Clemson University
TigerPrints

All Theses

Theses

12-2018

Characterization of *Botrytis cinerea* from Commercial Cut Flower Roses and Evaluation of Current Crop Management Practices

Melissa Muñoz
Clemson University, melimunoza@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Recommended Citation

Muñoz, Melissa, "Characterization of *Botrytis cinerea* from Commercial Cut Flower Roses and Evaluation of Current Crop Management Practices" (2018). *All Theses*. 3003.
https://tigerprints.clemson.edu/all_theses/3003

CHARACTERIZATION OF *BOTRYTIS CINEREA* FROM COMMERCIAL CUT FLOWER ROSES AND EVALUATION OF CURRENT CROP MANAGEMENT PRACTICES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

by
Melissa Muñoz
December 2018

Accepted by:
Dr. James E. Faust, Committee Chair
Dr. Guido Schnabel
Dr. Paula Agudelo

ABSTRACT

Botrytis cinerea Pers. is a necrotrophic fungal pathogen that infects over 235 different plant species around the world. In cut flower roses, *B. cinerea* causes gray mold disease which leads to large economic losses during greenhouse production and in the post-harvest environment. Disease symptoms are often not visible during the production stage but are observed after storage and/or transportation. Fungicide applications are the primary strategy for gray mold management. However, fungicide resistance has been observed in several crops around the world, resulting in lack fungicide efficacy. Cultural practices such as plant nutrient management, cultivar selection, and crop sanitation are also important aspects of the disease management program. Despite the extensive efforts to control this pathogen, gray mold disease remains a persistent threat for cut rose production.

In the first part of this research project, *Botrytis cinerea* Pers. *sensu stricto* was confirmed as the unique causal agent of gray mold in cut roses from Colombia. Other pathogenic fungi were identified, e.g., *Alternaria alternata, Cladosporium cladosporoides, Epicoccum nigrum, Penicillium citrinum, Aspergillus brasiliensis* and *Diplodia* sp. These fungi may become problematic in the future. Gray mold incidence and severity were evaluated in different rose tissue from the *Botrytis* susceptible cultivar 'Orange Crush'. Six commercial shipments from two different greenhouses at the same farm in Colombia were evaluated. The petals showed the highest disease incidence and severity. A total of 49 *B. cinerea* isolates were collected during the severity and incidence evaluation. Fungicide resistance profiling was performed using ten fungicides. The

isolates showed high occurrence of resistance to boscalid, cyprodinil, iprodione and thiophanate-methyl; moderate frequency of resistance to isofetamid, fenhexamid, fluopyram, and penthiopyrad, low resistance to fludioxonil, and no resistance to pidiflumetophen. Variation in the fungicide resistance profiles were observed between greenhouses and shipments. Isolates with simultaneous resistance to different chemical classes were also observed.

In the second part of this research project, *B. cinerea* spore count was recorded using two spore collectors installed in commercial cut flower greenhouses. The relationship between different production activities and conidia count was also evaluated via hierarchical cluster analysis. From the total 26 activities evaluated, 14 of them were related with a high spore count. A higher spore count was observed during the week days compared to the weekend, which coincided with the days with 50.5% more activities in the greenhouse. Possible relationships between spore count and disease incidence were also evaluated; however, no correlation was observed. The results of this research suggest: 1) fungicide resistance management practices should be implemented to improve the effective life of different fungicides and their efficacy against *B. cinerea* infection, 2) timely removal of plant debris from the production greenhouses may reduce inoculum proliferation, and 3) avoidance of free water in the greenhouse may reduce inoculum dispersal and germination.

DEDICATION

To God, who put on my path this wonderful opportunity, and gave to me more than I

asked. I feel very blessed and grateful every day of my life

To my parents for all the unconditional love and sacrifices that they have been doing for

my whole life, to them I owe all that I am right now.

To my husband for his patience, dedication and love, because he has been my support

throughout this process.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDICES

CHAPTER ONE

LITERATURE REVIEW

The rose crop

Botanical history

Roses are one of the most important ornamental crops in human history. Before roses were a popular cut flower and one of the most important garden plants, roses were appreciated for the beauty of their flowers and as a source of perfume and edible hips (Gudin 2000). Roses are woody perennial shrubs that have been cultivated for over 5000 years. The first reports date from 3000BC in China, western Asia, and northern Africa (Bendahmane et al. 2013). The genus *Rosa* belongs in the Rosaceae family and contains about 200 different species (Soules 2009). Most of the modern cultivated roses are named *Rosa x hybrida* (Gudin 2001). Cultivars are usually tetraploids and come from continuous hybridizations and polyploidizations of many species (Gudin 2000) including *R. moschata*, *R. wichurana*, *R. multiflora*, *R. gallica* , *R. chinensis*, *R. gigantean*, *R. foetida*, and *R. damascene* (Smulders et al. 2011). The cultivars are usually propagated asexually by bud-grafting or with soft-wood or semi-hardwood cuttings (Zlesak 2007). The most desired cultivars and colors depend on the market trends, season, and time of the year. However historically, the most dominant roses in the market are red, color (yellow, pink, orange and purple) and white roses respectively, additionally single roses predominate over spray roses (Blom and Tsujita 2003).

Economic importance

Cut roses are one of the most important ornamental crops in the world with more than 15 billion stems produced in approximately 8500 hectares of protected cultivation (Bendahmane et al. 2013) and annual wholesale value of over $11 billion. (Zlesak 2007). The protected cultivation facilities range from unheated, wooden structures with a single layer polyethylene covering to computer-controlled climate technology supplemented with artificial lighting in glass and aluminum structures. The more sophisticated greenhouses are used in northern latitudes where the ambient temperatures are less hospitable, while simpler structures are used in high altitude equatorial regions where the structure is primarily needed to protect the plants from wind and rain. (Blom and Tsujita 2003). Since the 1990's the cut rose production has moved from northern latitudes to equatorial regions as postharvest technology and transportation systems have allowed for long distance transport. Additionally, the lower cost of labor, the higher availability of labor and the lack need for energy inputs for climate control in equatorial regions have made production in these regions more profitable. The largest countries for cut rose production are Netherlands, Colombia, Ecuador and Kenya (Blom and Tsujita 2003).

In Colombia the cut rose industry represents one of the most important agronomic and economical crops, with a total estimated area of 1900 hectares of production located principally in Sabana Cundi-Boyacence Colombia. Over 70% of the roses that imported to the U.S. (Gómez 2013) come from this region, and this growth in production is considered to be one of the major development success stories of the 20th century (Mendez 1991).

Diseases of roses in greenhouses

Several plant diseases can affect the yield and quality of cut rose production, including fungal, oomycetes, bacterial and virus diseases. The most important fungi and oomycetes diseases include: powdery mildew (*Podosphaera pannosa* (Wall.:Fr.) de Bary), downy mildew (*Peronospora sparsa* Berk), black spot (*Diplocarpon rosae* Wolf) and gray mold *(Botrytis cinerea* Pers*).* While crown gall (*Agrobacterium tumefaciens)* is one of the most important bacterial diseases on roses (Gullino and Garibaldi 1996).

Both, powdery and downy mildew are caused by biotrophic pathogens that mainly affect the plants under cold and humid conditions (Debener and Byrne 2014). Powdery mildew often occurs on foliage, stems and buds reducing photosynthesis, respiration and transpiration which may result in yield losses (Kumar et al. 2010). Downy mildew affects greenhouse production of roses around the world (Xu and Pettitt 2003). The fungi requires free water for conidia germination (Debener and Byrne 2014). Symptoms on leaves appear as irregular purple, red or dark brown spots and leaf abscission that results in decreased productivity (Horst and Cloyd 2007). Black spot is a foliage disease with infection symptoms that are described as dark spots surrounded by chlorotic areas, followed by defoliation and weakened of the plant (von Malek 1998) the most susceptible stage is when the leaves are between 6 to 14 days old (in expansion) (Horst and Cloyd 2007). Gray mold disease is caused by *Botrytis cinerea* Pers., it is considered the most destructive disease for cut roses world-wide affecting both, production and post-harvest stages, the pathogen is present in the greenhouses and it sporulate profusely under high humidity and cool to mild temperature conditions (Gleason and Helland 2003).

Crown gall disease is caused by *Agrobacterium tumefaciens,* the disease affects the plant parenchyma and symptoms described as overgrowths or gals can be shown on the crown region right beyond the soil surface, roots or in aerial plant parts (Horst and Cloyd 2007). The bacteria enters via wounds to the plant tissue, for this reason proper sanitation of the crop and the tools used during cultural practices is determinant for maintaining the crown gal disease controlled (Gullino and Garibaldi 1996).

Botrytis species as plant pathogens

The genus *Botrytis* includes about 25 different species that are important pathogens of many crops around the world. This genus as well as the genera *Sclerotinia* and *Monilinia* belong to the family Sclerotiniaceae (Ascomycete). The fungi in this family are necrotrophic and for this reason the infected plant tissue results in a progressive decay and even plant death (Andrew et al. 2012). *Botrytis* species are divided in two phylogenetic clades (Staats et al. 2005). The first clade include species that principally affect monocots. The second clade has a total of five species, including *B. cinerea*, and these species primarily affect dicots Staats et al. (2005). *B. cinerea* (Teleomorph: *Botryiotinia fuckeliana*) is a filamentous, ubiquitous pathogen that causes gray mold in more than 235 plant species around the world, including vegetables, field and orchard crops, nursery plants and ornamentals (Andrew et al. 2012). Infection can occur during production or post-harvest stages. The economic impact of this pathogen is tremendous, and management strategies for the control are essential to the commercial viability of many crops (Elad et al. 2007).

The polyphagic behavior of *B. cinerea* results in this pathogen infecting different plant organs and a wide range of plant species (Fekete et al. 2011). *B. cinerea*

4

disseminates principally by multinucleate conidia (Holz, et al. 2007) resulting in constant genetic variations and mutations even through the reproduction is primarily asexual (Elad et al. 2007; Hahn, et al. 2014) suggest that ascospores produced in apothecium occur more frequently than expected, resulting in genetic variability. When sexual reproduction occurs, apothecia develop from sclerotia (resistant structures constituted by melanized mycelium) formed in soil or plant debris, and then ascospores will be released (Staats et al. 2005).

*Botryti*s in greenhouse production of cut roses

Botrytis cinerea is also a significant problem that affects commercial cut flower rose (*Rosa hybrida* L.) crops around the word leading to significant economical loses due to reduced productivity and post-harvest quality (Vrind 2005). This pathogen affects different plant tissues, such as flowers, leaves, and stems, causing gray mold and canker symptoms (Host and Cloyd 2007), although the greatest damage occurs on the flower petals (Elad 1988b). The disease is not always observed in the production greenhouses as it is often expressed after transportation or storage. This indicates that latent infections wait for the proper environmental conditions to develop (Hammer 1988). Symptoms on petals appear as small decolorated or necrotic flecks that enlarge to form big necrotized areas that may affect entire petals, sepals and receptacle. The infection may also lead to petal abscission and collapse of the flower head (Gleason and Helland 2003).

Botrytis *life cycle*

Conidia are the primary inoculum source or infection but sclerotia also provide an alternative method for pathogen dissemination. Conidia are produced on gray mycelia in

necrotized, infected plant tissue (Williamson, et al. 1995) while sclerotia can survive for a long period time in soil or plant debris (Hahn, et al., 2014; Williamson et al. 1995).

The disease cycle starts in the greenhouse when conidia are released into the air from conidiophores produced over decaying and necrotized tissue after rapid changes in temperature and humidity (Jarvis 1962a). Once released, conidia can spread amongst plants though the air, water, insects, or via human manipulation trough cultural practices (Daughtrey et al. 1995). After conidia land on the plant surface, the pathogen germ tube emerges if the environmental (relative humidity: >94%, and temperature from 15 to 25 ºC), and plant metabolic conditions (water and nutrients supply) are adequate (Williamson et al. 2007). The germ tube penetrates the plant cuticle with or without appressorium development (Tenberge 2007). Then, colonization and invasion of the sub-epidermal and intracellular tissue occurs, involving a series of enzymes and toxins that promote the death of host cells (Clark 1976); subsequently, the first visual symptoms will appear. The final stage of *B. cinerea* infection involves lesion expansion, tissue maceration and sporulation which makes inoculum available for new infections (Choquer et al. 2007).

The disease cycle in rose petals may occur in two different stages depending on the environmental conditions. The first stage includes the initial tissue penetration followed by growth cease. In this stage the disease remains as a latent infection in the rose tissue. At the second stage, under the proper environmental and host conditions rapid colonization occurs, and invasion of the tissue results in the development of necrotic lesions. Because of this, non-symptomatic, infected roses may not exhibit symptoms until the post-harvest environment (Elad 1988b).

Biological and metabolic mechanisms of *B. cinerea* infection

The entire infection process involves four stages. The first stage is host recognition, which is based on signals that occur during the plant-pathogen interaction. The second stage is the penetration of the plant surface. This stage involves a series of different genes codifying for enzymes that act in the host cuticle and cell wall to facilitate the penetration process. The third stage is the formation of the primary lesion that involves the death of the host tissue. In this stage, several secondary metabolites such as reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), extracellular Cu-Zn superoxide dismutase, necrosis-inducing proteins of the NLP family, and different toxins are released allowing the pathogen to colonize the plant. These secondary metabolites make nutrients available for the pathogen, resulting in cell collapse and chlorosis (Colmenares et al. 2002; Hahn, et al. 2014). While, ROS and H_2O_2 are also produced by the plant as a defensive response called the hypersensitive response, there is evidence that *B. cinerea* takes advantage of the hypersensitive response to achieve full pathogenicity (Rossi et al. 2017; Hahn, et al. 2014). The fourth stage is the lesion expansion and sporulation which completes the cycle (van Kan 2006).

Botrytis cinerea requires a series of different signal transducers to complete various cellular process involved in pathogenicity. Mitogen-activated protein kinase (MAPK) cascades, which consist in a series of three interconnected kinases activated by phosphorylation and cAMP- dependent pathway as part of the protein receptors system, play important roles in the regulation of the infection process (Hahn, et al. 2014; Schumacher et al. 2008). For example, during spore germination, a series of chemical and physical signals are released, and both cAMP-dependent and MAP-kinase cascade

7

pathways are part of the signal transduction process that leads to conidia germination. Other chemical signals as nutrients concentration such as glucose that are present in free water on the epidermis of the plants and are involved in the pathogen metabolism (Williamson et al. 1995). Additionally, different sugar sources as well as hydrophobicity responses are an important part of the fungi recognition system of the plant surface (Doehlemann, et al. 2005).

Management approaches

Cultural

Sandón (2005) described essential practices in rose production for the reduction of inoculum dispersal and reproduction such as crop sanitation, microclimate manipulation (reduction of relative humidity), air circulation, and plant nutrient management.

Nutrition management is highly related to crop susceptibility to *B. cinerea* infection, for example extremely high or low nitrogen concentration in the crop is directly correlated with gray mold incidence (Hobbs and Waters 1964). Additionally, calcium has been highly associated with reduced susceptibility to *B. cinerea*, since it has an essential role enhancing cell wall strength and reducing the *B. cinerea* enzymatic degradation (Elad and Volpin 1993). Baas *et al*., (2000) showed a negative correlation between calcium concentration in flowers and *Botrytis* incidence suggesting calcium as an essential nutrient for gray mold disease management in roses.

Biological

The role of bacteria and fungi as biocontrol agents (BCA) has been studied in the last decade as a component of gray mold management (Heyens et al. 2011). Different modes of action are attributed to the effect on BCAs on *B. cinerea* including synthesis of anti-fungal metabolites, nutrient and niche competition, and induction of host resistance (Haidar et al. 2016). Research has shown an effect of *Bacillus amyloliquefaciens, B. subtillilis, Halomonas sp., Aureobasidium pullans* inhibiting *B. cinerea* germination and penetration (Sylla et al. 2015). However, the considerable variability in the response has been reported in the production and post-harvest environments (Haidar et al. 2016).

Chemical

The principal strategy for *Botrytis* control in cut rose production is based on fungicide applications (Heyens et al. 2011) that include multi-site inhibitors, such as captan, chlorothalonil and dithiocarbamates, as well as site-specific fungicides with different modes of action such as succinate dehydrogenase inhibitors, dicarboxamides, anilinopyrimidines, methyl benzimidazoles carbamates, hydroxyanilides, phenylpyrroles and strobilurins. The multi-site fungicides have broad-spectrum activity and have been used since the 1950's, while single-site fungicides were first used in the 1960's as benzimidazoles (Hahn, Viaud, and Kan 2014a). On average, between 52 to 104 fungicide foliar applications per hectare are applied in commercial cut rose greenhouses over the course of one year. This results in high production costs associated with fungicide applications (until 60% of the total annual budget) which involve the fungicides value and labor cost. Additionally, the re-entrance period associated with some fungicide

applications, limits the accomplishment of other production activities in the greenhouse (Álvarez 2012).

Botrytis fungicide resistance development

Several studies have described _Botrytis_ resistance development to site-specific fungicide chemical classes (Saito, Michailides, and Xiao 2016); however, no resistance has been documented for multi-site fungicides. Additionally, several _B. cinerea_ isolates have shown chemical class resistance (CCR) that is defined as a simultaneous resistance development to more than one chemical class (Katan 2007; Grabke _et al._ 2012).

Several factors are important for resistance development which include the biology, reproductive and adaptive ability of the target fungi, structural or chemical class of the fungicide, and the specific mode of action of the fungicide (Brent and Hollomon 1998). According to the Fungicide Resistance Action Committee (FRAC), _B. cinerea_ is a pathogen with a high risk of fungicide resistance development because it has a short life cycle, high genetic and phenotypic variability, and a high reproduction rate. Thus, _Botrytis_ was one of the first fungi reported for resistance development to different chemical classes (Elad et al. 1992; Forster et al. 2007; Jiang, Ding, Michailides et al. 2009).

Factors driving genetic diversity of _Botrytis_

Although sexual recombination is not the primary reproduction strategy _of B. cinerea_ isolates, a high genetic variability is attributed to this pathogen. Asexual reproduction though multinucleate conidia has been associated with the phenotypical and genotypical variation observed in _B. cinerea_ (Alfonso et al. 2000). However, putative

sexual reproduction is still considered as a potential source of genetic variability due to recombination of strains with different mating types.

