by the application of pre-harvest fungicides and their effect on resident microflora. According to Sholberg and Boulé (2008), the application of Nova®, a fungicide used to control powdery mildew, reduced apple leaf and fruit microflora in two years of study. In the current study, Nova® was applied to Field 12 on May 15, 2007 which may help to explain the reduced pathogen loads when compared to the 2008 results (Figure 6). It should not be forgotten that the majority of post-harvest diseases are

a result of pathogens entering the fruit via wounds, all of which should be considered in accurate disease forecasting models.

4.2 Biological control of post-harvest pathogens

Five putative bacterial control agents were applied to apples harvested from commercial and experimental orchards in the Okanagan Valley, BC. It was hypothesized that one or more of the bacterial antagonists would provide significant pathogen control in semi-commercial and commercial storage conditions. This study showed that the four *P. fluorescens* isolates and one *S. plymuthica* isolate exhibited some control and antagonistic efficacy against post-harvest apple pathogens (Table 8). As indicated, *Serratia plymuthica* isolate 6-25 provided significant control in the greatest number of treatments (57.6%) (Table 8). These results suggest that this bacterium may provide control against a variety of post-harvest diseases and storage conditions and its use may be applied to other crops. Using *S. plymuthica* as a BCA, however, is a challenging endeavour as concerns surround its biosafety, a major consideration when selecting an antagonist (Droby et al. 2009). *Serratia plymuthica* belongs to the family Enterobacteriaceae. Members of this family, such as *Escherichia coli* strain O157:H7, have been known to cause human infections (Health Protection Agency 2007). Although opportunistic pathogenic strains of *S. plymuthica* have been isolated (Carrero et al. 1995; Clark and Janda 1985; Domingo et al. 1994) infection is rare compared to other members of the genus, *Serratia*, or of the family, Enterobacteriaceae.

Despite concerns surrounding its use as a biological control agent, *S. plymuthica* isolate 6-25 provided significant control in this study, against apple post-harvest pathogens, *P. expansum*, *B. cinerea* and *M. piriformis*. *S. plymuthica* biological control

efficacy has also been demonstrated by other research groups, although not on post-harvest apple pathogens. *S. plymuthica* strain HRO-C48 has been studied to control *Verticillium dahliae,* the causal agent of Verticillum wilt in oil-seed rape (Müller and Berg 2008). HRO-C48 also suppressed growth of *V. dahliae* and *Phytophthora cactorum* in strawberries (Kurze et al. 2001) and a commercial product based on strain HRO-C48 has been developed, RhizoStar® (e-nema GmbH, Raisdorf, Germany) (Müller and Berg 2008). *S. plymuthica* strain 5-6 also showed significant control of dry rot of potato caused by *Fusarium sambucinum* (Gould et al. 2008). The post-harvest biological control provided by *S. plymuthica* isolate 6-25 of this study should not be disregarded as the potential exists, as evident by other studies, for commercial application.

This study also showed that pseudomonads, specifically *P. fluorescens*, are also capable of providing control against post-harvest pathogens. According to Stockwell and Stack (2007), *Pseudomonas* spp., have been studied for decades as model organisms for biological control of plant diseases. In this study the greatest reduction in IS, in cases where significant control was exhibited, was with *P. fluorescens* isolate 1-112 followed by 1100-6 (Table 8). Other pseudomonads are also being studied for their efficacy against post-harvest pathogens. Mikani et al. (2008) reported significant control of *Botrytis mali*, formerly thought of as *B. cinerea* and recently revived by O'Gorman et al. (2008), using 10 strains of *P. fluorescens* isolated from leaf surfaces and apple fruit. Similar to the level of control found in this study, the results of Mikani et al. (2008), reported approximately 34.9% to 95.9% *B. mali* inhibition on Golden Delicious apples after 25 d at 5°C. In addition, Zhou et al. (2001) studied four strains of *Pseudomonas syringae* that were isolated from the phyllosphere of apple trees and controlled blue

