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Fungicide sensitivity and spatial and temporal dynamics of *Botrytis cinerea* and *Colletotrichum* spp. in conventional and organic strawberry fields

Juliana Silveira Baggio

Thesis presented to obtain the degree of Doctor of Science. Area: Plant Pathology

Piracicaba
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Juliana Silveira Baggio
Agronomist

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Advisor:
Prof. Dr. **LILIAN AMORIM**
Co-advisor:
Prof. Dr. **NATALIA APARECIDA PERES LAURETTI**

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To God,

My parents, Rosangela e Natale

My sister, Mariana

My family

I DEDICATE

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"True wisdom comes to each of us
when we realize how little
we understand about life,
ourselves, and the world around us."
Socrates

CONTENTS

RESUMO

Sensisbilidade a fungicidas e dinâmicas espacial e temporal de *Botrytis cinerea* e *Colletotrichum* spp. em campos de morangueiro convencionais e orgânicos

Mofo cinzento e antracnose do fruto, causados por *Botrytis cinerea* e *Colletotrichum* spp., respectivamente, são as mais importantes doenças em morangueiro. Esse estudo objetivou comparar sistemas de produção orgânico e convencional em morangueiro através da avaliação da sensibilidade de isolados de *B. cinerea* e *Colletotrichum* spp. aos principais fungicidas sítio-específicos utilizados no Brasil, e da caracterização das dinâmicas espacial e temporal das doenças com o uso de ferramentas epidemiológicas. Isolados foram coletados em campos convencionais e orgânicos em quatro diferentes Estados brasileiros nos anos de 2013 a 2015. Resistência à azoxistrobina, iprodiona e tiofanato-metílico foi encontrada em isolados de *B. cinerea* com valores de CE_{50} maiores que 71,9, 688 and 1,2 µg/ml, respectivamente. Resistência aos fungicidas acima mencionados foi observada em 87,5, 76,6 and 92,2 % dos isolados provenientes de campos convencionais e em 31,4, 22,9 and 51,4 % dos isolados oriundos de campos orgânicos, respectivamente. Além do mais, frequências de populações com resistência múltipla aos três fungicidas foram 75 e 8,6 %; e com nenhuma resistência a qualquer dos produtos, 6,25 e 34,3 % para isolados coletados em campos convencionais e orgânicos, respectivamente. Análise molecular dos genes do citocromo *b*, beta-tubulina e *bos1* revelaram, respectivamente, a presença das mutações G143A, E198A e I365N/S, Q369P ou N373S em populações resistentes. Fungicidas aplicados preventivamente em morangos inoculados com *B. cinerea* falharam em controlar isolados resistentes. Isolados de *C. acutatum* resistentes à azoxistrobina e ao difenoconazole não foram observados. Valores médios de CE_{50} para isolados coletados em campos orgânicos foram 0,44 e 0,95 µg/ml; e para isolados de áreas convencionais, 0,629 e 0,107 µg/ml para azoxistrobina e difenoconazole, respectivamente. Populações de *C. acutatum* demonstraram insensibilidade ao tiofanato-metílico e valores de CE_{50} não puderam ser determinados. Nenhum dos isolados analisados continha as mutações comumente associadas à resistência a fungicidas. As dinâmicas temporal e espacial das doenclas causadas por esses patógenos foram caracterizadas na safra de 2015 em campos de morangueiro convencional e orgânico. Modelos de progresso temporal foram ajustados à incidência cumulativa de frutos ou plantas doentes no tempo com regressões não-lineares. O padrão espacial das doenças foi analisado de acordo com o índice de dispersão (*D*), índice de agregação (V/M) e lei de Taylor. A relação incidência-densidade de frutos doentes foi analisada com o ajuste de um modelo linear. Frutos com sintomas de *Colletotrichum* spp. não foram observados na área orgânica e foram encontrados em poucos dias de avaliação na área convencional. Incidência de mofo cinzento em frutos e plantas foi bem descrita pelo modelo logístico. A área orgânica apresentou incidência de doença e taxa de progresso diária maiores que da área convencional. A agregação de frutos doentes em plantas foi observada para ambos os locais. O mofo cinzento apresentou padrões epidemiológicos semelhantes para as áreas convencional e orgância, porém com maior intensidade de doença no campo orgânico, provavevelmente em decorrência do manejo adotado para controle de doenças. Esse estudo reforça a importância da

implementação de programas de manejo integrado de doenças em viveiros de mudas e campos de produção de morangueiro.

Palavras-chave: *Fragaria* x *ananassa*, Mofo cinzento; Resistência a fungicidas; Epidemiologia; Sistemas de produção

ABSTRACT

Fungicide sensitivity and spatial and temporal dynamics of *Botrytis cinerea* and *Colletotrichum* spp. in conventional and organic strawberry fields

Botrytis and Anthracnose fruit rots, caused by *Botrytis cinerea* and *Colletotrichum* spp., respectively, are major strawberry diseases. This study aimed to compare organic and conventional strawberry production systems by evaluating the sensitivity of *B. cinerea* and *Colletotrichum* spp. isolates to the main single-site fungicides used in Brazil and by characterizing the spatial and temporal dynamics of the diseases caused by these pathogens using epidemiological tools. Isolates were collected from conventional and organic fields in four different Brazilian states from 2013 to 2015. Resistance to azoxystrobin, iprodione and thiophanate-methyl was found in *B. cinerea* isolates with EC_{50} values higher than 71.9, 688 and 1.2 µg/ml, respectively. Resistance to the aforementioned fungicides was observed in 87.5, 76.6 and 92.2 % of isolates from conventional fields, and 31.4, 22.9 and 51.4 % of isolates from organic fields, respectively. Moreover, frequencies of populations with multiple fungicide resistance to the three active ingredients were 75 and 8.6 %, with no resistance to any of the fungicides were 6.25 and 34.3 % for isolates collected from conventional and organic areas, respectively. Molecular analyses of the cytochrome *b*, beta-tubulin and *bos1* genes revealed, respectively, the presence of G143A, E198A and I365N/S, Q369P or N373S mutations in resistant populations of *B. cinerea*. Fungicides sprayed preventively on strawberry fruit inoculated with *B. cinerea* failed to control resistant isolates. Isolates of *C. acutatum* resistant to azoxystrobin and difenoconazole were not observed. Mean EC_{50} values for isolates collected from organic fields were 0.44 and 0.95 µg/ml, and from conventional areas were 0.629 and 0.107 µg/ml for azoxystrobin and difenoconazole, respectively. Populations of *C. acutatum* showed insensitivity to thiophanate-methyl rather than resistance and EC_{50} values could not be determined. None of the isolates contained the most common mutations associated with fungicide resistance. The temporal and spatial dynamics of Botrytis and Anthracnose fruit rots were characterized in the 2015 strawberry season in conventional and organic farms. Temporal progress models were fitted to the cumulative incidence of diseased strawberry fruit or plants over time with non-linear regressions. The spatial pattern of the diseases was analyzed according to the index of dispersion (*D*), the index of aggregation (V/M), and the Taylor's power law. The incidence-diseased fruit density relationship was analyzed by fitting a linear model. Fruit with symptoms of *Colletotrichum* spp. were not observed in the organic area and were found in few assessment dates in the conventional area. Incidence of Botrytis fruit rot in symptomatic fruit and plants were generally well described by the logistic model. The organic area presented disease incidence and daily progress rate higher than the conventional area. The aggregation of diseased fruit on strawberry plants were observed for both areas. Botrytis fruit rot presented similar epidemiological patterns for both conventional and organic areas, however, disease intensity was greater for the organic area; this was likely due to differences in disease control management. This study reinforces the importance for the implementation of integrated management programs in strawberry nurseries and production fields.

Keywords: *Fragaria* x *ananassa;* Gray mold; Fungicide resistance; Epidemiology; Production systems

1 INTRODUCTION

1.1 Strawberry production

Strawberry (*Fragaria* x *ananassa* Duch) production has grown and expanded worldwide. The five largest producers are China, the United States of America (USA), Mexico, Turkey and Spain, and they produced 2,997,504, 1,360,869, 379,464, 372,498 and 312,500 metric tons, respectively, in 2013 (FAO, 2016). South America, especially Brazil, Argentina and Chile, is believed to be responsible for the production of 318,686 metric tonnes of strawberries on 11,884 hectares (ANTUNES; PERES, 2013). In Brazil, the estimated annual area is approximately 3,500 hectares with 105,000 metric tons of harvested fruit, which supplies mainly the fresh market (98 % of the production) (ANTUNES; PERES, 2013). In 2015, the estimated yield was about 30 tons per hectare and 8,500 tons of fruit were sold at CEAGESP, the São Paulo wholesale market, in 2014 (FNP, 2016).

In Brazil, strawberries are grown in many temperate and subtropical areas on small farms using family labor with high yield: more than 60 % of the fruit is harvested by growers with up to 10 hectares (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2009). Minas Gerais, Rio Grande do Sul and São Paulo States are responsible for more than 75 % of the Brazilian strawberry production: Minas Gerais is responsible for 55 % of the national production (IBGE, 2009; RADMANN et al., 2006). In Minas Gerais, strawberries are grown in the south region of the State; in Rio Grande do Sul, the growers are located near the cities of Caxias do Sul and Farropilha; and in São Paulo, production takes place in the Atibaia, Campinas and Jundiaí regions (EMBRAPA, 2005).

‘Albion’, ‘Aleluia’, ‘Aromas’, ‘Camarosa’, ‘Camino Real’, ‘Campinas’, ‘Dover’, ‘Festival’, ‘Portela’ and ‘Oso Grande’ are the main cultivars used in Brazil. Some of them were developed by breeding programs in the USA, and others by Brazilian institutions like the EMBRAPA Temperate Climate and the Agronomic Institute of Campinas (IAC) (ANTUNES; PERES, 2013; OLIVEIRA; SCIVITTARO, 2006). Most of the plants used by the growers come from transplants produced by local nurseries or individual growers and only 15 % of them are imported from Argentina and Chile (ANTUNES; PERES, 2013). Through the introduction of new cultivars and depending on the region of Brazil where strawberries are grown, the production is possible 12

months of the year, with harvest peaks from June to November (ANTUNES; PERES, 2013).

Strawberries can be grown using conventional or organic production practices. Conventional agriculture has been based on dependence of non-renewable resources, intensive use of synthetic chemical inputs, machines and mechanical equipment, inorganic fertilizers and pesticides (TRIVELLATO; FREITAS, 2003). Organic agriculture seeks farming through practices of nutrients and organic material recycling, replacing crop residuals to the soil, using crop rotation and proper practices of soil preparation. Moreover, genetic modified organisms (GMOs) and pesticides and other artificial inputs are not allowed (TRIVELLATO; FREITAS, 2003).

Strawberry conventional production demands high amount of chemical products to control insects, mites and diseases. It is estimated that 30 to 40 fungicide applications are made per season all over Brazil (ZAMBOLIM; COSTA, 2006). In Brazilian conventional systems, it is estimated that costs with agricultural inputs and labor represent 44 and 29 %, respectively, of total investment; on the other hand, in organic farms, they represent 36 and 50 %, respectively (DONADELLI; KANO; FERNANDES JUNIOR, 2012). In organic production, yield represents about 60 to 70 % the yield of conventional production, however market prices of organic strawberries are at least 40 % higher than conventional fruit prices (DONADELLI; KANO; FERNANDES JUNIOR, 2012; SOUZA, 2006;).