Two of the principal reasons for the success of *B. cinerea* as a pathogen are the efficiency in the production of very large amounts of conidia (Nicot et al. 1996), and the occurrence of multiple infection cycles (involving primary and secondary inoculum) of the gray mold disease in infected plant tissue which may result in a variable genetic population with different biological efficacy and resistance qualities (Decognet et al. 2009). Spontaneous mutations are considered as another very important source of genetic variation leading to resistance development, e.g., Delcán (1997) described spontaneous mutations for resistance development occurring with a frequency that oscillates between 1×10^{-8} to 4.4×10^{-6} individuals .

Fungicide selection pressure over *B. cinerea* isolates may lead genetical and phenotypical changes in the pathogen population, i.e. the extensive use of fungicides, especially single-site fungicides had been related with fungicide resistance development to several chemical classes and modes of action (Brent and Hollomon 1995). Additionally, mixture of geographically distant spore populations between greenhouses and neighboring areas also has implications for genetic differentiation in terms of allelic frequency at specific locations (Alfonso et al., 2000).

Molecular mechanisms of fungicide resistance

Four different mechanisms have been attributed to resistance development in *B. cinerea*: 1) Point mutations on the gene encoding for the target site of the fungicide which is the most common and most important mechanism for resistance development (Hahn 2014). 2) Fungicide detoxification, including a series of different enzymes as

cytochrome P450 monooxygenases, hydrolases, and glutathione S-transferases, to quickly metabolize the fungicide and transform it in to a less harmful compound to the fungi cells (Leroux et al. 2002a). 3) Overexpression of the target site thought mutations that increase the transcription of the target site. In this case higher doses of the fungicide may compensate the target overproduction, however this is not recommended due to the increased potential to enhance fungicide resistance (Hahn 2014). 4) Efflux-based resistance that excludes the fungicide from the *Botrytis* mycelium and is based on overproduction of transported molecules described as ABC and MFS in *B. cinerea* isolates (Leroux et al. 2002b) making the fungicide less effective due to the inability to reach the target site (Kretschmer et al. 2009). This last mechanism has been described as a non-specific fungicide resistance and is considered to be the principal responsible for CCR (Hahn 2014).

Resistance to different FRAC codes

The fungicides belonging to the methyl benzimidazole carbamates (FRAC 1) and dicarboxamides (FRAC 2) chemical classes are site-specific fungicides that have been used for *B. cinerea* management for a longer period of time than the other chemical classes. This partially explains the higher rate for fungicide resistance development in *B. cinerea* reported for FRAC 1 and 2 (Leroux 2007). The methyl benzimidazole carbamates mode of action focuses on the pathogen cytoskeleton and motor proteins and is related to the ß- tubulin assembly during the meiosis process. Resistance to methyl benzimidazole carbamate fungicides has been related to point mutations mostly at codon E198 in the ß- tubulin gen (Table 1.1), resulting in a lack of binding between the fungicide and the fungi target places (Ma and Michailides 2005; Liu et al. 2016).

Resistance to the methyl benzimidazole carbamate group persists through long periods of cessation of application of these fungicides (Georgopoulos and Skylakakis 1986).

Dicarboxamides (FRAC 2) affect the pathogen signal transduction, specifically MAP/histidine kinase in the osmotic signal transduction, and involve different genes, e.g., Os1, Daf1 and Bos1. Mutation in these genes, mostly in the codon I365S (Table 1.1), is responsible for fungicide resistance development to dicarboxamides (Sun et al. 2010).

The anilinopyrimidine fungicides (FRAC 9), such as cyprodinil and pyrimethanil, are considered to be very effective against *Botrytis*. Their mode-of-action involves the inhibition of amino acid synthesis and protein secretion by the fungus. Resistance has been reported for this chemical class; however, the specific point of mutation is not clear (Table 1.1) (Hahn, et al 2014).

Phenylpyrrole fungicides (FRAC 12), such as fludioxonil, have a similar mode-of-action as the dicarboxamides group, inhibiting the signal transduction process of mitogen-activated protein (MAP)/histidine-kinase in the osmotic pathway. While, the mode-of-action of FRAC 2 and FRAC 12 are similar, cross resistance between both chemical classes is rare (Fernández-Ortuño et al. 2012). Isolates of *Botrytis* with low to medium resistance level to phenylpyrroles are sporadically found in the field (Kojima et al. 2004). This resistance development is a result of a drug efflux mechanism.

The hydroxyanilides chemical class (FRAC 17), represented by fenhexamid, acts through the inhibition of the ergosterol biosynthesis of the 3-ketoreductase enzyme which is involved in the C-4 demethylation of this pathway and is encoded by the erg27 gene, which is the point of mutation in *Botrytis* isolates showing resistance development

to this FRAC class (Table 1.1). The drug efflux mechanism has also been related to resistance development to this chemical class (Fillinger et al. 2008)

The mode-of-action of the strobilurins, also known as the quinone outside inhibitors (FRAC 11), is to inhibit pathogen respiration interacting at the Qo site (cyt b gene) in the mitochondrial cytochrome bc1 complex. Strobilurins are not usually used for *Botrytis* management because this fungus is able to bypass this respiratory inhibition by using an alternative enzyme as part of the respiratory chain. Nevertheless, quinone outside inhibitors are used and very effective against other fungi and oomycetes. *Botrytis* can be influenced by strobilurins because the crops are often treated with strobilurins for powdery or downy mildew, pathogens that often co-exist with *Botrytis* in several crops (Wood and Hollomon 2003).

The succinate dehydrogenase inhibitors fungicides (FRAC 7) have become a very important part of the fungicide pool since their release in 2003 (Sierotzki and Scalliet 2013). They are used alone or in mixtures with great efficacy for *Botrytis* control. However, mutations in the SdhB, SdhC and SdhD subunits in the ubiquinone binding point have been reported to confer resistance to different fungicides from this chemical group, especially boscalid (Amiri, et al. 2013). Four different patterns that represent different resistance responses to several fungicides in this group have been described as patterns A, B, C, and D. Pattern A represents *Botrytis* isolates resistant to boscalid. Pattern B contains isolates with resistance to boscalid and penthiopyrad. Pattern C isolates are resistant to boscalid, fluxapiroxad and penthiopyrad; and Pattern D represents *Botrytis* isolates that are resistant to boscalid, fluxapiroxad, penthiopyrad and fluopyram. These resistance responses are associated with the alleles H272R, H272Y, P225 and

N230I at the locus SdhB (Table 1.1), and resistance management practices are an important tool to preserve the efficacy of these fungicides (Hu, et al. 2015).

Fitness and resistance

Fitness is defined as "the capacity of a variant type to invade and displace the resident population in competition for available resources" or "an organism's capacity to survive and reproduce" (Demetrius and Ziehe 2007). Fitness is considered to be an essential component for the evaluation of resistance development since competitivity of resistant isolated might be affected if a "fitness penalty" (mutation confering resistance have an impact decreasing the pathogen reproductive rate, virulence or severity with non resistant isolates (Leach et al. 2001)) is associated . The relationship between fungicide resistance and *B. cinerea* fitness remains a point of discussion, and it depends on the fungicide to which resistance is expressed. Leroux (2007) suggested that in the absence of selection pressure (fungicide application), the frequency of fungicide resistance tends to decrease; however, if there is fitness penalty associated with the mutation that confers the resistance, the resistant response may persist.

The fitness of *B. cinerea* isolates has been evaluated using mycelial growth, sporulation, conidial germination, sclerotia production, and symptom severity as indicators to compare possible fitness penalties between resistant and non-resistant isolates to several fungicides. For example, Markoglou *et al.* (2006) analyzed *B. cinerea* isolates resistant to quinone outside inhibitors (FRAC 11) and determined a fitness cost associated with the resistant isolate expressed as a reduction on the sporulation rate, conidial germination and sclerotia production. Methyl-benzimidazole fungicides (FRAC 1) are a clear example of no fitness cost being associated with the resistance response,

because even when these fungicides have not been applied for several years, resistant isolates continue to dominate the population. Thus, methyl-benzimidazole-resistant isolates show a high fitness in the field (Malandrakis, Markoglou, and Ziogas 2011a). Resistance development to dicarboxamide and succinate dehydrogenase inhibitor fungicides (FRAC 2 and 7) has a fitness penalty for *Botrytis* populations. For this reason, once fungicide applications are interrupted for a period of time and then re-applied, the fungicide will recover efficacy because the resistance strains are replaced with sensitive strains amongst the *Botrytis* population (Elad, et al. 1992; Leroux et al. 2002b).

Chen *et al.* (2016) described a fitness penalty associated with *B. cinerea* isolates that expressed CCR. Isolates with simultaneous resistance to five or six fungicides were less competitive than sensitive isolates in terms of mycelial growth rate and spore production resulting in an increase in the population of sensitive isolates in the absence of the fungicide selection pressure. However, under continuous fungicide applications, the frequency of multi-fungicide resistance isolates tends to increase rapidly.

Fungicide resistance management approaches

Resistance management should be consider as a long-term series of strategies incorporating different tools (Walker et al. 2013), including good crop management practices as prophylaxis and sanitation, plant nutrition, humidity reduction, and removal of plant debris (Fig. 1.1) (Hahn 2014; Walker et al. 2013). Population monitoring is a basic step to determine the best management strategies, considering the evolution of resistance in the crop (Walker et al. 2013). The effectiveness of fungicide application and the rate of resistance development are affected by the following strategies: 1) Management of the application dose, because increasing dosage has been directly related

with higher rates of resistance development (van den Bosch et al. 2015). 2) Management of the number of applications, because increasing the number of fungicide applications increases the selection for resistance development. Additionally, split dose applications also increase the risk of resistance development since they increase the exposure time of the fungicides (van den Bosch et al. 2015). 3). Fungicide mixtures, because the effective life of highly risk-resistance fungicides can be improved by adding a second fungicide with a different mode of action (multi-site or single-site without evidence for resistance development) because it may increase the coverage of the pathogen population resulting in a better efficacy. However, the viability of this strategy depends of the availability of effective fungicides with different modes of action (Walker et al. 2013). 4) Fungicide (mode of action) rotation, because it decreases the exposure to selection pressure (Fig. 1.1) (van den Bosch et al. 2015).

Purpose of this study

The overall goal of this study was to assess the efficacy of current gray mold management practices on cut rose production. For that purpose, the first part of this research focused on evaluating: 1) the current disease status in a commercial cut rose production greenhouses, considering disease causal agent identification, and disease incidence and severity evaluation, and 2) fungicide resistance profiling for *B. cinerea* isolates obtained in the disease and incidence evaluation. The second part of the project focused on determining possible relationships of *B. cinerea* spore count with cultural practices and disease incidence.

Table 1.1. Most commonly used fungicides against *Botrytis* and their resistance components.

Fungicide group name	FRAC code	Year of first use	Target site/mode of action	Molecular mechanism	Target site of resistance	Resistance levels
MBC Methyl benzimidazole carbamates	1	1968	β-tubulin	Monogenic resistance due to mutation in single major gene and multiallelic resistance	E198A, E198X, F200	High
Dicarboximides	2	1975	Osmosensing MAP/histidine kinase	Monogenic resistance due to mutation in single major gene	I365S/N, Q369P, N373S	Low to high
SDHI Succinate dehydrogenase inhibitors	7	2004	Respiration, complex II or succinate dehydrogenase	Monogenic resistance due to mutation in single major gene and multiallelic resistance. Partial cross resistance between SDHI	H272R/Y, H272L, N230I, P225X	Medium to high
AP Anilinopyrimidines	9	1994	Methionine biosynthesis	Monogenic resistance due to mutation in single major gene and alternative overexpression of efflux transporters in MDR mutants	Unknown	High
PP Phenylpyrroles	12	1995	Osmoregulation	Monogenic resistance due to mutation in single major gene and overexpression of efflux transporters in MDR mutants	Unknown	Medium
Hydroxyanilides	17	1999	3-Keto reductase (sterol biosynthesis)	Monogenic resistance due to mutation in single major gene and detoxification P450-mediated	F412S, F412X, T631	Low to high

Adapted from Walker et al. (2013); Hahn (2014); De Miccolis Angelini et al. (2015)

Resistance management

Indirectly related with fungicide management:

| Preventive tactics from crop management: balanced nutrition (especially nitrogen fertilizers), humidity reduction when possible, sanitation, removal of plant debris and infected plant tissue. | → | **Effect:** Reduction on viable inoculum, delay on resistance appearance |

Directly related with fungicide management, implies population monitoring :

Management strategy

Resistance undetected
- Multi-site fungicide broadly used e.g. Ziram and mancozeb → No particular restriction, dose management
- Recent introduction of the fungicide, however resistance shown in other crops and locations. e.g. Fludioxonil → Fungicide alternation, and fungicide mixture (when no resistance present and negative cross-resistance)

Resistance detected
- Individual single-site resistance Overall cross-resistance within the same mode of action
  - Highly frequent resistant strains Greatly reduced fungicide efficacy
    - No fitness cost e.g. Methyl-thiophanate
    - Fitness cost e.g. Iprodione → Management of number of applications, fungicide alternation, and fungicide mixture (when no resistance present and negative cross-resistance)
  - Low frequency of resistant strains Low impact on fungicide efficacy
    - Fitness cost e.g. Boscalid, fluopyram, fenhexamid, cyprodinil → Fungicide alternation, and fungicide mixture (when no resistance present and negative cross-resistance)
- Multi-fungicide resistance Positive cross-resistance between different mode of action → All botrycides except thiram → Full dose application, management of number of applications, increased use of multisite fungicides

Figure 1.1. Decision tree of fungicide resistance management in B. cinerea populations based on monitoring of resistance status. Adapted from Walker et al. (2013)

Literature Cited

Alfonso, C., Raposo, R., and Melgarejo, P. 2000. Genetic diversity in *Botrytis cinerea* populations on vegetable crops in greenhouses in south-eastern Spain. Plant Pathol. 49:243–251.

Álvarez, H.A. 2012. Efecto del calcio en la expresión de *Botrytis cinerea* en flores y tallos de Rosa sp. (Master's thesis). Repositorio institucional UN. Universidad Nacional de Colombia. Facultad de Agronomía.

Amiri, A., Heath, S. M., and Peres, N. A. 2013. Resistance to fluopyram, fluxapyroxad, and penthiopyrad in *Botrytis cinerea* from strawberry. Plant Dis. 98:532–539.

Andrew, M., Barua, R., Short, S. M., and Kohn, L. M. 2012. Evidence for a common toolbox based on necrotrophy in a fungal lineage spanning necrotrophs, biotrophs, endophytes, host generalists and specialists. PLOS ONE. 7:29943.

Baas R, Marissen N, Dik AJ, 2000. Cut rose quality as affected by calcium supply and translocation. Acta Hortic. 518:45–54.

Bendahmane, M., Dubois, A., Raymond, O., and Bris, M. L. 2013. Genetics and genomics of flower initiation and development in roses. J. Exp. Bot. 64:847–857.

Blom, T. J., and Tsujita, M. J. 2003. Production and marketing- cut rose production. In *Encyclopedia of Rose Science*, ed. Andrew V. Roberts. Oxford: Elsevier, p. 594–600.

Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? 2nd ed. FRAC Monograph No. 2. Fungicide Resistance Action Committee, United Kingdom. p. 1–50.

Chen, S. N., Luo, C. X., Hu, M. J., and Schnabel, G. 2016. Fitness and competitive ability of *Botrytis cinerea* isolates with resistance to multiple chemical classes of fungicides. Phytopathology. 106:997–1005.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.-M., Simon, A., Viaud, M. 2007. *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiol. Lett. 277:1–10.

Clark, C. A. 1976. Comparative histopathology *of Botrytis squamosa* and *B. cinerea* on onion leaves. Phytopathology. 66:1279.

Colmenares, A., Aleu, J., Duran-Patron, R., Collado, I., and Hernández-Galán, R. 2002. The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. J. Chem. Ecol. 28:997–1005.

Daughtrey, M., Wick, R. L., and Peterson, J. L. 1995. Compendium of Flowering Potted Plant Diseases. APS Press. p. 20–21.

Debener, T., and Byrne, D. H. 2014. Disease resistance breeding in rose: Current status and potential of biotechnological tools. Plant Sci. 228:107–117.

Decognet, V., Bardin, M., Trottin-Caudal, Y., and Nicot, P. C. 2009. Rapid change in the genetic diversity of *Botrytis cinerea* populations after the introduction of strains in a tomato glasshouse. Phytopathology. 99:185–193.

Delcán J (1997) *Botrytis cinerea* Pers. en los cultivos protegidos del sureste español (Ph.D. thesis disertación). Universidad Politécnica de Madrid. Madrid, Spain

Demetrius, L., and Ziehe, M. 2007. Darwinian fitness. Theor. Popul. Biol. 72:323–345.

Doehlemann, G., Molitor, F., and Hahn, M. 2005. Molecular and functional
characterization of a fructose specific transporter from the gray mold fungus
Botrytis cinerea. Fungal Genet. Biol. FG B. 42:601–610.

Elad, Y. 1988. Latent infection of *Botrytis cinerea* in rose flowers and combined
chemical physiological control of the disease. Crop Prot. 7:361–366.

Elad, Y., and Volpin, H. Effect of nutrition on susceptibility of cucumber, eggplant,
and pepper crops to *Botrytis cinerea*. Can. J. Bot. 71:602–608

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 Pathol. 41:41–46.

Elad, Yigal, Williamson, B., Tudzynski, P., and Delen, N. 2007. *Botrytis* spp. and
diseases they cause in agricultural systems – An introduction. In *Botrytis:
Biology, Pathology and Control*, Springer, Dordrecht, p. 1–8.

Fekete, É., Fekete, E., Irinyi, L., Karaffa, L., Árnyasi, M., Asadollahi, M., Sándor, E.
2011. Genetic diversity of a *Botrytis cinerea* cryptic species complex in Hungary.
Microbiol. Res. 167:283–91.