mold, following inoculation by pipette, by 64 – 70% at 4°C after 28 d. The bacterial isolates also controlled grey mold, but to a lesser extent. Similar to methods used in this study, Zhou et al. (2001) also inoculated apples by dipping and found that a *P. syringae* isolate controlled blue mold by 93% after 133 d at 1°C in air, with the overall incidence of blue mold at 30%. In many cases, (Figure 12a) the bacterial isolates used in the present study exhibited similar and greater levels of control than those reported by Mikani et al. (2008) and Zhou et al. (2001). The effectiveness of these isolates may be due to the unique environment in which they were isolated. Many BCAs are found on the fruit surface or directly within a wound. The microbial agents used in this study, however, were isolated from the rhizosphere of legumes grown in cold Saskatchewan soils and may be more adapted to cold storage conditions. Droby et al. (2009) outlined the importance of antagonist selection and the use of a variety of screening procedures in finding effective BCAs. An assortment of selection techniques would increase biocontrol species diversity and therefore the potential for a successful product. These experiments illustrate the potential of *S. plymuthica* and *P. fluorescens* isolates for biological control application and commercialization.

4.2.1 Bacterial antagonists and CA storage

One type of commercial storage application used in this study was controlled atmosphere (CA). This research confirmed the hypothesis that one or more antagonists would provide control in commercial conditions. In all experiments, the lowest disease levels were found in apples stored in CA storage for 4.5 months following harvest in 2008. Many suggestions have been made regarding the selection criteria for potential BCAs (Wilson and Wisniewski 1989). One such criterion is the ability of the antagonist

to be compatible with current post-harvest commercial processing procedures such as CA (Janisiewicz and Korsten 2002; Wilson and Wisniewski 1989). This current study indicated that the selected antagonists were compatible with CA storage practices and may actually extend apple storage time due to enhanced pathogen suppression when compared to storage at 1°C. For example, the highest disease incidence recorded in CA was in Jonagold apples which reached an average IS of 10.39 ± 1.6 after 4.5 months in storage (Figure 10). The biological antagonists provided significant control against naturally occurring diseases in all orchards but Field 12 and the Reekie orchard (data not shown). In comparison, for Jonagold and Gala apples grown in Fields 3 and 12 then stored at 1°C in air for 4 months, non-inoculated controls had IS levels of 15.65 ± 5.0 (Figure 16b) and 19.90 ± 4.8 (Figure 16e), respectively. These values were almost double the infection severities found in CA. In both Jonagold and Gala apples harvested from Fields 3 and 12, respectively, the bacterial antagonists did not provide a significant reduction in IS after 4 months in storage (Figure 16b and e). However, isolate 6-25 reduced IS in Jonagold apples grown in Field 3 after 6 months in storage (Figure 16c). The decrease in apple disease supports the findings of Smock (1979) as CA effectively delayed the onset of storage decay.

In the CA-stored apples, natural disease incidence was very low even after 4.5 months of storage, except in the case of Jonagold apples from Field 3 which appeared to be due primarily to *P. expansum* (based on phenotypic observation). The gas composition of a packinghouse can directly or indirectly suppress fungal decay. Direct inhibition affects mycelium development and spore germination of resident pathogens. Alternatively, storage conditions also affect senescence and ripening of hosts, which, in

turn, affects their ability to resist infections, thereby indirectly controlling fungal decay (Barkai-Golan 2001). The implementation of CA is a balance between O_2 and CO_2 concentrations. General recommendations include 1 – 2 % O_2 and 0.5 – 2% CO_2. Concentrations outside of these ranges may lead to fruit injury which can affect fruit flavour and appearance (Kupferman 2003). However, post-harvest pathogens are only strikingly suppressed after O_2 concentrations have been lowered to less than 1% and CO_2 concentrations increased to greater than 10% (El-Goorani and Sommer 1981). The CA storage conditions in this study were approximately 1.5% O_2 and 1.5% CO_2, a range in which, if the conditions were right, may lead to the fungal outbreak that was observed in the Jonagold apples.