Recent reports from the Brazilian National Health Surveillance Agency (ANVISA) demonstrated that strawberry fruit rank first in among foods with high amount of samples contaminated by agrochemical products. According to ANVISA, pesticide residue monitoring has shown that 59 % of analyzed strawberry samples produced unsatisfying results due to agrochemical levels above those permitted and the use of pesticides not registered for strawberries (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA - ANVISA, 2013). The presence of residues on food may put human health at risk and have a negative environmental impact. Moreover, the indiscriminate use of pesticides can lead to pathogen resistance in the field.

Societal concern about sustainable food production can explain the increasing consumer demand for organic products, leading to the growth of organic market and, as a result, the increase in farms that adopt organic practices (DE MOURA; NOGUEIRA; GOUVÊA, 2012). An exploratory survey conducted in Brazil has shown that absence of chemical products used in the farming process was considered the

most important attribute of organic production of food (DE MOURA; NOGUEIRA; GOUVÊA, 2012). Brazilian domestic market of organic products has recorded an expansion of 40 % in 2010 and international sales increased by 30 %, especially with soybean products, sugar, coffee, cocoa and fruits (IBD CERTIFICAÇÕES, 2011). As markets are increasing their organic offerings, organic options have become more available than before. Furthermore, quality and safety studies of strawberries showed no physical-chemical differences between fruit from conventional and organic production systems, although fruit from conventional areas presented residues of pesticides not registered for the crop (LIMA et al., 2011).

1.2 Strawberry diseases

Diseases are the main problem of strawberries and can cause losses during all aspects of strawberry production, from nursery to postharvest. They decrease yield and increase production costs such as the purchase of fungicides for disease control. The main diseases are caused by bacteria and fungi and can affect the whole plant: roots, crowns, petioles, leaves, flowers and fruits. Botrytis fruit rot or Gray Mold, caused by *Botrytis cinerea*, and Anthracnose fruit rot, caused mainly by *Colletotrichum acutatum* and *C. gloeosporioides*, are the most important diseases of strawberries and can cause severe pre- and postharvest losses due to infections of flowers and fruits (MAAS, 1998; TANAKA; BETTI; KIMATI, 2005). These pathogens are distributed worldwide and demand intensive chemical control during flowering and fruiting phases that occur simultaneously in strawberry production (COSTA; VENTURA; LOPES, 2011; DOMINGUES et al., 2001; HOWARD et al., 1992; JARVIS, 1962; MERTELY; MACKENZIE; LEGARD, 2002; MERTELY; PERES; CHANDLER, 2009).

1.2.1 Botrytis Fruit Rot

Botrytis fruit rot is a widespread disease and causes fruit to rot wherever strawberries are grown. Postharvest damage is considered the largest inflicted by *Botrytis cinerea* since the fruit tissue is more susceptible to infection, and can be considered a great limiting factor in marketing of strawberries (DROBY; LICHTER, 2007). However, disease symptoms can be found on immature and mature

strawberry fruit, and the pathogen also attacks leaves, petioles and flowers (MAAS, 1998). Light-brown lesions appear on any portion of the fruit at any developmental stage and are frequently associated with infected stamens or petals that stick to the fruit or fruits touching other diseased fruits (BRISTOW; MCNICOL; WILLIAMSON, 1986; SUTTON, 1990). Lesions can be covered with a grayish structure of the pathogen, usually the powdery spores produced on conidiophores. In a more advanced stage, whole fruits rot and become dry and tough with little or no leakage of the fruit content (DROBY; LITCHER, 2007; MAAS, 1998).

Botrytis cinerea Pers., the causal agent of Botrytis fruit rot, is the anamorph stage of the fungus *Botryotinia fuckeliana* (de Bary) Whetzel and belongs to the phylum Ascomycota, order Helotiales and family Sclerotiniaceae. The fungus is an airborne plant pathogen, has a necrotrophic life cycle and affect several hosts, including over 200 cultivated crops (WILLIAMSON et al., 2007). It is known especially for causing postharvest rots and affecting different plant organs such as flowers, fruits, leaves, shoots and storage organs of several vegetables (beans, broccoli, cabbage, carrots, sweet potatoes, tomatoes), ornamentals (roses, sunflowers) and small fruit crops (blackberries, grapes, raspberries, strawberries) (DROBY; LICHTER, 2007).

B. cinerea can survive as mycelia and/or spores and, for longer periods it overwinters as sclerotia and mycelium in crop debris, such as dead leaves, petioles and stolons, straw mulches and weeds, serving as a primary source of inoculum within a crop (BRAUN; SUTTON, 1987). These sources of primary inoculum are responsible for spore production which is regulated by temperature and humidity fluctuation, following a diurnal cycle of initiation, production and dissemination. These spores can then be dispersed by air currents and water from rain or overhead-irrigation from neighboring crops and mainly from primary sources within the crop (BRISTOW; MCNICOL; WILLIAMSON, 1986; DROBY; LITCHER, 2007; WILLIAMSON et al., 2007).

Infection via flowers in the early stages and disease development after harvest when fruit reaches full maturity is very important in strawberries and can be considered as the main cause of fruit decay (DROBY; LICHTER, 2007). On a susceptible host, the pathogen can penetrate petals, sepals, stamens or receptacles (BRISTOW; MCNICOL; WILLIAMSON, 1986; POWELSON, 1960). Free-water over the flower and fruit surface leads to spore germination in few hours and if located

between the fruit and the petal it can persist for longer periods leading to fruit infection as soon as the buds open (JARVIS, 1962). Spores germinate and germ tubes grow and follow the pathway used by pollen tubes to the ovules, where the pathogen can survive for up to four weeks as a saprophyte until fruit maturity (MCNICOL; WILLIAMSON; DOLAN, 1985). Following penetration, the pathogen establishes in flower parts and can invade the region near the receptacle of developing fruit, causing them to rot or remaining inactive until fruit ripens or has been harvested (JARVIS, 1962). Direct infection of the fruit through surface injuries such as growth cracks, insect wounds and lesions can also occur (DROBY; LITCHER, 2007).

Once in the plant, *B. cinerea* sporulates on diseased parts of strawberry plants, which become a source of secondary inoculum. Under favorable conditions, the pathogen will infect flowers and fruit during the whole season where there is multiple flowering, fruiting and harvest cycles, leading to successive secondary cycles of infection and sporulation (BRAUN; SUTTON, 1988; DROBY; LICHTER, 2007; WILLIAMSON et al., 2007). Low to moderate temperatures (15 to 25 °C), high humidity and prolonged surface wetness are favorable for Botrytis fruit rot development (BULGER; ELLIS; MADDEN, 1987; WILCOX; SEEM, 1994). *B. cinerea* infections were already registered over temperatures ranging from 12 to 30 °C and after only four hours of wetness; however, maximum disease incidence in grapes (90 %) was reached after 24 hours of wetness at 12 to 20 °C (BROOME et al., 1995). In Brazil, temperatures around 20 °C are favorable to disease occurrence (TANAKA; BETTI; KIMATI, 2005). Frequent rains are also favorable to disease development and induce maximum incidence, causing severe yield losses to strawberry growers. Incidence increases especially after periods of light rains before harvest (COSTA; VENTURA; LOPES, 2011).

Botrytis fruit rot in strawberries can be controlled by using, resistant cultivars, biological control, cultural practices and, mainly, chemical control. Although there are no fully resistant cultivars, there are significant differences in susceptibility among strawberry cultivars, such as ‘Camarosa’ considered less susceptible to Botrytis fruit rot (LEGARD et al., 2000). Recent works on biological control demonstrated that Botrytis fruit rot development after strawberry harvest can be managed by a pre-harvest application of the yeast *Metschnikowia fructicola* (KARABULUT et al., 2004). The bacteria *Bacillus pumilus* can also be used in strawberry fields and is registered

by the Ministry of Agriculture, Livestock and Food Supply (MAPA) in Brazil (AGROFIT, 2016). Nonetheless, the combination of chemical and cultural control is very important for an effective management program. For instance, the acquisition of healthy strawberry transplants is the first step for the management of the disease (OLIVEIRA, 2015). Furthermore, a series of cultural practices may be adopted for disease control, such as the removal of plant debris within the crop, diseased and unmarketable fruit and senescent foliage within the plant canopy is critical for disease management, (MERTELY et al., 2000). Although marketable yields may be reduced, the use of wider spacing leads to the reduction of disease incidence (LEGARD et al., 2000). The use of drip-irrigation instead of overhead-irrigation prevents pathogen dispersion by water-splash and reduce infection risk by decreasing free moisture on strawberries (DROBY; LICHTER, 2007). Growing strawberries under plastic tunnels also reduces leaf wetness duration and disease incidence in the field and postharvest (COSTA; VENTURA; LOPES, 2011; XIAO et al., 2001). After all, chemical control is traditionally the standard and most used management method for Botrytis fruit rot in strawberries. The disease can be controlled by applying fungicides beginning before or at bloom and continuing until harvest (MERTELY; MACKENZIE; LEGARD, 2002; XIAO et al., 2001). Single- and multi-site fungicides are widely sprayed and their alternate usage as well as the use of fungicides belonging to groups with different modes of actions must be adopted by growers, since the emergence of *B. cinerea* resistance to systemic fungicides have been widely reported by several authors (AMIRI; HEATH; PERES, 2013; FERNÁNDEZ-ORTUÑO et al., 2015; FORCELINI et al., 2016; GRABKE et al., 2014).

1.2.2 Anthracnose Fruit Rot

Colletotrichum spp. are broad-range pathogens, have wide geographical distribution and cause diseases, mainly anthracnoses, in numerous important annual, fruit and ornamental plants all over the world (AGRIOS, 2005; SMITH, 2008). Many hosts can be affected by a single species and many species can cause disease in a single host (FREEMAN, 2008).

Three species complex of *Colletotrichum* cause several types of symptoms and make anthracnose diseases the most important in strawberries: *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. &

Sacc (DOMINGUES et al., 2001; HOWARD et al., 1992). *C. acutatum* is the anamorphic stage of the fungus *Glomerella acutata* J.C. Guerber & J.C. Correll, and *C. fragariae* and *C. gloeosporioides* are the anamorphic stages of *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk. They belong to the phylum Ascomycota, order Glomeralles and family Glomerellaceae. *Colletotrichum* spp. are known by having four types of host-pathogen interactions or infection strategies and can possess necrotrophic, hemiobiotrophic, biotrophic and epiphytic phases (PERES et al., 2005). The pathogens can infect several parts of strawberry plants, such as the leaves, petioles, crowns, roots, flowers, and green and ripe fruits during nursery, growing and postharvest stages (MERTELY; PERES; CHANDLER, 2009; UREÑA-PADILLA et al., 2002). *Colletotrichum* spp. are responsible for causing anthracnose crown and fruit rots and flower blight. Anthracnose crown rot, known as “chocolate” or “red heart” among Brazilian strawberry growers, is mainly caused by *C. fragariae* and *C. gloeosporioides* (TANAKA; BETTI; KIMATI, 2005). The pathogens are responsible for producing a light-brown to reddish necrosis of crown tissue causing plants to wilt and collapse. The disease may also affect stolons and petioles, producing dark-brown sunken lesions (MACKENZIE; MERTELY; PERES, 2009). Anthracnose crown rot development is most severe during warm (temperatures about 30 to 35 °C), and humid conditions and is mainly observed in nurseries and after the plants are transplanted in the production fields (SMITH; BLACK, 1987). Flower blight, mainly caused by *C. acutatum*, is widely spread through the Brazilian growing regions. The pathogen attacks flowers, petioles and young green fruit, producing dark-brown rot lesions with pink or orange masses of spores whenever humidity is high (COSTA; VENTURA; LOPES, 2011; TANAKA; BETTI; KIMATI, 2005). Anthracnose fruit rot, mainly incited by *C. acutatum*, may also be caused by *C. gloeosporioides* and *C. fragariae.* Disease symptoms occur in green and ripe fruit and lesions may expand and entirely cover the fruit surface producing a mass of spores, especially on highly susceptible cultivars (MERTELY; PERES; CHANDLER, 2009; SEIJO et al., 2008).