Fernández-Ortuño, D., Chen, F., and Schnabel, G. 2012. resistance to cyprodinil and lack
of fludioxonil resistance in *Botrytis cinerea* Isolates from strawberry in North and
South Carolina. Plant Dis. 97:81–85.

Fillinger, S., Leroux, P., Auclair, C., Barreau, C., Al Hajj, C., and Debieu, D. 2008.
Genetic analysis of fenhexamid-resistant field isolates of the phytopathogenic
fungus *Botrytis cinerea*. Antimicrob. Agents Chemother. 52:3933–3940.

Forster, H., Su, H., Vilchez, M., Gubler, W., and Adaskaveg, J. 2007. Non-persistent captan- and fenhexamid-resistance in *Botrytis cinerea* populations in California strawberries. Phytopathology. 97:S36–S36.

Georgopoulos, S. G., and Skylakakis, G. 1986. Genetic variation in the fungi and the problem of fungicide resistance. Crop Prot. 5:299–305.

Gleason, M. L., and Helland, S. J. 2003. Disease: *Botrytis*. In *Encyclopedia of rose science*, ed. Andrew V. Roberts. Oxford: Elsevier, p. 144–148.

Gómez, T. 2013. Caracterización de aislamientos *Botrytis cinerea* de rosa en la Sabana de Bogotá. Repositorio institucional UN. Universidad Nacional de Colombia. Facultad de Ciencias Agrarias.

Grabke, A., Fernández-Ortuño, D., and Schnabel, G. 2012. Fenhexamid resistance in *Botrytis cinerea* from strawberry fields in the carolinas is associated with four target gene mutations. Plant Dis. 97:271–276.

Gudin, S. 2000. Rose: genetics and breeding. Plant Breed. Rev. 17:159–189

Gudin, S. 2001. Rose breeding technologies. Acta Hortic. 547:23–33.

Gullino, M. L., and Garibaldi, A. 1996. Diseases of roses: evolution of problems and new approaches for their control. Acta Hortic. 424:195–202.

Hahn, M. 2014. The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. J. Chem. Biol. 7:133–141.

Hahn, M., Viaud, M., and Kan, J. A. L. van. 2014. The genome of *Botrytis cinerea*, a ubiquitous broad host range necrotroph. In *Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens*, Berlin Heidelber: Springer Verlag, p. 19–44.

Haidar, R., Fermaud, M., Calvo-Garrido, C., Roudet, J., and Deschamps, A. 2016. Modes of action for biological control of *Botrytis cinerea* by antagonistic bacteria. Phytopathol. Mediterr. 55:301–322.

Hammer, P. E. 1988. Postharvest control of *Botrytis cinerea* on cut roses with picro-cupric-ammonium formate. Plant Dis. 72:347.

Heyens, K., Aerts, R., Seels, B., Vogels, L., and Bruno, P. A. 2011. Biological control of *Botrytis cinerea* in tomato. Acta Hortic. 914:361–363.

Hobbs. E.L., and Waters, W.E. 1964. Influence of nitrogen and potassium susceptibility of *Chrysantemum morifolium to Botrytis cinerea.* Phytopathology. 54:674–676

Holz, G., Coertze, S., and Williamson, B. 2007. The ecology of *Botrytis* on Plant Surfaces. In *Botrytis: Biology, Pathology and Control*, Springer, Dordrecht, p. 9–27.

Horst, R.K. and Cloyd, R.A. 2007. Compendium of rose diseases and pests. Second Edition. APS Press. St. Paul, MN. p. 20–22

Hu, M.-J., Fernández-Ortuño, D., and Schnabel, G. 2015. Monitoring resistance to SDHI fungicides in *Botrytis cinerea* from strawberry fields. Plant Dis. 100:959–965.

Jarvis, W. R. 1962. Splash dispersal of spores of *Botrytis cinerea* Pers. Nature. 193:599.

Jiang, J., Ding, L., J. Michailides, T., Li, H., and Ma, Z. 2009. Molecular characterization of field azoxystrobin-resistant isolates of *Botrytis cinerea*. Pestic. Biochem. Physiol. 93:72–76.

Katan, T. 2007. Resistance to 3,5-dichlorophenyl-N-cyclic imide ('dicarboximide')

    fungicides in the grey mould pathogen *Botrytis cinerea* on protected crops. Plant

    Pathol. 31:133–141.

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.-S., Fillinger, S., Mernke, D.,

    Schoonbeek, H.-J., Pradier, J. -M., Leroux, P., De Waard, M.A., and Hahn, M.

    2009. Fungicide-driven evolution and molecular basis of multidrug resistance in

    field populations of the grey mould fungus *Botrytis cinerea*. PLOS Pathog.

    5:e1000696.

Kumar, S., Shah, R. and Mali, B.L. 2010. Epidemiology and management of powdery

    mildew of red rose caused by *Sphaerotheca pannosa* var. rosae. J. Pl. Dis. Sci.

    5:68–72

Leach, J. E., Vera Cruz, C. M., Bai, J., and Leung, H. 2001. Pathogen fitness penalty as a

    predictor of durability of disease resistance genes. Annu. Rev. Phytopathol.

    39:187–224.

Leroux, P. 2007. Chemical control of *Botrytis* and its resistance to chemical fungicides.

    In *Botrytis: Biology, pathology and control*, Springer, Dordrecht, p. 195–222.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. 2002a.

    Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest

    Manag. Sci. 58:876–888.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. 2002b.

    Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest

    Manag. Sci. 58:876–888.

Liu, C., Cui, Z., Yan, X., Qi, Z., Ji, M., and Li, X. 2016. Synthesis, fungicidal activity
and mode of action of 4-phenyl-6-trifluoromethyl-2-aminopyrimidines against
Botrytis cinerea. Molecules. 21:828.

Ma, Z., and Michailides, T. 2005. Advances in understanding molecular mechanisms of
fungicide resistance and molecular detection of resistant genotypes in
phytopathogenic fungi. Crop Prot. 24:853–863.

Malandrakis, A., Markoglou, A., and Ziogas, B. 2011. Molecular characterization of
benzimidazole-resistant *B. cinerea* field isolates with reduced or enhanced
sensitivity to zoxamide and diethofencarb. Pestic. Biochem. Physiol. 99:118–124.

Markoglou, A. N., Malandrakis, A. A., Vitoratos, A. G., and Ziogas, B. N. 2006.
Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI
fungicides. Eur. J. Plant Pathol. 115:149–162.

Méndez, José. 1991. The Development of the Colombian Cut Flower Industry. Working
Papers on Trade Policy, WPS 660. The World Bank.

Nicot P.C., Baille A., (1996). Integrated control of *Botrytis cinerea* on greenhouse
tomatoes. pp. 169–189 in C.E. Morris, P.C. Nicot and C. NguyenThe (eds.)
Aerial Plant Surface Microbiology, Plenum Press, New York.

Rossi, F. R., Krapp, A. R., Bisaro, F., Maiale, S. J., Pieckenstain, F. L., and Carrillo, N.
2017. Reactive oxygen species generated in chloroplasts contribute to tobacco
leaf infection by the necrotrophic fungus *Botrytis cinerea*. Plant J. Cell Mol. Biol.
92:761–773.

Saito, S., Michailides, T. J., and Xiao, C. L. 2016. Fungicide resistance profiling in
Botrytis cinerea populations from blueberry in California and Washington and
their impact on control of gray mold. Plant Dis. 100:2087–2093.

Sandón, C.M. 2005. Efecto de la limpieza de fuentes de inoculo del "moho gris"
sobre la presencia de conidias aéreas del agente causal de la enfermedad en un
cultivo de rosa variedad Classy (B.S. Thesis). Pontificia Universidad
Javeriana. Facultad de Ciencias Básicas. p. 15–66.

Schumacher, J., Kokkelink, L., Huesmann, C., Jimenez-Teja, D., Collado, I. G., Barakat,
R., et al. 2008. The cAMP-dependent signaling pathway and its role in conidial
germination, growth, and virulence of the gray mold *Botrytis cinerea*. Mol. Plant-
Microbe Interact. 21:1443–1459.

Sierotzki, H., and Scalliet, G. 2013. A review of current knowledge of resistance aspects
for the next-generation succinate dehydrogenase inhibitor fungicides.
Phytopathology. 103:880–887.

Smulders, M. J. M., Arens, P., Koning-Boucoiran, C. F. S., Gitonga, V. W., Krens, F. A.,
Atanassov, A., Atanassov, I., Rusanov, K.E., Bendahmane, M., Dubois, A.,
Raymond, O., Caissard, J.C., Baudino, S., Crespel, L., Gudin, S., Ricci, S.C.,
Kovatcheva, N., van Huylenbroeck, J., Leus, L., Wissemann V., Zimmermann,
H., Hensen, I., Werlemark, G., Nybom, H. 2011. Rosa. In *Wild Crop Relatives:
Genomic and Breeding Resources: Plantation and Ornamental Crops*, ed.
Chittaranjan Kole. Berlin, Heidelberg: Springer Berlin Heidelberg, p. 243–275.

Soules, V.A. Analysis of genetic diversity and relationships in the china rose group
(Master's thesis). Texas A&M University. 1–20

Staats, M., van Baarlen, P., Kan, V., and L, J. A. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol. Biol. Evol. 22:333–346.

Sun, H.-Y., Wang, H.-C., Chen, Y., Li, H.-X., Chen, C.-J., and Zhou, M.-G. 2010. Multiple resistance of *Botrytis cinerea* from vegetable crops to carbendazim, diethofencarb, procymidone, and pyrimethanil in China. Plant Dis. 94:551–556.

Sylla, J., Alsanius, B. W., Krüger, E., and Wohanka, W. 2015. Control of *Botrytis cinerea* in strawberries by biological control agents applied as single or combined treatments. Eur. J. Plant Pathol. 143:461–471.

Tenberge K.B. 2007. Morphology and cellular organization in *Botrytis* interactions with Plants. In *Botrytis: Biology, Pathology and Control*. Springer, Dordrecht. p. 67–73

van den Bosch, F., Paveley, N., Fraaije, B., van den Berg, F., and Oliver, R. 2015. Evidence-based resistance management: A review of existing evidence. In *Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management*, eds. Hideo Ishii and Derek William Hollomon. Tokyo: Springer Japan, p. 63–76.

van Kan, J. A. L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11:247–253.

Von Malek, B., and Debener, T. 1998. Genetic analysis of resistance to blackspot (*Diplocarpon rosae*) in tetraploid roses. Theor. Appl. Genet. 96:228–231

Vrind, T. A. 2005. The *Botrytis* problem in figures. Acta Hortic. 29:99–102.

Walker, A.-S., Micoud, A., Rémuson, F., Grosman, J., Gredt, M., and Leroux, P. 2013. French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). Pest Manag. Sci. 69:667–678.

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*. Mycol. Res. 99:1303–1310.

Williamson, B., Tudzynski, B., Tudzynski, P., and A L van Kan, J. 2007. *Botrytis cinerea*: The cause of grey mould disease. Mol. Plant Pathol. 8:561–80.

Wood, P. M., and Hollomon, D. W. 2003. A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. Pest Manag. Sci. 59:499–511.

Xu, X., and Pettitt, T. 2003. Disease: Downy Mildew. In *Encyclopedia of Rose Science*, ed. Andrew V. Roberts. Oxford: Elsevier, p. 154–158.

Zlesak, D.C., 2007. Rose. *Rosa x hybrida*. In: *Flower Breeding and Genetics.* N.O. Anderson ed. Springer, The Netherlands:695–740.

CHAPTER TWO

CHARACTERIZATION OF *BOTRYTIS CINEREA* FROM COMMERCIAL CUT FLOWER ROSES

Abstract

Botrytis cinerea Pers. infects cut flower roses (*Rosa* x *hybrida* L.) during greenhouse production and gray mold symptoms are often expressed in the post-harvest environment resulting in significant economic losses. Disease management is based on cultural practices and preventative chemical treatments, however gray mold outbreaks continue to occur. Rose tissues from six commercial shipments from two greenhouses in Colombia were evaluated to determine the *Botrytis* species composition as well as identify other pathogens present, gray mold incidence and severity, and fungicide resistance profiles. *Botrytis* isolates (49 total) were grouped into six morphological phenotypes, and all were identified to be *B. cinerea* sensu stricto. Disease incidence was higher in the petals than in the stem, stamen and ovary, sepal, or leaf tissues. Other pathogens were isolated infrequently and included *Alternaria alternata*, *Cladosporium cladosporoides*, *Epicoccum nigrum*, *Penicillium citrinum*, *Aspergillus tubingensis*, and *Diplodia* sp. Fungicide resistance profiles were determined using previously established discriminatory doses. Isolates resistant to thiophanate-methyl, iprodione, boscalid, and cyprodinil were found frequently in all shipments and in both greenhouses. The frequency of resistance to penthiopyrad, fenhexamid, fluopyram, isofetamid and fludioxonil varied between shipments and greenhouses. No resistance to pydiflumetofen was observed at the discriminatory doses tested. Isolates with resistance to multiple chemical classes were commonly found. These results indicate that fungicide resistance

management practices may improve preharvest and postharvest gray mold control of cut flower roses.

Introduction

Gray mold is a destructive fungal disease caused primarily by *Botrytis cinerea* Pers.*,* a ubiquitous fungal pathogen affecting more than 235 plant species around the world (Andrew et al. 2012), including roses (*Rosa* x *hybrida* L.). The disease reduces cut flower rose yield and post-harvest quality (Vrind 2005). *B. cinerea* is disseminated mainly by airborne conidia, which are produced on conidiophores (Holz, Coertze, and Williamson 2007). The pathogen can affect different plant tissues, including leaves, stems, and flowers. However, the most severe economic damage occurs when the pathogen infects the flowers petals (Elad 1988). Diseased flowers can be imperceptible at harvest because infections may remain latent until environmental conditions are favorable for tissue colonization. The favorable conditions for *B. cinera* growth include high relative humidity (>94%) and temperatures ranging from 15 to 25 ºC (Williamson et al. 2007). The initial symptoms appear as small lesions that develop into necrotic tissue leading to collapse of the petals and flower head (Elad 1988).

Botrytis cinerea has been described as a morphologically and genetically variable species (Elad et al. 2007b). Research done in several fruit crops has shown that gray mold disease caused by *B. cinerea* can be the result of a species complex with different genetic groups that may vary with the season, tissue, and host preference (Giraud et al. 1999; Fournier and Giraud 2008). Staats *et al.*, (2005) described a comprehensive DNA-sequence-based methodology to more accurately distinguish between *Botrytis* species using the nucleotide sequences of *G3PDH, HS60*, and *RPB2* genes. These genes used to

form the phylogenetic trees are essential for and often unique to the pathogen because they code for enzymes involved in different cellular processes (Staats et al. 2005). Based on these sequences, and considering the presence or absence of transposable elements (Walker 2016), and microsatellite amplifications (Váczy et al. 2008), *Botrytis pseudocinerea* was identified as a pathogen causing gray mold in different fruit crops including grapes, blackberries, and strawberries (Plesken et al. 2015). More cryptic species, including *B. caroliniana* and *B. fragariae,* were identified to cause gray mold of strawberry (Dowling and Schnabel 2017; Rupp et al. 2017b; Li et al. 2012). On ornamental plants different *Botrytis* species have been identified including, *Botrytis narcissicola* on narcissus; *Botrytis calthae* as a host-specific species on *Caltha,* occurring simultaneously with *B. pseudocinerea and B. cinerea; B. pelargonium* on geranium; and *B. convoluta* on iris (Walker 2016). *Botrytis* species have been previously identified in cut roses using PCR-RFLP and restriction enzymes to evaluate if *B. pseudocinerea* was part of the disease complex causing grey mold (Gomez 2013). The authors determined that only *Botrytis cinerea sensu stricto* was present in the roses, however this assessment was not based on key genes used for phylogenetic analysis including *G3PDH, HS60*, and *RPB2*

One of the principal strategies for gray mold management relies on weekly preventive fungicide applications including multi-site and site-specific fungicides (Hahn, Viaud, and Kan 2014a); however, the genetic plasticity and high adaptability of *B. cinerea* have resulted in resistance development to various single-site fungicides. Isolates may possess resistance to a single or multiple fungicide classes, termed chemical class resistance (CCR) (Elad, Yunis, and Katan 1992; Jiang et al. 2009; Grabke, Fernández-

Ortuño, and Schnabel 2012). *Botrytis cinerea* isolates with multiple CCR have been observed on different crops including cucumber, grapes, and strawberries (Elad, Yunis, and Katan 1992; Leroch et al. 2013; Amiri, Heath, and Peres 2013; Fernández-Ortuño et al. 2014). Resistance monitoring has been implemented to improve resistance management programs in order to preserve the efficacy of fungicides and improve disease control (Fernández-Ortuño et al. 2014; Hu, Cox, and Schnabel 2016). Whether *Botrytis* from Colombian roses is resistant to fungicides is unknown. The objectives of this study were to evaluate commercial shipments of cut flower roses to determine: (i) the *Botrytis* species and other pathogens present, (ii) the incidence and severity of gray mold on different cut flower tissues, and (iii) the occurrence of fungicide resistance in isolates from cut rose shipments.

Materials and Methods

Six shipments of commercial cut roses (*Rosa* x *hybrida* L.*)* from the *Botrytis* susceptible cultivar 'Orange Crush' were received between December 2016 and March 2017 from two greenhouses (A and B) located at Sabana Cundiboyacene in Colombia, South America (4º59'16.9" N, 73º59'36" W; 2650 m.a.s.l). The greenhouses were 400 m apart and 150 x 60 m in dimension. The roofs and sides were plastic and allowed for air ventilation during the day and night. The rose plants were produced in hydroponic systems using rice husks as a substrate. The production systems in both greenhouses were virtually identical, however, the setup of the two greenhouse floors varied slightly. Greenhouse A did not have any ground cover, while greenhouse B had brick paths installed in areas of highest human activity. Gray mold disease was managed in both

greenhouses with cultural practices, including removal of diseased or detached plant tissues on a regular basis, and forced air circulation using fans. Fungicides used during the experiment for gray mold management were the protectants captan, ziram, mancozeb, and chlorothalonil as well as site-specific fungicides belonging to FRAC codes 3, 7, 9,11, and 29. According to the producer, representatives of FRAC codes 1, and 2 were used in previous years. Also, FRAC 12 fungicides were used more recently. Protectant fungicides were applied and rotated on a weekly interval for gray mold management in both greenhouses. Additionally, weekly rotations of site-specific fungicides were based on FRAC codes. Not only the same FRAC code but also the same fungicides were applied in both greenhouses.