In the current study, the antagonistic effects of the bacterial isolates were difficult to observe because there was little natural disease present; if little disease was present, then there was little disease to control. However, the outbreak in Jonagold apples from Field 3 demonstrated the antagonistic potential of the bacteria because significant disease was present. While all of the antagonists demonstrated significant control, isolate 1100-6 and 1-112 were particularly effective, providing 94.9% and 83.8% reduction in disease symptoms in Jonagold apples from Field 3 (Figure 17). Although not directly inoculated with a pathogen, these BCAs performed better than the commercially registered BCA, Bio-Save 10 LP™, in studies conducted by Janisiewicz and Jeffers (1997) and Zhou et al. (2001). Using Bio-Save™ during 133 and 123 days in CA storage, Zhou et al. (2001) reported a 57% and a 73% reduction of blue mold in Empire and Delicious apples, respectively. It would be beneficial to test the efficacy of the bacterial antagonists against artificially inoculated pathogens under CA conditions as in Conway et al. (2007),

Janisiewicz et al. (2008) and Tian et al. (2002). However, during the present study, permission for pathogen inoculation within a commercial storage facility was not given.

4.2.2 Bacterial antagonists and 1-MCP

Another type of commercial storage application tested in this study was the use of 1-MCP. This research confirmed the hypothesis that one or more antagonists would provide control in commercial conditions, although to a lesser extent. 1-MCP was applied to Jonagold apples grown in Field 3 and Gala apples grown in Field 12 in the biological control experiments using apples from the 2007 harvest. It was postulated that the application of 1-MCP would result in a decrease in disease incidence and severity of non-inoculated wounded apples or apples inoculated with putative biological control agents and/or post-harvest pathogens. However, when the overall effect of 1-MCP was examined, a reduction in infection severity occurred in only 14% of the experimental treatments. For example, 1-MCP application reduced IS caused by natural disease incidence in Jonagold apples from Field 3 (Table 5) by 51.8% and in Gala apples from Field 12 (Figure 11) by 67.1%. Variable levels of control confirm findings of Saftner et al. (2003) who studied the effects of pre-storage heat, CA storage and pre- and post-application of MCP on inhibition of fungal decay and maintenance of Golden Delicious fruit quality. Pre-storage treatment of MCP reduced lesion size (mm) of fruit inoculated with *B. cinerea* and *P. expansum* by 34% and 17.9%, respectively. Post-storage application of MCP had no effect on reducing lesion diameter. Variable responses to 1-MCP application are not uncommon. Watkins (2008) indicated that the apple is a fruit with many cultivars that differ in their ripening rates, harvest criteria, post-harvest handling practices and storage periods in air and CA. In addition, time between harvest

and 1-MCP treatment and repeated application have variable effects in disease resistance responses. In the current study, when a decrease in infection severity was observed, it appeared to be primarily in experiments where there was no artificial inoculation of *P. expansum*, *B. cinerea* or *M. piriformis*; the only pathogens present were those that occurred naturally.

Contradictory to the intended outcome of 1-MCP application, some treatments in the present study resulted in an increase in infection severity of apples treated with 1-MCP, especially those apples inoculated with *B. cinerea* (Figure 12b). An increase in decay after 1-MCP application was also observed by Janisiewicz et al. (2003) and Leverentz et al. (2003) on Golden Delicious apple fruit and by Bedford et al. (2002) who reported an increase in *B. cinerea* decay when apples were fumigated with 1-MCP. Commercial application of 1-MCP as SmartFresh® is thought to delay apple ripening by preventing ethylene production via preferential binding to the ethylene receptor site and extending the action of natural defence mechanisms due to delayed ripening (Blankenship and Dole 2003). However, the reverse was observed in some cases as 1-MCP appeared to hinder plant defence mechanisms. According to Marcos et al. (2005), endogenous ethylene may be an important component in plant resistance and defence gene regulation; the application of 1-MCP may therefore compromise a plant's defence response system (Jiang et al. 2001).

Despite the controversy surrounding chemical control agents, fungicides still remain the preferred choice for the prevention of post-harvest diseases. Although fungicide-resistant pathogens have increased within the population, chemical control provides a consistent and effective reduction in disease. Within the agricultural

community, there is a relatively high level of scepticism surrounding the use of biological control products (Droby et al. 2009). Commercially acceptable decay levels are generally below 5% (Janisiewicz and Korsten 2002) and because of the inconsistency and variability of biofungicides, the packinghouse industry will often choose the most economically viable option that consistently reduces rot.