Anthracnose fruit rot causes yield and economic losses in several strawberry growing regions in Asia and South and North America (DOMINGUES et al., 2001; FREEMAN, 2008; SMITH, 2008). Although fruit is the most important part attacked by *C. acutatum*, it may also produce symptoms on roots, petioles, leaves and flowers (PERES et al., 2005). One or more light-brown, water-soaked lesions develop into

circular dark brown sunken lesions on green and mature fruit. Affected tissues may become firm and dry, and fruit can become mummified. On these lesions, spores may be produced in a salmon-pink or orange mucilage that erupt from subepidermal acervuli. (MAAS, 1998; MERTELY; PERES; CHANDLER, 2009; TANAKA; BETTI; KIMATI, 2005). Moreover, black, longitudinal and sunken lesions found in plant stolons may be associated with anthracnose fruit rot and, in more severe epidemics, plants can decline and die (MERTELY; PERES, 2005).

Colletotrichum spp. can be introduced in strawberry-growing areas where the pathogen is absent by infected transplants from nurseries. Some transplants may show symptoms, but most of the time the pathogen is found occurring on asymptomatic leaves and petioles, as appressoria or quiescent infections (HOWARD et al., 1992; MERTELY; LEGARD, 2004; PERES et al., 2005). *Colletotrichum* spp. possess different survival strategies, such as on or in the strawberry plant itself, in crop debris, or, alternatively, other crops and weeds, without showing any symptoms, as epiphytic stages or quiescent infections (PERES et al., 2005). *C. acutatum*, for example, may survive up to eight weeks on strawberry leaves in greenhouses (LEANDRO et al., 2003a) and its conidia survive up to 12 months in soil and crop debris in dry conditions (NORMAN; STRANDBERG, 1997). The fungus, inoculated on pepper, eggplant, tomato, bean and strawberry, was recovered from all the plants after a 3-month period, but only caused disease in strawberries (FREEMAN; HOROWITZ; SHARON, 2001). Survival on weeds was already reported for *C. acutatum* causing anthracnose fruit rot on strawberries and postbloom fruit drop on citrus (FRARE et al., 2016; FREEMAN; HOROWITZ; SHARON, 2001). Cross pathogenicity can also occur and isolates recovered from diseased strawberries and anemones showed genetic identity (FREEMAN; SHABI; KATAN, 2000). Moreover, *C. acutatum* isolates from blueberries were pathogenic to strawberry fruit, suggesting the pathogen may alternatively survive on the former crop in the absence of strawberries in the field (MACKENZIE et al., 2009). Furthermore, appressoria and secondary conidia produced by *C. acutatum* in asymptomatic parts of plants without infection or host penetration may become source of inoculum for strawberry fields (LEANDRO et al., 2001) and, as a consequence, contribute to inoculum availability during the growing season (LEANDRO et al., 2003a). The increase of inoculum levels during flowering period may be due to the production of secondary conidia on

strawberry leaves, which may be enhanced by the exposure to flowers extracts (LEANDRO et al., 2003b).

Colletotrichum spp. dissemination occurs mainly at short distances by water splash from rainfall and overhead-irrigation, but also by wind and insects (FREEMAN; HOROWITZ; SHARON, 2001; HOWARD et al., 1992; MADDEN; WILSON; ELLIS, 1993). The pathogens can be dispersed by contaminated soil, field equipment and farming and harvest operations (FREEMAN, 2008; PERES et al., 2005).

Colletotrichum spp. infection is highly dependent on wetness duration and temperature especially during bloom and fructification (COSTA; VENTURA; LOPES, 2011; TANAKA; BETTI; KIMATI, 2005). Temperatures between 25 and 30 °C are optimum for pathogen infection on immature and mature fruit and 80 % of anthracnose incidence can be reached after 13 hours of wetness (WILSON; MADDEN; ELLIS, 1990). Spores geminate by producing one or more germ tubes that terminate in appressorium formation. Appressoria may remain quiescent but infective under unfavorable conditions for infection. Under favorable conditions, direct penetration into the host after appressorium formation can occur (MAAS, 1998; SMITH, 2008). Plant infection, spore production and germination and appressorium formation are favored by temperatures from 17.6 to 27.7 °C and increasing wetness duration (LEANDRO et al., 2003a). However, the sequence and nature of *Colletotrichum* spp. infection from initial penetration to colonization vary among hosts and are not well understood (PERES et al., 2005). Very short rains followed by favorable conditions for *Colletotrichum* spp. infection can result in high levels of anthracnose incidence (MADDEN et al., 1992). The latent period of *Colletotrichum* spp., the time from infection to production of the first reproductive structures (sporulation), is important to determine the speed at which the disease may spread within a field and may vary from 2 to 3 days at 25 °C and from 6 to 17 days at 5 °C (KING et al., 1997). Therefore, temperature and wetness period is crucial for pathogen infection and establishment and disease progress.

As for Botrytis fruit rot, anthracnose diseases of strawberry can be managed by using a combination of genetic, cultural and chemical control. Although there are no cultivars fully resistant to anthracnose, ‘Strawberry Festival’, ‘Florida Radiance’, ‘Florida Eliana’ and ‘Sweet Charlie’ are considered moderately resistant, whereas ‘Albion’, ‘Camarosa’, ‘Camino Real’ and ‘Treasure’ are highly susceptible (SEIJO et

al., 2008). Unfortunately, in Brazil, the cultivars used by strawberry growers are highly susceptible to anthracnose, due to the absence of resistant cultivars in the Brazilian market (COSTA; VENTURA; LOPES, 2011).

As cultural management, the first prerequisite for disease control is the use of healthy propagation material. For instance, in Israel, transplants are tested twice a year to certify disease-free strawberry material (FREEMAN, 2008). Micropropagation of plants is also an alternative to rid strawberry of *Colletotrichum* spp. infection, however an active sanitation program throughout the nurseries must be applied by constant screenings for fungal, virus and bacterial pathogens and inspections for disease symptoms (DALE; HUGHES; DONNELLY, 2008). Treating transplants using heat also proved to be effective against anthracnose and plants can become free of disease (FREEMAN, 2008). Once in the field, the use of drip or subsurface irrigation instead of overhead irrigation reduces anthracnose severity on strawberry plants (SMITH, 2008). Therefore, overhead irrigation must be limited to the phase of transplant establishment (FREEMAN, 2008).

Growing strawberries in plastic mulches and keeping crop debris in row middles, typical procedures in Brazil, aim to establish a physical barrier between soil and fruit, avoiding splash dispersal of the pathogen from the soil to the plants (COSTA; VENTURA; LOPES, 2011). However, ground cover may affect spore dispersal by rain splash: the number of *C. acutatum* colonies in Petri plates with selective medium and fruit rot incidence were higher in plots with plastic cover than in plots covered with straw or noncovered and bare-ground plots (MADDEN; WILSON; ELLIS, 1993; YANG et al., 1990). Another method to reduce anthracnose fruit rot buildup in the field is the frequent harvest of fruit, removal of rotten fruit from the plants and the field, and harvest of infected areas last in the day (MERTELY et al., 2000; SMITH, 2008). The reduction of moisture accumulation, inoculum level and pathogen splash dispersal in strawberry fields can be achieved by growing the plants in walk-in greenhouses and low tunnels, which are adopted by growers in Minas Gerais and Espirito Santo State, in Brazil, and also in the USA and Israel (COSTA; VENTURA; LOPES, 2011; FREEMAN, 2008).

The use of fungicides to control strawberry anthracnose is a popular management adopted by growers in Brazil and USA (DOMINGUES et al., 2001; MERTELY; PERES; CHANDLER, 2009). However, chemical control can lead to the emergence of fungicide resistance of *Colletotrichum* spp. in strawberries and other

crops, such as previously reported (FORCELINI et al., 2016; MONDAL et al., 2005; YOUNG et al., 2010a).

1.3 Chemical control of Botrytis and Anthracnose fruit rots

Fungicides have been used by growers for more than 200 years to protect fields against fungal attack (BRENT; HOLLOMON, 2007). World population growth, food demand and need, low costs, higher yields and profits, and increase of food variety at low prices had an effect on the growth of the chemical industry market (HEWITT, 1998). Therefore, chemical control is the most common in controlling diseases in conventional production system and is based on fungicide sprays during the entire strawberry season. In organic production system, strawberry diseases are usually managed by using plant extracts, biological control, lime and sulfur compounds and Bordeaux and Viçosa mixtures (ANDRADE; NUNES, 2001).

All over the world, chemical control of diseases is based on the use of very old-established products, such as copper and sulfur-based formulation; ‘middle-aged’ fungicides, for example phthalimides and dithiocarbamates; more potent fungicides, with novel structure and systemic activity, introduced in the late 1960s and 1970s, such as benzimidazoles, dicarboximides and demethylation inhibitors; and fungicides in an advanced stage of development, launched in the 1990s, for example the quinone-outside inhibitors, anilinopyrimidines and succinate dehydrogenase fungicides (BRENT; HOLLOMON, 2007).

Fungicide Resistance Action Committee - FRAC (2016) classifies commercial fungicides according to their biochemical mode of action in the biosynthetic pathways of plant pathogens, grouping them according to processes in the metabolism and host plant defense inducers, and also to the risk of resistance development. Fungicides can be classified according to their target site and code, group name, chemical group, and FRAC code number (Table 1.1).

Table 1.1 – Mode of action, target site and code, group name, chemical group, common name and FRAC code of some fungicides registered for strawberries in Brazil

Mode of action	Target site and code	Group name	Chemical group	Common Name	FRAC Code
B: cytoskeleton and motor proteins	B1: β-tubulin assembly in mitosis	MBC (Methyl benzimidazole carbamates)	thiophanates	thiophanate-methyl	1
C: respiration	C3: complex III: cytochromoe bc1 at Qo site	QoI (Quinone outside inhibitors)	methoxy-acrylates	azoxystrobin	11
E: signal transduction	E3: MAP/Histidine-kinase in osmotic signal transduction	dicarboximides	dicarboximides	iprodione	2
G: sterol biosynthesis in membranes	G1: C14-demethylase in sterol biosynthesis	DMI (Demethylation inhibitors)	triazoles	difenoconazole	3

Source: FRAC (2016)

Active ingredients belonging to different chemical groups are registered for use in strawberries, such as azoxystrobin (quinone-outside inhibitor), different salts of copper (inorganic: multi-site contact activity), difenoconazole, imibenconazole, metconazole, tebuconazole and triforine (demethylation inhibitors), dodine (unknown mode of action), fluazinam (uncouplers of oxidative phosphorylation), iprodione and procymidone (dicarboximides), pyrimethanil (aniline-pyrimidines), thiophanate-methyl (methyl benzimidazole carbamates) (AGROFIT, 2016; FRAC, 2016).

