The Role of Fungicide Spray Coverage and Population Heterogeneity on the Selection for Fungicide Resistance in *Botrytis squamosa*

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TABLE OF CONTENTS

Abstract

The efficacy of fungicide is influenced by the presence of resistant individuals in the pathogen population and the quality of application (fungicide coverage). The overall objective was to study the relationship between fungicide spray coverage, proportion of resistance and Botrytis leaf blight control. Specifically, the first objective was to determine the threshold of resistant individuals in a population to cause a significant reduction in the efficacy of the fungicide. The second objective was to determine the influence of fungicide coverage (% leaf area sprayed) on the development of resistance. In the first experiment, five combinations of resistant/sensitive individuals were inoculated onto onion plants (0 % / 100 %, 25 % / 75 %, 50 % / 50 %, 75 % / 25 % and 100 % / 0 %). Following inoculation, the fungicide iprodione was applied, using at 0 %, 25 % or 50 % coverage. The number of lesions per inoculated leaves suggests that practical resistance arises when the population of the resistant isolates reaches 75 %. Furthermore, there was a significant correlation between the efficiency of disease control and fungicide coverage (0 % to 50 %). In the second experiment, field studies were conducted and the results suggest that repeated applications of the same fungicide have a more significant impact on the resistance selection. The significant increase of resistant individuals caused by the repeated applications will diminish the effect of the fungicide applied nonetheless the coverage applied. The results obtained put further emphasize on the importance of fungicide resistance control, and determined the threshold at which a particular fungicide will be no longer effective in treating the disease due to the increased presence of resistant populations (threshold of intervention).

Résumé

L'utilisation de produits chimiques contre les pathogènes des cultures est fortement compromise par la sélection pour la résistance causée par l'application excessive de ceux-ci. Dans cette étude, nous avons cherché à établir une corrélation entre l'hétérogénéité de pulvérisation de fongicide et la sélection de résistance chez *Botrytis squamosa*. *B. squamosa* un pathogène fongique important causant la brûlure de la feuille chez l'oignon. Le premier objectif était de déterminer le seuil de souches résistantes nécessaires dans une population provoquant une réduction drastique de l'efficacité du fongicide. Le deuxième objectif était de déterminer si la couverture de fongicide pouvait induire le développement d'une résistance. Afin de réaliser le premier objectif, cinq combinaisons de souches résistantes/sensibles ont été inoculées sur des plants d'oignon (0 % / 100 % 25 % / 75 %, 50 % / 50 %, 75 % / 25 % et 100 % / 0 %), suivit d'une application du fongicide iprodione, à trois différents niveaux de recouvrement (0 %, 25 % et 50 %). La quantité de lésions développées sur les plants oignon traité a été recueillie et les résultats suggèrent que la résistance pratique se développe lorsque la population des isolats résistants atteint plus de 75 %. De plus, il existe une corrélation entre les niveaux de contrôle de la résistance et la qualité du recouvrement en fongicide (entre 0 % à 50 %). Le second objectif de cette thèse a été réalisé en conditions de champ. Les résultats de cette étude suggèrent que l'application répétée du même fongicide a un impact significatif sur la sélection de la résistance. Il a été démontré que l'augmentation significative des individus résistants causés par des applications répétées a annulé l'impact des trois recouvrements d'iprodione testés. Les résultats obtenus mettent davantage l'emphase sur la nécessité de contrôler les niveaux de résistance aux fongicides et détermine

un seuil d'intervention auquel un fongicide particulier devient inefficace afin d'optimiser nos stratégies de lutte intégrées.

List of Tables and Figures

List of abbreviations

FRAC	Fungicide Resistance Action Committee
BLB	*Botrytis* leaf blight
BS	*Botrytis squamosa*
UIPP	L'Union des Industries de la Protection des Plants
PCR	Polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
RFLP	Restriction fragment length polymorphism
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
QoI	Quinone outside inhibitors
SHDI	Succinate dehydrogenase inhibitors
DMI	Demethylation inhibitors
MFS	Major facilitator superfamily
BcOS1	Osmosensor histidine kinase gene
PDA	Potato dextrose agar
UV	Ultraviolet
HK	Histidine kinase
MAPK	Mitogen-activated protein kinases
kPa	Kilopascal
EC_{50}	Half maximal effective concentration
SNP	Single nucleotide polymorphism

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largely in the field study aspect of this thesis, which include sampling the data as well as providing the analysis.

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Contributions of Authors

This thesis is manuscript based and conforms to the regulations of the McGill thesis preparation and submission guidelines. The research study was planned and conceived by Dr. Jean-Benoit Charron, Dr. Odile Carisse and Mr. Hervé Van Der Heyden. The following thesis comprise of 5 chapters in total. The first chapter consists of the general introduction, and the second chapter is the literature review pertinent to this study. Due to the complicated nature of this study, many tools and protocols have been developed in order to lay foundation and provide adequate understanding to the subject before conducting the main study. Therefore chapter 3 consists of the aforementioned Tools and protocols development which allowed us to determine the discriminated dosage if iprodione, the precision coverage analysis, as well as the effectiveness of the active compound of a fungicide compare to its commercial formula. Chapter 4 presents the impact of population heterogeneity of the selection for resistance in *Botrytis squamosa*. Lastly, chapter 5 contains the general discussion and a future studies and contribution to science section.

The manuscript of this thesis is co-authored by Dr. Odile Carisse, Dr. Jean-Benoit Charron, Mr. Hervé Van Der Heyden and myself. I contributed to the following aspect of the study: reviewing the relevant literature, conducting the experiment in laboratory, collecting the data, analysis of the data and writing the following thesis. Dr. Charron provided expertise on the molecular aspect of the study as well as helping reviewing the manuscript in question. Dr. Carisse provided expertise on the plant pathology aspect of the work and also helped designing the layout of the manuscript. Mr. Van Der Heyden carried out the fieldwork of this study and provided the results and helped with their analysis for that section. Dr. Charron has also provided the funds to carry out this study.

Chapter 1 General Introduction

1.1 Introduction

Fungicides are viable tools in terms of crop disease management (Brent et al., 1998). They allow us to increase crop yield significantly by protecting crops from pathogens that can ravage fields if left untreated. However the potency of these chemicals are compromised due to repeated and uncalculated applications, to a point where the pathogens are gaining mutations which allow them to bypass the fungicides, through the process called fungicide resistance (Gisi et al., 2008).

In order to maintain fungicide efficiency, committees such as the Fungicide Resistance Action Committee (FRAC) have been put in place to provide expertise and knowledge, as well as researchers all over the world are working in unison to have a better grasp at understanding fungicide resistance (Morton et al., 2008). Anti-resistance management programs are also conceived to delay the development of resistances, such as employing a mixture of fungicide and applying in a timely manner. However, there are still key components about fungicide resistance that we still do not fully understand, such as the correlation between the effectiveness of the fungicide and the ratio of resistant strains of pathogens present within an environment. Furthermore, it is also commonly believed that in order to achieve the optimal level of control, a high level of coverage must to be achieved. This belief contributes to renders agriculture unsustainable due to the ever-increasing costs and environmental risks associated with high levels of fungicides coverage. In addition, on a systemic standpoint it contributes to eliminate the coexistence of sensitive and resistant strains of pathogens, hindering the natural competition, which can in turn reduce the

population of resistant strains. We therefore hypothesized that by lowering the coverage, we expect to maintain heterogeneity in the population without compromising the disease control. These notions will be explored in the following thesis and *Botrytis squamosa* will be used as the host model.

Several *Botrytis* species are responsible for economically important diseases. *Botrytis cinerea* being the most problematic because it can affect up to 200 hosts and adaptability have made *Botrytis* a formidable pathogen that is regarded as the second most significant fungal plant pathogen worldwide (Dean et al., 2012). It is estimated that 20 % of crop loss are attributed to these filamentous fungi, and roughly 10 % of all fungicides are dedicated to Botrytis disease control, which equates to US$ 780 million (UIPP, Annual Report 2012). They can cause a wide array of disease including botrytis leaf bright and botrytis bunch rot which causes host tissue degradation. These pathogens are especially problematic because of their ability to stay quiescent following infection, thereby causing damage to their hosts several days or months after infection, often during post-harvest storage or transit (Jarvis, 1977).

The anti-*Botrytis* campaign has been marching on since the sixties, when numerous chemical fungicides were developed to limit the propagation of the pathogen (Elad et al., 2007). Great successes have been achieved through the employment of single-site fungicides, however it comes with increased risk of development of fungicide resistance in *Botrytis* species, nullifying the effect of the toxicants. There are currently many strategies in place to delay the occurrence of this phenomenon, such as using mixtures of different fungicides as oppose to a single type.

1.2 Hypotheses

1. There is a resistance threshold expressed as the proportion of resistant individuals in a *B. squamosa* population at which fungicide become inefficient.
2. There is a relationship between fungicide spray coverage and selection for fungicide resistance.

1.3 Objectives

1. To determine the proportion of *B. squamosa* resistant individuals at which application of the fungicide iprodione becomes ineffective (Threshold of resistance).
2. To measure the influence of the heterogeneity of the fungicide spray on the selection of *B. squamosa* resistance to iprodione.

Chapter 2 Literature review

2.1 Importance of Fungicide for Disease Management

2.1.1 Impact of fungal pathogens on crop production

Fungi are important in crop production in both beneficial and antagonistic manner. It is a decomposer, which increases the fertility of soil by decomposing organic matter. Its symbiotic relationship with plants also provides the necessary nutrient such as minerals and phosphate. However, despite its necessity to the survival of plants, fungi are is also an important group of pathogens, which can be catastrophic if left un-treated.

Fungal pathogens have the ability to affect hosts at different developmental stages, for instance during seedling or adult stages, such as *Fusarium* and *Sclerotium* species which affect the plants at the seedling stage and causes seed rot (Khan et al,. 2006). Fungal pathogens are also capable of affecting all parts of the plant, such as leaves, roots, flowers or stems. The ability of fungi to rapidly disseminate over wide area through spores brings complications in terms of control efforts. This allows the pathogen to rapidly propagate over a wide range of territories and put conservation efforts in jeopardy. This will ultimately lead to a severe economic damage as the pathogens reduce the crop yield significantly and also leave mark on societies. A famous case occurred in the mid-19 century, as the pathogen *Phytophthora infestans* left devastating damage to potato crops, as it causes potato late blight. The shortage of potatoes left some European counties in famine, especially in Ireland, as the Irish people are largely dependent on potatoes as food sources (Palm 2001). Even with the increasing knowledge against fungal pathogens, and the improvements of technologies in controlling the diseases, *Phytophthora infestans* still remain as the greatest pathogen against

potato crop (Kamoun et al., 2015), and can still lead to severe damages when control methods fail as fungicide resistances rise.

With rice being the main source of carbohydrates for more than half of the world's population, the significance of *Magnaporthe oryzae* fungus is put at the upmost importance (Dean et al,. 2012). This pathogen causes rice blast disease, which can lead to up to 30 % of losses in yield (Jean et al., 2007). Due to the magnitude of this disease, *M. oryzae* is widely studied, and scientists all over the world have put continuous efforts forward. Currently, a large amount of efforts have been focused on characterizing the genes responsible for avirulence, as well as the corresponding resistance genes (Chao and Elligboe, 1991).

Coming in second behind *M. oryzae* is *Botrytis cinerea*, a pathogen that is considered to be the second most significant pathogen (Dean et al., 2012), and 20 % of crop loss worldwide is attributed to this pathogen (UIPP, Annual Report 2012). *Botrytis cinerea* can infect up to 200 hosts, and causes grey mould in its hosts. Members of the *Botrytis genous* are important pathogens, which infect a wide variety of herbaceous annual and perennial plants, occurring in a large variety of climate settings throughout the world. However, this is not representative for the majority of *Botrytis spp.* which are generally restricted in their host range, mostly targeting monocotyledonous plants (Elad et al., 2007). The large host range, high reproduction rate, broad infections conditions and potential for dissemination make *Botrytis spp* difficult to control (Dean et al., 2012). Consequently, cost of *Botrytis* induced disease control is important (e.g. US$ 15-25 million in 2007, Elad et al., 2007).

Detection of *Botrytis cinerea* are traditionally achieved through the identification of fungal structures such as sclerotia or conidia through microscopy. Plating out on selective

medium would also allow us to identify different *Botrytis spp.*, however this process is time consuming and limits our ability to assess early stage infections (Dewey et al., 2004). *Botrytis spp.* also has ability to stay quiescent, lying dormant within the host for a prolonged period of time. This proves to be problematic, as the fungus can transforms into necrotroph in postharvest stages and promotes decay symptoms in its host (Williamson et al., 1995). Recent discoveries in molecular diagnostics such as the real time PCR have enabled us to circumvent this issue and accurately identify and quantify *Botrytis spp.* infections in a large population at an early stage where actions can be taken to limit the fungal propagation.

All in all, fungal pathogen poses great agricultural, economic and social importance. It can be devastating if left un-threated, and a continuous effort must be put forward constantly to improve our understanding in dealing with these pathogens.

2.1.2 Fungicides, key for sustainable agriculture

Having fungicide and sustainability in the same sentence might be contradictory to some, but in reality, one cannot exist without the other. As a matter of fact, our current agricultural system is largely dependent on the use of fungicides. For instance, more than 80 % of fruit and vegetables currently grown in the United States are sprayed at least once per season with a fungicide (Chen et al., 2014). The crop yield can be reduced from 50 to 95 percent if the disease were left un-treated, and it is estimated that using fungicides can increase the income of U.S. farms by $13 billion annually (Gianessi et al., 2006). Furthermore, the usage of fungicides have increased fourfold in the past 50 years, and the sales in 2007 alone are accounted up to $33 billion (De Sousa et al., 2011).

There are several ways which fungicides can maintain agricultural sustainability. First, fungicide will improve the productivity of the land by reducing the loss of yield due to fungal pathogen. Therefore, less land needs to be converted into agricultural land, thus more natural environment can be preserved. Furthermore, using fungicides can reduce the manual labour needed in field practices when compared to not using fungicides, since less manual work is needed to treat the diseases (Damalas and Eleftherohorinos, 2011).

With our increasing understanding of chemistry and pathogens response towards fungicides, we are able to create safer and more effective fungicides. We are also able to use more appropriate dosage and apply it at the fitting time window. All these efforts can lead to a more environmental and health friendly usage of fungicides.

2.1.3 Alternative control methods for fungal pathogen

Other methods of fungal pathogen control are also put in place to alleviate diseases, such as biocontrol, which is the use of biological interactions to our advantage. Three types of interspecies antagonisms are viable in terms of biological control, which include direct antagonism, mixed-path antagonism and indirect antagonism. A well-known example would be the usage of viruses to control the pathogen *Crypthonectria parasitica*, which causes chestnut blight in chestnut trees (Milgroom et al., 1992).

On the other hand, mixed-path antagonism makes use of several biological mechanism, including the use of antibiotics, lytic enzymes and physical interferences. Antibiotics are natural compounds produced by microbes that have effect on suppressing the development of fungal pathogens. For instance the fungi *Trichoderma virens* produces the mycotoxin

gliotoxin, which can be effective to treat root rots caused by *Rhizoctonia solani*, in many species (Whilhite et al., 2001).

Indirect antagonism makes use of natural competition that exist in nature. Un-harmful microbes are introduced in environments that compete with the pathogen for limited amount of nutrient and substrate present.

Much has been learned over the last couple of decades in terms of biological control. Its ability to control diseases while having the least amount of effect on human health and environment is attractive for the growers as well as the consumers. However, it is only used in small-scale operations, and rarely used on a commercial scale. This is mainly due to the excessive amount of cost, the unreliability in its consistency as well as the lack of convenience when compared to the usage of fungicides. Furthermore, the efficacy of biocontrol is also largely reliant on the environmental conditions in the field, leading to inconsistency (Heydari et al., 2010).

It is important to note that the efforts in combatting fungal pathogens should be multi-faceted. Long-term control of these diseases needs to be in mind when employing different methods of control. In order to achieve optimal control while maintaining sustainability involves the usage of correct cultural practices, such as proper water usage, tillage and crop rotations. Furthermore, using disease-resistant cultivars can also alleviate the pressure of the pathogen on crops while balancing the usage of chemicals and biological control agents (Staub, 1991). The aforementioned field practices provide a solid foundation for fungal pathogen control, and together with the usage of fungicides, make the control of diseases consistent and manageable.