Disease control was exhibited by the bacterial antagonists in this study, but efficacy was highly variable, as observed by other researchers. The tritrophic interaction between host, pathogen and antagonist is a highly complex one that demands a more sound and fundamental understanding. In addition to exploring the mechanism(s) of action for fungal suppression, combination and alternative treatments for enhanced efficacy should be explored. Such alternate biocontrol treatments, outlined by Sharma et al. (2009) include, for example, the addition of low doses of fungicides, salt additives, or nutrients and plant products, as well as the use of mixed cultures or manipulation of the physical environment. Rapid antagonist colonization of fruit wounds is critical to controlling decay; therefore, enhancements leading to improved wound colonization should be further explored (Janisiewicz and Korsten 2002).

4.3 Bacterial survival on apple

Acquiring basic knowledge about potential antagonists will provide a greater understanding into the mechanisms of actions, which will, in turn, offer insight into means for achieving more consistent control. Disease prevention relies on a quantitative relationship between antagonist and pathogen (Janisiewicz 1988) and knowledge of antagonist cell number during time in storage will assist in enhanced disease suppression (Etebarian and Sholberg 2005). In the present study, *Pseudomonas fluorescens* isolate 4-

6 was tagged with GFP to determine the survival characteristics of this antagonist alone and when challenged with *P. expansum*. It was hypothesized that green fluorescent protein would facilitate visualization of bacterial colonization and survival on apple surfaces. In this study, *gfp* did facilitate bacterial visualization, but indirectly as pAG408 provided antibiotic resistance of isolate 4-6 which permitted selective plating that excluded other organisms during survival analysis.

Bacterial concentrations were determined using direct scanning and colony counts. There was a strong linear relationship between fluorescent intensity and cell density (Figure 26). These results corresponded with those found by Etebarian and Sholberg (2005) who studied population levels of *P. fluorescens* isolate 1100-6 labelled with GFP using direct scanning, cell counts and microscopy. This and the current study illustrated that measuring fluorescent intensity as a means of estimating cell concentration was an effective method of population assessment. The initial inoculation concentration of 4-6-*gfp* (10^8 CFU/ml), however, combined with the method of recovery, resulted in fluorescent readings that were not high enough to quantify using the linear equation $y = 1.8041x + 6.5652$ (Figure 26). Using a Fast Prep machine for tissue maceration as in Etebarian and Sholberg (2005) or in Etebarian et al. (2005) may overcome issues incurred by extraction.

Despite low values, the raw microplate data were compared to plate counting data. Relative fluorescent units (RFUs) (Figure 27) and cell densities (CFU/ml) (Figure 28) of isolate 4-6-*gfp* followed similar patterns; when cell counts increased, so did RFUs. However, there was an inverse relationship between RFUs and CFU/ml for 4-6-*gfp* inoculated in the presence of *P. expansum*; RFUs increased while cell counts decreased.

Two possible explanations are that *P. expansum* auto-fluoresces or that the decayed apple tissue auto-fluoresces. The latter explanation corresponds with the formation of lesions at 30 d (Table 10). Increased antagonist populations after 30 d of storage were also found by Janisiewicz and Marchi (1992) who reported a 10 – 100 fold increase in populations of *Pseudomonas syringae* inoculated on pears stored at 1°C. Etebarian and Sholberg (2005) also found an increase in *P. fluorescens* isolate 1100-6 GFP on apples after 35 days in 5°C storage. However, unlike the survival characteristics of 4-6-*gfp* + *P. expansum* in this study, 1100-6 GFP + *P. expansum* maintained as high or higher cell numbers than 1100-6 GFP alone. Low cell counts after lesion development may be attributed to difficulties in extracting apple cores from completely decayed tissue. Even so, after 30 d at 1°C in air storage, 4-6-*gfp* appeared to lose some of its antagonistic capabilities (Table 10). Information such as this should be considered when implementing control strategies for optimal post-harvest disease management.