The active ingredients thiophanate-methyl, iprodione + pyrimethanil (Certus, Bayer S.A.), iprodione and procimidone are registered for strawberries and labeled for *B. cinerea* control in Brazil (AGROFIT, 2016). However, azoxystrobin, labeled for Mycosphaerella Leaf Spot management (*M. fragariae*) in strawberries, also suppresses Botrytis fruit rot, and it is on the list of pesticides that are allowed in the 'Integrated Production of Strawberry' program (INSTITUTO DE DEFESA AGROPECUÁRIA E FLORESTAL DO ESPÍRITO SANTO - IDAF, 2012). In addition, quinone-outside-inhibitor fungicides, such as azoxystrobin and pyraclostrobin, are also used in Asia, Europe and USA to control the pathogen (FERNÁNDEZ-ORTUÑO

et al., 2015; ISHII et al., 2009; WEBER; HAHN, 2011). In USA, in addition to the active ingredients registered in Brazil, there are other products labeled for control of Botrytis fruit rot, such as boscalid + pyraclostrobin (Pristine, BASF), cyprodinil + fludioxonil (Switch WG, Syngenta) and fenhexamid (Elevate WDG, Arysta LifeScience, Cary, NC) (AMIRI; HEATH; PERES, 2013). Few DMI fungicides, such as prochloraz and tebuconazole, effectively control *B. cinerea* under field conditions (LEROUX et al., 2002). Besides chemical fungicides, *Bacillus pumillus* (Sonata, Bayer S.A.) is registered as a microbiological fungicide and may be also used for biological control of Botrytis fruit rot in Brazil (AGROFIT, 2016).

Although there are no active ingredients labeled for controlling *Colletotrichum* species on strawberry in Brazil, azoxystrobin (Amistar 500 WG, Syngenta Crop Protection), difenoconazole (Score 250 EC, Sygenta Crop Protection), fluazinam (Frowncide 500 SC, ISK Biosciences do Brasil Defensivos Agricolas Ltda) and thiophanate-methyl (Cercobin 700 WP, Ihara), registered for other strawberry diseases, are used by growers for anthracnose control, especially preventively (DOMINGUES et al., 2001; D. JULIATO[1], *personal information*). Moreover, prochloraz a fungicide not registered for strawberries, has been shown to reduce the number of blighted flowers affected by *Colletotrichum* spp. in the field (KOSOSKI et al., 2001). Meanwhile, carbendazim, tebuconazole + trifloxystrobin (Nativo; Bayer S.A.) and fluxapyroxad + pyraclostrobin (Orkestra SC, BASF) are labeled for controlling postbloom fruit drop of citrus, caused by *C. acutatum* and thiophanate-methyl is registered for citrus and labeled for *C. gloeosporioides* control (AGORFIT, 2016). In Florida, USA, azoxystrobin and pyraclostrobin are the most commonly used fungicides for controlling anthracnose of strawberries as well as broad-spectrum protectant fungicides, such as captan, that are weekly applied (FORCELINI et al., 2016; MERTELY; PERES; CHANDLER, 2009). In Israel, difenoconazole may be also used for chemical control of *C. acutatum* in strawberries (FREEMAN et al., 1997).

The fungicides benomyl, carbendazim, thiabendazole and thiophanate-methyl belong to the group of the methyl benzimidazole carbamate fungicides (MBC fungicides) (FRAC, 2016). Benzimidazoles are broad-spectrum fungicides and have been used since 1960's in many crops, such as cereals, grapes, fruits and vegetables and in postharvest treatments (HEWITT, 1998). The fungicidal activity of

[1] D. JULIATO. Personal communication during visit to the grower farm on July 11, 2013.

benzimidazoles is inhibition of the nuclear division by preventing the formation of microtubules, which leads to the disruption of chromosome segregation and migration during mitosis and meiosis, by binding the beta-tubulin assembly, resulting in cell death (DAVIDSE, 1986). The target site of these fungicides are the beta-tubulin gene (BANNO et al., 2008; YARDEN; KATAN, 1993).

The group of the respiratory inhibitor fungicides, such as the quinone-outside inhibitors (QoI fungicides), includes azoxystrobin, pyraclostrobin, trifloxystrobin, picoxystrobin and kresoxim-methyl (FRAC, 2016). Strobilurins, one of the most common groups within the QoI fungicides, are an important class of chemicals used to manage a broad range of fungal diseases in several agricultural systems (BARTLETT et al., 2002). The fungicidal activity of QoI is the inhibition of ATP production during mitochondrial respiration by binding at the quinol-oxidase (Qo) site of cytochrome *b* (*cytb*) and blocking electron transfer between cytochrome *b* and *c1*, which is located in the inner mitochondrial membrane of fungi (complex III), disrupting the energy cycle (BARTLETT et al., 2002). Spore germination is the most sensitive development stage of fungi development to the QoI fungicides (HEWITT, 1998).

Dicarboximide fungicides (MAP/Histidine-Kinase in osmotic signal transduction) are represented by iprodione, procymidone and vinclozin (FRAC, 2016). This group of fungicides was originally introduced for controlling Botrytis fruit rot and the activity of iprodione was first reported in 1974 (LACROIX et al., 1974). Although the mode of action of dicarboximides is still uncertain, recent evidence suggests they interfere with the osmotic signal transduction pathway consisting of histidine kinase and MAP kinase cascades (YAMAGUCHI; FUJIMURA, 2005). After emergence of *B. cinerea* resistance to benzimidazole fungicides, dicarboximides became commercially interesting.

Triazoles, a subgroup of demethylation-inhibitor fungicides (DMI fungicides), include difenoconazole, imibenconazole, metconazole and tebuconazole (FRAC 2016). DMI fungicides are inhibitors of sterol biosynthesis, especially ergosterol that is considered a unique component in the maintenance of membrane integrity and function of fungi: interference in ergosterol availability results in disruption of the membrane and electrolyte leakage (HEWITT, 1998). These compounds are known to inhibit the cytochrom*e b* P450 dependent oxidative demethylation of eburicol in the ergosterol biosynthetic pathway (STEFFENS; PELL; TIEN, 1996). DMI fungicides

replaced benzimidazole fungicides in USA in 1980s after widespread resistance of pathogens to the benzimidazoles (HOLB; SCHNABEL, 2007).

1.4 Fungicide resistance

The indiscriminate and continuous use of fungicides can promote the selection of resistant pathogens, which were previously controlled and are not anymore, putting at risk the efficiency of the chemical control method (GHINI; KIMATI, 2000). Resistance development happens mainly by the emergence of mutant individuals/strains that have undergone mutation and multiplication and are resistant to a chemical product. Fungicides are not responsible for causing mutation on fungi, on the contrary, they select resistant strains that have been competing with sensitive strains (ECKERT, 1994; GHINI; KIMATI, 2000). As chemical control has been widely used in strawberry production, it has resulted in selection for resistance and the diseases have not been controlled efficiently. Several authors reported the emergence of resistance to *B. cinerea* and *Colletotrichum* spp. worldwide (AMIRI; HEATH; PERES, 2013; FERNÁNDEZ-ORTUÑO et al., 2016; FORCELINI et al., 2016; ISHII et al., 2009; MERCIER; KONG; COOK, 2010). For instance, some mechanisms of fungicide resistance can be identified in field strains of pathogens, such as reduced sensitivity of the fungicide target site; reduced penetration of toxicants; increased fungicide efflux; increased detoxification; decreased conversion to toxic metabolites; increased production of target enzyme; and target site detour through alternative pathway (SISLER, 1994).

Benzimidazole (MBC) fungicides, used to control *B. cinerea* and *Colletotrichum* spp., are included in the high risk list for resistance (FRAC, 2013). Resistance to benzimidazoles was reported to several fungal pathogens including *Venturia inaequalis* (KOENRAADT; SOMERVILLE; JONES, 1992), *Monilinia laxa* (THOMIDIS; MICHAILIDES; EXADAKTYLOU, 2009), *B. cinerea* in Japan, USA and Europe (BANNO et al., 2008; FERNÁNDEZ-ORTUÑO; CHEN; SCHNABEL, 2012; LEROCH et al., 2013) and *Colletotrichum* species from different hosts (CHUNG et al., 2010; PERES et al., 2004; YOUNG et al., 2010b). Resistance to MBC fungicides is conferred as a result of point mutations in the beta-tubulin (*β-tub*) gene, resulting in alteration of amino acids sequences at the binding sites (MA; MICHAILIDES, 2005). The most common substitutions in resistant pathogens occur at codons 167, 198 and

200 of beta-tubulin gene, i.e. F167Y, E198A/K and F200Y. The F167Y and F200Y mutations result in substitution of phenylalanine (F) by tyrosine (Y); and E198A/K is a replacement of glutamic acid (E) to alanine (A) and lysine (K), respectively (MA; MICHAILIDES, 2005). In *B. cinerea*, the most common mutations occur at positions 198 and 200 (BANNO et al., 2008; YARDEN; KATAN, 1993). In *C. gloeosporioides* from citrus and blueberries, substitution from glutamic acid (E) to alanine (A) at position 198 of beta-tubulin gene were found in resistant isolates (PERES et al., 2004). In *C. cereale*, the causal agent of turfgrass anthracnose, the same mutation was found in resistant isolates (WONG et al., 2008).

Quinone-outside inhibitor (QoI) fungicides also carry a high risk of pathogen resistance development (FRAC, 2013) with resistance occurring in different pathogens in different crops and countries, including *Alternaria alternata* on tangerine (VEGA; DEWDNEY, 2014), *Magnaporthe grisea* on several crops (AVILA-ADAME; KÖLLER, 2003), *Colletotrichum graminicola* from turf (AVILA-ADAME; OLAYO; WOLFRAM, 2003), *Colletotrichum cereale* on turfgrass (YOUNG et al., 2010a); *Colletotrichum acutatum* (FORCELINI et al., 2016) on strawberries, and *Botrytis cinerea* on several hosts (AMIRI; HEATH; PERES, 2013; CHATZIDIMOPOULOS; PAPAEVAGGELOU; PAPPAS, 2013; ISHII, 2009; ZHANG et al., 2011). Resistance to QoI fungicides is a result of point mutations in the cytochrome *b* (*cytb*) gene and the most common amino acid substitutions occur at positions 129, 137 and 143 (FRAC, 2013; GISI et al., 2002). A single point mutation at position 143 (G143A), leading to an amino acid change of glycine (G) to alanine (A), was described to govern the expression of high (complete) resistance of pathogens to QoI fungicides (BANNO et al., 2009; GISI et al., 2002). Two other amino acid substitutions may also occur, but express moderate (partial) resistance, which is usually controlled by the recommended field rates of QoI fungicides. These mutations are characterized by substitutions of phenylalanine (F) to leucine (L) at position 129 (F129L) and of glycine (G) to arginine (R) at position 137 (G137R) (BARTLETT et al., 2002; FERNÁNDEZ-ORTUÑO; CHEN; SCHNABEL, 2012). Resistance to QoI fungicides of *B. cinerea* in strawberries has been reported in USA, Europe and Asia and the G143A mutation in the cytochrome *b* is the most commonly found (AMIRI; HEATH; PERES, 2013; BANNO et al., 2009; FERNÁNDEZ-ORTUÑO et al., 2016; ISHII et al., 2009). Moreover, some *B. cinerea* isolates can possess an additional intron inserted between the codons 143 and 144 of the cytochrome *b* gene and the G143A mutation

is usually not found in this case (BANNO et al., 2009). The F129L substitution was found in *Phakopsora pachyrhizi*, the causal agent of soybean rust, in Brazilian isolates (KLOSOWSKI et al., 2016) as well on *Colletotrichum cereale*, the causal agent of turfgrass (YOUNG et al., 2010a) and *C. acutatum* from strawberries (FORCELINI et al., 2016). These isolates expressed reduced sensitivity and/or intermediate resistance to QoI fungicides. *C. cereale* and *C. acutatum* isolates possessing the mutation G143A were considered as highly resistant to QoI fungicides.