The roses were harvested and packaged as commercial bouquets of 25 roses per greenhouse. Each bouquet was wrapped in a clear plastic film covering the side of the flowers, and the bouquets were shipped in cardboard boxes. The shipments were received within one week at Clemson University and immediately processed. From each shipment and greenhouse, a set of five roses arbitrary selected from each bouquet. Each flowering stem was divided into five different groups of tissues: petals (3 outer petals, 3 mid petals positioned inside the outer petals, and 3 inner petals in direct contact with stamens and ovary), leaves, sepals, stamens and ovaries, and the entire stem cut into 15 cm sections. The tissues were surface sterilized for 1 min in a sodium hypochlorite solution (0.525%), immersed for 1 min in sterile deionized water, and air dried for 5 min to kill any spores that may have come in contact with the flowers during the transportation process. Then, each tissue type from each rose was individually placed in 15-cm-diameter petri dishes with moist filter paper covered with a lid and placed in a clear (3.78 L) sealable bag.

Bags were kept at -20 ºC for 24 h and then incubated for 7 d at 22 ºC and 100% relative humidity with 12 h light/12 h dark intervals. Gray mold incidence was determined as the percentage of samples that developed disease symptoms or signs. Gray mold severity was determined based on the percentage of symptomatic area per tissue type. Affected area was calculated based on the total area of the tissue showing symptoms or signs of the disease. Incidence of other fungi occurring in the rose tissues was also recorded, and the identity of the pathogens was determined as described below.

Morphological and phenotypic characterization of fungal isolates

Forty-nine *Botrytis* isolates and 20 non-*Botrytis* fungal isolates were collected from symptomatic rose tissue from the six shipments. Each isolate came from a different tissue sample. For single-spore isolation, spores were spread onto water agar and Petri dishes (9 cm dia.) and were placed in the dark at 22 ºC for 16 h to promote conidia germination. Individual germinated conidium were removed from the water agar under a dissection microscope using a sterile scalpel and then placed onto potato dextrose agar (PDA) medium (Difco Laboratories, Sparks, MD). Morphological characters of single spore colonies were assessed after 10 d at 22 ºC with intervals of 12 h of fluorescent light and 12 h darkness. Isolates were stored in the form of dried mycelium on filter paper. Koch's postulates were performed with each of the non-*Botrytis* isolates to verify pathogenicity.

DNA extraction, PCR amplification and sequencing

DNA sequencing was performed on single-spore *Botrytis* isolates from each morphological type and resistance profile, as well as non-*Botrytis* isolates. The isolates were cultured on PDA as described above for 7 d, except that cellophane paper (Research Products International Corp, Mount Prospect, IL) was used to cover the medium surface. Approximately 10 to 20 mg of aerial mycelia and conidia were collected using a sterile toothpick and placed into extraction buffer (1M KCl, 100mM Tris-HCl and 10 mM EDTA). Genomic DNA was extracted and purified as described previously (Chi et al. 2009)

For *Botrytis* species identification, a set of three different pairs of primers were used to amplify the *glyceraldehyde 3-phosphate dehydrogenase* (*G3PDH*), *heat-shock protein 60* (*HS60*), and *DNA-dependent RNA polymerase subunit II* (*RPB2*) regions of nuclear DNA (Staats et al. 2005). PCR amplifications were carried out in a 25 μl mixture reaction containing 50 to 100 ng of fungal DNA, 5 μl of 10X Thermo Pol buffer, 2.5mM dNTPs, 10 pmol of each primer and 0.25 μl of Taq polymerase (New England Biolabs). The PCR amplifications were accomplished using an iCycler Thermal Cycler (T100; Bio-Rad Laboratories Inc). The following thermocycling program was used to amplify the *G3PDH* gene fragment: 94 ºC for 3 min (1 cycle), denaturation at 94 ºC for 30 s, annealing at 56ºC for 30 s, extension at 72 ºC for 1 min (35 cycles), and then final extension at 72 ºC for 5 min (1 cycle). The thermocycling program used to amplify the genes *HS60* and *RPB2* was 94 ºC for 5 min (1 cycle); denaturation at 94 ºC for 30 s, annealing at 55ºC for 30 s, extension at 72 ºC for 90 s (35 cycles), and final extension at 72 ºC for 10 min (1 cycle). The PCR products were visualized in red gel (<100 ppm,

Biotum, Hayward, CA) stained with 1% agarose-Tris-borate-EDTA (1X TBE). The PCR products were purified and sequenced in both 3' and 5' directions at CORE laboratories at Arizona State University. The internal transcribed spacer regions from non-*Botrytis* fungal isolates were amplified using *ITS1F* and *ITS4* primers (White et al. 1990). The PCR amplifications were carried out in an iCycler Thermal Cycler (T100; Bio-Rad Laboratories Inc) using the following thermocycling pattern: 94 ºC for 5 min (1 cycle); denaturation at 94 ºC for 40 s, annealing at 55 ºC for 1 min, extension at 72 ºC for 2 min (35 cycles), and then final extension at 72 ºC for 10 min (1 cycle). The PCR products visualization, purification and DNA sequencing were performed as described above. Sequences were assembled and analyzed using Geneious version 11.1.3 (Biomatters Ltd.). Sequences of *Botrytis* and *non-Botrytis* fungi were entered into National center of Biotechnology Information (NCBI) BLAST tool. The accessions with higher homology were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov) and used as reference for comparisons.

Fungicide resistance profiling and chemical class resistance evaluation

Commercial formulations of the following 10 active ingredients were used to determine fungicide resistance of *Botrytis* isolates as previously described (Fernández-Ortuño et al. 2014; Dowling et al. 2016; Hu, Cox, and Schnabel 2016; Hu, Fernández-Ortuño, and Schnabel 2015): boscalid (Endura fungicide, 70% wt/wt; BASF Corporation, FRAC 7), cyprodinil (Vangard WG fungicide; Syngenta Crop Protection, FRAC 9), fenhexamid (Elevate 50 WDG; Arysta Life Science, FRAC 17), fluopyram (Luna Privilege fungicide; Bayer CropScience, FRAC 7), fludioxonil (Scholar SC fungicide;

Syngenta Crop Protection, FRAC 12), iprodione (Rovral 4 Flowable; Bayer Crop Sciences, FRAC 2), isofetamid (Kenja fungicide, 400 SC; SummitAgro, FRAC 7), penthiopyrad (Fontelis; DuPont Crop Protection, FRAC 7), pidiflumetophen (Adepidin; Syngenta, FRAC 7), and thiophanate-methyl (Topsin M 70WP; United Phosphorus, Inc., FRAC 1). The fungicide resistance profiles were identified for all the *Botrytis* isolates collected during the evaluation by testing for resistance to each of the 10 fungicides described above. The profiles were tested in 24-well plates (15 mm-diameter, 6 x 4 wells12.5 x 8.5 x 2 cm; Thermo Fischer Scientific, Rochester, NY) using spores present in different tissues as described previously (Fernández-Ortuño et al. 2014; Hu, Fernández-Ortuño, and Schnabel 2015; Hu et al. 2016). Discriminatory doses for FRAC 7 fungicides isofetamid and pidiflumetophen were chosen to be the same as was described previously for the FRAC 7 fungicide penthiopyrad (5 ug/ml) (Hu, et al. 2015). Conidia were transferred from actively growing colonies to the 24 well plates with a sterile toothpick. Mycelial growth was visually assessed after 4 d of incubation in the darkness at 22 ºC as described previously (Fernández-Ortuño et al. 2014).

The number of chemical class resistances (CCR) was determined for each of the 49 isolates. The isolates were classified as being simultaneously resistant to 0, 1, 2, 3, 4, 5, or 6 chemical classes, i. e. 0 CCR to 6 CCR isolates. The frequency of CCR isolates was evaluated across shipments and greenhouses.

Data analysis

Data analysis was made using JMP® Pro version 13.2.0 (SAS Institute Inc., Cary, NC). ANOVA and Fisher's LSD student's T test were used respectively to determine treatment effects and compare means between treatments at $p<0.05$.

For determination of disease incidence and severity, the data set consisted of a 2x6 factorial model consisting of two greenhouses, six shipments and five different tissue samples per combination. When the factors were significant, a Fisher's LSD student's T test was used to compare means for the factor levels at $p<0.05$. Additionally, multivariate analysis was performed to determine correlations between latent infections and the evaluated tissues.

A 2x6x10 unbalanced, full factorial ANOVA was made to analyze the effect of fungicide (10), shipment (6) and greenhouse (2) factors on the fungicide resistance for all 49 *Botrytis* isolates collected during the evaluation period. When the factors or interactions were significant a Fisher's LSD Student's T test was used for comparing means for the factor levels at $p<0.05$. For CCR evaluation a full factorial ANOVA was used to determine if the shipment, greenhouse or the interaction of both had an effect on CCR number. When the factors or interactions were significant, a Fisher's LSD Student's T test was used for comparing means for the factor levels at $p<0.05$.

Results

Morphological and molecular characterization of fungal isolates

The 49 single spore isolates were grouped into six morphology groups based on shape, amount of aerial mycelium, mycelium/conidia color, and distribution (Fig. 2.1).

Morphology groups were characterized as follows: group 1: irregular, aerial, umbonate formations and strong sporulation; group 2: a homogeneous colony with copious gray sporulation; group: profuse, raised white mycelium in the center and sporulation at the edges of the colony; group 4: variable mycelium elevation and spore distribution; group 5: white mycelium with variable elevation and rare sporulation; group 6: large sporulation evenly distributed and white umbonate mycelium at the center of the colony. Morphology group 4 was the most prevalent (20.9%), followed by groups 6, 5, 3, 2, and 1 with 20.9%, 14.0%, 14.0%, 14.0% and 9.3%, respectively. No differences were found in the resistance development of the different morphological groups.

Based on combined *G3PDH*, *HS60,* and *RPB2* gene sequence analysis, isolates from each morphological group were identified as *Botrytis cinerea* Pers. *sensu stricto*. The *G3PDH* and *HS60* sequences of our isolates revealed the highest identity with the reference sequences KY930944 and KY930945, respectively. Only *HS60* sequences from isolates S2GAR2M and S2GAR2O differed from the reference sequence and resulted in an amino acid (aa) change from cytosine to thymine at codon 644 resulting in the change at codon 215 from proline to leucine. Most nucleotide variations were found in gene *RPB2*, but all corresponded to silent mutations, including a change of guanine for adenine at the bases 276 and 348 for the isolate S2GBR3O and a change of guanine for thymine at base 849 for the isolates S5GBR1M, S5GBR4Sep, S1GBR3M, S5GBR4O, S4GBR4O, and S2GBR3O (data not shown).

Sequence analysis of the ITS regions from other fungi revealed their identity as *Alternaria alternata, Cladosporium cladosporoides, Epicoccum nigrum, Penicillium citrinum, Aspergillus brasiliensis* and *Diplodia* sp. The highest homologies corresponded

with the accession numbers MF564200, MG199960, KX869965, NR_121224, KT378129, and KC963918 respectively at the GenBank data base (https://www.ncbi.nlm.nih.gov). Incidence of *Alternaria alternata, Epicoccum nigrum*, *Penicillium citrinum*, and *Cladosporium cladosporiodes* in all shipments and greenhouses combined was 5.1%, 2.7%, 2.6%, and 1.7% respectively. Koch's postulates were confirmed for all of the fungi isolated from 'Orange Crush' roses. *Aspergillus brasiliensis* and *Diplodia* sp. were isolated from rose tissues, but they appeared to be avirulent because we were unable to confirm Koch's postulates (data not shown).

***Botrytis* incidence and severity on cut rose tissues**

Evaluation of latent infections on cut flower roses showed that *B. cinerea* was the most frequently isolated pathogen. A total of 14.6% of sampled rose tissue showed symptoms and/or signs of *Botrytis cinerea*. Greenhouse and shipment did not have an effect in the *B. cinerea* incidence or severity, but *B. cinerea* incidence and severity were differentially expressed ($p < 0.005\%$, and $p < 0.01\%$ respectively) on the different rose tissues. Incidence (Fig. 2.2a) in the petals (50%) was significantly higher than in stems (8.3%), sepal (3.3%), stamen and ovary (3.3%), and leaf (0%), and no significant differences were found amongst the last four tissues. Disease severity (Fig. 2.2b) was greater in the petals (9.5%) than in the stamen and ovary tissues (3.0%), sepals (1.7%) and leaves (0%).

The interaction between greenhouse and tissue was significant ($p < 0.05\%$) for disease incidence. Specifically, the percent affected petals was higher in greenhouse A

(63.3%) than greenhouse B (36.7%; p< 0.05%), but no differences were found for the other rose tissues between greenhouses. No differences were observed for the main effect of shipment or greenhouse on disease incidence or severity. Multivariate analysis showed no correlation between tissues for *B. cinerea* incidence or severity, i.e., the infection of one tissue was not a prerequisite to infection of another (data not shown).

Fungicide resistance profiling

Fungicide, shipment, greenhouse and their interactions were significant for the presence of fungicide resistance (p< 0.005). Across greenhouses and shipments *B. cinerea* isolates exhibited a high occurrence of resistance to thiophanate-methyl, iprodione, boscalid, and cyprodinil with average frequencies of 86.7%, 78.8%, 77.1%, and 75.4%, respectively (p= 0.05, Fig. 2.4). Differences in the resistance to isofetamid, fluopyram, penthiopyrad and fenhexamid were observed between greenhouses A and B. For greenhouse B a high percentage (66.7%) of the isolates was resistant to isofetamid, fluopyram, penthiopyrad, and a moderate percentage (47.5%) of the isolates from greenhouse B was resistant to fenhexamid. Resistance frequencies to isofetamid, fluopyram, penthiopyrad and fenhexamid in greenhouse A isolates were 19.2%, 22.5%, 33.8% and 26.3%, respectively.

The frequency of fludioxonil resistant isolates was relatively low for both greenhouses with an average frequency of 23.3%. No resistance was found to pydiflumetophen. The percentage of isolates displaying fungicide resistance varied with shipments and ranged from 0% at shipment 2 from greenhouse A to 73% at shipments 1 and 3 from greenhouse B (Fig. 2.3, Supplemental Table 2.1). Shipments 1, 3, 4, and 5

had the highest resistance frequencies and shipments from greenhouse B had higher fungicide resistance frequencies than greenhouse A in 4 of the 6 shipments.

Chemical class resistant evaluation

No differences were observed in numbers of isolates with multiple CCR between greenhouses; however, differences were observed amongst shipments ($p < 0.001$) (Supplemental Table 2.2). The isolates from shipment 2 revealed a smaller proportion of 4, 5, and 6 CCR isolates compared to other shipments. A one direction ANOVA was performed to evaluate if resistance profiles were related with morphological group, shipment, or greenhouse. No effect of morphological type or greenhouse was determined for the resistance profile response; however, shipment had an effect on the resistance and CCR ($p < 0.001$) (Supplemental Table 2.2). Shipments 3 and 4 had a higher proportion of isolates with simultaneous resistance to FRAC 1, 2, 7, 9, and 17 fungicides and FRAC 1, 2, 7, 9, 12, and 17 fungicides, respectively.

Discussion

This study showed that gray mold was the most common disease in cut roses from Colombia. This is consistent with previous studies that showed *Botrytis cinerea* to be the most frequent and limiting pathogen for production and postharvest management of cut rose flowers (Pie and De Leeuw 1991). We also confirmed PCR-RFLP results obtained by Gomez (2013) that the gray mold pathogen of roses is *Botrytis cinerea* Pers. *sensu stricto.* Other pathogens affecting postharvest quality of cut roses from Colombia were found in lower frequencies. *Alternaria alternata* was the second most frequently isolated pathogen which has been described as one of the main rose pathogens in Bangladesh

(Ghosh and Shamsi 2014), Pakistan (Abbas et al. 2017), and Ecuador (León and Andrés 2016), as well as in other flower crops such as sunflowers (Kgatle et al. 2018). However, this research is the first report of *Alternaria alternata* as a pathogen of roses from Colombia. *Cladosporium cladosporoides, Epicoccum* sp., *Penicillium* sp., *Aspergillus* sp., and *Diplodia* sp. have also been described as pathogens for roses and other ornamental plants (Yong 2004; Bensch et al. 2010; Ghosh and Shamsi 2014), but this is the first report of *Epicoccum nigrum*, *Penicillium citrinum* and *Aspergillus tubingensis* as rose pathogens.

Some studies have examined levels of susceptibility of rose cultivars to *Botrytis cinerea* (Hammer and Evensen 1991; Friedman et al. 2010) and differences in susceptibility amongst rose cultivars were found. Differences in the susceptibility have been attributed to the thickness of petal cuticles (Hammer and Evensen 1991), genetic background, polyphenolic compound levels (Lattanzio, et al. 2006; Nagpala et al. 2016), and pectin levels in the cell walls (Lionetti et al. 2007). The cultivar used in this study, Orange Crush, is a particularly susceptible cultivar according to the producers in Colombia. The petals of 'Orange Crush' roses yielded more *B. cinerea* infections that any other tissue. On grapes and strawberries flower petals have been characterized not only as the most susceptible tissue for *Botrytis* infection, but also the site of primary infections (Williamson et al. 2007). Host tissue susceptibility to *Botrytis* infection has been related with different factors such as tissue maturity (Droby and Lichter 2007), plant hormones, free radical levels (Elad 1997), phenolic compounds (Kretschmer et al. 2007), and plant nutrition (Elad 1988; Volpin 1991; Lattanzio et al. 2006). It is possible that the differential response of *B. cinerea* incidence in the evaluated rose tissues may be related

to some of these compounds; however, further investigation needs to be done to determine the cause of the variations on the *Botrytis* incidence in the different rose tissues.