CHAPTER 5: CONCLUSIONS

This thesis built upon preliminary research and addressed the following three objectives: 1) to validate the performance of DNA macroarrays under field conditions; 2) to assess bacterial antagonist performance under semi-commercial and commercial conditions and; 3) to determine how long and in what capacity a selected antagonist will colonize the fruit surface.

The first hypothesis addressed the first objective and stated that macroarray technology would accurately identify and quantify post-harvest apple pathogens throughout the growing season and that disease prevalence would correlate with disease incidence post-harvest. Results confirmed that in two years of field trials, the DNA macroarray is a fast and easy technique capable of detecting apple post-harvest pathogens throughout the apple growing season (Table 3). Commercial application of this diagnostic and quantitative technique would provide accurate information to assist growers and field personnel in crop management. A positive correlation existed between pathogen detection at the end of the growing season and natural disease incidence found post-harvest. More work is required to obtain information about the importance of monitoring pre-harvest pathogen loads and their contribution to post-harvest disease.

The second hypothesis addressed the second objective and stated that one or more of the bacterial antagonists would provide significant pathogen control in semi-commercial and commercial storage conditions. Results indicated that despite variability between years, variety and biological control efficacy, the potential BCAs demonstrated control at 1°C in air and particularly in CA storage (Table 8). Biocontrol efficacy in combination with 1-MCP was less effective and the application of 1-MCP greatly

increased disease levels with *B. cinerea* artificial inoculation (Figure 12b). Combining some or all of the bacterial control agents used in this study in conjunction with other organic amendments such as salts (ie. sodium bicarbonate) or manipulating the physical environment (ie. heat treatment) may provide consistency in control which the industry demands.

The third hypothesis addressed the third objective and stated that the use of green fluorescent protein would facilitate visualization of bacterial colonization and survival on apple surfaces. Results indicated that green fluorescent protein was an effective technique to monitor population levels as the *gfp*-marked strain behaved similarly to the wild-type strain (Figure 25; Table 9). The fluorescence levels obtained by direct scanning, however, were not sufficient to distinguish 4-6-*gfp* from background auto-fluorescence. The presence of the *gfp*-containing plasmid within *P. fluorescens* isolate 4-6 therefore provided an indirect method of monitoring population levels as pAG408 contained antibiotic resistant genes that allowed for strain-specific isolation from an inoculated apple wound. Bacterial populations were found to initially increase then decrease within an apple wound during 55 d in storage at 1°C (Figure 28). In the presence of *P. expansum*, 4-6-*gfp* populations declined, which coincided with *P. expansum* lesion development. The information obtained within this study will assist the fruit industry in detecting, quantifying and biologically controlling post-harvest pathogens of pome fruit.

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APPENDIX

Appendix A

Table A1. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	2628.336	4.585	0.0619
B. cinerea	2	384.596	1.857	0.2357
M. piriformis	2	573.896	0.429	0.6697

Table A2. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	327.860	0.134	0.8766
B. cinerea	2	66.667	1.0	0.4053
M. piriformis	2	5625.402	28.266	0.0001***

Table A3. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2007 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect
Growing season section (early, mid, late)

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	741.125	1.119	0.3681
B. cinerea	2	55.512	0.567	0.5863
M. piriformis	2	683.272	1.018	0.3994

Table A4. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	75.335	0.034	0.9668
B. cinerea	2	488.562	0.579	0.5800
M. piriformis	2	203.247	0.438	0.6583

Table A5. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	1702.334	4.590	0.0533
B. cinerea	2	148.328	12.683	0.0047**
M. piriformis	2	1336.256	4.316	0.0601

Table A6. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in the early, mid or late growing season in 2008 for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Growing season section (early, mid, late)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
P. expansum	2	182.052	0.171	0.8461
B. cinerea	2	76.432	0.262	0.7753
M. piriformis	2	503.152	1.681	0.2398

Table A7. Key data extracted from the one-way ANOVA tables for the % *P. expansum, B. cinerea* or *M. piriformis* detected from I-rod and plant tissue samples as indicated by DNA macroarrays in all growing season sections for Fields 3, 12 and the Kiran and Reekie orchards.