Dicarboximide (DC) fungicides are considered to be of medium-to-high risk of pathogen resistance development (FRAC, 2013). Resistance to dicarboximide fungicides was reported in *Alternaria alternata* on passionfruit (DRY; YUAN; HUTTON, 2004), *Monilinia fructicola*, the causal agent of brown rot in stone fruit (ELMER; GAUNT; FRAMPTON, 1998), *Sclerotinia minor* from lettuce (HUBBARD et al., 1997) and *Botrytis cinerea* on strawberries, blueberries, raspberries, ornamentals, tomatoes, cucumbers and eggplants (FERNÁNDEZ-ORTUÑO et al., 2014; LAMONDIA; DOUGLAS, 1997; MYRESIOTIS; KARAOGLANIDIS; TZAVELLA-KLONARIA, 2007; WEBER, 2011). Resistance of *B. cinerea* isolates from strawberries to dicarboximides was reported in Asia (BANNO et al., 2008), Europe (FERNÁNDEZ-ORTUÑO et al., 2016; MYRESIOTIS; KARAOGLANIDIS; TZAVELLA-KLONARIA, 2007; WEBER, 2011), and the USA (GRABKE et al., 2014; MA et al., 2007). In Brazil, Domingues et al. (2003) reported that *B. cinerea* isolates from strawberries showed reduced sensitivity to iprodione. Isolates of *B. cinerea* can possess low, moderate, or high levels of resistance to dicarboximide fungicides. Low to moderate levels are typically found in field isolates, whereas high levels of resistance are rarely seen in the field (GRABKE et al., 2014). Resistance to dicarboximide fungicides is usually related to point mutations in the *bos1* gene (FERNÁNDEZ-ORTUÑO et al., 2016; GRABKE et al., 2014; MA; MICHAILIDES, 2005). The most common mutations in reduced-sensitive or resistant isolates occur at positions 127, 365, 369, 373 and 1136 of *bos1* gene, i.e. F127S, I365N/R/S, Q369H/P, N373S and V1136I. The F127S mutation results in substitution of phenylalanine (F) to serine (S); I365N/R/S mutations are characterized by replacement of isoleucine (I) with asparagine (N), arginine (R) or serine (S); Q369H/P mutations are the result of substitutions of glutamine (Q) to histidine (H) or proline (P); N373S mutation is characterized by replacement of asparagine (N) to serine (S);

and V1136I results in substitution of valine (V) to isoleucine (I) (GRABKE et al., 2014; MA et al., 2007).

Demethylation-inhibitor (DMI) fungicides are included in the medium risk class in the list of pathogen resistance development (FRAC, 2013). Resistance or sensitivity reduction to DMIs has been reported in *Monilinia fructicola* from peaches (SCHNABEL et al., 2004), *Uncinula necator*, the causal agent of grape powdery mildew (DÉLYE; LAIGRET; CORIO-COSTET, 1997), *Sclerotinia homoeocarpa* from turfgrass (GOLEMBIEWSKI et al., 1995), *Venturia inaequalis*, the causal agent of apple scab (KÖLLER; PARKER; REYNOLDS, 1991), among others. *Colletotrichum gloeosporioides* isolates from grape vineyards showed significantly lower sensitivity to prochloraz and tebuconazole than isolates collected from strawberry fields (XU et al., 2014). Moreover, reduced sensitivity to DMI fungicides was reported for *Botrytis cinerea* isolates from grapevines (LEROUX et al., 1999). Resistance to DMI fungicides is usually related to mutations in the 14α-demethylase (*cyp51*) gene or alternative mechanisms, such as overexpression of the target gene (Ma and Michailides 2005). A single point mutation at position 136 (F136Y), leading to the substitution of phenylalanine (F) to tyrosine (Y), was associated with highly resistant isolates of *Uncinula necator*, whereas mutations were not found in low resistant phenotypes (DÉLYE; LAIGRET; CORIO-COSTET, 1997). Several other mutations in the *cyp51* gene related with amino acids substitutions are found in laboratory-resistant mutants (MA; MICHAILIDES, 2005).

Furthermore, besides the high risk of the fungicide resistance development, according to the Fungicide Resistance Action Committee (FRAC), *B. cinerea* is classified as having a high risk of development of resistance to fungicides, whereas *Colletotrichum* spp. represents a medium risk (FRAC, 2013). The high risk of development of fungicide resistance in *B. cinerea* may be related to its high genetic variability and its wide host range (polyphagy) (LEROUX et al., 2002).

1.5 Epidemiology of strawberry diseases

Epidemiology, which means “related to people or population”, is the science of population dynamics and has been presented with several definitions by some plant pathologists. Vanderplank (1963) defined epidemiology as the science of the diseases in populations. Kranz (1974) wrote that epidemiology is the study of

pathogen populations on host populations and the disease as a result of this interaction influenced by environmental factors and human interferences. Madden, Hughes and van den Bosch (2007) reported epidemiology as being the change of the disease intensity on a host population in time and space. Epidemiology usually demands a quantification of disease intensity, which can be divided in disease incidence and disease severity. Disease incidence refers to the number of plant units with disease symptoms in relation to the total number of units evaluated; whereas disease severity refers to the area of a symptomatic plant unit in relation to the total area (VANDERPLANK, 1963). Disease development in time is often represented by disease progress curves, whereas, spatial development is described by gradients or spatial patterns of dispersal. Moreover, considering the dynamic nature of plant diseases, they can be divided in monocyclic and polycyclic diseases. In monocyclic diseases, the disease has only one cycle per crop season and infected plants do not act as inoculum source to healthy plants in the same season. In polycyclic diseases, the disease has several cycles within the crop season, inoculum is increased and infected plants become source of inoculum to other plants (BERGAMIN FILHO; AMORIM, 2011).

The study of temporal and spatial patterns of plant disease development is very important to correctly understand and interpret disease occurrence and pathosystem behavior in the fields, as well as to compare the influence of different treatments and control measures adopted (CAMPBELL; MADDEN, 1990). Temporal progress of disease analysis has been possible with the aid of mathematical models of disease development over time and the most common models used are: linear, monomolecular, exponential, logistic and Gompertz (NUTTER JR., 1997). Selection of a model that best describes a disease must take into consideration the biological meaning of the model, values of coefficient of determination R^2, the mean square error, the standard deviation (error) of the parameters estimate, and the plot of the standardized residuals versus predicted values (CAMPBELL; MADDEN, 1990). Regarding the spatial disease development in a field, there are several methods to quantitatively analyze the spatial patterns of diseased plants, such as index of dispersion (*D*), Taylor's power law (MADDEN; HUGHES, 1995), ordinary runs tests (MADDEN et al., 1982), spatial autocorrelation (CAMPBELL; MADDEN, 1990), etc.

Monomolecular models are usually used to describe diseases with monocyclic patterns, whereas logistic models are employed to describe polycyclic diseases

(BERGAMIN FILHO; AMORIM, 2011). Diseases having only one cycle per season, which are increasing constantly over time, can be described by linear or monomolecular models. The monomolecular model is more common, since it takes in consideration the reduction of healthy tissue in the area within disease incidence increase: disease increasing is high at the beginning of the epidemic and decreases overtime due to healthy tissue decrease (CAMPBELL; MADDEN, 1990; NUTTER JR., 1997). In diseases that have more than one cycle per season, the number of new infected individuals is higher than the number of infected plants in the area, and the disease increase is directly related to disease incidence level and progress rate. Exponential, logistic and Gompertz models are common used to describe polycyclic epidemics, however, logistic and Gompertz models consider the reduction of healthy individuals within epidemic development (NUTTER JR., 1997; MADDEN; HUGHES; VAN DEN BOSCH, 2007).

Dispersion index (*D*) is characterized by the relation between observed variance and expected binomial variance. The presence of aggregation or randomness of disease spatial pattern can be determined by partitioning the plots into quadrats and preparing binary (presence/absence) spatial maps (BASSANEZI et al., 2003). The Taylor's power law relates the observed variance (Vobs) and the expected binomial variance (Vbin) for a random distribution of binary data and is represented by the equation log (Vobs) = log (*A*) + *b* log (Vbin). The randomness situation in the spatial distribution is inferred when observed variance is equal to binomial variance and log (*A*) = 0 and *b* = 1, whereas aggregation occurs when $b \geq 1$ and log (*A*) > 0. Moreover, when *b* > 1, the degree of aggregation varies within disease incidence (CAMPBELL; MADDEN, 1990; MADDEN; HUGHES; VAN DEN BOSCH, 2007).

The dynamics of leather rot of strawberries, caused by *Phytophthora cactorum*, in time and space were assessed considering the influence of temperature, rainfall, and wetness duration on the components of disease cycle, such as infection, sporulation and dissemination. This knowledge was essential to develop methods to control this disease on strawberries (MADDEN; WILSON; ELLIS, 1993). Effects of rain intensity and surface topography, such as plant canopy density and ground cover, on strawberries were studied to characterize *Colletotrichum acutatum* dissemination and to understand temporal and spatial patterns of disease development in the field, thereby helping to propose methods for controlling

anthracnose fruit rot (YANG et al., 1990). Other epidemiological studies have demonstrated the importance of inoculum source in the occurrence of strawberry infection by *Botrytis cinerea* (BOFF et al., 2001; MERTELY et al., 2000). It has been reported that in annual crop using waiting-bed transplants, a system widely adopted in the Netherlands, necrotic leaves are not a significant source of inoculum and protection of flowers and young developing fruit are recommended strategies for Botrytis fruit rot control (BOFF et al., 2001).

Analyses of temporal progress and spatial spread of diseases enable identification patterns of pathogen dissemination, mechanisms of disease dispersion, disease growth rate, environmental variables occurring in the field (MADDEN; HUGHES; VAN DEN BOSCH, 2007), and also compare systems and disease control measures adopted by growers, such as conventional and organic strawberry production. Epidemiological studies may also help characterize the developmental pattern of diseases in fields where fungicide application does not control disease efficiently anymore, due to reduction or loss of isolate sensitivity to the most common chemicals used in strawberry production. Disease development in time and space in conventional and organic production systems can be compared with the aid of epidemiological tools. Furthermore, little information on spatial and temporal progress of Anthracnose and Botrytis fruit rots in strawberries is available, especially regarding the emergence of fungicide resistance in Brazilian strawberry fields.