The cut roses were asymptomatic upon delivery to our research facility but developed disease on various tissues after incubation. This coincides with the two stages described for *Botrytis* infection on cut roses Elad, (1988). In the first stage, often referred to as latent infections, conidia germinate on rose tissue followed by limited colonization that is macroscopically invisible. In the second stage, mycelia continue to expand in the rose tissue under favorable environmental conditions, e.g., relative humidity >94%. Visible necrotic areas appear as the disease advances to finally develop mycelia that will produce new conidia. This second stage of gray mold development during shipment was likely suppressed by forced air cooling of the bouquets in shipping boxes soon after harvest and by keeping the boxes at 4ºC during shipping.

Fungicide resistance in *B. cinerea* from agricultural crops is common and was also confirmed in isolates from cut roses in this study. Fungicide resistance in *B. cinerea* isolates has been reported for different crops including strawberries, grapes, apples, and some vegetables (Weber 2011; Leroch et al. 2013; Grabke et al. 2012). In this study, *B. cinerea* isolates from cut roses of both greenhouses were frequently resistant to thiophanate methyl (FRAC 1), iprodione (FRAC 2), cyprodinil (FRAC 9), and boscalid (FRAC 7). Thiophanate-methyl or any other FRAC 1 had not been used by the Colombian rose producer in more than a decade out of resistance concerns. This underscores the stability of resistance to FRAC 1 fungicides in *B. cinerea* and confirms observations of other studies (Malandrakis et al 2011; Ma and Michailides 2005). FRAC

2 fungicides had not been used for several years according to the producer but resistance still persisted in a large proportion of the population. This persistence of resistance to FRAC 2, contradicts other studies showing that once applications of FRAC 2 fungicides are interrupted for a long period of time, the susceptible population will recover making the fungicide efficient again (Leroux et al. 2002; Elad et al 1992). It is possible that the routine applications of FRAC 12 fungicide fludioxonil maintained resistance to FRAC 2 fungicides in the population. Depending on the molecular basis of resistance some genotypes do reveal positive cross resistance between fludioxonil (FRAC 12) and iprodione (FRAC 2), as documented for *Alternaria alternata* from tomatoes (Malandrakis et al. 2015). FRAC 9 fungicides had still routinely been used in both greenhouses up to the onset of this study either as solo products or in mixture with fludioxonil, which may explain the high frequency of resistance in both greenhouses.

FRAC 7 boscalid was one of the first FRAC 7 fungicides introduced for gray mold control and had been used extensively in the operation in previous years. Resistance to this particular chemical is conferred by many point mutations in the SDHB subunits (Amiri et al 2013; Hu et al 2015). Many of the these genotypes can also be selected by carboxin, another SDHI fungicide (Avenot and Michailides 2010). A review of the spray history revealed that carboxin and fluxapyroxad had been used in the 7 weeks prior to the first shipment in both greenhouses, thus the populations were exposed to roughly to the same selection pressure. That may explain the consistent and high frequency of resistance across both greenhouses.

Resistance to newer generation FRAC 7 fungicides, including isofetamid, fluopyram, and penthiopyrad was identified but neither had been used ever at the farm.

Furthermore, resistance frequencies were significantly different between greenhouses. This can be explained by incomplete cross resistance among FRAC 7 fungicides, where certain point mutations in SDHI subunit B confer resistance to specific SDHIs (Hu et al 2016). It is also possible, that the pathogen populations in the two greenhouses were subject to different genetic influx from several sources such as from weeds, nearby greenhouses, or compost piles. Resistance frequencies were fewer in isolates from shipments 2 and 6, which coincided with a reduced number of site-specific fungicides applied prior to these shipments. According to the producer, the number of single-site fungicide applications made in the four weeks previous to each shipment were 5, 1, 5, 8, 5, and 2 (in form of rotations of FRAC codes 3, 7, 9, 11, and 29) from shipments 1 to 6, respectively. This would support the hypothesis that the selection of resistant genotypes is directly linked to the number of applications of selective fungicides. The differences in resistance frequencies among FRAC 7 fungicides is a function of genotype selection and differences in intrinsic activity. Pydiflumetofen has the highest intrinsic activity among the FRAC 7s tested and can inhibit mycelium of *B. cinerea* that at the same discriminatory dose (5 ug/ml ai) can overcome exposure to penthiopyrad, fluopyram, and isofetamid.

Farm managers in Colombia have been rotating fungicides to avoid or delay resistance development for years. However, the number of effective and available fungicides is limited to 4 to 5 single-site fungicides, which results in producers using these FRAC codes multiple times per year. In this study isolates with resistance to fungicides of multiple FRAC codes were found, which is consistent with observations in other crops (Leroch et al. 2013; Amiri et al. 2013; Chen et al. 2016). For example,

isolates of *B. cinerea* collected from strawberries in Europe and the United States revealed resistance to five and more chemical classes of fungicides (Leroch et al. 2013; Fernández-Ortuño et al. 2014). Research shows that the development of resistance to multiple chemical classes is a consequence of stepwise accumulation of resistance to fungicides often based on target gene modifications rather than a single mechanism of resistance such as ATP-Binding Cassette transporter activity (Li et al. 2012; Nakajima et al. 2001). Rotation of fungicides may therefore continue to select for such phenotypes and not lead to effective disease management (Hu et al. 2016).

Continual production of roses occurs throughout the year in humid greenhouses that provide optimal conditions for gray mold development. Under these conditions it is difficult to implement resistance management strategies without having to use fungicides frequently. In the absence of gray mold-resistant rose cultivars only an integrated approach including cultural practices, the integration of multisite and single-site fungicides, extended spray intervals, and perhaps integration of biological control options for gray mold management will result in sustainable disease management.
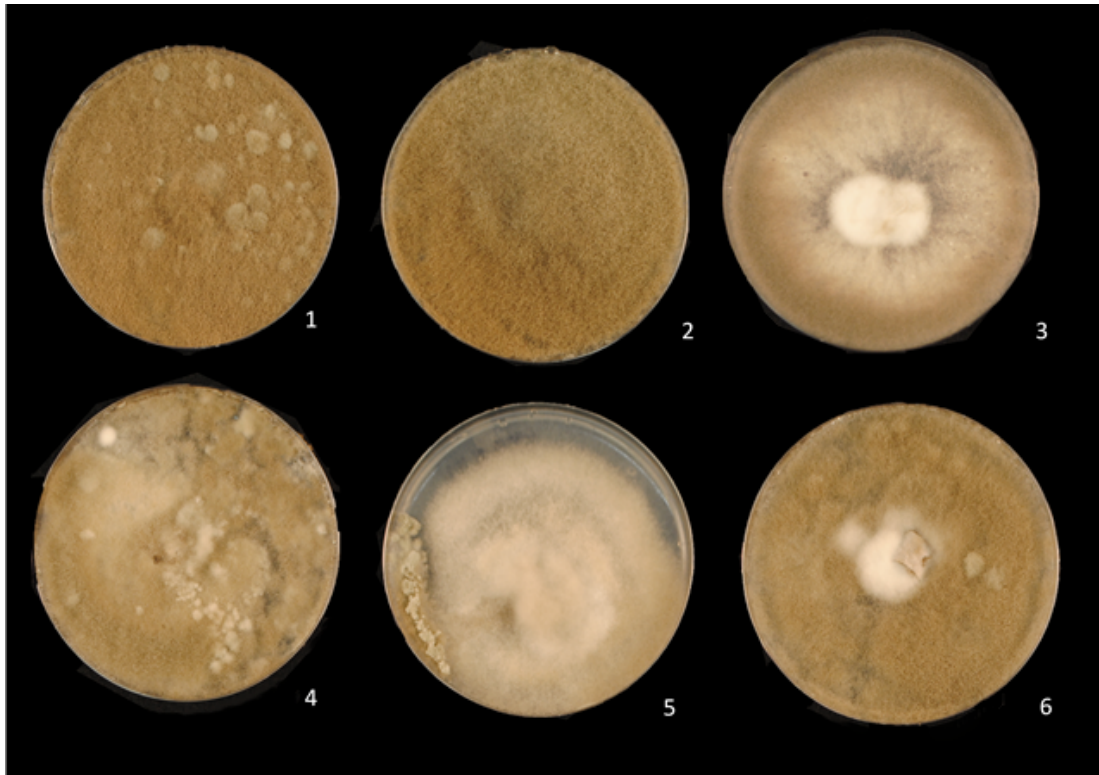
Figure 2.1. Morphology groups of *B. cinerea* cultures on PDA. Group 1: Gray, raised, sporulating mycelium with umbonate elevations non-uniformly distributed across the colony; Group 2: homogeneous raised elevation with uniform gray sporulation occurring evenly over the plate; Group 3: umbonate white mycelium without sporulation in the center of the plate; Group 4: Unevenly distributed mycelium and sporulation over the plate. Elevation varies within the same colony between raised, flat and umbonate; Group 5: Predominantly white colony with little sporulation; Group 6: large areas of sporulation with umbonate elevations of white mycelium frequently at the center of the plate.
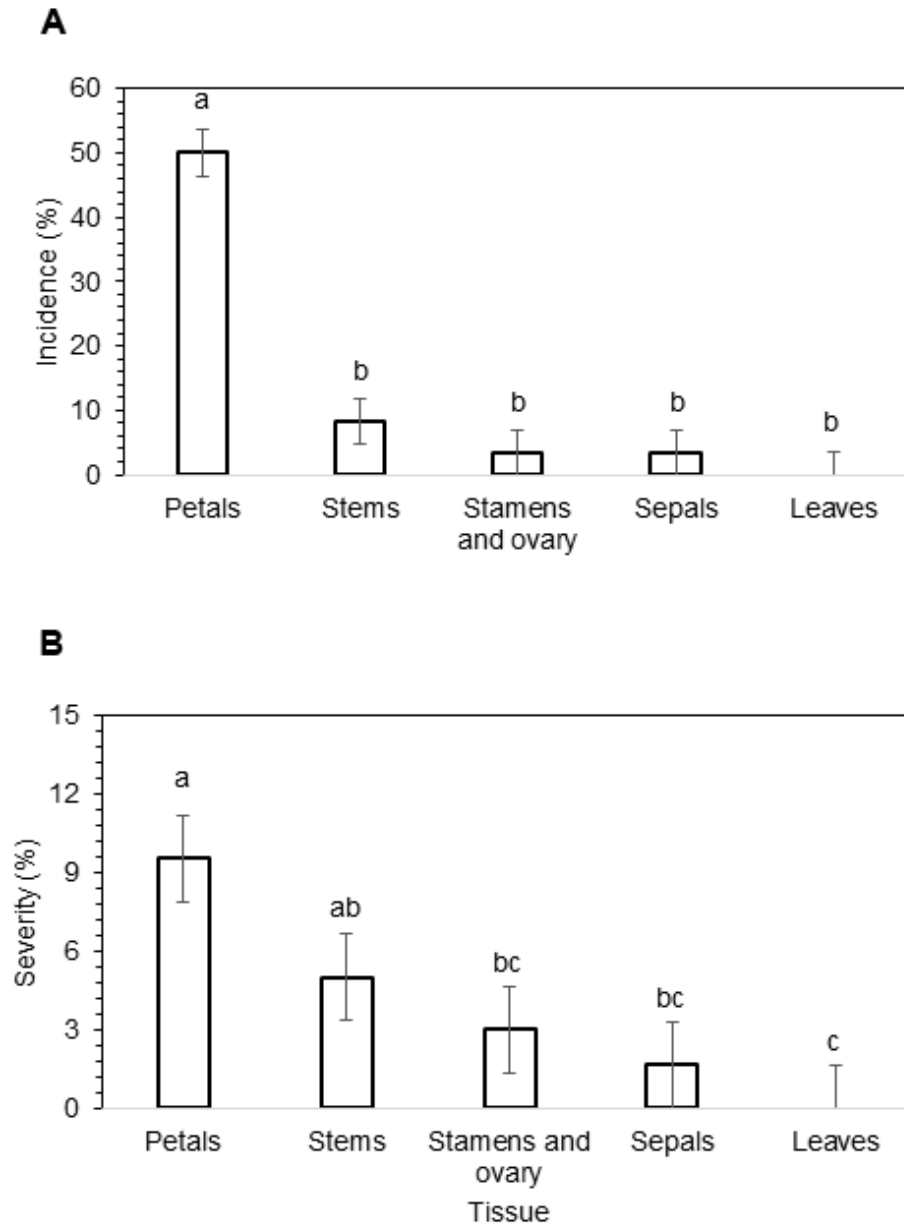
Figure 2.2. *B. cinerea* incidence (A) determined as the percentage of tissue with any symptoms or signs; and severity (B) determined as percentage of symptomatic area for each rose tissue from six shipments and two greenhouses. LSD test (α= 0.05), error bars represent ±1 SE. Different letters indicate significantly different responses within tissues
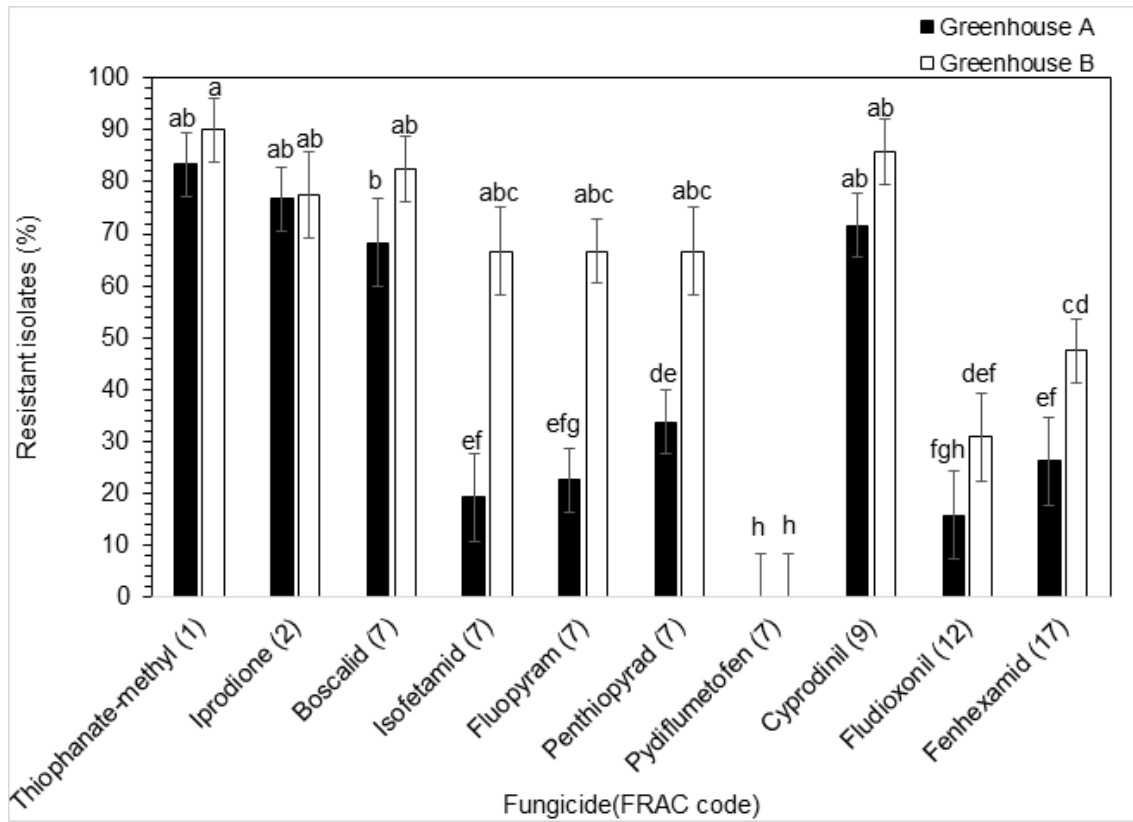
Figure 2.3. *B. cinerea* isolates resistant to fungicides from infected rose tissues from both greenhouse A (n=30) and B (n=19) averaged over six shipments. Least square means where calculated based on an LSD test (α= 0.05). Error bars represent +1 SE. Different letters indicate significantly different responses within fungicides and greenhouses.
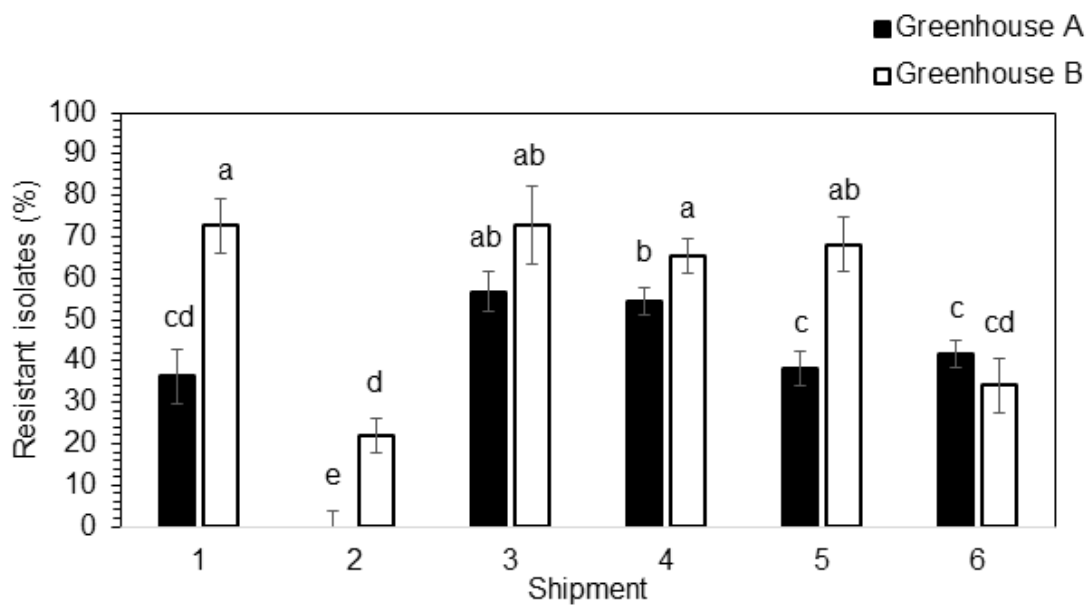
Figure 2.4. Percentage of fungicide resistant isolates to at least one fungicide recorded from six shipments from both greenhouse A (n=30) and B (n=19). LSD test (α = 0.05). Error bars represent ±1 SE. Different letters indicate significantly different responses within shipments and greenhouses.