Main Effect				
Detected pathogens (*P. expansum*, *B. cinerea* or *M. piriformis*)				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2007 growing season	2	896.063	4.116	0.0538
2008 growing season	2	1022.252	3.222	0.0880
2007/08 growing season	2	454.769	19.248	0.0194*

Table A8. Key data extracted from the two-way ANOVA table for infection severity data collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Incubation Period	1	3639.591	13.081	0.0004***
1-MCP+/-	1	3260.433	11.718	0.0008***
Interaction				
Incubation period x MCP+/-	1	1008.275	3.624	0.0584

Table A9. Key data extracted from the two-way ANOVA table for infection severity data collected from Gala apples grown in Field 12 analyzed for natural disease incidence after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Incubation Period	1	13.767	36.073	0.0000***
1-MCP+/-	1	0.175	0.458	0.4995
Interaction				
Incubation period x MCP+/-	1	0.286	0.750	0.3876

Table A10. The effect of incubation period (1, 2, 4 and 6 months) on natural disease incidence and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3, Gala apples grown in Field 12 and Kiran and Red Delicious apples grown in Reekie and analyzed for natural disease incidence from harvest after 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control Treatment

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3	3	61.3201	74.6611	0.0000***
Field 12	3	79.6754	276.1476	0.0000***
Kiran	3	0.8366	3.2372	0.0268*
Reekie	3	11.1988	17.9952	0.0000***

Table A11. Key data extracted from the one-way ANOVA table constructed from infection severity data collected from Fields 3, 12, Kiran and Reekie analyzed for natural disease incidence for apples stored in CA storage for 4.5 months from harvest 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1 month storage	3	0.9484	1.5636	0.1195
2 months storage	3	10.0842	7.1456	0.0001***
4 months storage	3	8863.9197	27.1892	0.0000***
6 months storage	3	40909.7401	48.0806	0.0000***

Table A12. Key data extracted from the one-way ANOVA table constructed from infection severity data collected from Fields 3, 12, Kiran and Reekie analyzed for natural disease incidence for apples stored in CA storage for 4.5 months from harvest 2008.

Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field	3	29.2208	55.0013	0.0000***

Table A13. Key data extracted from the two-way ANOVA table for infection severity data collected from Jonagold apples grown in Field 3 analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2007..

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	1	0.900	1.192	0.3182
1-MCP+/-	1	0.342	2.262	0.1355
Interaction				
Biological Control x MCP+/-	5	0.332	0.439	0.8202

Table A14. Key data extracted from the two-way ANOVA table for infection severity data collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2007..

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	1	3039.868	5.773	0.0001***
1-MCP+/-	1	3435.342	32.620	0.0000***
Interaction				
Biological Control x MCP+/-	5	2779.181	0.439	0.0002***

Table A15. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates from harvest 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	2310.748	18.214	0.0005***
1100-6	1	1674.926	11.450	0.0033**
1-112	1	7.358	0.243	0.6279
2-28	1	1542.633	26.057	0.0001***
4-6	1	0.020	1.49e-4	0.9904
6-25	1	130.302	0.966	0.3387

Table A16. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are constructed from IS results collected from Field 12 Gala apples and analyzed for natural disease incidence in combination with bacterial isolates from harvest 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	624.112	1.329	0.2659
1-MCP-	5	5194.937	8.902	0.0000***

Table A17. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and inoculated with *P. expansum* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	32.862	61.071	0.0000***
1-MCP+/-	1	0.015	0.413	0.7077
Interaction				
Biological Control x MCP+/-	5	5.626	1.125	0.0000***

Table A18. The effect of 1-MCP in six separate biological control categories and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0420	1.775	0.1994
1100-6	1	1.4724	7.422	0.0139*
1-112	1	0.0425	0.215	0.6482
2-28	1	0.0872	2.057	0.1687
4-6	1	0.0329	0.302	0.5893
6-25	1	3.9641	52.958	0.0000***

Table A19. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are constructed from infection severity results collected from Jonagold grown in Field 3 analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates from harvest in 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	16.7121	31.798	0.0000***
1-MCP-	5	21.7763	39.548	0.0000***

Table A20. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and inoculated with *B. cinerea* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	21.222	20.760	0.0000***
1-MCP+/-	1	15.998	78.249	0.0000***
Interaction				
Biological Control x MCP+/-	5	13.841	13.534	0.0000***

Table A21. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 20077. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	9.706	45.191	0.0000***
1100-6	1	0.3931	1.793	0.1973
1-112	1	0.2607	1.060	0.3169
2-28	1	13.3139	80.502	0.0000***
4-6	1	5.9336	29.726	0.0000***
6-25	1	0.2310	1.271	0.2743

Table A22. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 2007.