Therefore, the main objectives of this project were to characterize the sensitivity of *Botrytis cinerea* and *Colletotrichum* spp. isolates from conventional and organic strawberry fields to the main systemic fungicides used in Brazil, using *in vitro*, fruit assay and molecular techniques; and to compare the spatial and temporal development of Botrytis and Anthracnose fruit rots in conventional and organic strawberry fields using epidemiological tools.

References

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA. Programa de Análise de Resíduos de Agrotóxicos em Alimentos (PARA): relatório de atividades de 2011 e 2012. Brasília, 2013. 45 p.

AGRIOS, G.N. **Plant pathology.** 5th ed. San Diego: Elsevier Academic Press, 2005. 922 p.

AGROFIT - SISTEMA DE AGROTÓXICOS FITOSSANITÁRIOS. 2016. Disponível em: <http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons>. Acesso em: 05 maio 2016.

AMIRI, A.; HEATH, S.M.; PERES, N.A. Phenotypic characterization of multifungicide resistance in *Botrytis cinerea* isolates from strawberry fields in Florida. **Plant Disease**, Saint Paul, v. 97, n. 3, p. 393–401, 2013.

ANDRADE, L.N.T.; NUNES. M.U.C. **Produtos alternativos para controle de doenças e pragas em agricultura orgânica**. Aracaju: Embrapa-Tabuleiros Costeiros, 2001. 20 p. (Documentos, 281).

ANTUNES, L.E.C.; PERES, N.A. Strawberry production in Brazil and South America. **International Journal of Fruit Science**, Philadelphia, v. 13, n. 1/2, p. 156–161, Jan. 2013.

AVILA-ADAME, C.; KÖLLER, W. Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. **Pest Management Science**, Sussex, v. 59, n. 3, p. 303–309, 2003.

AVILA-ADAME, C.; OLAYO, G.; WOLFRAM, K. Characterization of Colletotrichum graminicola isolates resistant to strobilurin-related QoI fungicides. **Plant Disease**, Saint Paul, v. 87, n. 12, p. 1426–1432, 2003.

BANNO, S.; FUKOMORI, F.; ICHIISHI, A.; OKADA, K.; UEKUSA, H.; KIMURA, M.; FUJIMURA, M. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. **Phytopathology**, Saint Paul, v. 98, n. 4, p. 397–404, 2008.

BANNO, S.; YAMASHITA, K.; FUKUMORI, F.; OKADA, K.; UEKUSA, H.; TAKAGAKI, M.; KIMURA, M.; FUJIMURA, M. Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. **Plant Pathology**, Oxford, v. 58, n. 1, p. 120–129, 2009.

BARTLETT, D.W.; CLOUGH, J.M.; GODWIN, J.R.; HALL, A.A.; HAMER, M.; PARR-DOBRZANSKI, B. The strobilurin fungicides. **Pest Management Science**, Sussex, v. 58, n. 7, p. 649–662, 2002.

BASSANEZI, R.B.; BERGAMIN FILHO, A.; AMORIM, L.; GIMENES-FERNANDES, N.; GOTTWALD, T.R.; BOVÉ, J.M. Spatial and temporal analyses of citrus sudden death as a tool to generate hypotheses concerning its etiology. **Phytopathology**, Saint Paul, v. 93, n. 16, p. 502–512, 2003.

BERGAMIN FILHO, A.; AMORIM, L. Epidemiologia de doenças de plantas. In: AMORIM, L.; REZENDE, J.A.M.; BERGAMIN FILHO, A. (Ed.). **Manual de fitopatologia:** princípios e conceitos. 4. ed. São Paulo: Ceres, 2011, v. 1, cap. 18, p. 101-118.

BOFF, P.; KASTELEIN, P.; DE KRAKER, J.; GERLAGH, M.; KÖHL, J. Epidemiology of grey mould in annual waiting-bed production of strawberry. **European Journal of Plant Pathology**, Dordrecht, v. 107, n. 6, p. 615–624, 2001.

BRAUN, P.G.; SUTTON, J.C. Inoculum sources of *Botrytis cinerea* in fruit rot of strawberries in Ontario. **Canadian Journal of Plant Pathology**, Ontario, v. 10, p. 1–5, 1987.

_____. Infection cycles and population dynamics of *Botrytis cinerea* in strawberry leaves. **Canadian Journal of Plant Pathology**, Ontario, v. 10, n. 2, p. 133–141, 1988.

BRENT, K.J.; HOLLOMON, D.W. **Fungicide resistance in plant management:** how can it be managed? 2nd ed. Brussels: FRAC, 2007. 53 p.

BRISTOW, P.R.; MCNICOL, R.J.; WILLIAMSON, B. Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. **Annals of Applied Biology**, Warwick, v. 109, n. 3, p. 545–554, 1986.

BROOME, J.C.; ENGLISH, J.T.; MAROIS, J.J.; LATORRE, B.A.; AVILES, J.C. Development of an infection model for Botrytis bunch rot of grapes based on wetness duration and temperature. **Phytopathology**, Saint Paul, v. 85, p. 97–102, 1995.

BULGER, M.A.; ELLIS, M.A.; MADDEN, L.V. Influence of temperature and wetness duration on infection of strawberry flowers by *Botrytis cinerea* and disease incidence of fruit originating from infected flowers. **Phytopathology**, Saint Paul, v. 77, n. 8, p. 1225-1230, 1987.

CAMPBELL, C.L.; MADDEN, L.V. **Introduction to plant disease epidemiology.** New York: John Wiley, 1990. 532 p.

CHATZIDIMOPOULOS, M.; PAPAEVAGGELOU, D.; PAPPAS, A.C. Detection and characterization of fungicide resistant phenotypes of *Botrytis cinerea* in lettuce crops in Greece. **European Journal of Plant Pathology**, Dordrecht, v. 137, n. 2, p. 363–376, 2013.

CHUNG, W.H.; CHUNG, W.C.; PENG, M.T.; YANG, H.R.; HUANG, J.W. Specific detection of benzimidazole resistance in *Colletotrichum gloeosporioides* from fruit crops by PCR-RFLP. **New Biotechnology**, New York, v. 27, n. 1, p. 17–24, 2010.

COSTA, H.; VENTURA, J.A.; LOPES, U.P. Manejo integrado de pragas e doenças do morangueiro. **Horticultura Brasileira,** Brasília, v. 29, S5856-5877, 2011. **Anais...** Apresentado no CONGRESSO BRASILEIRO DE OLERICULTURA, 51., 2011, Viçosa.

DALE, A.; HUGHES, B.R.; DONNELLY, D. The role of micropropagation in producing specific pathogen-tested plants. **HortScience**, Alexandria, v. 43, n. 1, p. 74–77, 2008.

DAVIDSE, L.C. Benzimidazole fungicides: mechanisms of action and biological impact. **Annual Review of Phytopathology**, Palo Alto, v. 24, p. 43–65, 1986.

DE MOURA, F.A.; NOGUEIRA, C.M.; GOUVÊA, M.A. Atributos determinantes na decisão de compra de consumidores de alimentos orgânicos. **Agroalimentaria**, Caracas, v. 18, n. 35, p. 75–86, 2012.

DÉLYE, C.; LAIGRET, F.; CORIO-COSTET, M.F. A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. **Applied and Environmental Microbiology**, Washington, v. 63, n. 8, p. 2966–2970, 1997.

DOMINGUES, R.J.; TÖFOLI, J.G.; PINTO, R.F. Sensibilidade "in vitro" a iprodione, taxa de crescimento e patogenicidade de linhagens de *Botrytis cinerea* à *Rosa* sp. **Ecossistema,** Espírito Santo do Pinhal, v. 28, n. 1, p. 35-39, 2003.

DOMINGUES, R.J.; TÖFOLI, J.G.; OLIVEIRA, S.H.F.; GARCIA JÚNIOR, O. Controle químico da flor preta (*Colletotrichum acutatum* Simmonds) do morangueiro em condições de campo. **Arquivos do Instituto Biológico**, São Paulo, v. 68, n. 2, p. 37–42, 2001.

DONADELLI, A.; KANO, C.; FERNANDES JUNIOR, F. Estudo de caso: Análise econômica entre o custo de produção de morango orgânico e convencional. **Pesquisa & Tecnologia**, Campinas, v. 9, n. 2, p. 1-5, 2012.

DROBY, S.; LICHTER, A. Post-harvest *Botrytis* infection: etiology, development and management. In: ELAD, Y.; WILLIAMSON, B.; TUDZYNSKI, P.; DELEN, N. (Ed.). **Botrytis:** biology, pathology and control. Dordrecht: Springer, 2007. p. 349–367.

DRY, I.B.; YUAN, K.H.; HUTTON, D.G. Dicarboximide resistance in field isolates of *Alternaria alternata* is mediated by a mutation in a two-component histidine kinase gene. **Fungal Genetics and Biology**, Orlando, v. 41, n. 1, p. 102–108, 2004.

ECKERT, J.W. Historical development of fungicide resistance in plant pathogens. In: DELP, C.J. (Ed.) **Fungicide resistance in North America**. Saint Paul: APS Press, 1994. chap. 1, p. 1-3.

ELMER, P.A.G.; GAUNT, R.E.; FRAMPTON, C.M. Spatial and temporal characteristics of dicarboximide-resistant strains of *Monilinia fructicola* and brown rot incidence in stone fruit. **Plant Pathology**, Oxford, v. 47, n. 4, p. 530–536, 1998.

EMBRAPA. **Sistema de produção de morango.** EMBRAPA Clima Temperado, 2005. Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Morango/SistemaProducaoMorango/index.htm>. Acesso em: 15 maio 2016.

FAO. 2016. Disponível em: <http://faostat3.fao.org/browse/Q/QC/E>. Acesso em: 25 maio 2016.

FERNÁNDEZ-ORTUÑO, D.; SCHNABEL, G. First report of thiophanate-methyl

resistance in *Botrytis cinerea* on strawberry from South Carolina. **Phytopathology**, Saint Paul, v. 96, n. 11, p. 1700, 2012.

FERNÁNDEZ-ORTUÑO, D.; CHEN, F.; SCHNABEL, G. Resistance to pyraclostrobin and boscalid in *Botrytis cinerea* isolates from strawberry fields in the Carolinas. **Plant Disease**, Saint Paul, v. 96, n. 8, p. 1198–1203, 2012.

FERNÁNDEZ-ORTUÑO, D.; GRABKE, A.; LI, X.; SCHNABEL, G. Independent emergence of resistance to seven chemical classes of fungicides in *Botrytis cinerea*. **Phytopathology**, Saint Paul, v. 105, n. 4, p. 424–432, 2015.

FERNÁNDEZ-ORTUÑO, D.; TORÉS, J.A.; CHAMORRO, M.; PÉREZ-GARCÍA, A.; VICENTE, A. Characterization of resistance to six chemical classes of site-specific fungicides registered for gray mold control on strawberries in Spain. **Plant Disease**, Saint Paul, 2016. Available at: <https://www.researchgate.net/publication/303372062_Characterization_of_Resistance_to_Six_Chemical_Classes_of_Site-Specific_Fungicides_Registered_for_Gray_Mold_Control_on_Strawberries_in_Spain>. Accessed: May 21, 2016.