The combined efforts of pathogen management are what makes an agricultural system sustainable. The sole usage of fungicide is not the answer to the problem, but it is an integral part of the overall solution (Staub 1991). With our increasing knowledge on the fungicides and pathogens, we are now able to apply chemicals at a more accurate manner, where we are reducing the impact on health and environment as well as reducing the selective pressure on development of fungicide resistance.

2.2 Modes of action of fungicide

2.2.1 Single site and multi-site fungicides

Fungicides modes of action are either single-site or multi-, both having its pros and cons. For instance single site toxicants are very specific in their target, usually active against a critical enzyme or protein needed for the survival of the fungi (Fernandez et al., 2010). Their high specificity also renders relatively safe for the environment and human health.

On the other hand, multi-site fungicides do not target a specific gene like the single site fungicides do, instead, it has a broad range of point of control as its name suggest. These types of toxicants often work by staying on the surface of leaves rather than being absorbed into the plant tissue like the systemic fungicides. It is also less prone to selecting resistance in the pathogens, as it targets numerous metabolic sites within the fungus. Due to its nonspecific nature of targeting, it also means that it can be more harmful for the safety of human consumption as well as the environment.

2.2.2 Evolution of fungicides used against Botrytis spp.

The use of fungicides against pathogens saw a significant increase in the sixties (Couderchet, 2003). The first wave of fungicides was mostly composed of multi-site fungicides, including dichlofluanid and thiram, which are members of the dithiocarbamate family. Their mode of action are thought to be related to inhibition of essential enzymes of the pathogen, preventing the germination of the spores (Yang et al,, 2011). Currently these fungicides are being used against *Botrytis cinerea* infections, mainly as a preventive measure (Couderchet, 2003).

Later, in the seventies, fungicides of the benzimidazole family became available, for example benomyl and thiophanate. Thiophanate- mode of action involves binding the tubulins of the pathogen during mitosis, inhibiting cell division (Lyr, 1995). Benomyl bind to the microtubules of the pathogen that can interfere with intracellular transportation and meiosis (Dane and Dalgic, 2005). These fungicides were highly effective in the management of *Botrytis spp.,* however excessive and improper applications caused a rapid outbreak of resistant strains, up to 92 % resistance was reported in 1981 (Couderchet, 2003).

In the early 1980s, dicarboximide fungicides became available. These fungicides helped alleviate the selection for resistance for other fungicides, as the growers have more products available for fungicides rotation. Vinclozolin and iprodione are dicarboximide fungicides, which interfere with the pathogen's osmoregulation by inducing lipid peroxidation and the destructions of the membranes (Cui et al., 2002). Iprodione is currently used against *Botrytis squamosa* and *Botrytis Cinerea* infections, either as a standalone application or mixed with others fungicides. The nineties brought fungicides of the anilinopyrimidine (Pyrimethanil and cyprodinil) family in to the anti-botrytis crusade. Pyrimethanil's mode of action is to inhibit the secretion of cell wall degrading enzymes

produced by the pathogens, which is essential for infection (Milling and Richardson, 1995). During this era, the phenylpyrrole fungicides were also used for the first time, which includes fenpiclonil and fludioxonil. These phenypyrrole fungicides are related to pyrrolnitrin, which is the natural isolate with antibiotic property belonging to the bacteria *Pseudomas pyrrcinia* (Pillonel et al., 1997). These fungicides inhibit spore germination and mycelial growth (Couderchet, 2003 through the disruption of the signalling pathways present in the pathogen's cell, leading to the over stimulation of the histidine kinase (HK)-MAPK stress–response pathway (Kim et al., 2010).

2.2.3 Different group of fungicides

Several important groups of fungicides are used in terms of control against *Botrytis squamosa*. They differ in their chemical composition as well as their mode of action.

Dicarboximide fungicides are an important family of fungicides that is used to treat *Botrytis squamosa*. It includes commonly used fungicides such as iprodione and vinclozolin. Their exact mode of action is yet to be fully characterised, but it is believed that they affect osmotic regulation, as well as inhibiting triglyceride biosynthesis of pathogens (Copping et al., 1998). The general guidelines provided by the Fungicide Resistance Action Committee (FRAC) suggest minimising the number of applications in order to reduce the selective pressure on resistance, and ideally limit the number of applications to less than three times per season. It is also suggested to maintain prolonged times without the exposure to dicarboximides, in order to regain the natural balance between the resistant and sensitive isolates present in the environment (Raposo et al., 2000).

Dithiocarbamates is a multisite inhibitor, and although it is a weak botryticide due to its un-specified targeting mechanism, it still offers a decent protection as a protective measure when applied prior to the infection (Van der Heyden et al., 2012). Their modes of action rely in blocking the enzymes responsible for the respiration of spores (Leroux et al., 2010). They are commonly used in field practices for their low cost, and applied at the beginning of the growth season while the disease pressure is still relatively low (Carisse et al., 2007).

Quinone outside inhibitors (QoI) fungicides are also well known and used in treating diseases caused by *B. squamosa.* It includes group of fungicides such as strobilurins, which inhibits pathogen growth by targeting the mitochondrial complex III, therefore hindering the ability of the pathogen to produce energy. The FRAC guidelines recommends the usage of these fungicide as a preventive measure, therefore it should be applied as early as possible in the disease cycle. Furthermore, they should also be applied in a mixture with other types of fungicides that do not share the same mode of action (Gisi et al., 2002).

Succinate dehydrogenase inhibitors (SHDI) are a large group of fungicides that includes many commercial products such as Boscalid, Sedaxane and Fluopyram. Their modes of action affect the mitochondrial respiration of pathogens by disrupting the function of the subunit B, C and D of the complex II (Leroux et al., 2010). SDHI fungicides are one of first fungicides available in the market, therefore the development of resistance have been a long-term issue. FRAC recommends that SDHI fungicides should always be applied in mixtures, to alleviate the selective pressure on resistance emergence.

2.3 Fungicide resistance

2.3.1 Severity of fungicide resistance in agriculture

As mentioned in this literature review, the use of fungicides is crucial in terms of crop protection. The improper usage of these chemicals can lead to the accelerated development of pathogen resistance, therefore it is the upmost importance to put in place management procedures to monitor and hinder this phenomenon. However the lack of knowledge regarding the different resistant profiles of all the pathogens still led to the development of resistances throughout the past three decades. Our inability to no be able to prevent fungicide resistance can be troublesome, and it is even more problematic for irreversible resistances. This is because some compounds' effectiveness can be rendered null and withdrawn from the market, thus leading to financial damages to manufacturer which in turn will reduce their investment for future crop protection products (Russel, 2004).

The effort that we put into understanding pathogen resistance underlines the severity of the situation. Our struggle against fungicide resistance started ever since the usage of the first fungicide chemicals, started over 35 years ago. Researchers over the world collaborates to further out understanding on this subject and the Fungicide Resistance Action Committee have also been put in place in 1994 to deal with this issue (Brent 2007).

2.3.2 Development of fungicide resistance

Fungicides are vital tools that we possess in combating fungi infections in nowadays-agricultural practices. Agricultural yield and relating economic gains are largely dependent on the efficacy of the fungicides applied. Therefore prolonging the shelf life of a certain pesticide by avoiding the developing resistances of the pathogens will be crucial to our control of the enemies of the cultures. This issue have become apparent in the 1970s when

the large appearances of resistance towards systemic fungicides have been repetitively reported (Elad et al., 1992). An improper use of fungicide such as an excessive use or the incorrect timing of the application can all accelerate the selection for resistant strain in the field.

It is logical to presume that a high dosage of pesticide will have high efficiency in removing a certain pest, resulting in better control and limitation of the emergence of resistances. Higher dosage is also thought to eliminate any partially resistant strains, which have the possibility to mutate, evolve or recombined into a further resistant strain (Lucas et al., 2006). This concept however has been challenged by the fact that an increased dosage will also rapidly remove all the sensitive individuals from a gene pool, leaving no competitor for the unmanageable resistant strains. A study conducted by Metcalfe et al. (2000) have utilized three different measured quantity of DMI fungicides to treat spring wheat cv. Baldus inoculated by *Mycospharella graminicola*. They found out that the lower dose application did not select for resistance, but instead slightly reduced the ratio of resistant strains versus nonresistant compared to the high dose applications (Metcalfe et al., 2000).

The heterogeneity of a fungicide spray can be altered by the dosage, the pattern and the size of the droplet. Empirical evidences suggest that fungicide resistant and sensitive isolates coexist in the same environment (Murray et al 1996). Considering that there is a fitness cost associated with fungicide resistance, it is desirable to maintain an equilibrium between resistant and sensitive individuals. In other words, despite the generally admitted ideas that fungicide efficiency should be as close as possible to 100 %, it might be important, for fungicide resistance management purpose to keep a proportion of sensitive individuals (sub-optimal fungicide efficiency). Therefore selecting optimal spraying application

favouring the coexistence between both sensitive and resistant individuals could delay the selection for resistance. For instance, a high dose fungicide application with fine droplet onto an onion leaf, resulting in a near 100 % coverage can theoretically lead to the selection for resistant strains. This is due to the fact that the entirety of the onion leaf will be treated, eliminating all the sensitive individuals from the environment, leaving only the resistant counterpart behind to grow and propagate without any competitor. On the other hand, an optimal heterogeneity of fungicide spray favouring coexistence can be greatly beneficial in the post-treatment recovery of sensitivity regarding that particular fungicide employed (Parnell et al., 2005). A study conducted by Parnell et al (2005) based on a simple differentiation equation model showcase that in general the heterogeneity of fungicide sprays is not enough to create coexistence per se. Other factors such as the competitive ability of both resistant and the sensitive strains, and the fitness cost associated with the resistance influence the development of resistance.

This issue becomes even more intricate when the fitness cost associated with the acquisition of resistance is taking into consideration. If the fitness cost associated with resistance is high, it is expected that resistant individuals will not maintain their population, meaning that ceasing the application of that particular fungicide for a period of time will favour the sensitive strain. In this scenario, mixtures, alternations or discontinuation of that specific fungicide can be utilized to diminish the selection for resistance.

2.3.3 Mechanisms used by the pathogens against fungicides

Different mechanisms of resistance can be employed by the pathogen to lower the effectiveness of the fungicide in field and greenhouse applications. They can be generally classified into several distinct categories.

The pathogen can reduce uptake of fungicides into its cell or increase the expulsion of the toxicants. This can be achieved via transporters such as the ATP-binding cassette (ABC) transporters or the major facilitator superfamily (MFS) membrane proteins (Waard et al., 2007). For instance, the demethylation inhibitors (DMI) fungicides promotes accumulation of toxic compounds in the fungi's cell and can be countered by the enhanced toxicant efflux (Stehmann and De Ward, 1995). Unsuccessful binding of the fungicide can be a result of altered target site present in the pathogen. This is often caused by a single nucleotide polymorphism where a single nucleotide modification can lead to an amino acid substitution and ultimately to a peptide sequence that will be unrecognisable by the fungicide. Overstimulation of an enzyme or a metabolic pathway leading to the rapid breakdown of fungicide is also very effective in dealing with toxicants. For instance an enhanced level of catalase and superoxide dismutase are being produced by resistant strains of *Botrytis spp.* to deal with the damage caused by the lipid peroxides contained in fungicides such as the dicarboxides (Edlich and Lyr, 1992).

2.3.4 Fitness cost relating to fungicide resistance

A common strategy in terms of dealing with fungicide resistance is to halt the usage of that particular chemical for a period of time. This will allow the natural competition to occur between the sensitive and resistant isolates, where the resistant isolates will be flustered out eventually when there is lack of fungicide selection. However empirical

evidences indicates that even in the absence of the fungicide, resistance pathogens are still thriving in the environment, and this is due to the absence of fitness cost related to acquiring and maintaining the resistance (Enne et al. 2001).

The notion of fitness cost is a big factor on assessing the severity of fungicide resistance. Several scenarios can occur as a pathogen gains the ability to be resistant towards a particular chemical. Firstly, the resistance can be maintained within the population if there is no fitness cost associated with it, meaning that the mutants will be able to compete on the same level as the sensitive isolates in the absence of selective pressure, therefore coexistence will occur. On the other hand, when there is a fitness cost related to acquiring and maintaining the trait, then the mutants will be out-competed by the sensitive strains and eliminated through the process of natural competition in the lack of selective pressure by the fungicide. Lastly, the resistant individuals will be able to outcompete the sensitive isolates in the presence of selective pressure (Parnell et al., 2006).

2.3.5 Population heterogeneity in fungicide resistance

An intricate part to comprehending fungicide resistance is understanding how the coexistence between sensitive and resistant isolates can affect the severity of the disease. Coexistence is relevant due to the fact that it promotes natural competition between the sensitive and resistance isolates, and as previously stated, fitness cost can come in handy in terms of lowering the quantity of resistant isolates within an environment when in the absence of fungicide selective pressure. Therefore it is important to maintain a healthy level of sensitive isolates present within an environment, because it will aid the recovery of the sensitivity towards the fungicide posttreatment. This raises the question of what is the

optimal ratio of population heterogeneity where you can achieve an acceptable level of control while maintaining the level of sensitive and resistant ratio (Parnell et al. 2005). For instance, applying partial fungicide coverage would allow the survival of a sensitive population, which will not only compete for resources with the resistant population but also maintain the viability of the fungicide applied on a long-term basis (figure 2.1).

A study conducted by Parnell et al. (2005) has indicated that coexistence is possible when there is an incomplete coverage of the fungicide. However it is only based on statistical model, where more field and laboratory experiments are still needed to further explore the notion of population heterogeneity in affect the development of resistance (Parnell et al. 2005).

2.3.6 Fungicide resistance management

Several management methods are put in place to delay the development of fungicide resistance, such as proper practices and timely application. Depending on the type of the chemicals involved, the total times of applications per season should be closely monitored, and limit the number of applications when possible. Fungicide should also be applied in alternation with other fungicides with different mode of action in order to reduce the population of resistant strains. It is also suggested to use mixtures of fungicides with different modes of action as well as multi-site fungicides within a single application.

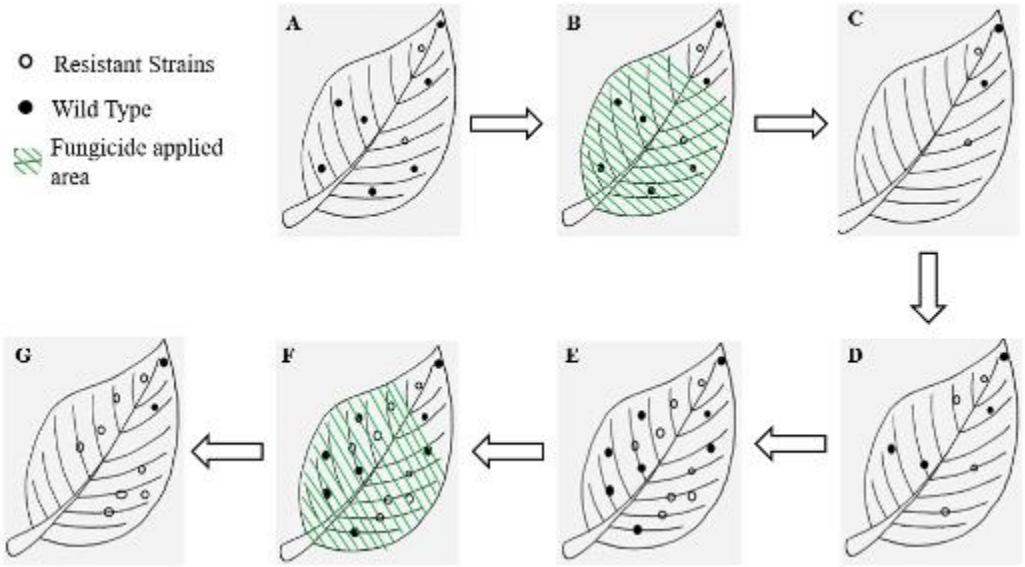

Figure 2.1 Effect of partial fungicide coverage on coexistence. The state of resistant and sensitive isolate prior to fungicide application A). A theoretical partial coverage of fungicide has been applied in B) and F). Clonal reproduction of the pathogen occurring between C), D) and E). The state of resistant and sensitive isolate at the end of the cycle G)

Fungicide applications should be an integral part of disease management and be used in harmony with other types of disease control methods that will in term create the integrated pesticide management. Fungicides should not be thought of as being the quick fix or the magic solution to controlling crop disease, but it should be thought as a part of a complex solution which will require proper cultural practices such as pruning, weeding and staking are essential to maintain the healthiness of the crop (Asami et al. 2003). Growers should also work closely with specialists in their area, as well certified crop advisors to create programs which will cater to the specific needs of their fields (Lester 2006). The specialist can forecast the severity of a specific disease for the upcoming season as well as generating the risk assessment for the grower. This will in turn provide an adequate timing for the application of the chemicals as well as its frequency (Lester 2006).