Literature Cited

Abbas, M. F., Khan A.-U.-D, Rafique, K., Qadir, A., Rashid, A., Qamar, M. I., Rafique, M., and Gleason, M. 2017. First report of Alternaria black spot of rose caused by *Alternaria alternata* in Pakistan. Plant Dis. 101:1676.

Amiri, A., Heath, S. M., and Peres, N. A. 2013. Resistance to fluopyram, fluxapyroxad, and penthiopyrad in *Botrytis cinerea* from strawberry. Plant Dis. 98:532–539.

Andrew, M., Barua, R., Short, S. M., and Kohn, L. M. 2012. Evidence for a common toolbox based on necrotrophy in a fungal lineage spanning necrotrophs, biotrophs, endophytes, host generalists and specialists. PLOS ONE. 7(1): e29943. https://doi.org/10.1371/journal.pone.0029943

Avenot, H. F., and Michailides, T. J. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Prot. 29:643–651.

Bensch, K., Groenewald, J. Z., Dijksterhuis, J., Starink-Willemse, M., Andersen, B., Summerell, B. A., Shin, H.-D., Dugan, F.M., Schroers, H.-D., Braun, U., and Crous P.W. 2010. Species and ecological diversity within the *Cladosporium cladosporioides* complex (Davidiellaceae, Capnodiales). Stud. Mycol. 67:1–94.

Chen, S. N., Luo, C. X., Hu, M. J., and Schnabel, G. 2016. Fitness and competitive ability of *Botrytis cinerea* isolates with resistance to multiple chemical classes of fungicides. Phytopathology. 106:997–1005.

Chi, M.-H., Park, S.-Y., and Lee, Y.-H. 2009. A Quick and safe method for fungal DNA extraction. Plant Pathol. J. 25:108–111.

Dowling, M. E., Hu, M.-J., Schmitz, L. T., Wilson, J. R., and Schnabel, G. 2016. Characterization of *Botrytis cinerea* isolates from strawberry with reduced sensitivity to Polyoxin D zinc salt. Plant Dis. 100:2057–2061.

Dowling, M. E., and Schnabel, G. 2017. First report of *Botrytis mali* causing gray mold on strawberry in the United States. Plant Dis. 101:1034.

Droby, S., and Lichter, A. 2007. Post-Harvest *Botrytis* Infection: Etiology, development and management. In *Botrytis: Biology, pathology and control*, eds. Yigal Elad, Brian Williamson, Paul Tudzynski, and Nafiz Delen. Dordrecht: Springer Netherlands, p. 349–367.

Elad, Y. 1988. Latent infection of *Botrytis cinerea* in rose flowers and combined chemical and physiological control of the disease. Crop Prot. 7:361–366.

Elad, Y. 1997. Responses of plants to infection by *Botrytis Cinerea* and novel means involved in reducing their susceptibility to infection. Biol. Rev. 72:381–422.

Elad, Y., Williamson, B., Tudzynski, P., and Delen, N. 2007. *Botrytis* spp. and diseases they cause in agricultural systems – An Introduction. In Elad, Y., Williamson, B., Tudzynski, P., and Delen, N. (eds) *Botrytis: Biology, Pathology and Control*. Springer Netherlands, Dordrecht p. 1–8.

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 Pathol. 41:41–46.

Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N. A., and Schnabel, G. 2014. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven southern U.S. states. Plant Dis. 98:825–833.

Fournier, E., and Giraud, T. 2008. Sympatric genetic differentiation of a generalist

    pathogenic fungus, *Botrytis cinerea*, on two different host plants, grapevine and

    bramble. J. Evol. Biol. 21:122–32.

Friedman, H., Agami, O., Vinokur, Y., Droby, S., Cohen, L., Refaeli, G., Resnick, N.,

    and Umiel, N. 2010. Characterization of yield, sensitivity to *Botrytis cinerea* and

    antioxidant content of several rose species suitable for edible flowers. Sci. Hortic.

    - SCI HORT-Amst. 123:395–401.

Ghosh, A., and Shamsi, S. 2014. Fungal diseases of Rose plant in Bangladesh. J.

    Bangladesh Acad. Sci. 38:225–233.

Giraud, T., Fortini, D., Levis, C., Lamarque, C., Leroux, P., LoBuglio, K., and Brugoo,

    Y. 1999. Two sibling species of the *Botrytis cinerea* complex, transposa and

    vacuma, are found in sympatry on numerous host plants. Phytopathology.

    89:967–973.

Gómez Rodríguez, T. 2013. Caracterizacion de aislamientos de *Botrytis cinerea* de rosa

    de la sabana de Bogota. Repositorio institucional UN. Available at:

    http://www.bdigital.unal.edu.co/44422/ [Accessed August 24, 2018].

Grabke, A., Fernández-Ortuño, D., and Schnabel, G. 2012. Fenhexamid resistance in

    Botrytis cinerea from strawberry fields in the carolinas is associated with four

    target gene mutations. Plant Dis. 97:271–276.

Hahn, M., Viaud, M., and Kan, J. A. L. van. 2014. The genome of *Botrytis cinerea,* a

    ubiquitous broad host range necrotroph. In *Genomics of Plant-Associated Fungi*

    and Oomycetes: Dicot Pathogens, Berlin Heidelber: Springer Verlag, p. 19–44.

Hammer, P. E., and Evensen, K. B. 1991. Differences between rose cultivars in

    susceptibility to infection by *Botrytis cinerea*. Phytopathology. 26:785–785.

Holz, G., Coertze, S., and Williamson, B. 2007. The ecology of *Botrytis* on plant

    surfaces. In *Botrytis: Biology, Pathology and Control*, Springer, Dordrecht, p. 9–

    27.

Hu, M.-J., Cox, K. D., and Schnabel, G. 2016. Resistance to increasing chemical classes

    of fungicides by virtue of "selection by association" in *Botrytis cinerea*.

    Phytopathology. 106:1513–1520.

Hu, M.-J., Fernández-Ortuño, D., and Schnabel, G. 2015. Monitoring resistance to SDHI

    fungicides in *Botrytis cinerea* from strawberry fields. Plant Dis. 100:959–965.

Jiang, J., Ding, L., Michailides, T. J., Li, H., and Ma, Z. 2009. Molecular characterization

    of field azoxystrobin-resistant isolates of *Botrytis cinerea*. Pestic. Biochem.

    Physiol. 93:72–76.

Kgatle, M. G., Truter, M., Ramusi, T. M., Flett, B., and Aveling, T. A. S. 2018.

    Alternaria alternata, the causal agent of leaf blight of sunflower in South Africa.

    Eur. J. Plant Pathol. 151:677–688.

Kretschmer, M., Kassemeyer, H.-H., and Hahn, M. 2007. Age-dependent grey mould

    susceptibility and tissue-specific defence gene activation of grapevine berry skins

    after infection by *Botrytis cinerea*. J. Phytopathology. 155:258–263.

Lattanzio, V., M T Lattanzio, V., and Cardinali, A. 2006. Role of polyphenols in the

    resistance mechanisms of plants against fungal pathogens and insects. In

    Phytochemistry, p. 23–67.

León, H., and Herrera, F.A. 2016. Aislamiento, caracterización molecular y análisis de patogenicidad de *Alternaria* spp. sobre botones de rosa (rosa sp) y plantas de brócoli (Brassica oleracea var. Italica). Available at: http://repositorio.usfq.edu.ec/handle/23000/6158.

Leroch, M., Plesken, C., Weber, R. W. S., Kauff, F., Scalliet, G., and Hahn, M. 2013. Gray mold populations in german strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. Appl. Environ. Microbiol. 79:159–167.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M., and Chapeland, F. 2002. Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Manag. Sci. 58:876–888.

Li, X., Kerrigan, J., Chai, W., and Schnabel, G. 2012. *Botrytis caroliniana*, a new species isolated from blackberry in South Carolina. Mycologia. 104:650–658.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., and Bellincampi, D. 2007. Overexpression of pectin methylesterase inhibitors in arabidopsis restricts fungal infection by *Botrytis cinerea*. Plant Physiol. 143:1871–1880.

Ma, Z., and Michailides, T.J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853–863.

Malandrakis, A. A., Apostolidou, Z. A., Markoglou, A., and Flouri, F. 2015. Fitness and cross- resistance of Alternaria alternata field isolates with specific or multiple

resistance to single site inhibitors and mancozeb. Eur. J. Plant Pathol. 142:489–499.

Malandrakis, A., Markoglou, A., and Ziogas, B. 2011. Molecular characterization of benzimidazole-resistant B. cinerea field isolates with reduced or enhanced sensitivity to zoxamide and diethofencarb. Pestic. Biochem. Physiol. 99:118–124.

Nagpala, E. G., Guidarelli, M., Gasperotti, M., Masuero, D., Bertolini, P., Vrhovsek, U., and Baraldi, E. 2016. Polyphenols variation in fruits of the susceptible strawberry cultivar alba during ripening and upon fungal pathogen interaction and possible involvement in unripe fruit Tolerance. J. Agric. Food Chem. 64:1869–1878.

Nakajima, M., Suzuki, J., Hosaka, T., Hibi, T., and Akuts U. K. 2001. Functional analysis of an ATP-Binding cassette transporter gene in *Botrytis cinerea* by gene disruption. J. Gen. Plant Pathol. 67:212–214.

Pie, K., and De Leeuw, G.T.N 1991. Histopathology of the initial stages of the interaction between rose flowers and *Botrytis cinerea*. Neth. J. Plant Pathol. 97:335–344.

Plesken, C., Weber, R. W. S., Rupp, S., Leroch, M., and Hahn, M. 2015. *Botrytis pseudocinerea* is a significant pathogen of several crop plants but susceptible to displacement by fungicide-resistant *B. cinerea* strains. Appl. Environ. Microbiol. 81:7048–7056.

Rupp, S., Weber, R. W. S., Rieger, D., Detzel, P., and Hahn, M. 2017. Spread of *Botrytis cinerea* strains with multiple fungicide resistance in german horticulture. Front. Microbiol. 7:2075.

Staats, M., van Baarlen, P., and van Kan, J.A.L. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol. Biol. Evol. 22:333–346.

Váczy, K. Z., Sándor, E., Karaffa, L., Fekete, E., Fekete, E., Arnyasi, M., Czegledi, L., Kovics, G.J., Druzhinia, J.S., and Kubicek, C.P. 2008. Sexual recombination in the *Botrytis cinerea* populations in Hungarian vineyards. Phytopathology. 98:1312–1319.

Volpin, H. 1991. Influence of calcium nutrition on susceptibility of rose flowers to *Botrytis* blight. Phytopathology. 81:1390.

Vrind, T. A. 2005. The *Botrytis* problem in figures. Acta Hortic. 29:99–102.

Walker, A.S. 2016. Diversity within and between species of *Botrytis*. In *Botrytis – the Fungus, the Pathogen and its Management in Agricultural Systems*, eds. Sabine Fillinger and Yigal Elad. Cham: Springer International Publishing, p. 91–125.

Weber, R. W. S. 2011. Resistance of *Botrytis cinerea* to multiple fungicides in northern German small-fruit production. Plant Dis. 95:1263–1269.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In PCR- protocols and applications, a laboratory manual: Academic Press, p. 315–322.

Williamson, B., Tudzynski, B., Tudzynski, P., and van Kan, J.A.L. 2007. *Botrytis cinerea*: The cause of grey mould disease. Mol. Plant Pathol. 8:561–80.

Yong, A. 2004. El cultivo del rosal y su propagación. Cultiv. Trop. 25:53–57.

S. Table 2.1. List of morphological types and fungicide resistance profile for isolates collected from six cut flower rose shipments from each of two greenhouses.

Shipment	Greenhouse	Morphology type	FRAC					
			1	2	7	9	12	17
1	A	1	●	●		●	●	
1	A	3*	●	●	●			
1	A	5						
1	B	2*	●	●	●	●	●	
1	B	5						
2	A	5*						
2	A	4*						
2	A	6*						
2	B	5						
2	B	3						
2	B	3*	●	●	●	●		●
2	B	6						
3	A	2	●	●	●	●		●
3	A	4	●	●	●	●		●
3	A	5	●	●	●	●		
3	A	6	●	●	●	●		●
3	B	2	●	●	●	●		●
3	B	5	●	●	●	●		
4	A	4	●	●	●	●		●
4	A	4	●	●	●	●		
4	A	5	●	●	●	●		
4	A	5	●	●	●	●		●
4	A	6	●	●	●	●	●	●

4	B	3	•	•	•	•	•	•
4	B	4	•	•	•	•		
4	B	5	•	•	•	•		
4	B	6	•	•	•	•		
4	B	6*	•	•	•	•	•	•
5	A	1*	•	•	•	•		
5	A	1						
5	A	2	•	•	•	•		•
5	A	2	•	•	•	•		
5	A	4	•	•	•	•		
5	B	2*	•	•	•	•		•
5	B	4*	•	•	•	•		
5	B	6	•	•	•	•		
6	A	1	•	•	•	•		
6	A	4	•	•	•	•		
6	A	4	•			•		
6	B	3	•			•		
6	B	3	•		•	•		•
6	B	4	•	•	•	•		
6	B	4	•	•	•	•	•	•
6	B	6	•		•	•		•

* Indicates that the isolate from that specific combination was sequenced for genetic identification.

S.Table 2.2. Distribution of isolates with different levels of chemical class resistance

(CCR) for each of the six shipments of cut flower roses. LSD test (p= 0.05%).

CCR	CCR isolates (%) observed within each shipment					
	Shipment number					
	1	2	3	4	5	6
0	0	82	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	22
3	25	0	0	0	0	0
4	25	9	40	31	86	56
5	50	9	40	54	14	11
6	0	0	20	15	0	11
	ab[z]	c	ab	a	ab	b

[z] Different letters indicate significantly different distribution of CCR responses between

shipment numbers (α= 0.05).

CHAPTER THREE

EVALUATION OF *BOTRYTIS CINEREA* CONIDIA COUNT IN THE ROSE CANOPY AND ITS RELATIONSHIP WITH THE PRODUCTION ACTIVITIES OCCURING IN COMMERCIAL ROSE GREENHOUSES

Abstract

Gray mold disease caused by *Botrytis cinerea* Pers. affects cut rose quality in production and postharvest environments, but inoculum for infection is mostly produced during production. Production activities may increase spore dispersal which may increase the *B. cinerea* infection risk. Over the course of one-year, daily spore count data was obtained from spore traps located in two commercial greenhouses. Production activities and disease incidence data were compared with daily spore count. The correlation between the different production activities and spore count was evaluated via hierarchical cluster analysis and possible relationships between spore count and disease incidence were assessed using transfer functions analysis in time series. The spore count was 49.5% during the week days, were most activities take place compared to the weekends. The highest spore count was observed across both greenhouses when "dumping refreshing solutions", "fungicide application", "lime application", "harvest", "pinch", "removal of powdery mildew symptomatic tissue" , "removal of downy mildew symptomatic tissue" "supplemental watering", "sweeping of the central aisle" activities occurred. Additionally, for greenhouse A "changing greenhouse plastic" and "cleaning leachate trays" activities were also related with a high spore count, while a high spore count for greenhouse B was observed during "NaOCl application", "dead heading" and "manual plant debris removal" were performed. No correlation was observed between spore count

and the duration of leaf wetness and the temperature during leaf wetness. No correlation was observed between *B. cinerea* spore count and gray mold incidence as was previously described for gray mold infection in strawberry flowers (Blanco, de Santos, and Romero 2006). The results indicate that the number and type of production activities have an effect in the movement of inoculum y cut rose greenhouses. Activities handling and increasing free water in the greenhouses were related with a high spore count.

Introduction

Botrytis cinerea is the fungal pathogen that causes gray mold in cut roses in both production and post-harvest environments (Vrind 2005). The disease primarily affects flower petals, but other plant tissues can also be infected (Elad 1988a). Dissemination of *B. cinerea* occurs primarily by spread of multinucleate conidia through air movement and in rain splash (Holz et al. 2007). Conidia are produced in conidiophores over gray mycelia in infected and necrotized plant tissue (Hahn, et al. 2014). Plant debris, senescing stems in the canopy, and growing substrate in the greenhouses are considered to be the most important sources of *B. cinerea* inoculum for rose crops while environmental conditions play a critical role in the conidia availability and movement (Sandón 2005). Inoculum can also be produced in sclerotia in the soil, plant debris and weed, as well as mycelium on decaying plant material (Beever and Weeds 2007).

B. cinerea spore dispersal has been related to a series of different factors including environmental and non-environmental conditions. Rapid changes in the environment humidity may lead to conidia release from the conidiophores by hygroscopic movements of the conidiophore (Jarvis 1962b). While, leaf wetness period

and temperature during leaf wetness are important climatic variables for predicting *B. cinerea* infection risk (Bulger 1987).

Sampling of airborne conidia to evaluate inoculum availability has been done to better understand different plant-pathogen systems (West, et al. 2009). Luo et al. (2005) used spore traps to monitor *Monilinia fruticola* spores in plum orchards and determined differences in the spore population during the season. Jarvis (1962) observed that the number of *B. cinerea s*pores present on raspberry fields was affected by the removal of berries. Later, Hausbeck and Pennypacker (1991) showed that *B. cinerea* conidia concentration in a geranium stock plant greenhouse was associated with different 'grower activities' including fertilization, irrigation, fungicide applications and harvest. In geranium pot production, cultural practices such as cuttings manipulation, plant sanitation and watering influenced the amount of *B. cinerea* conidia present in the greenhouses (Daughtrey, et al. 1995). The production stage of cut roses also involves a series of different activities inside the greenhouses that may move or disturb a large amount of plant material or substrate through various processes such as harvesting flowering stems, plant debris removal, and plant growth regulator applications. The objectives of this research were: 1) To determine the daily variation in the *B. cinerea* spore count in commercial cut rose production greenhouses over the course of one year, 2) To identify the relationship between the *B. cinerea* spore count and the activities used during crop production, and 3) To identify if there is a relationship between high spore count and high *B. cinerea* infection risk.