Main Effect Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	33.7372	53.215	0.0000
1-MCP-	5	1.3253	0.940	0.4630

Table A23. Key data extracted from the two-way ANOVA table constructed from infection severity data collected from Jonogold apples grown in Field 3 and inoculated with *M. piriformis* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	14.346	10.660	0.0000***
1-MCP+/-	1	0.784	2.913	0.0908
Interaction				
Biological Control x MCP+/-	5	7.164	5.323	0.0002***

Table A24. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data is are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect 1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0020	0.0181	0.8943
1100-6	1	0.6318	1.3963	0.2527
1-112	1	0.0194	0.0729	0.7902
2-28	1	0.6870	4.3055	0.0526
4-6	1	6.5402	13.1253	0.0019**
6-25	1	0.0677	0.5298	0.4761

Table A25. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007.

Main Effect				
Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	1.1507	0.9683	0.4455
1-MCP-	5	20.3591	13.5439	0.0000

Table A26. Key data extracted from the two-way ANOVA table constructed from infection severity data collected from Gala apples from Field 12 and inoculated with *P. expansum* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	192.271	1.940	0.0936
1-MCP+/-	1	182.887	9.226	0.0030**
Interaction				
Biological Control x MCP+/-	5	164.951	1.664	0.1494

Table A27. Key data extracted from the two-way ANOVA table from infection severity data collected from Gala apples grown in Field 12 and inoculated with *M. piriformis* after harvest in 2007.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	4	3.015	1.729	0.1505
1-MCP+/-	1	6.578	1.508e-5	0.9969
Interaction				
Biological Control x MCP+/-	4	8.469	4.856	0.0014***

Table A28. The effect of 1-MCP in six separate biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect 1-MCP+/-				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
No biocontrol	1	0.0242	0.0487	0.8278
1-112	1	1.9035	4.7987	0.0419*
2-28	1	6.0306	14.5425	0.0013**
4-6	1	0.4423	1.1814	0.2914
6-25	1	0.0683	0.1370	0.7156

Table A29. The effect of the biological control treatments in two categories, 1-MCP+ and 1-MCP- and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2007.

Main Effect Biological Control Treatment				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
1-MCP+	5	7.0326	3.9878	0.0075**
1-MCP-	5	4.4516	2.5814	0.0498*

Table A30. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2008.

	DF	**Type III SS**	**F-value**	***p*-value**
Main Effects				
Biological Control	5	1.2214	0.8840	0.4926
Incubation Period	3	105.9743	127.8366	0.0000***
Interaction				
Biological Control x Incubation Period	15	14.3647	3.4656	0.0000***

Table A31. Key data extracted from the two-way ANOVA table from infection severity data collected from Gala apples grown in Field 12 and analyzed for natural disease incidence in combination with bacterial isolates after harvest in 2008.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	5	0.5763	0.8804	0.4950
Incubation Period	3	149.6138	380.9182	0.0000***
Interaction				
Biological Control x Incubation Period	15	3.6138	1.8400	0.0309*

Table A32. The effect of the incubation period on biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3 and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2 months	5	5.5753	4.3605	0.0021**
4 months	5	5067.4972	3.9609	0.0039**
6 months	5	15646.1017	5.2559	0.0005***

Table A33. The effect of the incubation period on biological control treatments and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Gala apples grown in Field 12 and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
2 months	5	2.1850	2.8870	0.0221*
4 months	5	1876.4999	2.5715	0.0370*
6 months	5	743.3109	0.7762	0.5782