2.4 *Botrytis squamosa*

2.4.1 Importance of the disease caused by B. squamosa

Onions production is amongst the biggest vegetable commodities produced in the world. Quebec is a large producer of onions in Canada along with Ontario, in 2011, 4,608 acres of onions were being planted which equates to 58,004 tons of onions produced generating 36 million CND of revenue (Statistic Canada, agricultural sector 2011). In several production areas, onions are affected by Botrytis leaf blight (BLB), caused by *Botrytis squamosa*. The disease can reduce up to 47 % of onion production in Quebec (Carisse et al., 2011) and up to 27 % in the Netherlands (De Visser, 1996).

2.4.2 Symptoms of the disease

Botrytis squamosa causes botrytis leaf bright (onion leaf bright) in onions and it is specific to *Alium* spp.. Tiny yellow-whitish spots will appear at the site of lesion along with green halo surroundings (Hancock and Lorbeer 1963). This can be misunderstood as mechanical or insect damage but the symptoms will become evident of *Botrytis squamosa's* infection at the development of additional lesions, which ultimately causes blight and premature death of the onion leaf starting at the tip (Carisse et al., 2009). The primary inoculum of *Botrytis squamosa* are generated from cull piles and seed production fields in late May to early June (Ellerbrock and Lorbeer, 1977). BLB causes great economic in areas that have a vast agricultural sector relating to onion cultures, and it is crucial to have the recognition of the infection at its early stages in order to put in place optimal management strategies.

2.4.3 Epidemiology

The pathogen survives as sclerotia produced on crop debris left on the ground following harvest in the fall. In the spring, conidia will be produced on the sclerotia and overwinters and serve as primary inoculum for outbreaks. The majority of the spores produced by this fungus are asexual (conidia), and only towards the end the infectious season that the sexual spores will start to be produced. Sexual ascospores are created through recombination, creating a wider diversity to adapt and survive in a less optimal environment. The symptoms of infection can appear 24-48 hours after inoculation as whitish color lesions, with usual length of 1 to 5 mm (Carisse et al., 2009). The rate of infection depends on many factors, both from the efficiency of the primary and secondary inoculums as well as the

environmental factors. The primary inoculum provides the foundation for the infection of the secondary inoculums by generating initial lesions and causing dieback onto the leaf. Dead leaves are then an ideal growing environment for the necrotrophic pathogens (Lorbeer 1992). The humidity and the temperature are two environmental factors that are essential in the pathogen's infection. An optimal temperature would range from 18^{o}C to 21^{o}C and a humidity level that permits a 6 to 8 hours of leaf wetness (Shoemaker and Lorbeer, 1977).

2.4.4 Control and management

The controls of B*otrytis squamosa* infections rely largely on the proper selection and application of fungicides. Multi-site fungicides, such as dithiocarbamate, are currently being used on a seven to ten day-interval by eastern Canadian onion producers (Carisse et al., 2011). It is mostly used as a preventative measure before lesions appearance (Carisse and Tremblay 2007). Even though these multi-site fungicides are not prone to resistance development, they are still not the perfect answer to *Botrytis squamosa* infections because of their impact on human environment health (Van der Heyden, 2012). Single-site fungicides such as iprodione can be used at a later stage of the infection process. This fungicide inhibits the germination of spores and growth of fungal mycelium by interfering the DNA and RNA synthesis of the pathogen. Boscalid have been put in use in the last decade as a systemic fungicide that has a wide spectrum of mode of action (Carisse et al., 2011).

2.5 Iprodione

2.5.1 Modes of Action

Iprodione of the dicarboximide family fungicides have been introduced in the late 1970s as a response to the surge of resistant strains to benzimidazole fungicides (Steel and Nair., 1993). Iprodione have a relatively narrow spectrum of activity when compared to other fungicides available in the market. The precise mode of action of this fungicide is not fully understood, but it has been shown that it interferes with the two component histidine kinase encoded by the *BcOS-1* gene, which regulates the pathogen's ability to deal with osmotic and oxidative stress (Leroux et al., 2002).

2.5.2 Mechanism of resistance towards iprodione

The mechanism towards this particular fungicide involves a point mutation in the *bos1* (also known as *BcOS1 or Daf1*) gene that encode for the class III histidine kinase. The histidine kinase III plays a role in the fungal osmosensing system that aids the pathogen to adapt to various environmental stresses such as osmotic or oxidative stresses (Grabke et al., 2014). The mutation was recorded to be located at the 86th codon of the second repeat of the gene from isoleusine to serine (I86S) (Oshima et al., 2006). Other point mutation have also been reported to cause resistance towards iprodione, including a three amino acid substitution group V368F, Q369H, T447S and a two amino acid substitution group Q369P, N373S (Oshima et al., 2002). Grabke et al (2014), have recently published a study that characterized iprodione resistance in *Botrytis cinerea* from strawberries and blackberries in southern United States, in which he identified Q369P and N373S to be also related to QoI resistance. Gathering from all these data we can conclude that this resistance can be a result

of several point mutations, however there is one that appears be common in many of the studies published, which is I365S.

2.5.3 Fitness cost relating to iprodione resistance

Raposo et al (2000) reported a negative correlation coefficient between the survival of the sclerotia and the resistance towards iprodione. This suggests that, in absence of selection pressure due to iprodione application, the resistant individuals may not compete well with the sensitive ones. This information provides a good basis for the management of iprodione resistance, as ceasing the applications can possibly reduce the proportion of resistant individuals in the populations. Cui et al (2002) also investigated the sensitivity of the mutants to sodium chloride, which can greatly affect the osmotic pressure of the pathogen. A mutated functional gene would lead to osmotic stress, which ultimately lead to being out competed by the sensitive individuals in the absence of selective pressure.

Connecting Statements for Chapter 3

The following chapter describes the development of several tests and methods needed prior to the completion of the main study. This includes growing the necessary onions plants at the correct development stages and producing inoculums *Botrytis squamosa*. We also carried out tests in this chapter to verify that there is no discrepancy between the commercial formula Rovral and the active compound iprodione in regards to disease control.

The development of a protocol outlining the use of a custom made spraying bench necessary to apply iprodione onto onion plants is also presented. This equipment allowed for accurate adjustment of the coverages that are normally applied by field machinery on the onion plants.

The experimental procedures was carried out by Zhe Jia and supervised by Dr. Jean Benoit-Charron, Dr. Odile Carisse and Hervé Van Der Heyden. Audrey Levasseur provided expertise as well as teaching the necessary techniques. Annie Lefebvre carried out the growth inhibition analysis of the *Botrytis squamosa* strains collected. Dr. Bernard Panneton provided the expertise in regards to fungicide coverage as well as providing the necessary analytical tools.

Experimental procedures described in section 3.2.4 were performed by Hervé Van Der Heyden, which includes the fieldwork related to this study. Mr. Van Der Heyden also supervised the analysis of the data collected from the aforementioned section and participated in the redaction of section 3.3.3, which describes the results relating to assessment of the resistance profile of *Botrytis* towards various fungicides.

Chapter 3 Methodology and tools development

3.1 Introduction

Currently, all fungicide spray technologies are unable to achieve a complete coverage (100 %), and this is especially true for field applications (Dahmen and Staub, 1992). This is mainly due to the fact that there are many factors that we cannot control, which can interact with the spraying process. Fungicide drifts, runoffs, rains, winds and plants positions can all be problematic for achieving a uniform coverage (Pimentel 1995). A very important basis of this study was to precisely create different level of fungicide coverages onto onion plants. This is crucial because one aspect of this study was to determine how different levels of coverage could affect the control of diseases. Therefore, we were faced with the challenge to create three distinct levels of coverage, to be used in the main study (Chapter 4). To do so, we calibrated a custom made spraying bench by tuning its parameters, including height of the bench, pressure, type of nozzle, and at which the speed the spraying head moves.

One of the most common methods to determining the sensitivity of a fungal pathogen towards a certain chemical is by carrying out growth inhibition assays (Raposo et al. 1995). Through the use of such assays, we can determine the discriminating dosage of fungicide that can eliminate any wild-type (non-resistant) isolates while not affecting the normal growth of the mutant (resistant) isolates (Lehner et al. 2015). However, it is problematic to interpret these data for crops, since the pathogen will most probably react differently towards a chemical when tested on an actual host than on potato dextrose agarose (PDA) plates. Therefore, we set goal to determine the discriminating dosage by testing the sensitivity of *Botrytis squamosa* towards iprodione on actual onion plants.

The one remaining issue needed to be resolved was to investigate the difference in efficacy between using the commercial product Rovral® (FMC Corporation) and using only the active compound (Iprodione) without any adjuvants. This was necessary because as stated previously, we are studying the impact of different coverages on disease control, therefore the adjuvants might alter the fungicide coverages in a manner that we will not be able to control, and deviate from out goal. Furthermore, working with Rovral proved to be difficult at high concentrations, since the spraying machinery lack the capability to blend the mixture while spraying, which led to a clogged filter due to sedimented solid adjuvants. However, the purpose of this experiment is to be representative of what would naturally occur in field practices, therefore, it was necessary to test the difference in lesion rates between using Rovral and iprodione.

Lastly, we also described the method used to determine the EC_{50} of *Botrytis* against iprodione and several other active ingredients. This information provided the basis for determining the discriminating dosage of iprodione that we applied in the study. It also underlined why iprodione was used as oppose to other fungicide due to the fitness cost associated with iprodione resistance. Therefore, it becomes advantageous to optimise its application in order to maximize its long-term efficiency (Raposo et al., 1995).

The objectives of the preliminary tests were as follows:

1. To achieve consistently the desired level of coverages (0 %, 25 % and 50 %) by calibrating the custom made spraying chamber.

2. To determine if there is a difference in efficacy between Rovral and Iprodione, and determine the discriminating dosage of iprodione which inhibits growth of sensitive strains while un-affecting the resistant strain.
3. To determine the EC_{50} of *Botrytis* towards several active ingredients and determine whether there is a presence of cross resistance between boscalid and other active ingredients of group 7.

3.2 Materials and methods

3.2.1 Onion plant production

Onion seedlings (Frontier) were planted in 9 X 9 cm square pots with approximately 40 g of general purpose Promix BX soil (Premier Horticulture). Plant were grown for four to six weeks in growth chamber under the following conditions: 20°C, 70 % relative humidity and 16 hours photoperiod. Under these conditions the plant produced four leaves, which is the optimal phenological stage for Botrytis inoculation (Carisse et al., 2012).

3.2.2 Production of inoculum

Two primary inoculums were produced from ten isolates of *B. Squamosa*. The first inoculum consists of five strains resistant to iprodione (BS02, BS04, BS05, BS07 and BS19) and the second one consist of the strains sensitive to iprodione (BS06, BS09, BS25, BS31 and BS42). These strains were taken amongst the 45 characterised strains collected from onion producing fields in Quebec (Figure 3.1A and 3.1B). Spores of the same resistance profiles were equally mixed to create the inoculum in order to prevent any biases toward more aggressive isolates.

3.2.3 Fungicide application and inoculation of Botrytis squamosa on onion plant

Fungicide applications were carried out in a custom made spraying chamber shown in figure 3.1I. The different levels of coverage (0 %, 25 % and 50 %) were attained by adjusting the air pressure as well as the speed at which it the spraying head assembly moves horizontally. The fungicide iprodione was diluted in 100 % isopropanol and further diluted in water to achieve the appropriate concentration.

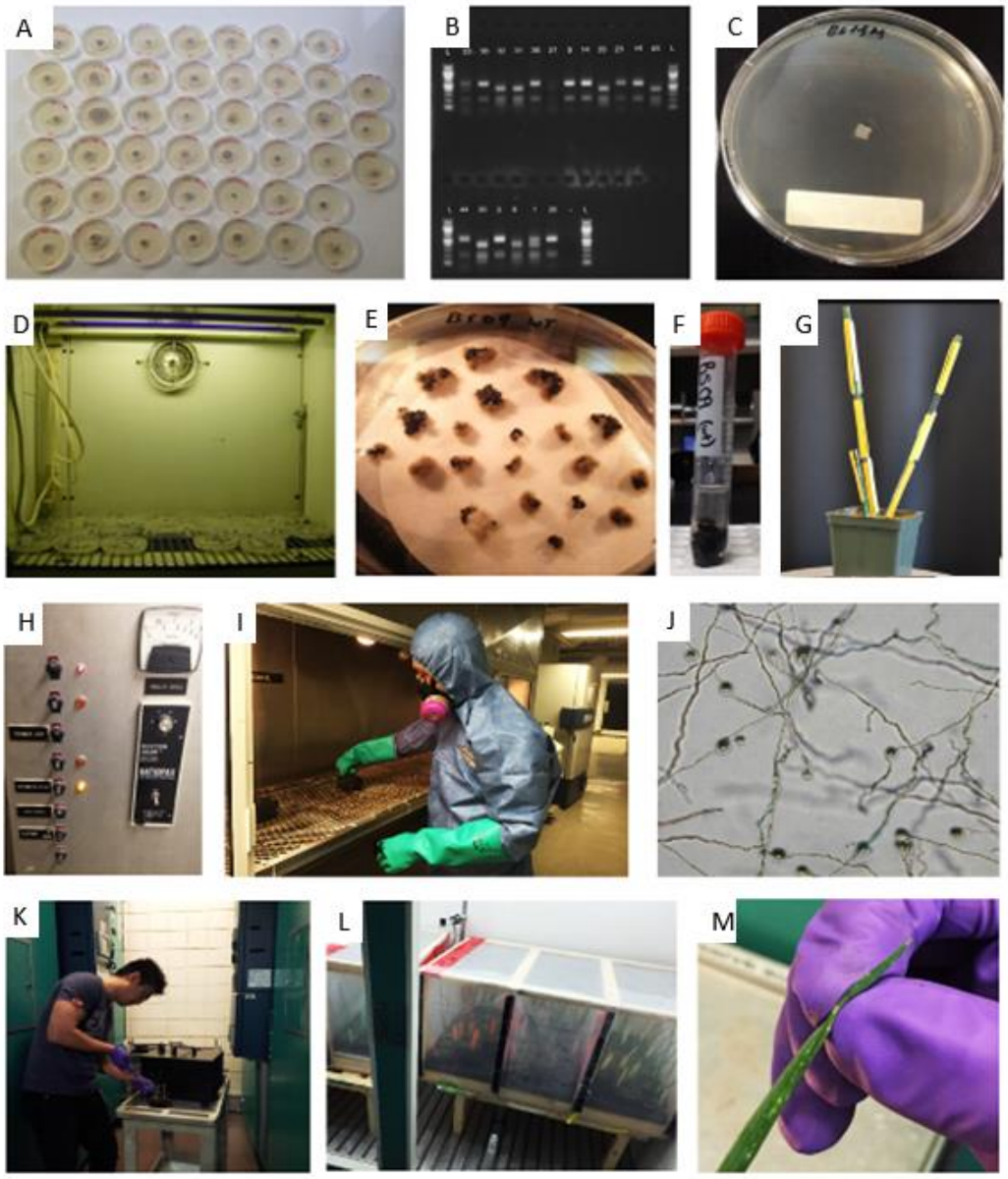

Figure 3.1 Experimental procedures for *Botrytis squamosa* inoculation and iprodione application. A) Total of 45 *B. squamosa* strains are collected from several onion producing sites and isolated on PDA petri supplemented with novobiocin. B) The sensitivity of the collected isolates towards iprodione was assessed using PCR-RFLP. C) Isolates repicked and grown on new PDA medium for sclerotia production. D) Sclerotium in sporulation chamber under UV lights and 14^{o}C temperature to stimulate sporulation. E) Sclerotia of *B. squamosa* covered with spores which were collected in F) 5 % glycerol solution. G) Stick and pot model to simulate the onion

plant. Water sensible papers are attached on the sides to test for coverage. H) Control panel of the custom built spraying machinery. I) Spraying bench with onion plants laid horizontally onto it, prepped for iprodione application. J) Water germination assay of *B. squamosa* in water agar. Black dots are spores and the filaments are the mycelium grown from the spores. K) Inoculation of onion plants with *B. squamosa*, and put immediately in L) the inoculation chamber, which have 100 % humidity level. M) A typical lesion caused by *B. squamosa* on onion plants.