Materials and Methods

Two spore traps (Burkard multi-vial cyclone samplers, Burkard Manufacturing Co Ltd, Hertfordshire, UK) were installed inside two commercial greenhouses similar in structure and size, identified as A and B, both located at the same farm in Sabana Cundiboyacene in Colombia, South America (4º59'16.9" N, 73º59'36" W; 2650 m.a.s.l). Each spore trap was placed inside the canopy of a commercial bed of 'Orange Crush' roses at a height of 1.5 m above the ground. Air samples with solid particulate material were collected daily between 06:30 am and 06:30 pm using an air inflow rate of 16.5 L/min. Daily samples were contained in 1.5 ml Eppendorf tubes (USA scientific, Ocala, FL) and 14 tubes (one from each day) were sent every two weeks to Clemson University for conidia quantification. The spores were counted in a hemocytometer (Bright-line 3110, Hausser Scientific, Horsham, PA) under the microscope (Olympus BX41-YX, Olympus America Inc., Melville, NY). To verify that the spores observed where from *B. cinerea*, macroscopic evaluation where done on Petri dishes (9 cm dia.) with potato dextrose agar medium (PDA) (Difco Laboratories, Sparks, MD) amended with 1% of lactic acid (Honeywell Fuka™ - Thermo Fischer Scientific, Rochester, NY) and then molecular confirmation using the partial gene *glyceraldehyde 3-phosphate dehydrogenase* (*G3PDH*) were performed, which confirmed that the spores belong to *B. cinerea*.

Date and type of production activities were recorded by the greenhouse managers. A total of 29 different activities were recorded (Table 3.1) and only the ones conducted five or more times per greenhouse during the year of evaluation were included in the analysis to reduce the possibility of random chance associations. Daily spore count

data was categorized as low, medium and high based on >22, 22-50, and >50 spores counts per day respectively, considering that 22 spores/day was the average of the spore count observed on the evaluation. The effect of production activities on the spore count was evaluated on same-day and day-after merged.

Relative humidity, air temperature, and duration of continuous leaf wetness data were recorded during the evaluated period using an iMethos 3.3 weather station (Pess Instruments, Loxahatchee, FL). Two on-farm monitoring techniques and one scouting technique were used to evaluate gray mold incidence: "Flower quality index" was obtained daily after evaluation of 40 arbitrarily selected roses per cultivar and greenhouse as the percentage of flowers with necrotic, discolored, or pink flecks. "*Botrytis* humid chamber index" was recorded after placing 5 arbitrarily-selected flowers per cultivar per greenhouse in a humid chamber and evaluating the proportion of flowers with signs of *Botrytis*. Finally, "Plant quality index" was obtained weekly by scouting to the upper two thirds of the plants and determining the percentage of necrotic, or discolored flecks symptoms in flowers, stems and leaves per cultivar, greenhouse and bed. These datasets were used separately for correlation analysis between spore count and gray mold occurrence.

Data analysis

Data analysis was performed using JMP® Pro version 13.2.0 (SAS Institute Inc., Cary, NC). ANOVA and Fisher's LSD student test were performed to compare spore count means between greenhouses, days of the week, and weeks of the year at *p < 0.05*. Hierarchical clustering using the Ward method was used to group production activities

with similar spore count profiles, ANOVA and Fisher's LDS student test ($p < 0.05$) were used to determine significance of the clusters. Transfer functions analysis using time series evaluation was made to determine the correlation between weather variables (leaf wetness and air temperature during the leaf wetness period), and gray mold incidence data with spore count.

Results

Temporal variation in the spore count

A low, medium and high spore count was observed for 72%, 20.3% and 8% of the year of evaluation, respectively (Fig. 3.1a). The maximum, minimum, and average spore count values over the evaluation were 215, 0 and 22 spores/day respectively (Fig. 3.2a). Spore count varied throughout the year of observation with the highest values occurring from the last week of December 2017 to the first week of January 2018 (Fig. 3.2a). Differences were also observed between days of the week (p < 0.013 %), with 49.5% higher spore count values occurring during weekdays (Mon.-Fri.) (Fig. 3.3) which are the days with an average of 50.5% more activities happening in the greenhouses. No difference was observed between greenhouses.

Relationship between Botrytis spore count and the greenhouse environment

No relationship between spore count and leaf wetness duration (p = 0.38) or average temperature during leaf wetness (p = 0.81) was observed. The peaks on the year of evaluation with longer period of continuous leaf wetness and higher temperatures were observed during June-July 2017 and March-April 2018 (Fig. 3.1a and 1b), while the

highest peaks for spore count occurred between December 2017 and March 2018 (Fig. 3.1a).

Relationship between *Botrytis* spore count and gray mold incidence reported by the growers

No relationship was observed between *Botrytis* spore count and the daily or weekly reports of *Botrytis* incidence including *"Flower quality index"* ($p = 0.89$), "*Botrytis* humid chamber index" ($p = 0.11$), and *"Plant quality index"* ($p = 0.15$). The highest *"Flower quality index"* (71%) was observed during the first two weeks of April 2018 (Fig. 3.1d). *"Flower quality index"* was below 35% during the rest of the evaluated period. "*Botrytis* humid chamber index" showed two peaks over 50% during June to July 2017 and November 2017 to January 2018 (Fig. 3.2b), while the highest values reported *"Plant quality index"* were observed from November 2017 to January 2018 and during the first two weeks of April 2018 (Fig 3.2c).

Relationship between *Botrytis* spore count and production activities

A total of 23 and 22 production activities were analyzed for its relationship with the spore count for greenhouses A and B, respectively. For greenhouse A, 11, 4 and 8 production activities grouped into clusters 1, 2 and 3 respectively (Fig. 3.4). For greenhouse B, clusters 1, 2 and 3 contained 13, 5 and 4 production activities, respectively (Fig. 3.5). In both greenhouses, activities at cluster 1 were most related with high spore counts (Table 3.2, and Table 3.3) and there were 9 production practices applied to both greenhouses including "dumping refreshing solutions", "fungicide application", "lime application", "harvest", "pinch", "removal of powdery mildew symptomatic tissue",

"removal of downy mildew symptomatic tissue" "supplemental watering", "sweeping of the central aisle". In greenhouse A, activities in cluster 2 were most related with a medium spore count, and activities at cluster 3 with a low spore count (Table 3.2). At greenhouse B, clusters 1 and 2 showed the highest values of medium spore count, and activities at cluster 3 were most related with a low spore count (Table 3.3).

Discussion

In this study variations in the *B. cinerea* spore count were observed suggesting constant changes in the inoculum availability and movement in the cut rose commercial greenhouses. An overall higher number of spores was collected between Monday through Friday, where about 50% more production activities were performed. This is in agreement with Buttner and Stetzenbach (1993) who used four aerobiological samplers in an enclosed experimental room to determine the effects of human activity on air sampling. They described a direct correlation between human activities and movement of airborne fungal conidia.

Of production activities associated with a high spore count in greenhouses A and B respectively, nine activities were shared between both greenhouses including: "dumping refreshing solutions", "fungicide application", "lime application", "harvest", "pinch", "removal of powdery mildew symptomatic tissue", "removal of downy mildew symptomatic tissue", "supplemental watering", and "sweeping of the central aisle". These practices involve movement of *B. cinerea* inoculum in rose greenhouses in form of infected substrate or plant debris. The movement of this material could be related with the disturbance of the principal spore deposits in the greenhouse leading to conidia liberation into the air (Sandon 2005). Other high risk activities involved water handling

70

practices in the greenhouse that had previously been related with high spore counts in greenhouses as well. For example, Jarvis (1962) described the "water-and-spore projectiles" theory where handling and splashing water was associated with horizontal conidia movement followed by immediate germination after landing on the new host. Also, Williamson et al. (2007) described free water in greenhouses as a high-risk condition for *Botrytis* development if the optimal temperature (15 to 25 ºC) is also present. Thus, these activities can affect both spore dispersal and germination. Hausbeck and Pennypacker (1991) also showed an increase in the concentration of *B. cinerea* conidia in geranium stock plant greenhouses after fungicide applications, and these results were attributed to the "water-and-spore projectiles" theory.

Additionally, for greenhouse A, "changing the greenhouse plastic" and "cleaning of leachate trays" were activities related with a high spore count. For the first case "changing the greenhouse plastic" is an activity that move large amounts of dust in the greenhouse and generate air disturbance that likely increases aerial dispersal of fungal spores (Aylor and Flesch 2001). "Cleaning of leachate trays" involves wet plant debris movement, however, this activity was not related with a high spore count when it was done at greenhouse B.

Conidia development, mycelial growth, and infection development are directly related with climatic conditions such as leaf wetness, relative humidity and temperature (Jarvis 1962b), in the current study no correlation was shown between climatic conditions and spore count. This may be a result of time delay that between conducive weather conditions and sporulation. Also, uneven spore distribution and movement in the

greenhouses, i.e., single location spore traps may be insufficient to accurately describe the spore count in a commercial greenhouse. For example, stationary spore collectors may underestimate vertical profile of spore movement and actual number of spores produced in the greenhouse (Eversmeyer and Kramer 1992), since spore collectors are more reliable for horizontal conidia movement (Savage et al. 2012).

In this study no correlation was shown between *B. cinerea* spore count and disease incidence observed on roses from these greenhouses which agrees with (Blanco, de Santos, and Romero 2006) who observed a similar response between *B. cinerea* conidia concentration and gray mold incidence in strawberry flowers. In that study, no correlation was shown between conidia concentration and gray mold incidence. In some cases, the highest incidence for gray mold was observed when low conidia concentrations were present in the field. Underestimation of real spore production as was described above may had influence these results. Inoculum sources external to the greenhouse may also contribute to alter the infection risk since they may change the inoculum influx and also affect the genetic variability of the inoculum (Kerssies et al. 1997). Additionally, Xu et al., (2000) showed that relying on spore monitoring is not the most accurate method to predict *Botrytis* infection risk, but weather data, such as vapor pressure deficit and temperature, are more reliable indicators, because only a few infective spores are required to start a new disease cycle. This was confirmed by MacKenzie and Peres (2011) and Pavan *et al*. (2009) who developed an accurate model to predict *B. cinerea* infection risk only based on climatic variables such as leaf wetness and temperature for strawberry crops. Additional research is needed to determine if an infection risk model can be developed for cut rose production based on environmental data.

The results of this study have several implications for improving gray mold management practices. For example, fungicide applications prior to the performance of high-risk activities may improve *Botrytis* disease management. Reducing free water in the greenhouse has the potential to reduce spore dispersal, movement and germination. Successful gray mold management should involve sanitation, opportune removal of the primary inoculum sources, avoidance of free water, and proper use of fungicides.

Table 3.1. Descriptions of the production activities performed commercial greenhouses.

| Production activities	Description
Aligning	Shoot reallocation into the canopy.
Blowing (mechanical plant debris removal)	Plant debris are removed from the beds using a mechanical blower.
Changing greenhouse plastic	Plastic of the walls and roofs of the greenhouse are removed and changed by new plastic.
Cleaning of leachate trays	Remoting of substrate and plant debris from the plastic leachate trays underneath crop beds.
Deadheading	Removal of decaying flower heads.
Dumping refreshing solutions	Flower rehydrating solutions used right after harvest are dumped in the greenhouse floor and replaced for a new solution.
Foliar fertilizer application	Fertilizer sprays supplementary to fertigation .
Fungicide applications	Fungicides (site-specific and multisite) are applied as canopy sprays.
Harvest	Recollection of ready flowers (correct age and size).
Harvest zone cleaning	Removal of decaying plant tissue from the highest third of the plants.
Lime application	Dry lime application in the principal aisle.
Manual plant debris removal	Plant debris are collected by hand form the beds.
Mite control	Scouting of mites and insecticide application using high volume spray equipment.
NaOCl application	Application of sodium hypochlorite (NaOCl) solution in the principal aisle and between beds using a venturi injector.
Pinch	Removal of apical flower buds.
Pruning	Trimming plant tissue to renew the canopy.
Pruning of dead stems	Trimming decaying and dead stems.