FERNÁNDEZ-ORTUÑO, D.; GRABKE, A.; BRYSON, P.K.; AMIRI, A.; PERES, N.A.; SCHNABEL, G. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven southern U.S. states. **Plant Diseae**, Saint Paul, v. 98, n. 6, p. 825–833, 2014.

FNP CONSULTORIA & COMÉRCIO. Morango. In _____. **Agrianual 2016:** anuário da agricultura brasileira. São Paulo, 2016. p. 378-379.

FORCELINI, B.B.; SEIJO, T.E.; AMIRI, A.; PERES, N. A. Resistance in strawberry isolates of *Colletotrichum acutatum* from Florida to quinone-outside inhibitor fungicides. **Plant Disease**, Saint Paul, 2016. doi: http://dx.doi.org/10.1094/PDIS–01–16–0118–RE.

FRARE, G.F.; COUTO, H.T.Z.; CIAMPI-GUILLARDI, M.; AMORIM, L. The causal agent of citrus postbloom fruit drop, *Colletotrichum acutatum*, can survive on weeds. **Australasian Plant Pathology**, Collingwood, v. 45, n. 4, p. 339–346, 2016.

FREEMAN, S. Management, survival strategies, and host range of Colletotrichum acutatum on strawberry. **HortScience**, Alexandria, v. 43, n. 1, p. 66–68, 2008.

FREEMAN, S.; HOROWITZ, S.; SHARON, A. Pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. **Phytopathology**, Saint Paul, v. 91, p. 986–992, 2001.

FREEMAN, S.; SHABI, E.; KATAN, T. Characterization of *Colletotrichum acutatum* causing anthracnose of anemone (*Anemone coronaria* L.). **Applied and Environmental Microbiology**, Washington, v. 66, n. 12, p. 5267–5272, 2000.

FREEMAN, S.; NIZANI, Y.; DOTAN, S.; EVEN, S.; SANDO, T. Control of *Colletotrichum acutatum* in strawberry under laboratory, greenhouse, and field conditions. **Plant Disease**, Saint Paul, v. 81, n. 7, p. 749–752, 1997.

FUNGICIDE RESISTANCE ACTION COMMITTEE. **Pathogen risk list.** 2013. Disponível em: <http://www.frac.info/docs/default-source/publications/pathogen-risk/pathogen-risk-list.pdf> Acesso em: 26 maio 2016.

_____ **FRAC code list © 2016:** fungicides sorted by mode of action (including FRAC Code numbering). 2016. Disponível em: < http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2015-finalC2AD7AA36764.pdf?sfvrsn=4>. Acesso em: 26 maio 2016.

GHINI, R.; KIMATI, H. **Resistência de fungos a fungicidas**. Jagariúna: EMBRAPA Meio Ambiente, 2000. 78 p.

GISI, U.; SIEROTZKI, H.; COOK, A.; MCCAFFERY, A. Mechanisms influencing the evolution of resistance to Qo-inhibitor fungicides. **Pest Management Science**, Sussex, v. 58, n. 9, p. 859–867, 2002.

GOLEMBIEWSKI, R.C.; VARGAS JR., J.M.; JONES, A.L.; DETWEILER, A.R. Detection of demethylation inhibitor (DMI) resistance in *Sclerotinia homoeocarpa* populations. **Plant Disease**, Saint Paul, v. 79, p. 491–493, 1995.

GRABKE, A.; FERNÁNDEZ-ORTUÑO, D.; AMIRI, A.; LI, X.; PERES, N.A.; SMITH, P. Characterization of iprodione resistance in *Botrytis cinerea* from strawberry and blackberry. **Phytopathology**, Saint Paul, v. 104, p. 396–402, 2014.

HEWITT, H.G. **Fungicides in crop protection**. Wallingford: CAB International, 1998. 221 p.

HOLB, I.J.; SCHNABEL, G. Differential effect of triazoles on mycelial growth and disease measurements of *Monilinia fructicola* isolates with reduced sensitivity to DMI fungicides. **Crop Protection**, Guidford, v. 26, n. 5, p. 753–759, 2007.

HOWARD, C.M.; MAAS, J.L.; CHANDLER, C.K.; ALBREGTS, E.E. Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. **Plant disease**, Saint Paul, v. 76, n. 10, p. 976–981, 1992.

HUBBARD, J.C.; SUBBARAO, K.V.; KOIKE, S.T. Development and significance of dicarboximide resistance in *Sclerotinia minor* isolates from commercial lettuce fields in California. **Plant Disease**, Saint Paul, v. 81, n. 2, p. 148–153, 1997.

IBD CERTIFICAÇÕES. 2011. Disponível em: <http://ibd.com.br/pt/NoticiasDetalhes.aspx?id_conteudo=44>. Acesso: 15 junho 2016.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. **Censo Agropecuário 2006.** 2009. Disponível em: <http://www.ibge.gov.br/>. Acesso 16 maio 2016.

INSTITUTO DE DEFESA AGROPECUÁRIA E FLORESTAL DO ESPÍRITO SANTO. **Grade de agrotóxicos:** agrotóxicos cadastrados no Instituto de Defesa Agropecuária e Florestal do Espírito Santo (IDAF) para uso em morango. Vitória: Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural; IDAF, 2012. 3 p.

ISHII, H.; FOUNTAINE, J.; CHUNG, W.H.; KANSAKO, M.; NISHIMURA, K.; TAKAHASHI, K. Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. **Pest Management Science**, Sussex, v. 65, n. 8, p. 916–922, 2009.

JARVIS, W.R. The infection of strawberry and raspberry fruits by *Botrytis cinerea* Fr. **Annals of Applied Biology**, Warwick, v. 50, n. 3, p. 569–575, 1962.

KARABULUT, O.A.; TEZCAN, H.; DAUS, A.; COHEN, L.; WIESS, B.; DROBY, S. Control of preharvest and postharvest fruit rot in strawberry by *Metschnikowia fructicola*. **Biocontrol Science and Technology**, Oxford, v. 14, n. 5, p. 513–521, 2004.

KING, W.T., MADDEN, L.V.; ELLIS, M.A.; WILSON, L.L. Effects of temperature on sporulation and latent period of *Colletotrichum* spp. infecting strawberry fruit. **Plant Disease**, Saint Paul, v. 81, n. 1, p. 77–84, 1997.

KLOSOWSKI, A.C.; MAY-DE-MIO, L.L.; MIESSNER, S.; RODRIGUES, R.; STAMMLER, G. Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi*. **Pest Management Science**, Sussex, v. 72, n. 6, p. 1211–1215, 2016.

KRANZ, J. **Epidemics of plant disease:** mathematical analysis and modeling. Berlin: Springer, 1974. 170 p.

KOENRAADT, H.; SOMMERVILLE, S.; JONES, A.L. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. **Phytopathology**, Saint Paul, v. 82, p. 1348–1354, 1992.

KÖLLER, W.; PARKER, D.; REYNOLDS, K. Baseline sensitivities of *Venturia inaequalis* to sterol demethylation inhibitors. **Plant Disease**, Saint Paul, v. 75, p. 726–728, 1991.

KOSOSKI, R.M.; FURLANETTO, C.; TOMITA, C.K.; CAFÉ FILHO, A.C. Antracnose do Morangueiro. **Fitopatologia Brasileira**, Brasília, v. 26, n. 3, p. 662–666, 2001.

LAMONDIA, J.A.; DOUGLAS, S.M. Sensitvity of *Botrytis cinerea* from Connecticut greenhouses to benzimidazole and dicarboximide fungicides. **Plant Disease**, Saint Paul, v. 81, n. 7, p. 729–732, 1997.

LEANDRO, L.F.S.; GLEASON, M.L.; WEGULO, S.N.; NUTTER, F.W. Survival and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. **Phytopathology**, Saint Paul, v. 91, p. 659–664, 2001.

LEANDRO, L.F.S.; GLEASON, M.L.; NUTTER, F.W.; WEGULO, S.N.; DIXON, P.M. Influence of temperature and wetness duration on conidia and appressoria of *Colletotrichum acutatum* on symptomless strawberry leaves. **Phytopathology**, Saint Paul, v. 93, n. 4, p. 513–520, 2003a.

_____. Strawberry plant extracts stimulate secondary conidiation by *Colletotrichum acutatum* on symptomless leaves. **Phytopathology**, Saint Paul, v. 93, n. 10, p. 1285–1291, 2003b.

LEGARD, D.E.; XIAO, C.L.; MERTELY, J.C.; CHANDLER, C.K. Effects of plant spacing and cultivar on incidence of Botrytis fruit rot in annual strawberry. **Plant Disease**, Saint Paul, v. 84, n. 5, p. 531–538, 2000.

LEROCH, M.; PLESKEN, C.; WEBER, R.W.S.; KAUFF, F.; SCALLIET, G.; HAHN, M. Gray mold populations in German strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. **Applied and Environmental Microbiology**, Washington, v. 79, n. 1, p. 159–167, 2013.

LEROUX, P., CHAPELAND, F.; DESBROSSES, D.; GREDT, M. Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. **Crop Protection**, Guidford, v. 18, n. 10, p. 687–697, 1999.

LEROUX, P.; FRITZ, R.; DEBIEU, D.; ALBERTINI, C.; LANEN, C.; BACH, J. Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. **Pest Management Science**, Sussex, v. 58, n. 9, p. 876–888, 2002.

LIMA, M.A.; CALEGARIO, F.F.; SILVA, A.V.C.; SCAPIM, M.S. Qualidade do morango cultivado sob diferentes sistemas de produção. In: CONGRESSO IBÉRICO DE AGRO-ENGENHARIA, 2011, Évora. **Anais...** Évora: Universidade de Évora, 2011.

MAAS, J.L. **Compendium of strawberry diseases**. 2nd ed. Saint Paul: American Phytopathological Society Press, 1998.128 p.

MA, Z.; MICHAILIDES, T.J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. **Crop Protection**, Guidford, v. 24, n. 10, p. 853–863, 2005.

MA, Z.; YAN, L.; LUO, Y.; MICHAILIDES, T.J. Sequence variation in the two-component histidine kinase gene of *Botrytis cinerea* associated with resistance to dicarboximide fungicides. **Pesticide Biochemistry and Physiology**, San Diego, v. 88, n. 3, p. 300–306, 2007.

MACKENZIE, S.J.; MERTELY, J.C.; PERES, N.A. Curative and protectant activity of fungicides for control of crown rot of strawberry caused by *Colletotrichum gloeosporioides*. **Plant Disease**, Saint Paul, v. 93, n. 8, p. 815–820, 2009.

MACKENZIE, S.J.; PERES, N.A.; BARQUERO, M.P.; ARAUZ, L.F.; TIMMER, L.W. Host range and genetic relatedness of *Colletotrichum acutatum* isolates from fruit crops and leatherleaf fern in Florida. **Phytopathology**, Saint Paul, v. 99, p. 620–631, 2009.

MADDEN, L.V; HUGHES, G. Plant disease incidence: distributions, heterogeneity, and temporal analysis. **Annual Review of Phytopathology**, Palo Alto, v. 33, p. 529–564, 1995.

MADDEN, L.V.; HUGHES, G.; ELLIS, M.A. Spatial heterogeneity of the incidence of grape downy mildew. **Phytopathology**, Saint Paul, v. 85, p. 269–275, 1995.