In order to achieve the aforementioned coverage, stick and pot models that simulate the biological model of the onion plant was created (Figure 3.1G). These models had water-sensitive paper (QInstruments, Germany) strips attached to the sticks that turns blue once in contact with water. These water-sensible papers can be scanned and analysed using surface color detection program (Hydropap), provided by Dr. Bernard Panneton, from Agriculture Canada, in Saint-Jean-sur-Richelieu. The stick and pot models were laid out on the platform in the same fashion that the onion would, in order to have accurate representation (Figure 3.1I). The test was repeated until the coverage obtained were consistent and had no overlap in between different levels. The following parameters combinations were tested: 0.5 km per hour nozzle speed/ 30 pound per square inch of pressure, 0.5 km per hour nozzle speed/ 40 pound per square inch of pressure, 2km per hour nozzle speed/ 30 pound per square inch of pressure, 2km per hour nozzle speed/ 30 pound per square inch of pressure, 2 km per hour nozzle speed/ 40 pound per square inch of pressure, 1.5 km per hour nozzle speed / 40 pound per square inch of pressure, 1km per hour nozzle speed / 40 pound per square inch of pressure. For all the applications, the plants were positioned 50 cm away from the spraying nozzle, equipped with a TeeJet 60 nozzle for all the applications.

Using the parameters determined with the stick and pot models shown in figure 3.1G, the onions plants were placed horizontally onto the platform, and sprayed uniformly on one side (Figure 3.1I). Given enough time for the fungicide droplets to dry, the plants were turned over to have their backside pulverized. This was done to make sure to have a consistent level of coverage onto the whole plant. After the fungicide application, the plants were placed into a growth chamber (CONVIRON E15) for 24 hours under a 10 hours photoperiod with 60 % relative humidity and at 21^{o}C.

Prior to every plant inoculation, a germination assay was conducted to verify the viability of the pathogen (Figure 3.1J). Three droplets of approximate 20 μl of inoculum were deposited onto water agar plates at room temperature for 24 hours. The germination rates were determined by counting 50 conidia, and calculating the ratio between conidia with no mycelium and conidia with growth of mycelium that is at least half of their size.

The inoculation process occurs 24 hours after the application of fungicide iprodione, to ensure that the fungicide was fully dried and absorbed onto the leaves surfaces and also reduce health hazards while handling. The inoculum ($7.5x10^4$ conidia mL^{-1}) was applied using an airbrush compressor apparatus, at 172 kPa (Figure 3.1K). Immediately after the inoculation, the plant was placed into a custom-built inoculation cage located in a growth chamber (CONVIRON model E15). The condition within the chamber were 10 hour of photoperiod, 18ºC ± 1°C during daylight and 20ºC ± 1°C during nighttime at 100% relative humidity. After 84 hours, the RH was decreased to 60 % permanently without any change in temperature or photoperiod. In order to reach 100 % relative humidity, we used the inoculation chamber shown in figure 3.1L. The capacity for humidity level of the CONVIRON chamber was only 95 %, therefore we created these boxes to saturate the humidity level.

The plants were placed in a randomized fashion within the chamber in order to avoid any biases. The lesions rate were collected one week after the inoculation of the onion plants. Typical lesions caused by *Botrytis squamosa* grown on the first three leaves of the onion were counted.

3.2.4 Resistance profile of Botrytis towards various fungicides

The sampling of *Botrytis* strains was carried out each season using BBL culture swabs (Fisher Scientific) on symptomatic plants. The different strains were isolated using 90 mm petri dishes containing PDA medium (Potato Dextrose agar at 39 g / L) supplemented with 100 µg / µl of novobiocin. After being cultured for 72 hours, monocolonies were isolated and repicked onto new novobiocin supplemented PDA plates. This process was repeated until a pure culture was obtained. The strains to be tested were allowed four days incubation in order to prepare for the following resistance assays. Each isolate was tested for the following active ingredients: Azoxystrobin, Boscalid, Fluazinam, Fluopyram, Fluxapyroxad, Iprodione and Pyrimethanil (Sigma-Aldrich). Prior to the test, PDA medium described initially were prepared and supplemented with the aforementioned active ingredients. The active ingredient concentrations used were 0, 0.5, 1.0, 5.0, 10.0 and 50 ppm. At the time of the test, 6 mm culture disks were taken from the periphery of the petri dish containing the "mother" culture and placed on the center of the petri supplemented with various active ingredients. The tests were all done in duplicate. At the time of the repicking process, all strains were also placed in 2 ml tubes filled with sterile soil for long term conservation purposes.

The cultures grown in petri containing pesticides were then incubated in the dark for a period of 48 hours until further assessment. During the evaluation, the average growth diameter was noted and also compared to the control, which was supplemented with no active ingredients (0 ppm). The percent inhibition versus control was then calculated for each dose used. In order to calculate the EC_{50} (concentration needed to inhibit 50 % of radial growth), a logistic regression was used. A linear regression was also used to study cross-resistance between fungicides belonging to the same group.

3.3 Results

3.3.1 Achieving precise coverage

Our initial goal was to create 5 different levels of coverage; 0 %, 25 %, 50 %, 75 % and 100 % allowing us to study the gradual increase of fungicide coverage on the development of lesions as well as the selection for resistant strains. However, we quickly realised for the reasons discussed later that only 0 %, 25 % and 50 % coverages could be used for our study. The 0 % coverage were achieved by not subjecting the onion plants to fungicide, however they were still moved around with the other plants in order to not create biases during the handling process. The 25 % coverage was attained using the following parameters on the custom built spraying machinery: 1.5 km/h for nozzle speed, 305 kPa of pressure for the application while the plants were positioned 50 cm away from the spraying nozzle. The 50 % coverage was attained using the following parameters on the custom built spraying machinery: 1km/h for nozzle speed, 275 kPa of pressure for the application while the plants are positioned 50 cm away from the spraying nozzle. The coverages obtained were checked for consistency prior to each spraying session and we made sure that there was no overlap in-between the different sets of coverage (Fig. 3.5). We also kept the variation level to a minimum in between the different replicates.

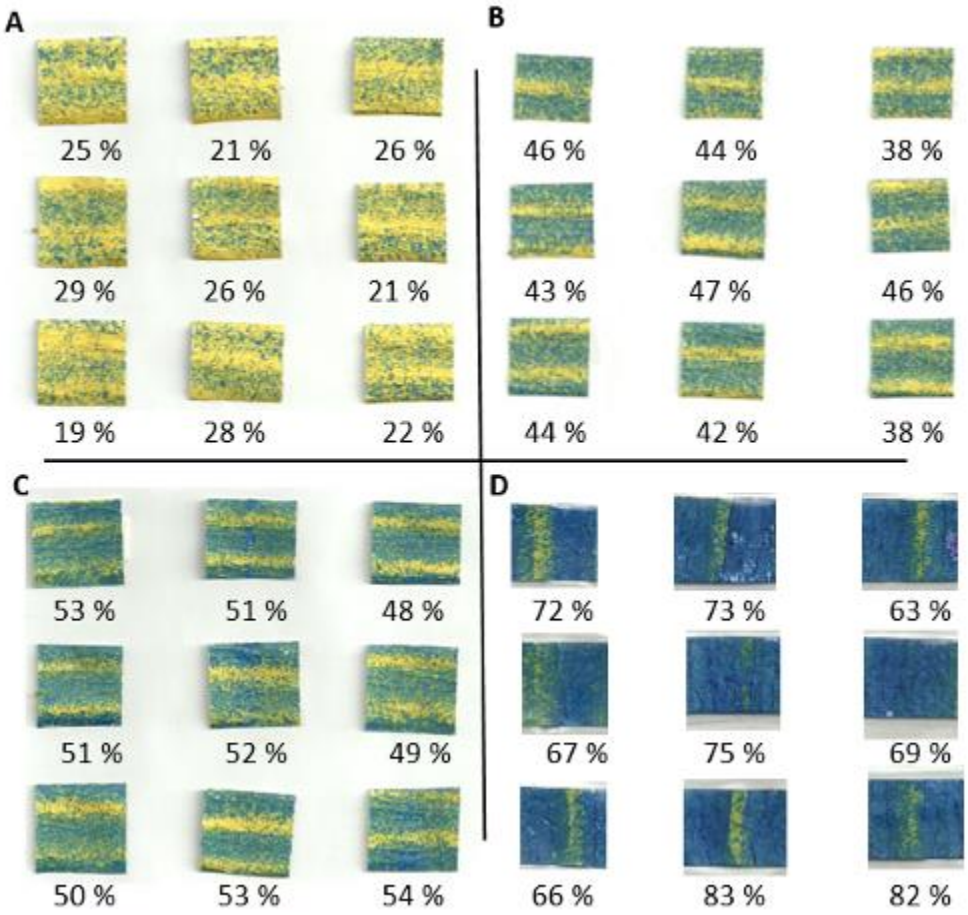

Figure 3.2 Fungicide coverage assay on water sensible paper. Four levels of coverages attained using the custom made spraying machinery. Coverage of A) 25 %, B) 43%, C) 50 % and D) 75 % were reached.

3.3.2 Determining the Discriminating Dosage of Iprodione

In order to determine the discriminating dosage of iprodione, we inoculated onions plants with 100 % sensitive *B. squamosa* isolates as well as 100 % resistant isolates on separate events. These sensitive and resistant isolates are prepared using a mixture of five different strains to prevent any biases. The sensitive inoculum consisted of five strains sensitive to iprodione (BS06, BS09, BS25, BS31 and BS42) and the resistant inoculum consisted of five strains resistant to iprodione (BS02, BS04, BS05, BS07 and BS19) these strains were characterized using PCR-RFLP and chosen from 45 isolates collected by La compagnie de recherche Phytodata shown in table 3.1. Furthermore, growth inhibition analyses were also conducted on these particular strains to confirm their resistance profile and also determine their EC_{50} value in regards to active ingredient iprodione (Table 3.2).

The discriminating dosage points where the lesion rate of the pathogen would reduce drastically while un-affecting the resistant isolates indicate the discriminating dose. This allowed us to determined that the discriminating dosage of iprodione is in-between 0 ppm and 50 ppm. By knowing this general range of discriminating dosage, we were able to repeat the assay a second time in order to narrow down the options with smaller increments. Therefore, we repeated the process with 0 ppm, 25 ppm, 50 ppm and 100 ppm. The results indicate a discriminating dosage between 25 and 50 ppm (Figure 3.3). Thus, we used 25 ppm of iprodione for the subsequent experiments described in this thesis unless stated otherwise.

Table 3.1 Characterization of 45 strains of *Botrytis squamosa* collected in several muck land onion-producing sites located in the South-Western part of Quebec province.

Strain number	R / S	Strain number	R / S	Strain number	R / S	Strain number	R / S
BS1	Resistant	**BS13**	Resistant	**BS25**	*Sensitive*	**BS37**	*Sensitive*
BS2	Resistant	**BS14**	Resistant	**BS26**	Resistant	**BS38**	Resistant
BS3	Resistant	**BS15**	Resistant	**BS27**	Resistant	**BS39**	*Sensitive*
BS4	Resistant	**BS16**	Resistant	**BS28**	*Sensitive*	**BS40**	Resistant
BS5	Resistant	**BS17**	Resistant	**BS29**	Resistant	**BS41**	Resistant
BS6	*Sensitive*	**BS18**	Resistant	**BS30**	*Sensitive*	**BS42**	*Sensitive*
BS7	Resistant	**BS19**	Resistant	**BS31**	*Sensitive*	**BS43**	Resistant
BS8	Resistant	**BS20**	Resistant	**BS32**	*Sensitive*	**BS44**	Resistant
BS9	*Sensitive*	**BS21**	Resistant	**BS33**	*Sensitive*	**BS45**	*Sensitive*
BS10	Resistant	**BS22**	Resistant	**BS34**	Resistant		
BS11	Resistant	**BS23**	Resistant	**BS35**	*Sensitive*		
BS12	Resistant	**BS24**	Resistant	**BS36**	Resistant		

Table 3.2 EC_{50} *Botrytis squamosa* strains used in this study including five sensitive strains (BS 6, 9, 25, 31 and 42) and five resistant (BS 2, 4, 5, 7 and 19) strains (BS 2, 4, 5, 7 and 19).

	Iprodione dosage (ppm)				
	0	0.5	1	5	10
***Botrytis squamosa* strain**	Radial growth (mm)				
BS 06	50.27	11.59	9.80	5.00	5.00
BS 09	36.56	11.40	10.05	5.00	5.00
BS 25	50.28	12.16	10.30	5.00	5.00
BS 31	25.72	8.60	7.01	5.00	5.00
BS 42	42.13	13.90	8.18	5.00	5.00
BS 02	58.65	55.78	39.59	12.31	5.00
BS 04	57.89	56.44	38.57	11.59	5.00
BS 05	46.85	55.81	31.25	11.08	5.00
BS 07	50.63	57.85	21.87	10.73	5.00
BS 19	70.80	62.93	27.66	11.42	5.00

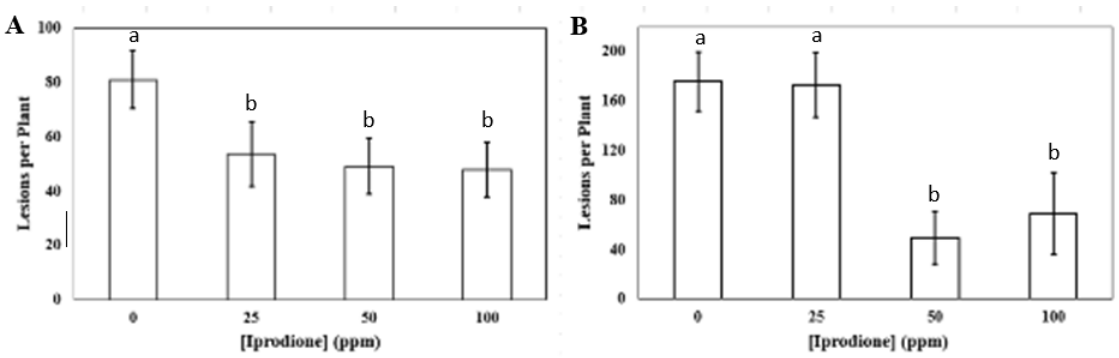

Figure 3.3 Discriminating dosage of iprodione for resistant individuals. A) Different iprodione concentrations used on sensitive isolates of *B. squamosa*. The coverage used here was 50 %. B) Different iprodione concentrations used on the resistant isolates of *B. squamosa*. The coverage used here was 50 %.