|	
Removal of *Botryti*s symptomatic tissue in the harvest zone	Removal of any plant tissue with symptoms or signs of gray mold especially in the highest third of the plant.
Removal of dead stems	Recollection of trimmed dead stems.
Removal of downy mildew symptomatic tissue	Scouting and elimination of any plant material symptomatic of downy mildew.
Removal of powdery mildew symptomatic tissue	Scouting and elimination of any plant material symptomatic of powdery mildew.
Supplemental watering	Water application with a hose in addition to the drip irrigation when plants are looking water stressed.
Sweeping between beds	Waste and plant debris removal between beds using a broom.
Sweeping of the central aisle	Waste and plant debris removal from the greenhouse principal aisle (people, equipment and plant material movement) using a broom.
Thermo-fogging	Quaternary ammonium salts application by vaporization into the canopy and substrate.
Weeding	Weed removal from the surface of the crop beds.
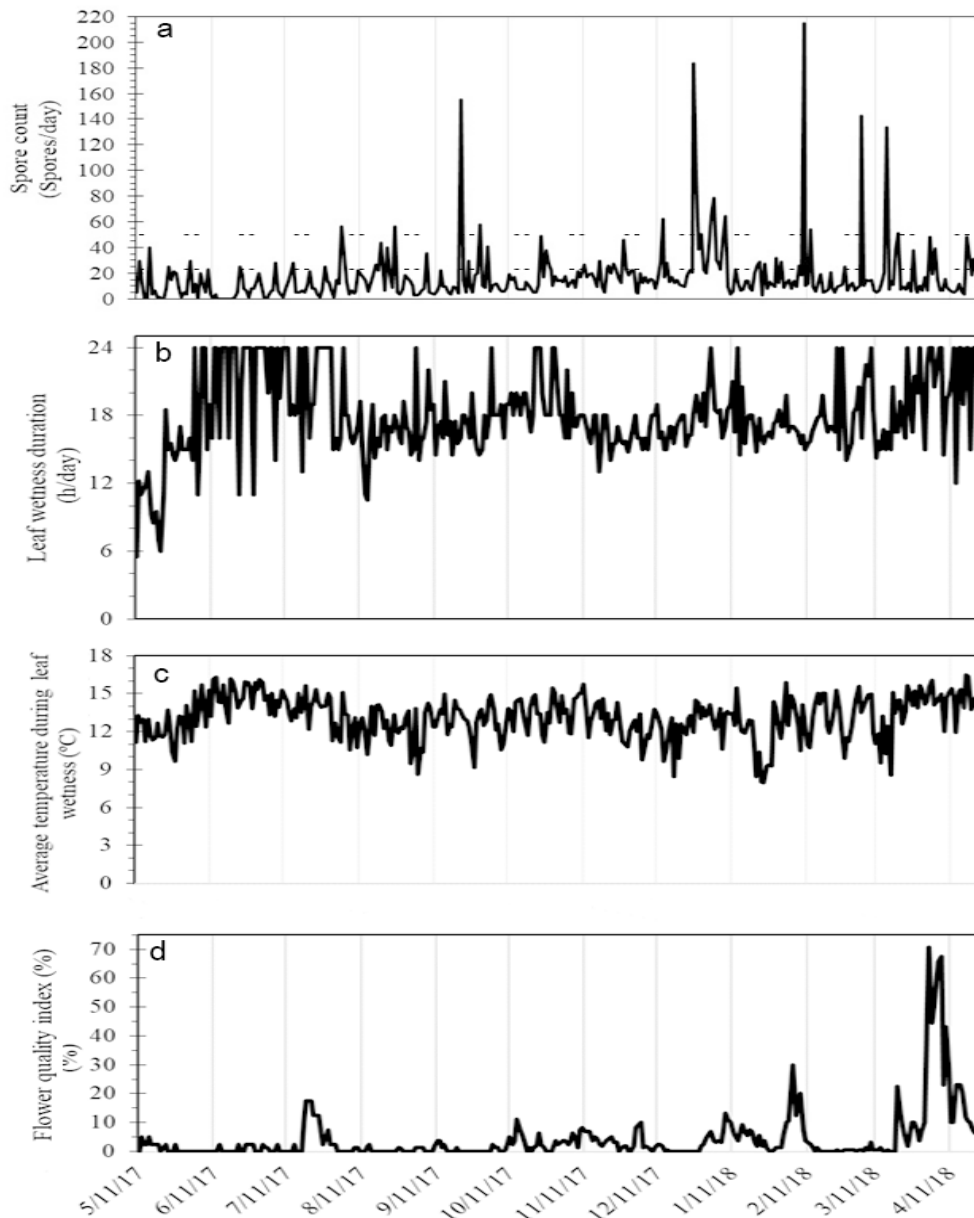
Figure 3.1. a) Spore count recorded from a spore trap placed in a rose canopy over one year. Dashed lines separate the three spore count classifications: low (<22 spores/day), medium (22-50 spores/day), and high (>50 spores/day), **b)** daily leaf wetness duration per day, **c)** average temperature during leaf wetness, and **d)** *Botrytis* incidence as evaluated during daily flower scouting. Data averaged across both evaluated greenhouses.
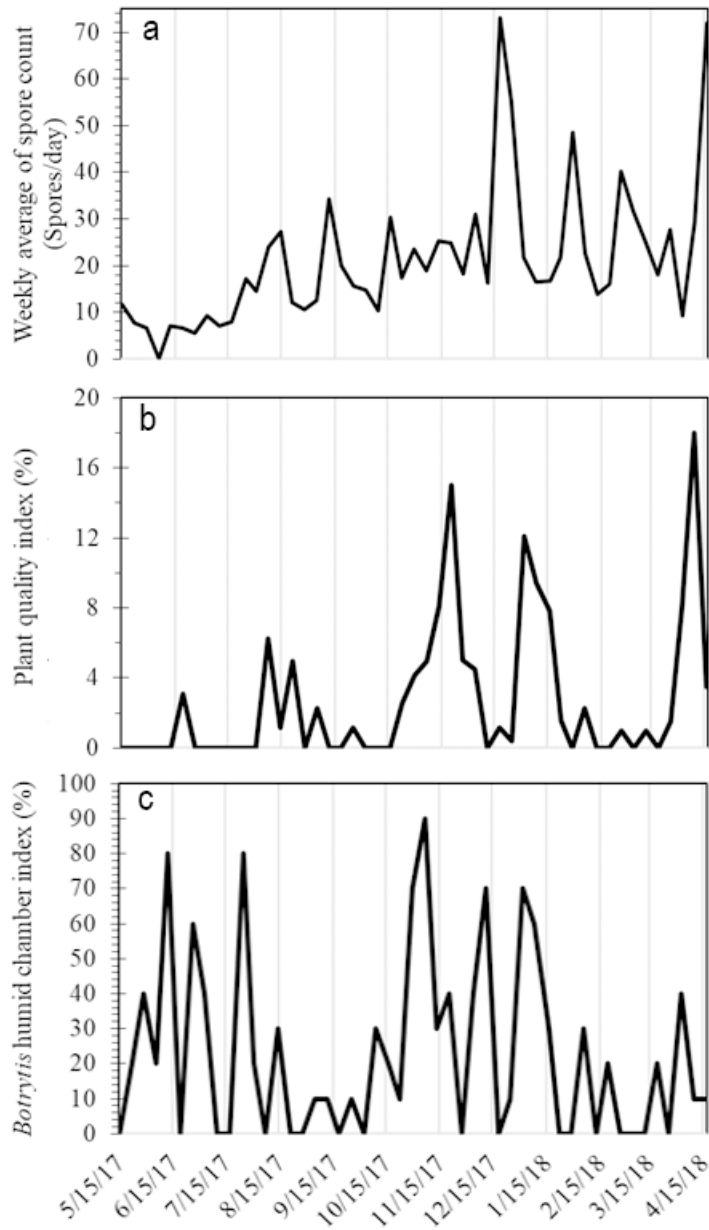
Figure 3.2. Average weekly data of **(a)** spore count recorded in a cut flower rose canopy, **(b)** *Botrytis* presence in the harvest zone, e.g., the top one third of a cut flower rose canopy, and **(c)** *Botrytis* incidence in the humid chamber (containing harvested cut flower roses). Data averaged across greenhouses.
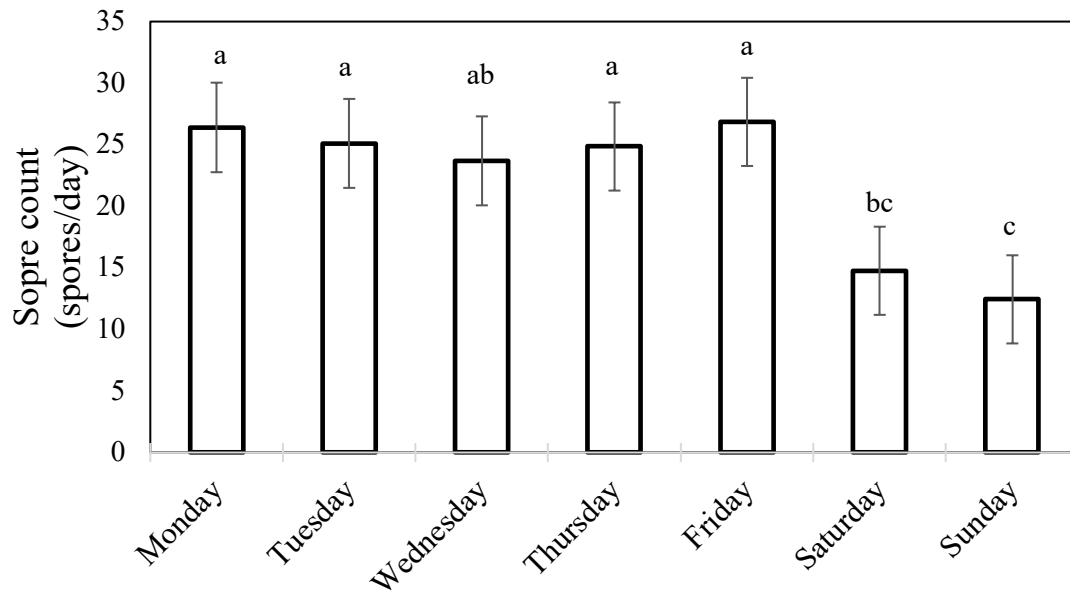
Figure 3.3. Variation in the spore count between days of the week recorded in a cut flower rose canopy. Data were averaged across greenhouses. Different letters indicate statistically different values between days. Least square means where calculated based on an LSD test ($\alpha= 0.05$). Error bars represent ±1 SE.
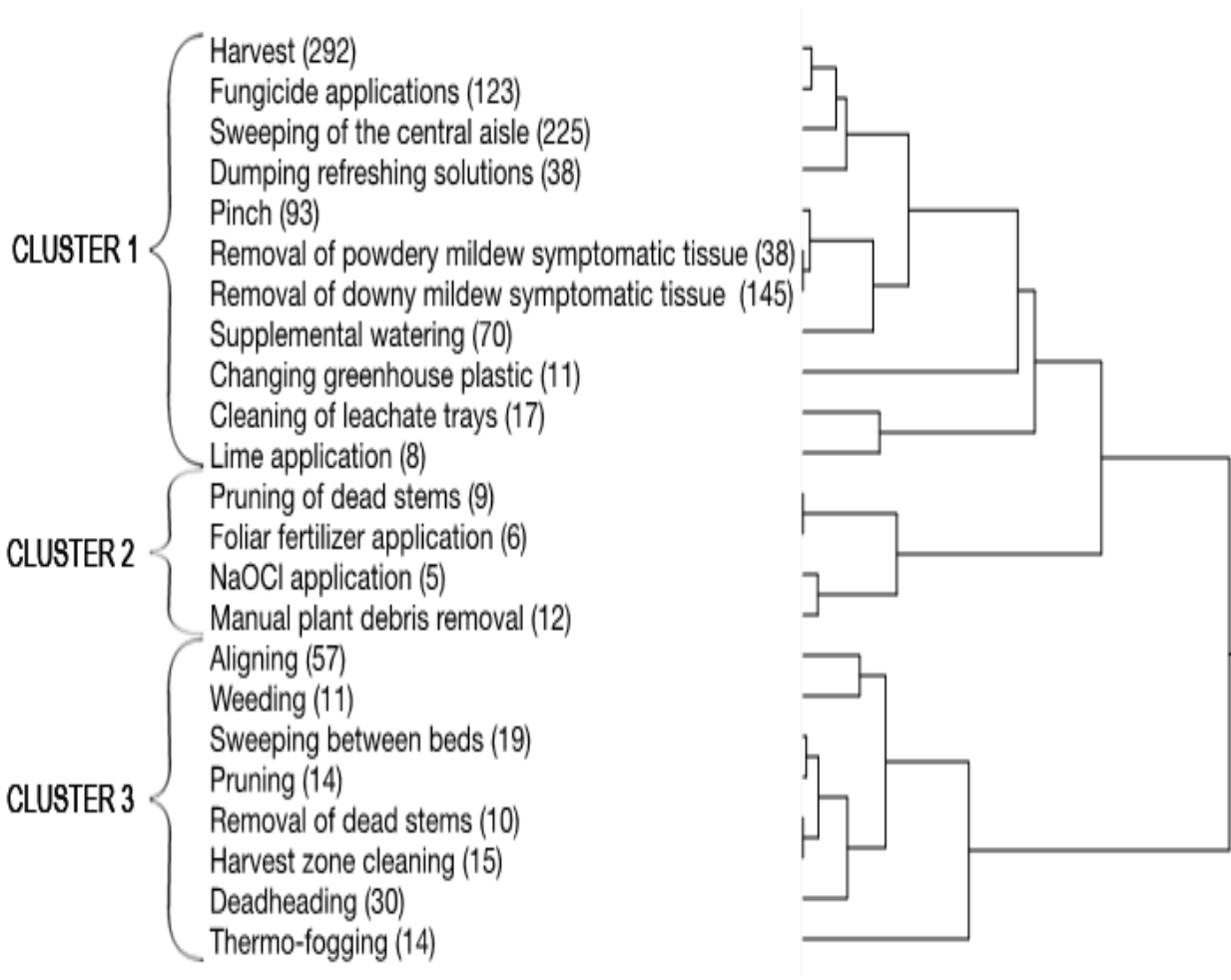
Figure 3.4. Hierarchical cluster distribution of production activities and their relationship to spore count when comparisons were made for the cultural practices performed on the same day and the day before spore count was recorded at greenhouse A. The number of times that each activity was performed is shown inside the parentheses.
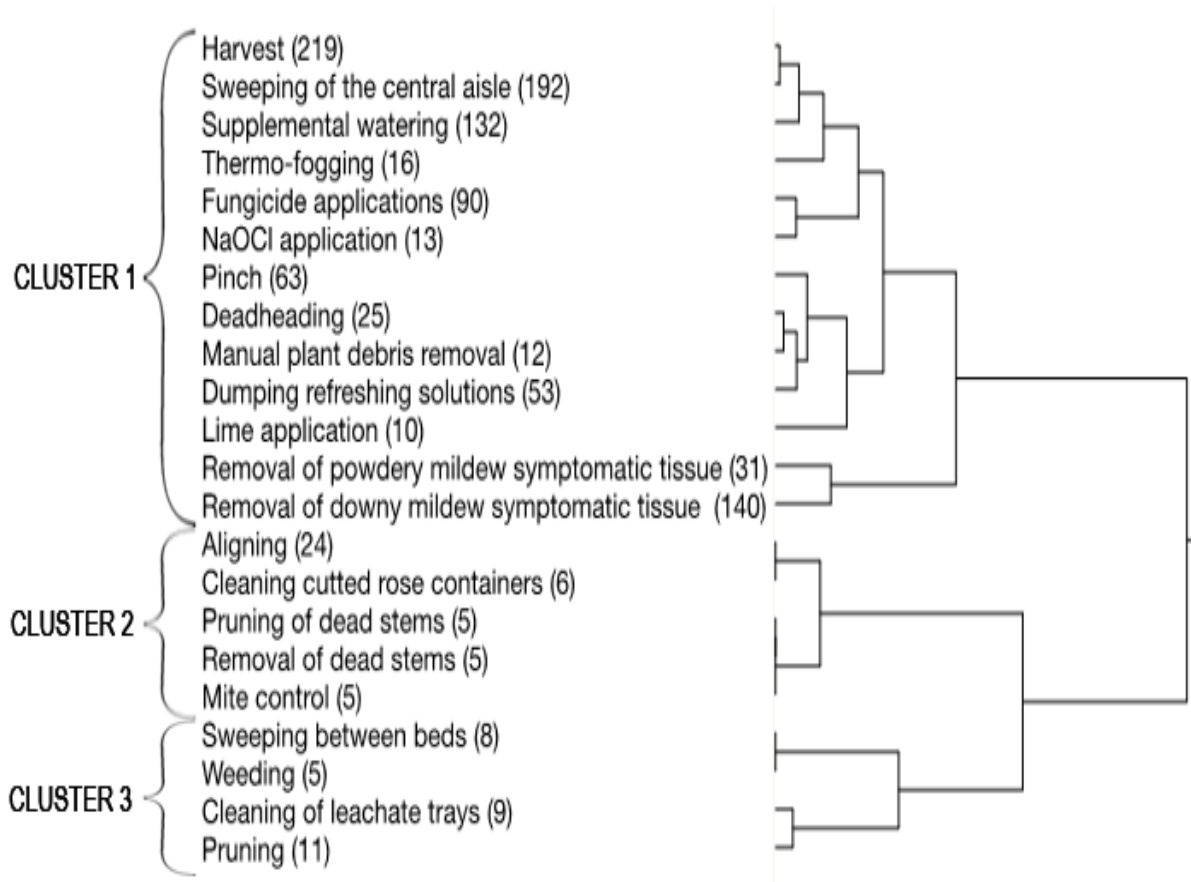
Figure 3.5. Hierarchical cluster distribution of production activities and their relationship to spore count when comparisons were made for the cultural practices performed on the same day and the day before spore count was recorded at greenhouse B. The number of times that each activity was performed is shown inside the parentheses.

Table 3.2. The number of production activities performed in a cut flower rose greenhouse included within clusters, that encompass unique spore count distribution at greenhouse A. Low <22 spores/day; Medium = 22-50 spores/day; High >50 spores/day.

Cluster	Number of cultural practices	Spore count (%)		
		Low	Medium	High
1	11	64.4 b[z]	25.9 b	9.7 a
2	4	62.9 b	37.1 a	0.0 b
3	8	80.2 a	19.3 c	0.4 b

[z] Different letters indicate statistically different values for the clusters within spore count levels (within columns). Least square means where calculated based on an LSD test (α= 0.05).

Table 3.3. The number of production activities performed in a cut flower rose greenhouse included within clusters, that encompass unique spore count distributions at greenhouse B. Low <22 spores/day; Medium = 22-50 spores/day; High >50 spores/day.

Cluster	Number of cultural practices	Spore count (%)		
		Low	**Medium**	**High**
1	13	73.8 c[y]	19.9 a	7.0 a
2	5	81.3 b	18.7 a	0.0 b
3	4	94.9 a	5.1 b	0.0 b

[y] Different letters indicate statistically different values for the clusters within spore count levels (within columns). Least square means where calculated based on an LSD test (α= 0.05).

Literature Cited

Aylor, D. E., and Flesch, T. K. 2001. Estimating spore release rates using a lagrangian stochastic simulation model. J. Appl. Meteorol. 40:1196–1208.

Beever, R. E., and Weeds, P. L. 2007. Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In *Botrytis: Biology, pathology and control*, eds. Yigal Elad, Brian Williamson, Paul Tudzynski, and Nafiz Delen. Dordrecht: Springer Netherlands, p. 29–52.

Blanco, C., de Santos, B., and Romero, F. 2006. Relationship between concentrations of *Botrytis Cinerea* conidia in air, environmental conditions, and the incidence of grey mould in strawberry flowers and fruits. Eur. J. Plant Pathol. 114:415–425.

Bulger, M. A. 1987. Influence of temperature and wetness duration on infection of strawberry flowers by *Botrytis cinerea* and disease incidence of fruit originating from infected flowers. Phytopathology. 77:1225.

Buttner, M. P., and Stetzenbach, L. D. 1993. Monitoring airborne fungal spores in an experimental indoor environment to evaluate sampling methods and the effects of human activity on air sampling. Appl. Environ. Microbiol. 59:219–226.

Sandón, 2005. Efecto de la limpieza de fuentes de inoculo del "moho gris" sobre la presencia de conidias aéreas del agente causal de la enfermedad en un cultivo de rosa variedad Classy (B.S. Thesis). Pontificia Universidad Javeriana. Facultad de Ciencias Básicas. p. 15-66

Chi, M.-H., Park, S.-Y., and Lee, Y.-H. 2009. A Quick and Safe method for fungal DNA Extraction. Plant Pathol. J. p. 25.

Daughtrey, M., Wick, R. L., and Peterson, J. L. 1995. Compendium of Flowering Potted
Plant Diseases. APS Press. p. 20-21.

Elad, Y. 1988. Latent infection of *Botrytis cinerea* in rose flowers and combined
chemical and physiological control of the disease. Crop Prot. 7:361–366.

Eversmeyer, M. G., and Kramer, C. L. 1992. Local dispersal and deposition of fungal
spores from a wheat canopy. Grana. 31:53–59.

Hahn, M., Viaud, M., and Kan, J. A. L. van. 2014. The genome of *Botrytis cinerea*, a
ubiquitous broad host range necrotroph. In *Genomics of Plant-Associated Fungi
and Oomycetes: Dicot Pathogens*, Berlin Heidelber: Springer Verlag, p. 19–44.

Hausbeck, M.K., and Pennypacker, S.P. Influence of grower activity and disease
incidence on concentrations of airborne conidia of *Botrytis cinerea* among
geranium stock plants. Plant Dis. 75:798-803

Holz, G., Coertze, S., and Williamson, B. 2007. The ecology of *Botrytis* on plant
surfaces. In *Botrytis: Biology, Pathology and Control*, eds. Yigal Elad, Brian
Williamson, Paul Tudzynski, and Nafiz Delen. Dordrecht: Springer Netherlands,
p. 9–27.

Jarvis, W. R. 1962. The dispersal of spores of *Botrytis cinerea* fr. in a raspberry
plantation. Trans. Br. Mycol. Soc. 45:549–559.

Jarvis, W. R (b). 1962. Splash dispersal of spores of *Botrytis cinerea* Pers. Nature.
193:599.

Kerssies, A., Bosker-van Zessen, A. I., Wagemakers, C. A. M., and van Kan, J. A. L. 1997. Variation in pathogenicity and DNA polymorphism among *Botrytis cinerea* Isolates Sampled Inside and Outside a Glasshouse. Plant Dis. 81:781–786.

Luo, Y., Michailides, T. J., Morgan, D. P., Krueger, W. H., and Buchner, R. P. 2005. Inoculum dynamics, fruit infection, and development of brown rot in prune orchards in california. Phytopathology. 95:1132–1136.

MacKenzie, S. J., and Peres, N. A. 2011. Use of leaf wetness and temperature to time fungicide applications to control *Botrytis* fruit rot of strawberry in florida. Plant Dis. 96:529–536.

Pavan, W., Fraisse, C. W., Cordova, L. G., and Peres, N. A. The strawberry advisory system: a web-based decision support tool for timing fungicide applications in strawberry. Department of Agricultural and Biological Engineering, UF/IFAS Extension, University of Florida.1-4

Savage, D., Barbetti, M. J., MacLeod, W. J., Salam, M. U., and Renton, M. 2012. Mobile traps are better than stationary traps for surveillance of airborne fungal spores. Crop Prot. 36:23–30

Staats, M., van Baarlen, P., Kan, V., and L, J. A. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol. Biol. Evol. 22:333–346.

Vrind, T. A. 2005. The *Botrytis* problem in figures. Acta Hortic. 29:99–102.

West, J. S., Atkins, S. D., and Fitt, B. D. L. 2009. Detection of airborne plant pathogens; Halting epidemics before they start. In: Outlooks of Pest Management. 20:11-14.

Williamson, B., Tudzynski, B., Tudzynski, P., and van Kan, J.A. 2007. *Botrytis cinerea*: The cause of grey mould disease. Mol. Plant Pathol. 8:561–80.