Table A34. Key data extracted from the two-way ANOVA table from infection severity data collected from Jonagold (Field 3), Gala (Fields 12 and Kiran) and Red Delicious (Reekie) apples after 4.5 months in CA storage and analyzed for natural disease incidence following inoculation with bacterial isolates after harvest in 2008.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Biological Control	5	3.3247	5.4749	0.0001***
Field	3	12.3636	33.9323	0.0000***
Interaction				
Biological Control x Field	15	5.8261	3.1980	0.0000***

Table A35. The effect of the biological control treatments on infection severity and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from Jonagold apples grown in Field 3, Gala apples grown in Field 12 and Kiran and Red Delicious apples grown in Reekie after 4.5 months in CA stroage and analyzed for natural disease incidence when inoculated the bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control Treatment

Category	DF	Type III SS	*F*-value	*p*-value
Field 3	5	7.7543	4.8907	0.0009***
Field 12	5	0.1933	1.1827	0.3298
Kiran	5	0.1136	2.2402	0.0633
Reekie	5	1.0897	1.7312	0.1432

Table A36. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *P. expansum* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Biological Control

Category	DF	Type III SS	*F*-value	*p*-value
Field 3, Jonagold	5	3041.4892	6.8050	0.0001***
Field 12, Gala	5	401.2654	3.1239	0.0151*

Table A37. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *B. cinerea* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3, Jonagold	5	5981.4974	4.9960	0.0008***
Field 12, Gala	5	1962.7067	6.4391	0.0001***

Table A38. The effect of the biological control treatments in two categories, Field 3 Jonagold apples and Field 12 Gala apples and the resulting key data extracted from the one-way ANOVA results. Data are from IS results collected from apples analyzed for disease incidence when inoculated with *M. piriformis* and bacterial isolates after harvest in 2008. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect				
Biological Control				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Field 3, Jonagold	5	2115.5372	4.7271	0.0012**
Field 12, Gala	5	3.0721	4.1326	0.0030**

Table A39. Growth rate (μ) comparisons of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* and the resulting key data extracted from the one-way ANOVA table.

Main Effect				
Bacterial Isolate 4-6-wt vs 4-6-*gfp*				
Category	**DF**	**Type III SS**	***F*-value**	***p*-value**
Growth Rate (μ)	1	0.0022	0.6492	0.4656

Table A40. Key data extracted from the two-way ANOVA table constructed from cell densities (CFU/ml) of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* stored for 55 d at 1°C in air.

	DF	Type III SS	F-value	*p*-value
Main Effects				
Bacterial strain	1	50.5367	670.6401	0.0000***
Time	5	72.3075	191.9023	0.0000***
Interaction				
Bacterial strain x Time	5	72.7250	171.9775	0.0000***

Table A41. Cell density comparisons determined by dilution plating method of *P. fluorescens* isolates 4-6-*gfp* and 4-6-*gfp* + *P. expansum* stored for 55 d at 1°C in air and the resulting key data extracted from the one-way ANOVA table. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Bacterial Isolate 4-6-*gfp* vs 4-6-*gfp* + *P. expansum*

Category	DF	Type III SS	*F*-value	*p*-value
0 d storage	1	0.1061	2.3329	0.1610
10 d storage	1	8.4137e-4	0.0157	0.9024
20 d storage	1	0.5121	4.0261	0.0758
30 d storage	1	2.3762	36.5429	0.0005***
45 d storage	1	16.6408	120.1450	0.0000***
55 d storage	1	4.418e13	8.8646	0.0206*

Table A42. Infection severity comparisons of *P. fluorescens* isolates 4-6-wild-type and 4-6-*gfp* stored for 55 d at 1°C in air and the resulting key data extracted from the one-way ANOVA table. Individual one-way ANOVAs were determined for each category and combined into a single table.

Main Effect
Bacterial Isolate 4-6-*gfp* vs 4-6-*gfp* + *P. expansum*

Category	DF	Type III SS	*F*-value	*p*-value
30 d storage	1	21.6680	7.6629	0.0040**
40 d storage	1	46.8838	14.1739	0.0093**
45 d storage	1	18.9113	1.3841	0.2840
55 d storage	1	13.9568	0.5615	0.4820