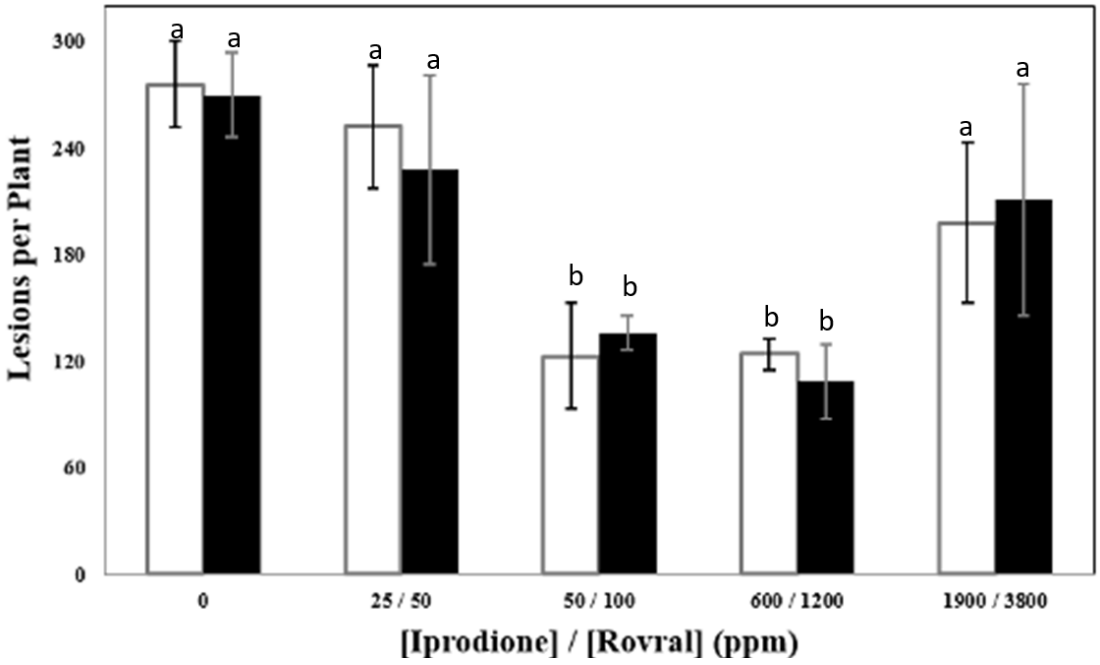

Figure 3.4 Difference between iprodione and Rovral for lesion control. The 100 % resistant *B. squamosa* isolate was used in this scenario with a 50 % iprodione coverage that for both treatments.

We also determined that we could use the active material (iprodione) instead of the commercial product (Rovral) without affecting the outcomes of the sassays. This was tested using the same gradient of concentration of Rovral as oppose to iprodione and by calculating the end resulting lesion rate remain, which nonetheless remained the same at each concentrations tested (Fig.3.4).

3.3.3 Resistance profile of Botrytis towards various fungicides

The most noticeable observation from the resistance tests carried out on *Botrytis* samples assessed from 2014 and 2016 is the average EC_{50} of 159 ppm for the active ingredient boscalid. This is surprising because isolates collected in 2004-2005 had an EC_{50} of 0.29 ppm (Figure 3.5). For other active substances belonging to group 7, the EC_{50} were 23 ppm, 20.47 ppm and 18.22 ppm for fluopyram, fluxapyroxad and penthiopyrad respectively. Correlation analyzes showed no cross-resistance between the active ingredients belonging to group 7 (Figure 3.6 A, B and C). For active ingredients azoxystrobin, iprodione, pyrimethanil and fluazynam, the EC_{50} were 0.123 ppm, 0.98 ppm, 20.5 ppm and 0.021 ppm respectively (Figure 3.5).

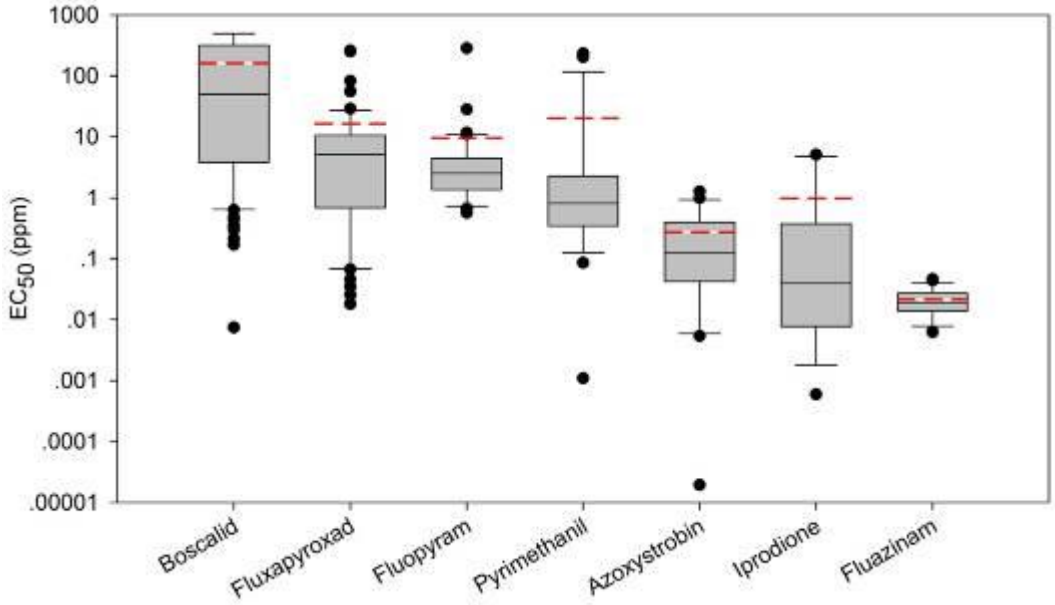

Figure 3.5 Resistance tests carried out on *Botrytis* samples assessed between 2014 and 2016. The red dotted line on each Tukey box represents the mean value for each active ingredient, and the black dots represent the outliers.

3.4 Discussion

A reliable and consistent spraying method was needed for the purpose of this study. In this chapter, we were able to consistently produce 25 % and 50 % iprodione coverages in a replicable manner. The initial goal of this study was to also test 75 % and 100% coverages, but we were unable to achieve those levels for the following reasons. To have an output of such coverage requires a high level of pressure, however as the pressure increases pass 40 psi the experimental setup showed signs of inconsistencies and caused a great amount of variances in-between replicates. Furthermore, fungicide runoff was inevitable for iprodione coverages above 60 % limiting the amount of fungicide onto onion leaves. Lastly, the custom built spraying machine lacked the capability to mix the solution while praying, which resulted in the blockage of the nozzle. Therefore, we chose not to achieve coverages of more than 50 %.

Determining discriminating dosages might seem redundant since there are already results available from radial growth inhibition assays (Lorbeer, 1992). For instance, Grabke et al. (2014) used a discriminating dosage of 5 and 50 mg/L of Rovral, which was assessed using a conidial germination assay. In addition, results we obtained from growth inhibition assays on samples that are used for this study show an EC_{50} value of 5 mg/L to 10 mg/L for the resistance strains (table 3.2). The issue with such data is the fact that these tests are done on fungicide supplemented PDA plates, and the conclusions are not applicable to situations where the fungicide is applied directly on the plants. For instance, the recommended concentrations for Rovral is 3,600 mg/L, whereas the concentration determined by conducting a EC_{50} analysis on the sample that we are testing in this study is between 1 and 5 mg/L for sensitive strains and 5 to 10 mg/L for the resistant strains. Determining

discriminating dosages might seem redundant since there are already results available from radial growth inhibition assays (Lorbeer, 1992). For instance, Grabke et al. (2014), used a discriminating dosage of 5 and 50 mg/L of Rovral, which was assessed using a conidial germination assay. these tests are done on fungicide supplemented PDA plates, and the conclusions are not applicable to situations where the fungicide is applied plants.he recommended concentrations Rovral 3,600 mg/L, determined 5 mg/L.. Furthermore, by looking at the results in section 3.3.3 (figure 3.5), we can notice that the EC_{50} for iprodione is merely 1 mg/L, and this was achieved by analysing samples that were collected between 2014 and 2016. Even for the most severe case of resistance, which was at 159 mg/L for the active ingredient boscalid, is still far off from the dosage that the product's label would recommend. Thus, it was necessary to determine the actual discriminating dosage prior to conducting the main experiment, to which we determined it to be in-between 20 and 50 mg/L of iprodione.

The reason why iprodione was chosen to be the fungicide of choice is due to the fact that there is fitness costs associated with its resistance (Raposo et al., 1995). As a matter of fact, an EC_{50} value higher than 1.6 mg/L was considered to be field resistant to iprodione back in 1992 when Raposo and al conducted his study on this subject. The fact that there has not been a strong selection for resistant population in 20 years is due to the fitness cost associated with iprodione resistance, meaning that the resistant individuals will be out competed against the wild type individuals when there is a lack of selective pressure (Carisse and Tremblay, 2007). On the other hand, fungicide boscalid have seen an increase of EC_{50} value from 0.29 mg/L in 2005 to 159 mg/L to 2016. This is due to the fact that unlike iprodione, there is no fitness cost associated with boscalid, meaning that the mutation which

allows pathogen to bypass the chemical will not impair its survival, therefore exhibits no competitive disadvantage against the wild type individuals when there is no boscalid present (Kim and Xiao, 2011). In brief, by studying a fungicide for which we can see a decrease of resistance in the fields would be more practical as oppose to a fungicide which will only increase in resistance despite the change in practices.

This led us to investigate the next issue, which is to determine whether we should use the active compound of the commercial product itself or to use the commercial formula as it is, which is the active compound plus the adjuvants. There are arguments for both sides. Firstly, it would be logical to use the commercial product since it is the most representative, because it is actually used by the growers in field practices. However, the commercial formulation contains adjuvants that enhance the effectiveness of the product, through increasing adhesiveness, absorption and coverage. This is problematic from our point of view as it could create possible biases. Using the active compound iprodione only, without using any of the adjuvants, circumvents this potential source of variability by limiting the possibility that other chemicals present in the formulation could affect the outcome of our study. Logically the flaw in this strategy is that it would not be representative of what happens in the field. Therefore, the just thing to do in this case would be to do an assay where we would compare the level of control between using Rovral and iprodione at the same amount of dosage and we determined that there is no significant difference between the two options. We chose to use iprodione in this case because it is easier to handle, and it is easier to dilute in liquids, and has lesser chance to sediment on the bottom.

In this chapter, we established a framework of fungicide coverages to be applied, as well as developing a strategy to achieve it on a consistent basis. We were able to determine

that there is no difference in disease control between using the active compound of the commercial formula. Furthermore, we successfully characterised 45 strains of *B. squamosa* and selected the resistant and sensitive strains to be used in the following experiment. To further confirm the characterization, we also performed a growth inhibition assay on the selected strains. We also determined the discriminating dosage, which was tested on onion plants, as oppose to a controlled PDA medium. Lastly, our field results collected from 2014 to 2016 indicated the EC_{50} values of various fungicide in actual field setting. Taken together, these results provide a sound foundation for the studies to follow, and will be key in the control of resistance propagation in current agricultural practices.

Connecting Statement to chapter 4

The previous chapter describes the methods development needed for the completion of the main study. With the knowledge of the discriminating dosage as well as the difference between using iprodione or Rovral, we can now confidently proceed to investigate the objectives of this study, which are to determine the proportion of resistant strains needed where a fungicide application will be rendered ineffective (Threshold of intervention), and to measure the impact of the heterogeneity of the fungicide spray on the selection of resistance. Furthermore, we can also be confident that the levels of coverage achieved are precise and consistent.

The experimental procedures were carried out by Zhe Jia and supervised by Dr. Jean-Benoit Charron, Dr. Odile Carisse and Hervé Van Der Heyden. Audrey Levasseur provided expertise as well as teaching the necessary techniques to the author. Dr. Jean-Benoit Charron, Dr. Odile Carisse and Hervé Van Der Heyden also helped writing the manuscript and provided constructive criticism. Hervé Van Der Heyden carried out the fieldwork and analyzed the data presented in section 4.4.3.

The following chapter is written in the format of manuscript, and the results will be submitted for publication in the future.

Chapter 4: The Role of Fungicide Spray Coverage and population Heterogeneity on the Selection for Resistance in *Botrytis squamosa*

4.1 Abstract

The usage of fungicides to treat or prevent crop diseases has always been riddled with pathogen resistance ever since its employment on an industrial scale. In this study, we aimed to investigate how fungicide is influenced by the presence of resistant individuals in the pathogen population as well as by the quality of application (fungicide coverage). To do so, onion plants were used as hosts for their capability to produce countable lesions that provided a source a quantitative assessment of *Botrytis squamosa*, lesions. The first objective was to determine the threshold of resistant individuals in a population needed to cause a significant reduction in the efficacy of the fungicide. To do so, a total of five ratios of resistant/sensitive individuals were inoculated onto onion plants (0 %/100 %, 25 %/75 %, 50 %/50 %, 75 %/25 % and 100 %/0 %), and sprayed with three coverages of iprodione. The number of lesions were counted and suggested that practical resistance arises when the population of the resistant isolates reaches 75 %. The second objective was to determine the influence of fungicide coverage (% leaf area sprayed) on the development of resistance. The results suggest that despite the coverage of iprodione applied, repeated application of the same fungicide will have a more significant effect on the selection for resistance. Taken together, the results obtained provided knowledge in regards to how fungicide coverage can affect disease control, and that an incomplete coverage can lead to coexistence between sensitive and resistant individuals, which ultimately lead to natural competition and superior disease control.

4.2 Introduction

In order to cope with a rapidly growing population, we need now more than ever to produce safe and reliable crops. Although technologies involving the usage of chemicals such as pesticides and fungicide have its flaws, we cannot deny how crucial they are in our agricultural practices (Hashemi et al., 2010). Therefore, we need to further our knowledge on this subject to achieve sustainable agricultural production. A sustainable agricultural system consist of multi-faceted methods to not only produce crops, but also cause the least amount of damages to the environment and human health while doing so (Pretty, 1995). Not only the usage of chemicals associate with costs for the growers, but are also detrimental for the environment. Therefore, we need to gage the amount and the frequencies at which chemicals are applied in order to maximise their effects and sustain high yield. However, the increased occurrence of fungicide resistances will either nullify the effectiveness of the chemicals, or will demand a higher application dosage.

Fungicide resistance is the process where a fungal pathogen has the ability to survive and reproduce in the presence of fungicides (Brent et al., 2002). This is a problematic issue in the field of agriculture as it can compromise our ability to manage pests and diseases on a long-term basis. Pathogens acquire mutations through the process of selection that allow them to circumvent the mode of action of the chemicals (Rosslenbroich et al., 2000). For instance pathogens can obtain single nucleotide mutation (SNP) at the site that the fungicide is targeting, leading to the un-specificity of the chemical, ultimately nullifying its effect.

The cause of fungicide resistance can be multifaceted. Many factors such as the mode of actions, the timing and the dosage of toxicants applied can all contribute to the

development of resistance (Staub 1991). It is also important to take into consideration the inherent biology and genetics of the pathogen and assess its susceptibility towards mutation. Some less obvious factors can also accelerate the development of pathogen resistance, such as the coverage of the particular pesticide/fungicide, and the heterogeneity of the pathogen population present within the same environment (Brent and Hollomon, 2007). For instance, achieving coverage of 100 % can lead to the selection for resistant strains. This is due to the fact that high coverage could eliminate and filter out the sensitive strains, which is crucial to promote a healthy natural competition between the two types of strains. On the other hand, a lower level of coverage could potentially favour co-existence between sensitive and resistant strains and benefit the post-treatment recovery of sensitivity (Parnell et al., 2005). Therefore, it becomes imperative to investigate the proper level coverage that can provide adequate level of control while maintaining a healthy biome. Past studies have also found similar effect where by Metcalfe et al. (2000) have utilized three different measured quantity of DMI fungicides to treat spring wheat cv. Baldus inoculated by *Mycospharella graminicola*. They showed that the lower dose application did not select for resistance, but instead slightly reduced the ratio of resistant strains versus nonresistant compared to the high dose applications.

Our ability to detect and gather information about pathogen resistance has greatly increased over the past decades with the advancement of technology in molecular biology (Staub, 1991). Techniques such as pyrosequencing and Real-Time PCR analysis have trivialised our ability to sample and profile resistance of pathogens towards a specific fungicide. However, challenges in the case of field practices still exist, where these data do not necessarily correlate with the level of control in the actual fields. For instance, it would

be practical to establish a threshold where the frequency of resistant strains reach above a tipping point (threshold of intervention) which cause a drastic effect on the control of the fungicide. This threshold of intervention would be a practical tool at the disposition of the growers, where they can predict the success rate of the fungicide application by knowing the resistance profile in their fields (Parnell et al., 2005)

Many pathogens have seen a great deal of increased resistance, such as *Botrytis squamosa,* which is a host specific necrotrophic pathogen that causes Botrytis leaf bright (BLB) in onions plants (Carisse et al., 2007). Continuous efforts have been put forward in the past 30 years in order to properly control and manage BLB (Carisse et al., 2011). The overarching goal of these efforts was to maintain an adequate level of control while reducing the amount of fungicide applied. The understanding of the impact of fungicide coverage on the selection for resistance as well as identifying the threshold of intervention will greatly contribute to the cause. Therefore, the aim of this study is to investigate the impact of fungicide spray coverage and population heterogeneity on the selection for resistance *Botrytis squamosa*.

4.3 Materials and Methods

4.3.1 Collection and generation of Botrytis Squamosa inventory

Forty-five isolates of *B. Squamosa* were obtained from various onion fields located in the Montreal mucklands situated near the western-southern part of Quebec province. The BBL culture swabs (Fisher Scientific) were used to collect randomly selected single sporulating colonies from onion leaves. The colonies were then placed on potato dextrose agar (PDA) plates supplemented with 100 µg / ml novobiocin for further preservation and

purification. A qPCR reaction was carried out in order to verify the content of the colonies collected, and to ensure that the recipient is in fact *B. Squamosa,* using a specific set of primers and TaqMan probe: B_squa_up221 (5-GCCGAACCAATACACCT-3), B_squa_lo361 (5-ATTGGATCACTT GGCGTG-3) and B_squa_Taqman_up282 (5-(6-FAM)TCTGTCCTGCAGAGACGCCC(BHQ1)-3) (Integrated DNA Technologies). Carisse et al., 2009).

4.3.2 DNA extraction & PCR amplification of BcOS1 histidine kinase gene

A small sample of mycelium or sclerotium was collected from PDA plates (Difco Laboratories) and put into a screw-cap test tube containing 100 mg of glass beads (Sigma, 425-600 um) and 80 ul of isopropanol. In order to release DNA from scleotium, the Fastprep-24 (MP Biomedicals) machine was used to agitate the tubes at 4.0 m/s during 20 seconds. The solution was then spun down for 5 seconds at 10,000g and the isopropanol evaporated using the SpeedVac centrifugal evaporator (Savant Instruments) at 55°C for 20 minutes. The chelex-100 solution (BioRad) was used as the extraction buffer, which consists of nuclease free water and 5 % chelex 100 molecular-biology-grade resin. A total of 300 ul of this buffer was added in the test tubes that were then incubated at 105°C for 15 minutes in a dry bath. Following the incubation, the tubes were vortexed for 5 seconds and spun down at 15,000 g for 5 min at 4°C. The supernatant that consist of extracted DNA is now ready for PCR analysis.

The PCR primers used to amplify the BcOS1 histidine kinase gene were: BcOS5 (5'-GAGGCTTTCCAAAAAGCTCT-3') and BcOS10R (5'-TCTTGGTCAAATCTCCTCTGGCGACA-3'), (Oshima et al., 2006). This particular

primer set amplified a fragment size of 1030 base pairs. The total reaction volume was 25 ul and consisted of 12.5 ul (1X reaction) of DNA buffer, 1ul (0.4 uM) of foward and reverse primer, 0.50 ul of Titanium taq DNA polymerase (Clontech Laboratories), and 7 ul of double-distilled water (Integrated DNA Technologies). The reaction was carried out in a Biometra T-gradient (Montréal Biotech Inc), and the parameters were set as follows: 98°C for 2 minutes, followed by 39 cycles of 98°C for 12 seconds each, then 62°C for 15 seconds each and 68°C for 1 minute each.

4.3.3 Characterization of resistance profile through RFLP-PCR

The total volume for each restriction reaction was 20 ul, which consisted of 5ul of PCR amplified product of the BcOS1 gene, 2 ul of CutSmart buffer, 0.2ul of bovine serum albumin, 0.5 ul of TAQa1 restriction enzyme (New England BioLabs), and 12.3 ul of double-distilled water (Integrated DNA Technologies). The reaction was incubated in a Biometra T-gradient (Montréal Biotech Inc) for 1 hour at 65 °C. The digested product were processed on an electrophoresis agarose gel (1.7 %) and visualized on a UV imagining machinery.

4.3.4 Production of conidia from sclerotia and preservation of sporulating sclerotia

The sclerotia were obtained from 45 *B. Squamosa* isolates, which were grown naturally on PDA plates supplemented with novobiocin. Sclerotia aged of one to two months were collected and transferred into a nutrient free medium in order to stimulate sporulation. Prior to the transfer into petri dish containing sterile cotton covered by filtered paper, the sclerotiums were sterilized in 1 % javex solution for 2 minutes and rinsed in nanopure distilled water for 2 minutes. A total of 25ml of nanopure distilled water was added into the plates to maintain humidity. The petri dishes were then sealed with parafilm and kept at 15°C

in a UV neon light incubator with a photoperiod of 18 hours. Theses plates were supervised and the appearance of conidiophore was noted.

The sporulating sclerotia were preserved in falcon tubes containing 5 % glycerol solution and kept at -20°C in a freezer. For long-term conservation, plugs of 4x4mm mycelium were collected from the initial plates of the isolates and put into a tube containing sterile pro-mix soil. These tubes were vortexed for 5 seconds and kept at room temperature for 2 to 5 days and transferred into a 4°C preservation chamber.

4.3.5 Onion plant production

Onion seedlings (Frontier) were planted in 9 X 9 cm square pots with approximately 40 g of general purpose Promix BX soil (Premier Horticulture). Plant were grown for four to six weeks in growth chamber under the following conditions: 20°C, 70 % relative humidity and 16 hours photoperiod. Under these conditions the plant produced four leaves, which is the optimal phenological stage for Botrytis inoculation (Carisse et al., 2012).

4.3.6 Production of inoculum

Two inoculums were produced from ten isolates of *B. Squamosa*. The first inoculum consisted of five strains resistant to iprodione (BS02, BS04, BS05, BS07 and BS19) and the second one consisted of the strains sensitive to iprodione (BS06, BS09, BS25, BS31 and BS42). In order to prevent any biases toward more aggressive isolates, spores of the same resistance profiles were equally mixed to create the inoculum. Aliquots of 30 mL of the inoculum were stored in -20^{o}C for future uses for no more than 18 months of storage. Mixtures of primary inoculums were used to generate the following sensitive / resistant secondary inoculums: 100/0, 75/25, 50/50, 25/75 and 0/100.

4.3.7 Fungicide application and inoculation of Botrytis squamosa on onion plant and experimental design

Fungicide applications were carried out in a custom made spraying chamber. Iprodione was diluted in 100 % isopropanol and further diluted in water to achieve the appropriate concentration. The different levels of coverage (0 %, 25 % and 50 %) were attained by adjusting the air pressure of the spraying nozzle as well as the speed at which it the spraying head assembly moves horizontally. In order to achieve the aforementioned coverages, stick and pot models that simulate the biological model of the onion plant was created. These models had water-sensitive paper (QInstruments, Germany) strips attached to the sticks that turns blue once in contact with water. These water-sensible papers can be scanned and analysed using surface color detection program (Hydropap), provided by Dr. Bernard Panneton, from Agriculture Canada, in Saint-Jean-sur-Richelieu. The stick and pot models were laid out on the platform in the same fashion that the onion would, in order to have accurate representation. The test was repeated until the coverage obtained were consistent and had no overlap in between different levels. The following parameters combinations were tested: 0.5 km per hour nozzle speed/ 30 pound per square inch of pressure, 0.5 km per hour nozzle speed/ 40 pound per square inch of pressure, 2 km per hour nozzle speed/ 30 pound per square inch of pressure, 2 km per hour nozzle speed/ 30 pound per square inch of pressure, 2 km per hour nozzle speed/ 40 pound per square inch of pressure, 1.5 km per hour nozzle speed / 40 pound per square inch of pressure, 1km per hour nozzle speed / 40 pound per square inch of pressure. For all the applications, the plants were positioned 50 cm away from the spraying nozzle, equipped with a TeeJet 60 nozzle for all the applications.

Using the parameters determined with the stick and pot models, the onions plants were placed horizontally onto the platform, and sprayed uniformly on one side. Given enough time for the fungicide droplets to dry, the plants were turned over to have their backside pulverized. This was done to make sure to have a consistent level of coverage onto the whole plant. After the fungicide application, the plants were placed into a growth chamber (CONVIRON E15) for 24 hours under a 10 hours photoperiod with 60 % relative humidity and at 21^{o}C.

Prior to every plant inoculation, a germination assay was conducted to verify the viability of the pathogen. Three droplets of approximate 20 µl of inoculum were deposited onto water agar plates at room temperature for 24 hours. The germination rates were calculated by counting 50 conidia, and calculating the ratio between conidia with no mycelium and conidia with growth of mycelium that is at least half of their size.

The inoculation process occurs 24 hours after the application of fungicide iprodione, to ensure that the fungicide was fully dried and absorbed onto the leaves surfaces. The inoculum ($7.5x10^{4}$ conidia mL^{-1}) was applied using an airbrush compressor apparatus (TBV), at 172 kPa. Immediately after the inoculation, the plant was placed into a custom-built inoculation cage located in a growth chamber (CONVIRON model E15). The condition within the chamber were 10 hour of photoperiod, 18^{o}C ± 1°C during daylight and 20^{o}C ± 1°C during nighttime at 100 % relative humidity. After 84 hours, the RH was decreased to 60 % permanently without any change in temperature or photoperiod. The plants were placed in a randomized fashion within the chamber in order to avoid any biases. The lesions rate were collected one week after the inoculation of the onion plants. Typical lesions caused by *Botrytis squamosa* grown on the first three leaves of the onion were counted.

The complete fungicide and inoculation frequencies used are shown in Figure 4.1. A total of three different levels of coverage are tested (0 %, 25 % and 50 %) as well as five different unique sets of ratios (100%S, 25%S/75%R, 50%S/50%R, 75%S/25%R, 100%R). Each sample consists of five biological replicates, which comprise of 60 plants in total. The experience will be repeated three times (repetition in time).

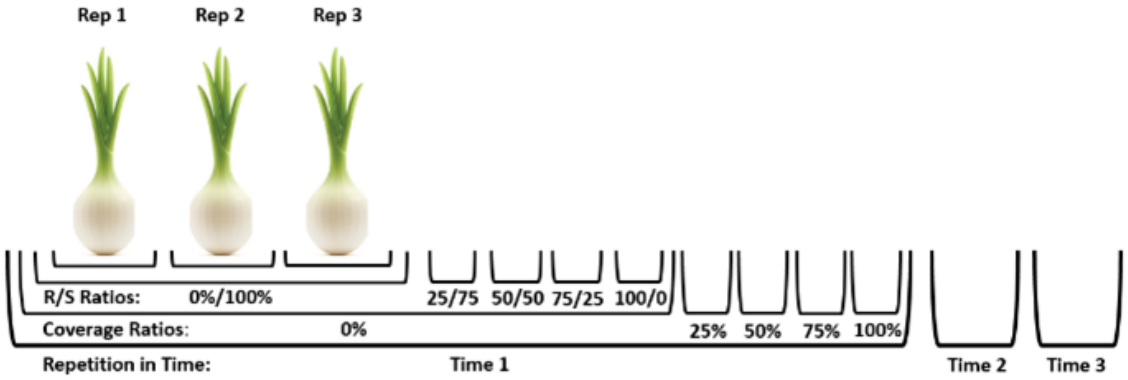

Figure 4.1 Frequencies of iprodione applications and *B. squamosa* used in this study. The experimental design of the experiment consists of a total of three different levels of coverage (0 %, 25 % and 50 %) as well as five different unique sets of ratios (100%S, 25%S/75%R, 50%S/50%R, 75%S/25%R, 100%R). Each sample consists of 5 biological replicates that comprise of 60 plants in total.

4.3.8 Generation of Botrytis squamosa spores from infected onion leaves and qPCR analysis

Botrytis squamosa lesions were counted from the previous steps and onions plants were put back into growth chambers (CONVIRON model E15) to senesce. The plants were left in growth chambers at 20°C, 60 % relative humidity and 16 hours photoperiod. They were not watered at this stage to speed up the senescence process. Dead onion leaves were collected and deposited onto filter papers (Fisher Sientific) within a petri dish. Five millilitres of water were added into the petri dishes and sealed with parafilm. Three days later, the onion leaves covered with *Botrytis squamosa* spores were collected with a BBL culture swab (Fisher Scientific), and deposited into a 15 ml falcon tube containing 300 µl of 100 % isopropanol. The spores were kept at 4^{o}C until DNA extraction. The methods for DNA extraction are described in sub-section 4.2.2, and the primers used for RT-PCR analysis were B_squa_up221 (5-GCCGAACCAATACACCT-3), B_squa_lo361 (5-ATTGGATCACTT GGCGTG-3) and the TaqMan probes used is B_squa_Taqman_up282 (5-(6-FAM)TCTGTCCTGCAGAGACGCCC(BHQ1)-3) (Integrated DNA Technologies. Carisse et al., 2009).

4.3.9 Assessment of varying iprodione coverages and repeated applications on the selection for resistance in field trials

The tests were carried out under commercial settings in 2015 and 2016 using a completely randomized block design. The design consisted of seven treatments and four repetitions. Three treatments involved using commercial fungicide application regimes, comprising several fungicides, including Rovral (commercial formulation of iprodione), at

30 %, 50 % and 75 % coverages. Three other treatments involving 30 %, 50 % and 75 % coverages were achieved using only Rovral as the fungicide applied. Experimental control data obtained from field sections not treated with any fungicide was also included in the analysis.

At the end of the sample collection process, ten samples of plants / plant parts or fruits affected by *Botrytis* were isolated according to the method described in section 4.3.3. Therefore, in each plot, a total of ten isolates of *Botrytis* were harvested and tested for resistance to iprodione. To do so, a PCR analysis was carried out followed by an enzymatic restriction. This procedure is described in Chapter 4 of the thesis submitted as the main document of the report. An analysis of variance was performed (PROC GLM) and when it revealed a significant difference, a Tukey multiple comparison test was used to determine which treatments were found to be different.

4.4 Results

4.4.1 Determining the threshold of intervention

One goal of this study was to determine the parameters at which the effectiveness of the fungicide would be drastically lowered, which is also known as the threshold of intervention. In order to do so, it was necessary to investigate several unknown factors, which played an intricate role in this process. These factors were determined in a set of preliminary experiments (chapter 3), where we determined the appropriate fungicide coverage. Based on these tests we decided to use 0 %, 25 % and 50 % coverages. Furthermore, we determined that there is no significant difference between using iprodione and its commercial formulation Rovral (Iprodione plus adjuvants), in regards to disease control in a controlled

laboratory setting. Lastly, we determined the dosage of iprodione to use in the present study, a dosage that would eliminate any *Botrytis squamosa* individuals sensitive to iprodione, while being ineffective against resistant individuals. This value was set out to be 50 ppm.

These preliminary tests thus provided us with the tools necessary to investigate the threshold of intervention. By applying a precise dosage of iprodione at the appropriate levels of coverage (figure 4.1 is shown in figure 4.2) we observed that the impact of different coverages as well as the ratio of sensitive and resistant individuals can affect severity of the disease by modulating the lesion rate.

As expected, the result demonstrates that the lesion rate for all five ratios of sensitive/ resistant *Botrytis* populations with 0 % coverage (no iprodione applied) are high and showed no significant difference (Fig 4.2). Similarly, the number of lesions per plant following 25 % iprodione coverage also showed no significant difference between all different ratios of sensitive over resistant isolates. However, the lesion rates for the isolates composed of 100 % sensitive and the 75 % / 25 % resistant strains were significantly different when an iprodione coverage of 50 % is achieved. In addition, these results revealed that increasing ratios of resistant individuals can affect the effectiveness of the fungicide iprodione. Interestingly, we noticed that when a 50 % iprodione coverage is achieved, a significant difference in lesion rate is observed for populations containing resistant individuals below 25 % (Figure 4.2).

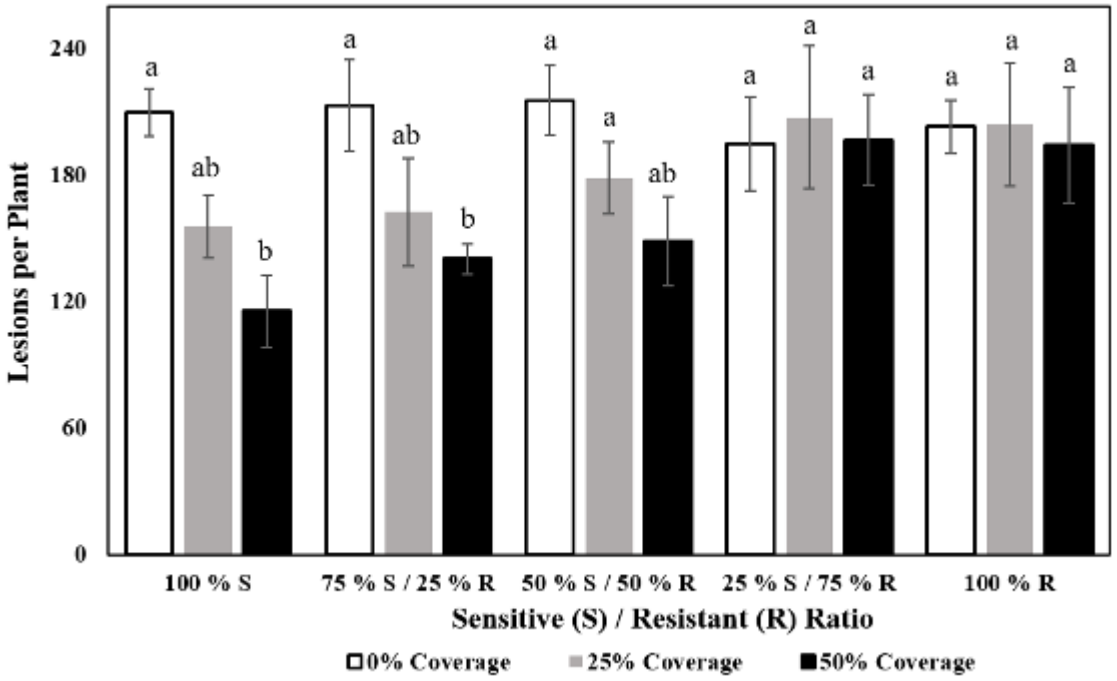

Figure 4.2 Effect of iprodione coverage variations on populations of *Botrytis Squamosa* composed of increasing ratios of sensitive vs resistants isolates. Onion plants were inoculated with five ratios of sensitive/resistant isolates of *Botrytis squamosa* and sprayed with 50 ppm of iprodione or water only to attain 0 %, 25 % and 50 % coverages.

Table 4.1 Sum of squares analysis (Type III) showcasing effect of variables tested on development of the disease

Sources	Type	DF	Sum of squares	Mean squares	E Mean squares)	F	Pr > F
Proportion	Fixed	4	12968.133	3242.033	∑ 2 + 5 * ∑ 2 (proportion * coverage) + 15 * Q (proportion)	2.630	0.114
Coverage	Fixed	2	8886.480	4443.240	∑ 2 + 5 * ∑ 2 (proportion * coverage) + 25 * Q (coverage)	3.604	0.077
Proportion * Coverage	Random	8	9861.787	1232.723	∑ 2 + 5 * ∑ 2 (proportion * coverage)	4.140	0.001
Error		60	17865.600	297.760	∑ 2		

Consequently, when the resistant individuals exceed 25 % in a population, the differences observed are becoming less significant. This experiment thus suggests that practical resistance arises at around 75 % of resistant isolates present within a population. Furthermore, this experiment revealed that the control of *Botrytis* leaf blight improved as a result of increased fungicide leaf area coverage (0 % to 50 %) but only when the sensitive isolates were exceeding the resistant ones (Figure 4.2).

Interestingly, mixed model analysis of the resistant / sensitive *Botrytis squamosa* isolate ratio and the iprodione coverage showed that the level of coverage and the ratios of sensitive and resistant isolates both have insignificant effects on the development of the disease on their own (Table 4.1). However, this analysis revealed that a significant effect on onion lesion rates could be observed when the isolate ratio and fungicide coverage are considered together.

4.4.2 Effect of fungicide spray coverage on the selection for resistance

In order to determine molecularly whether fungicide coverage can select for resistance, we analyzed the shift in sensitive and resistant ratio after they were subjected to different level of coverages using a quantitative PCR approach after one cycle of fungicide application, which measured the presence of SNP, which indicates the presence of resistance. Figure 4.3 shows variation in lesion rates caused by different ratios of sensitive and resistant *Botrytis* strains after being subjected to different fungicide coverages.

On panel A of figure 4.3, we can notice that the percentage of 100 % sensible individuals remains above 75 % when subjected to all three levels of coverages. The presence of resistant individuals for this scenario is abnormal and will be further discussed in the

following section. Similarly, in panel E the percentage of 100 % resistant individuals remain above 70 % when subjected to all three levels of coverage and abnormal presence of sensitive individuals is also noted. Any significant changes in the sensitive and resistant ratio after one set of iprodione application were not detected when ratios of 75 % sensitive / 25 % resistant or the 25 % sensitive / 75 % resistant individuals where used. Overall, the interpretation of the variation in the sensitive / resistant population after being subject to iprodione proved to be difficult, as the statistical analysis did not show any significant variations due to inconsistent results, high degree of variation within the treatment, and also lack of repeated applications of fungicide.

This analysis also revealed that onion plants exposed to a Botrytis population composed of 100 % resistant isolates have lesion rates above 70 % at all three levels of fungicide coverages (Fig 4.3E). The abnormal presence of sensitive individuals in the 100% resistant scenario is also noted. No significant changes in lesion rates were noted when onion plants were exposed to ratios of sensitive / resistant *Botrytis* isolates of 75 % / 25 % and 25 % / 75 % after one round of iprodione application at the three levels of coverage (Figure 4.3 B – D). Overall, the interpretation of the variation in the sensitive / resistant Botrytis population after being subject to iprodione by the quantitative PCR approach proved to be difficult, as the statistical analysis did not show any significant variations due to inconsistent results, high degree of variation within the treatment, and also lack of repeated applications of fungicide.

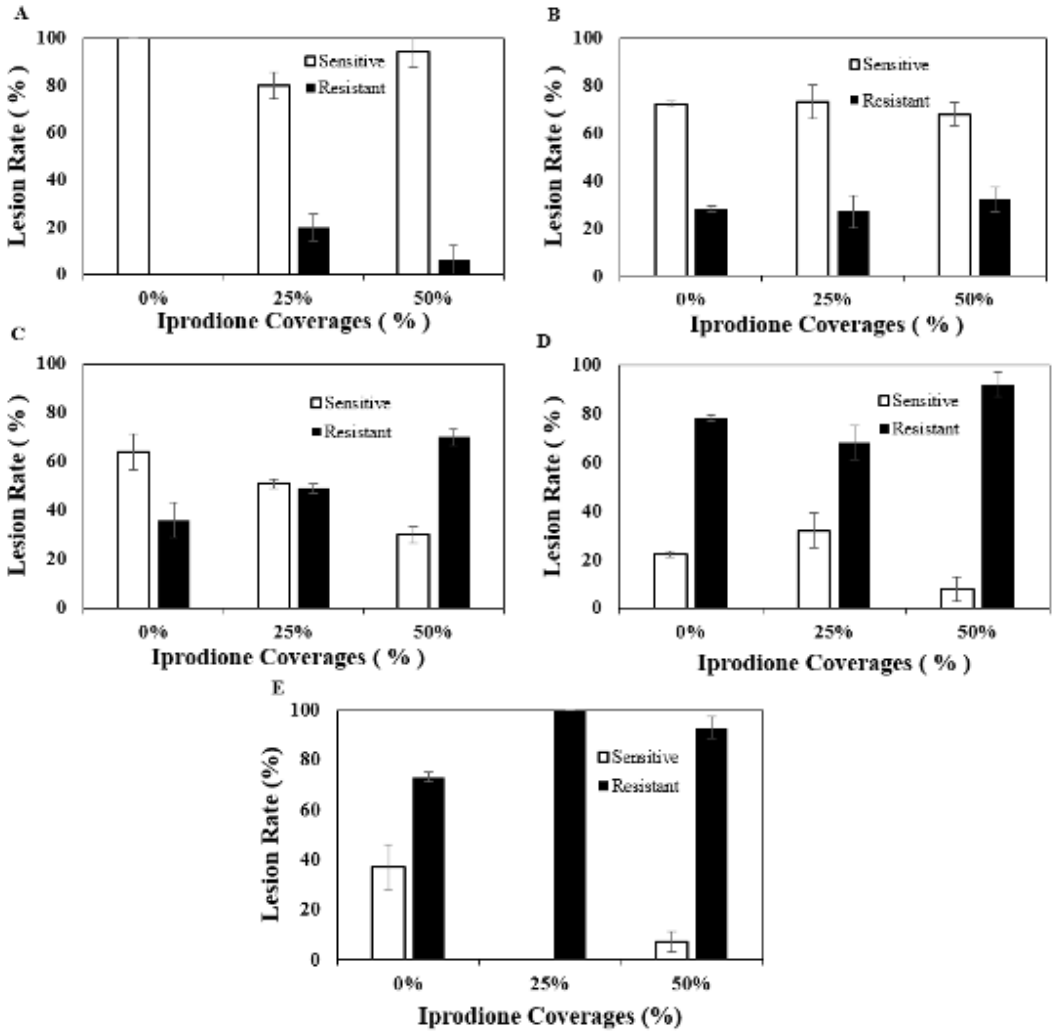

Figure 4.3 Variation in sensitive/resistant B. squamosa ratio after being subjected to different iprodione coverages. A) Lesion rate for 100 % sensitive individuals subjected to 0 %, 25 % and 50 % of coverage. B) Lesion rate for 75 % sensitive / 25 % resistant individuals subjected to 0 %, 25 % and 50 % of coverage. C) Lesion rate for 50 % sensitive / 50 % resistant individuals subjected to 0 %, 25 % and 50 % of coverage. D) Lesion rate for 25 % sensitive / 75 % resistant individuals subjected to 0 %, 25 % and 50 % of coveages. E) Lesion rate for 100 % resistant individuals subjected to 0 %, 25 % and 50 % of coverage.

4.4.3 Impact of coverage and repeated application of iprodione on the development of field resistances

A significant difference between fungicide treatments and the resistance percentages of *Botrytis* strains found on onion plants grown in the field was observed in 2015 (Psite1> 0.0001; Psite2> 0.0001; Fig 4.4). The percentages of resistant Botrytis individuals were significantly lower for treatments 1 to 4 when compared to treatments 5 to 7 (Figure 4.4A). Damage assessment results also revealed a significant difference between treatments (Figure 4.4B). In this case, treatments 3 and 4 were significantly different from the untreated control (Psite1> 0.0001, Psite2> 0.0001).

In addition, a significant difference between fungicide treatments and resistance percentages was identified for onion growing fields assessed in 2016 (Psite1> 0.0001; Psite2> 0.0001). The grower's regime aforementioned in figure 4.4 indicates a set of fungicide application which the grower apply in his practices, which are conceived to accommodate the level disease present in his fields. Therefore a lower presence of resistance is expected for treatment 1 (control) to 4 (grower's regime at 30 %, 50% and 75% coverage) when compared to treatment 4 and 7, which only used Rovral as the fungicide of choice (Figure 4.5A). However, following the assessment of obtained results, no significant differences are shown between the treatments.

The results suggest that regardless of the fungicide coverage, the repeated usage of same fungicide will increase significantly the selection for resistance in *Botrytis* and also increases the number of lesions per plant, which will in turn negate the effect of coverage.

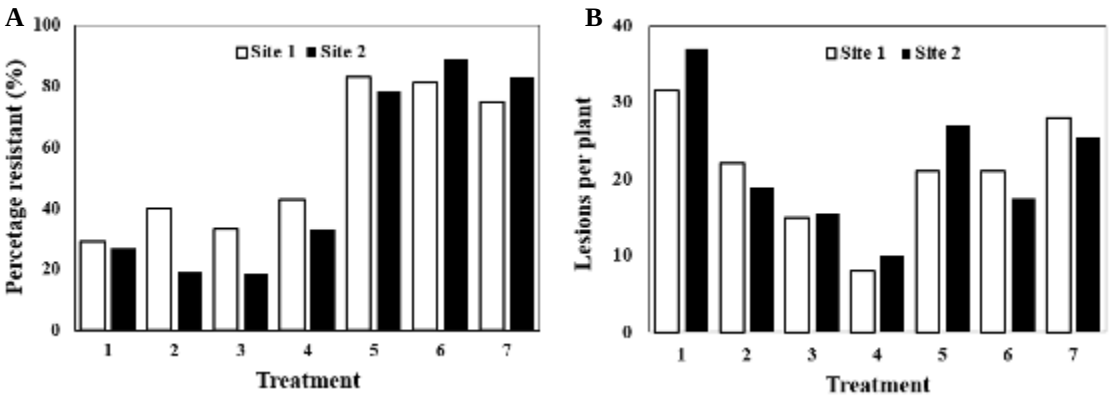

Figure 4.4 Results of field trials for onion sites 1 and 2 assessed in 2015. A) Percentage of resistant isolates per treatment and B) number of lesions per plant. The corresponding treatments are as follows: (1) control, (2) grower's regime at 30 % coverage, (3) grower's at 50 % coverage, (4) grower's regime at 75 % coverage, (5) Rovral only at 30 % coverage, (6) Rovral only at 50 % coverage, (7) Rovral only at 75 % coverage

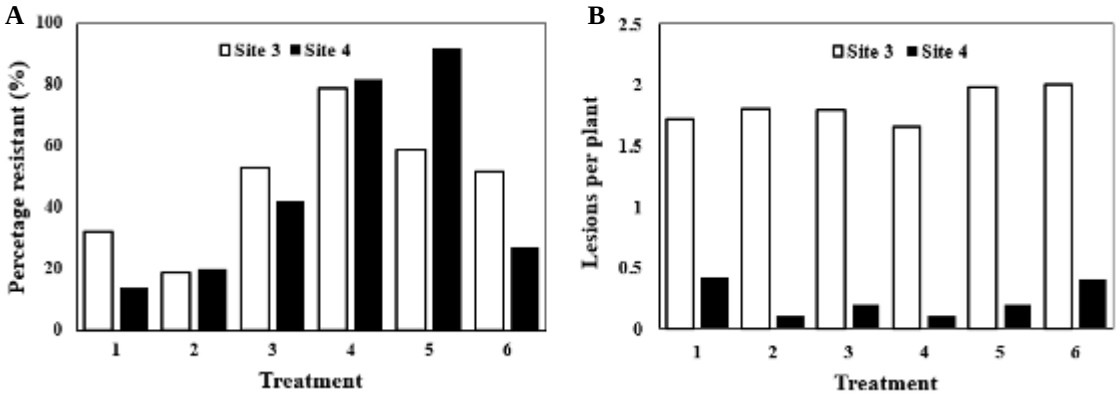

Figure 4.5 Results of field trials for onion sites 3 and 4 assessed in 2016. A) Percentage of resistant isolates per treatment and B) number of lesions per plant. The corresponding treatments are as follows: (1) control, (2) grower's regime at 30 % coverage, (3) grower's at 50 % coverage, (4) grower's regime at 75 % coverage, (5) Rovral only at 30 % coverage, (6) Rovral only at 50 % coverage, (7) Rovral only at 75 % coverage

4.5 Discussion

The overall goal of this study was to provide interpretation to data collected in the fields. While there are many tools available for gathering and sampling severity of crop diseases, we still lack the knowledge to interpret them (Hobbelen et al., 2014). For instance, Carisse et al. (2009), have developed many tools over the years to detect the presence of *Botrytis squamosa* as well as assess the severity of their resistance against fungicides. Tools such as qPCR analysis and monitoring based indicators are accurate in data collection and interpretation on a basic level (Van Der Heyden et al., 2012). This is why, in this study, we aimed to interpret these data, and give relevance so that the growers can take adequate measures to gage the emergence of resistant crop pathogens

To do so, we first determined the threshold of intervention, which is when the resistant strains reach above a tipping point within the population, causing a loss of effectiveness of the fungicide (Zadoks, 1985). According to the data obtained in chapter 4, we determined that the threshold of intervention was attained when 75 % of resistant *Botrytis* individuals are present within a population (Figure 4.2) Therefore, actions should be taken when fungicide field studies indicate over 75 % resistance of *Botrytis squamosa* towards iprodione. Furthermore, a warning threshold can also be established between 50 % and 75 % resistant individuals. This mean that if a resistance profile lands within this range, pre-emptive measures should be taken to slow down on the usage of iprodione. Our results suggest that increases in iprodione coverage significantly impact the level of control in scenarios where resistant individuals are below 25 %. However, varying iprodione coverages will no longer affect the quality of disease control in scenarios where resistant populations are above 25 %. The latter statement is applicable for field practices, since many surveys

have been done in regards to resistance of *Botrytis* against iprodione, which indicate that the resistance have nearly doubled from 2010 to 2014, and is now in some cases over 80 % (Carisse et al., 2007 and Van der Heyden et al., 2014).

Our second objective was aimed at investigating whether an increasing coverage of fungicide can select for resistance. Understanding this concept would help in achieving a co-existence between sensitive and resistant individuals, which has been proven to alleviate the selection for pressure (Parnell et al., 2009). From the data that we have gathered from field trials, we can conclude that different levels of coverage play a small role in terms of selection for resistance when compared to repeated application of the same fungicide. This is due to the fact that repeated applications of Rovral will eliminate sensitive individuals at each cycle, leaving less and less opportunity for them to replicate (Brent and Hollomon, 2007). Furthermore, the lack of other fungicides with different modes of action also means that the individuals resistant to Rovral will never be at risk (Brent and Hollomon, 2007). Given enough time and repeated applications of Rovral, the plant will be completely infected with *B. squamosa strains* resistant to iprodione regardless of the coverage. Thus, a higher coverage can only expedite this process. This procedure was also carried out in a controlled laboratory setting, where we used custom made fungicide application machinery and growing chambers with conditions optimised for *B. squamosa* infection. However the results were inconclusive due to limited timeframe that prevented us to repeat the experiments. Furthermore, the results obtained are only from one cycle of iprodione application, which limits their comparison with those of the filed study.

Taken together, our results put further emphasize the importance of fungicide resistance control, and determined a threshold of intervention at which iprodione is no longer

effective in treating *Botrytis squamosa*, which provides growers an invaluable tool to control this disease. Furthermore, the field data gathered also underlines the importance of staggering different types of fungicides with different modes of action between applications as it can retardate the development of resistance.

Chapter 5 General Discussion and Future Studies

5.1 General Discussion and Contribution to Knowledge

Crop pathogens and diseases have existed ever since human species started living a settled lifestyle and engaged in the practice of agriculture. Our struggles to fend off crop diseases have evolved through the ages with the evolution of technology and the increased understanding of biology and chemistry. However, there are still many unknown factors that we encounter today that can disturb the balance that we achieved throughout the ages. Pathogen resistance is in fact one of the imminent threat, as the carelessness in the application of chemicals can accelerate this process. The immergence of resistance in *Botrytis squamosa* towards iprodione is one of the resulting effects of over-usage of this chemical.

Chapter 3 of this study consisted of tools and protocols development that enabled us to complete the main study. The first experiment consisted of calibrating the spraying bench, which allowed us to achieve a precise and consistent coverage of fungicide, this was a very important step since we were working very closely with precise fungicide coverages, and having overlapping and inconsistent coverage could have compromised the whole procedure. Furthermore, we established in this chapter the discriminating dosage of iprodione against *Botrytis squamosa* and we were able to establish for the first time a link between the growth inhibition of a pathogen in a lab setting and on actual plants. Lastly, we determined that we could use as the chemical agent in this study a solution of iprodione instead of the commercial formulation Rovral without affecting scope of this study.

Chapter 4 of this study consisted of the manuscript part of this dissertation, where we investigated the role of fungicide coverage and heterogeneity on the selection for resistance in *Botrytis squamosa*. In the first objective, we established that the threshold of intervention

is 75 % resistant isolates, and we see an increasing rate of resistance development in-between 50 % and 75 % resistant individual present within the population. This means that if growers have access to fungicide surveys of their fields, and notice the presence of the iprodione resistant *Botrytis* population is over 75 %, then they should consider stopping the usage this chemical. The field data obtained in the second objective suggest that repeated applications of the same fungicide have a significant impact on the selection for resistance. Therefore, it is the upmost importance to have a fungicide application regime that incorporates different chemicals with different modes of action to reduce the development of resistance. The laboratory trials were non-conclusive at this time due to result inconsistency and variation within the treatment and will have to be repeated.

Together, the results obtained in this study suggest that there is a way to control the emergence of resistance in crop pathogens. This is especially useful in the case of reversible resistance, such as the case of iprodione resistance. With this knowledge, we can adapt our method of fungicide and pesticide application, by applying the correct chemical at the right dosage and time. The knowledge gained from this project leads us one step closer to a sustainable agricultural system, which is the future of crop production.

5.2 Future Studies

The Establishment of the correlation between resistance profile and level of control in the *Botrytis squamosa* - iprodione system, have opened up opportunities to unravel our understanding in the resistance phenomenon as a whole. Many additional researches can be built on the foundation that is laid with the outcome of this study.

Firstly, we only had the chance to complete this experience by investigating one pathogen, which is *Botrytis squamosa*, which is a host specific pathogen. Therefore it would be advantageous to also replicate this study on other pathogens, which are not host specific, or pathogens that infect other variety of crops.

Secondly, using other types of fungicides or pesticides with different modes of action will also contribute to the better understanding of this process. Since we looked at a fungicide that has a fitness cost associated with it, it would be also interesting to use a fungicide with no associated fitness cost in the future.

Lastly, due to technical limitations associated with the spraying bench, we were unable to work with higher coverage. Therefore, it would be interesting to study the implications of higher coverage on the selection for resistance, as well as working with different plant hosts, which are more difficult to spray with the standard equipment.

References

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M. and Parr-Dobrzanski, B. (2002). The strobilurin fungicides. Pest Management Science, 58: 649-662.

Brent, K. J. and Hollomon, D.W. (1998). Fungicide resistance: The assessment of risk. Global Crop Protection Federation, Brussels. FRAC Monograph No. 2.

Carisse, O. and Tremblay, D. M. (2007). Incidence and significance of iprodione-insensitive isolates of Botrytis squamosa. Plant Disease, 91:41-46.

Carisse, O., Tremblay, D.M., Lévesque, C.A., Gindro, K., Ward, P. and Houde, A. (2009). Development of a TaqMan real-time PCR assay for quantification of airborne conidia of Botrytis squamosa and management of Botrytis leaf blight of onion. Phytopathology, 99:1273-1280.

Carisse, O., Tremblay, D.M., McDonald, M.R., Brodeur, L., and McRoberts, N. (2011). Managemant of Botrytis leaf blight of onion: The Québec experience of 20 years of continual improvement. Plant Disease, 95:504-514.

Chin, K. M., Chavaillaz, D., Kaesbohrer, M., Staub, T. and Felsenstein F. G. (2001). Characterizing resistance risk of *Eryphe gaminis* f.sp. tritici to strobilurins. Crop Protection, 20:87-96.

Couderchet, M. (2003). Benefits and problems of fungicide control of *Botrytis cinerea* in vineyards of Champagne. Vitis, 42:165-171.

Dane, F. and Dalgic, O. (2005). The effects of fungicide benomyl (benlate) on growth and mitosis in onion (Allium cepa L.) root apical meristem. Acta Biologica Hungarica, 56:119-128.

De Visser. C. L. M. (1996). Field evaluation of a supervised control system for *Botrytis* leaf blight in spring sown onions in The Netherlands. European Journal of Plant Pathology, 102:795-805.

Dewey, F.M. and Meyer, U. (2004) Rapid, quantitative tube immunoassays for on-site detection of Botrytis, Aspergillus and Penicillium antigens in grape juice. Analytica Chimica Acta, 513:11-19.

Edlich, W. and Lyr, H. (1992). Target sites of fungicides with primary effects on lipid peroxidation. Target Sites of Fungicides. Florida, USA

Elad Y, Yunis H and Katan T (1992) Multiple fungicide resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of Botrytis cinerea in Israel. Plant Pathology, 41:4-44.

Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (2007) Botrytis: Biology. Pathology and Control. Springer, Dordrecht, Netherlands, 29-52.

Ellerbrock, L. A. and Lorbeer, J.W. (1977). Sources of primary inoculum of *Botrytis squamosa*. Phytopathology, 67:219-225.

Faretra, F., Antonacci, E. and Pollastro, S. (1998). Sexual Behaviour and Mating System *of Botryotinia fuckeriana*, Teleomorph of *Botrytis cinerea*. Journal of General Microbiology, 134:2543-2550.

Fernández-ortuño, D., Torés, J. A., Vicente, A. D. and Pérez-garcía A. (2010). The QoI Fungicides, the Rise and Fall of a Successful Class of Agricultural Fungicides, Fungicides, Odile Carisse (Ed.). Intech, Rijeka, Croatia, 204-210.

Gisi, U., and Sierotzki, H. (2008). Fungicide modes of action and resistance in downy mildews. European Journal of Plant Pathology, 122:157-167.

Grabke, A., Fernández-Ortuño, D., Amiri, A., Li, X., Peres, N. A., Smith, P. and Schnabel, G. (2014). Characterization of iprodione resistance in Botrytis cinerea from strawberry and blackberry. Phytopathology, 104:396-402.

Hancock, J. G., and Lorbeer, J. W. (1963). Pathogenesis of *Botrytis cinerea*, *B. squamosa*, and *B. allii* on onion leaves. Phytopathology, 53:669-673

Ishii, J., Izawa, K., Matsumura, S., Wakamura, K., Tanino, Tanaka, T., Ogino, C., Fukuda, H. and Kondo, A. (2009). A simple and immediate method for simultaneously evaluating expression level and plasmid maintenance in yeast. *Journal of Biochemistry,* 145:701-708.

Janisiewicz, W.J. (1994). Enhancement of biocontrol of blue mold with nutrient analog 2-deoxy-D-glucose on apples and pears. Applied Environmental Microbiology, 60:2617-2676.

Jarvis, W. R. (1977). Botryotinia and Botryis species: taxonomy physiology and pathogenicity. Ottawa, Canada Departement of Agriculture, 15-33.

Kim, Y., Coppey, M., Grossman, R., Ajuria, L., Jimenez, G., Paroush, Z. and Shvartsman, S. Y. (2010) MAPK substrate competition integrates patterning signals in the *Drosophila* embryo. Current Biology, 20:446–451

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J. and Chapeland, F. (2002). Mechanisms of resistance to fungicides in field strains of Botrytis cinerea. Pest Management Science, 58:876-888.

Leroux, P. (2007). Chemical control of Botrytis and its resistance to chemical fungicides. Botrytis: Biology, Pathology and Control, Springer. Dordrecht, Netherlands, 10-21.

Lorbeer, J. W. (1992). *Botrytis* leaf blight in onions. Plant Diseases of Internation Imporance. Volume II. Diseases of Vegetables and Oil Seed Crops. New Jersey, USA.

Lucas, J. (2006). Avoiding and managing fungicide resistances. HGCA conference. Harpenden, England, paper 8.1.

Lyr, H. (1995). Modern Selective Fungicides: Properties, Applications, Mechanisms of Action, Villengang, Germany, 39-52.

Metcalfe, R. J., Shaw, M. W. and Russell, P.E. (2000). The effect of dose and mobility on the strength of selection for DMI fungicide resistance in inoculated field experiments. Plant Pathology 49:546-557.

Milling, R. J. and Richardson, C. J. (1995). Mode of action of the anilino-pyrimidine fungicide pyrimethanil. 2. Effects on enzyme secretion in *Botrytis cinerea.* Pest Management Science. 45:43-48.

Murray, A. E., Hollibaugh, J. T. and Orrego, C. (1996). Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Applied Environmental Microbiology, 62:2676–2680.

Oshima, M., Fujimura, M., Banno, S., Hashimoto, S., Motoyama, T., Ichiishi, A. and Yamaguchi, I. (2002). A Point Mutation in the Two-Component Histidine Kinase *BcOS-1* Gene Confers Dicarboximide Resistance in Field Isolates of*Botrytis cinerea.* Phytopathology, 92:75-80.

Oshima, M., Banno, S., Okada, K., Takeuchi, T., Kimura, M., Ichiishi, A., Yamaguchi, I. and Fujimura, M. (2006). Survey of mutations of a histidine kinase gene BcOS1 in dicarboximide-resistant field isolates of Botrytis cinerea. Journal of Genetic Plant Pathology, 72:65-73.

Parnell, S., Gilligan, C. A. and van den Bosch, F. (2005). Small-scale fungicide spray heterogeneity and the coexistence of resistant and sensitive pathogen strains. Phytopathology, 95:632-639.

Pillonel, C. and Meyer, T. (1997). Effect of phenylpyrroles on glycerol accumulation and protein kinase activity of *Neurospora crassa*. Pesticide Science, 49: 229-236.

Raposo, R., Gomez, V., Urrutia, T. and Melgarejo, P. (2000). Fitness of Botrytis cinerea Associated with Dicarboximide Resistance. Phytopathology, 90:1046-1249.

Rosslenbroich, H. J. and Stuebler, D. 2000. Botrytis cinerea -history of chemical control and novel fungicides for its management. Crop Protection, 19:557-561.

Seddon, B. and Edwards, S. G. (1993). Analysis of and strategies for the biocontrol of *Botrytis cinerea* by *Bacillus brevis* on Chinese cabbage. Biological Control of Foliar and Postharvest Diseases, 16:38.

Shoemaker, P. B. and Lorbeer, J.W. (1977). Timing initial fungicide application to control botrytis leaf blight epidemics on onion. Phytopathology, 67:412-413.

Sierotzki, H., Wullschleger, J., and Gisi, U. (2000). Point-mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Eryphe gaminis* f.sp. tritici field isolates. Pesticide Biochemistry and physiology. 68:107-112.

Staats, M., Van Baarlen, P. and Van Kan, J. A. L. (2004). Molecular Phylogeny of the Plant Pathogenic Genus *Botrytis* and the Evolution of Host Specificity. Molecular Biology and Evolution, 22:333-346.

Staub, T. (1991). Fungicide resistance: practical experience with anti-resistance strategies and the role of integrated use. Annual Review Phytopathology 29, 421-42.

Statistique Canada, Division de l'Agriculture, Section des cultures. Productions de fruits et légumes. (2011). Statistique Canada, Ottawa, Canada, 79:2.

Steel, C. C. and Nair, N. G. (1993). The physiological basis of resistance to the dicarboximide fungicide iprodione in Botrytis cinerea. Pesticide Biochemistry and Physiology, 47:60-68.

Stehmann, C. and De Ward, M. A. (1996). Sensitivity of population of *Botrytis cinerea* to triazoles, benomyl and vinclozolin. European Journal of Plant Pathology, 102:171-180.

Van der Heyden, H., Carisse, O. and Brodeur, L. (2012) Comparison of monitoring based indicators for initiating fungicide spray programs to control Botrytis Leaf Blight of onion. Crop Protection, 33:21-28.

Veloukas, T., Kalogeropoulou, P., Markogolou, A. N. and Karaogalanidis, G. S. (2014). Fitness and competitve ability of *Botryitis cinerea* field isolates with dual resistance to SDHI and QoI fungicides associated with several sdhB and the cytb G143A mutations. Phytopathology, 104:347-356.

Vincelli, P. and Dixon E. (2002) Resistance to QoI (Strobilurin-like) fungicides in isolates of Pyricularia grisea from perennial ryegrass. Plant Disease 86:235-240.

Williamson, B., Duncan, G. H., Harrison, J. G., Harding, L. A., Elad, Y. and Zimand, G. (1995). Effect of humidity on infection of rose petals by dry-inoculated conidia of *Botrytis cinerea.* Mycological Research, 88:1303-1310.

Ward, A., Reyes, C. L., Yu, J., Roth, C. B. and Chang, G. (2007). Flexibility in the ABC transporter MsbA: Alternating access with a twist. National Academy of Science of USA, 104:19005-19010.

Yang, C., Hamel, C., Vujanovic, V. and Gan, Y. (2011). Fungicide: Modes of Action and Possible Impact on Nontarget Microorganisms. ISRN Ecology, 8.

Zhang, Y., Lamm, R., Pillonel, C., Lam, S. and Xu, J. R. (2002) Osmoregulation and fungicide resistance: the Neurospora crassa os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. Appl Environ Microbiol, 68:532-538.