MADDEN, V.; HUGHES, G.; VAN DEN BOSCH, F. **The study of plant disease epidemics**. Saint Paul: APS Press, 2007. 421 p.

MADDEN, L.V.; WILSON, L.L.; ELLIS, M.A. Field spread of anthracnose fruit rot of strawberry in relation to ground cover and ambient weather conditions. **Plant Disease**, Saint Paul, v. 77, n. 9, p. 861–866, 1993.

MADDEN, L.V.; LOUIE, R.; ABT, J.J.; KNOKE, J.K. Evaluation of tests for randomness of infected plants. **Phytopathology**, Saint Paul, v. 72, p. 195–198, 1982.

MADDEN, L.V.; WILSON, L.L.; YANG, X.; ELLIS, M.A. Splash dispersal of *Colletotrichum acutatum* and *Phytophthora cactorum* by short-duration simulated rains. **Plant Pathology**, Oxford, v. 41, n. 4, p. 427–436, 1992.

MCNICOL, R.J.; WILLIAMSON, B.; DOLAN, A. Infection of red raspberry styles and carpels by *Botrytis cinerea* and its possible role in post-harvest grey mould. **Annals of Applied Biology**, Warwick, v. 106, n. 1, p. 49–53, 1985.

MERCIER, J.; KONG, M.; COOK, F. Fungicide resistance among Botrytis cinerea isolates from California strawberry fields. **Plant Health Progress**, Saint Paul, 2010. doi:10.1094/PHP–2010–0806–01–RS.

MERTELY, J.C.; LEGARD, D.E. Detection, isolation, and pathogenicity of *Colletotrichum* spp. from strawberry petioles. **Plant Disease**, Saint Paul, v. 88, p. 407–412, 2004.

MERTELY, J.C.; PERES, N.A. **Root necrosis of strawberries caused by *Colletotrichum acutatum*.** Gainesville: University of Florida; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences, 2005. (Publication, PP-211).

MERTELY, J.C.; MACKENZIE, S.J.; LEGARD, D.E. Timing of fungicide applications for *Botrytis cinerea* based on development stage of strawberry flowers and fruit. **Plant Disease**, Saint Paul, v. 86, n. 9, p. 1019–1024, 2002.

MERTELY, J.C.; PERES, N.A.; CHANDLER, C.K. **Anthracnose fruit rot of strawberry**. Gainesville: University of Florida, Plant Pathology Department; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences, 2009. 4 p.

MERTELY, J.C.; CHANDLER, C.K.; XIAO, C.L.; LEGARD, D.E. Comparison of sanitation and fungicides for management of Botrytis fruit rot of strawberry. **Plant Disease**, Saint Paul, v. 84, n. 17, p. 1197–1202, 2000.

MONDAL, S.N.; BATHIA, A.; SHILTS, T.; TIMMER, L.W. Baseline sensitivities of fungal pathogens of fruit and foliage of citrus to azoxystrobin, pyraclostrobin, and fenbuconazole. **Plant Disease**, Saint Paul, v. 89, n. 11, p. 1186–1194, 2005.

MYRESIOTIS, C.K.; KARAOGLANIDIS, G.S.; TZAVELLA-KLONARIA, K. Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. **Plant Disease**, Saint Paul, v. 91, n. 4, p. 407–413, 2007.

NORMAN, D.J.; STRANDBERG, J.O. Survival of *Colletotrichum acutatum* in soil and plant debris of leatherleaf fern. **Plant Disease**, Saint Paul, v. 81, n. 10, p. 1177–1180, 1997.

NUTTER JR., F.W. Quantifying the temporal dynamics of plant virus epidemics: a review. **Crop Protection**, Guidford, v. 16, n. 7, p. 603–618, 1997.

OLIVEIRA, M.S. **Fungicide resistance profile of quiescnet Botrytis cinerea infections on strawberry transplants**. 2015. 100p . Thesis (Master of Science) - University of Florida, Gainesville, 2015.

OLIVEIRA, R.P.; SCIVITTARO, W.B. Desempenho produtivo de mudas nacionais e importadas de morangueiro. **Revista Brasileira de Fruticultura**, Cruz das Almas, v. 28, p. 520–522, 2006.

PERES, N.A.R.; SOUZA, N.L.; PEEVER, T.L.; TIMMER, L.W. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus. **Plant Disease**, Saint Paul, v. 88, n. 2, p. 125–130, 2004.

PERES, N.A.; TIMMER, L.W.; ADASKAVEG, J.E.; CORRELL, J.C. Lifestyles of *Colletotrichum acutatum*. **Plant Disease**, Saint Paul, v. 89, n. 8, p. 784–796, 2005.

POWELSON, R.L. The initiation of strawberry fruit rot caused by Botrytis cinerea. **Phytopathology**, Saint Paul, v. 50, p. 491-494, 1960.

PRIMIANO, I.V. **Adaptabilidade de isolados de Monilinia fructicola com diferentes níveis de sensibilidade à azoxistrobina.** 2015. 82 p. Dissertação (Mestrado em Fitopatologia) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2015.

RADMANN, E.B.; BIANCHI, V.J.; OLIVEIRA, R.P.; FACHIELLO, J.C. Caracterização e diversidade genética de cultivares de morangueiro. **Horticultura Brasileira**, Brasília, v. 24, n. 1, p. 84–87, 2006.

SCHNABEL, G.; BRYSON, P.K.; BRIDGES, W.C.; BRANNEN, P.M. Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. **Plant Disease**, Saint Paul, v. 88, n. 9, p. 1000–1004, 2004.

SEIJO, T.E.; CHANDLER, C.K.; MERTELY, J.C.; MOYER, C.; PERES, N.A. Resistance of strawberry cultivars and advanced selections to Anthracnose and Botrytis fruit rots. **Proceedings of the Florida State Horticultural Society**, Tallahassee, v. 121, p. 246–248, 2008.

SMITH, B.J. Epidemiology and pathology of strawherry anthracnose: a North American perspective. **HortScience**, Alexandria, v. 43, n. 1, p. 69–73, 2008.

SMITH, B.J.; BLACK, L.L. Resistance of strawberry plants to *Colletotrichum fragariae* affected by environmental conditions. **Plant Disease**, Saint Paul, v. 71, p. 834–837, 1987.

STEFFENS, J.J.; PELL, E.J.; TIEN, M. Mechanisms of fungicide resistance in phytopathogenic fungi. **Current Opinion in Biotechnology**, London, v. 7, n. 3, p. 348–355, 1996.

SUTTON, J.C. Epidemiology and management of Botrytis leaf blight of onion and gray mold of strawberry: a comparative analysis. **Canadian Journal of Plant Pathology**, Ontario, v. 12, n. 1, p. 100–110, 1990.

TANAKA, M.A.S.; BETTI, J.A.; KIMATI, H. Doenças do morangueiro (*Fragaria* x *ananassa*). In: KIMATI, H.; AMORIM, L.; REZENDE, J.A.M.; BERGAMIN FILHO, A.; CAMARGO, L.E.A. (Ed.). **Manual de fitopatologia:** doenças das plantas cultivadas. São Paulo: Ceres, 2005. p. 489-499.

THOMIDIS, T.; MICHAILIDES, T.; EXADAKTYLOU, E. Contribution of pathogens to peach fruit rot in northern Greece and their sensitivity to iprodione, carbendazim, thiophanate-methyl and tebuconazole fungicides. **Journal of Phytopathology**, Berlin, v. 157, n. 3, p. 194–200, 2009.

TRIVELLATO, M.D.; FREITAS, G.B. Panorama da agricultura orgânica. In: STRINGHETA, P.C.; MUNIZ, J.N. (Ed.). **Alimentos orgânicos:** produção, tecnologia e certificação. Viçosa: UFV, 2003. p. 9-36.

UREÑA-PADILLA, A.R.; MACKENZIE, S.J.; BOWEN, B.W.; LEGARD, D.E. Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. **Phytopathology**, Saint Paul, v. 92, n. 11, p. 1245–1252, 2002.

VANDERPLANK, J.E. **Plant diseases:** epidemics and control. New York: Academic Press, 1963. 349 p.

VEGA, B.; DEWDNEY, M.M. Distribution of QoI resistance in populations of tangerine-infecting *Alternaria alternata* in Florida. **Plant Disease**, Saint Paul, v. 98, n. 1, p. 67–76, 2014.

WEBER, R.W.S. Resistance of *Botrytis cinerea* to multiple fungicides in Northern German small-fruit production. **Plant Disease**, Saint Paul, v. 95, n. 10, p. 1263–1269, 2011.

WEBER, R.W.S.; HAHN, M. A rapid and simple method for determining fungicide resistance in *Botrytis*. **Journal of Plant Diseases and Protection**, Berlin, v. 118, n. 1, p. 17–25, 2011.

WILCOX, W.F.; SEEM, R.C. Relationship between strawberry gray mold incidence, environmental variables, and fungicide applications during different periods of the fruiting season. **Phytopathology**, Saint Paul, v. 84, p. 264–270, 1994.

WILLIAMSON, B.; TUDZYNSKI, B.; TUDZYNSKI, P.; VAN KAN, J. *Botrytis cinerea*: the cause of grey mould disease. **Molecular Plant Pathology**, London, v. 8, n. 5, p. 561–580, 2007.

WILSON, L.; MADDEN, L.; ELLIS, M. Influence of temperature and wetness duration on infection of immature and mature strawberry fruit by *Colletotrichum acutatum*. **Phytopathology**, Saint Paul, v. 80, n. 1, p. 111–116, 1990.

WONG, F.P.; CERDA, K.; HERNANDEZ-MARTINEZ, R. Detection and characterization of benzimidazole resistance in California populations of *Colletotrichum cereale*. **Plant Disease**, Saint Paul, v. 92, p. 239–246, 2008.

XIAO, C.L.; CHANDLER, C.K.; PRICE, J.F.; DUVAL, J.R.; MERTELY, J.C.; LEGARD, D.E. Comparison of epidemics of Botrytis fruit rot and powdery mildew of strawberry in large plastic tunnel and field production systems. **Plant Disease**, Saint Paul, v. 85, n. 8, p. 901–909, 2001.

XU, X.F.; LIN, T.; YUAN, S.K.; DAI, D.J.; SHI, H.J. Characterization of baseline sensitivity and resistance risk of *Colletotrichum gloeosporioides* complex isolates from strawberry and grape to two demethylation-inhibitor fungicides, prochloraz and tebuconazole. **Australasian Plant Pathology**, Collingwood, v. 43, n. 6, p. 605–613, 2014.

YAMAGUCHI, I.; FUJIMURA, M. Recent topics on action mechanisms of fungicides. **Journal of Pesticide Science**, Tokyo, v. 30, n. 2, p. 67–74, 2005.

YANG, X.; MADDEN, L.V.; WILSON, L.L.; ELLIS, M.A. Effects os surface topography and rain intensity on splash dispersal of *Colletotrichum acutatum*. **Phytopathology**, Saint Paul, v. 80, p. 1115–1120, 1990.

YARDEN, O.; KATAN, T. Mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin that correlate with benomyl-resistance phenotypes of field strains of *Botrytis cinerea*. **Phytopathology**, Saint Paul, v. 83, n. 12, p. 1478–1483, 1993.

YOUNG, J.R.; TOMASO-PETERSON, M. CERDA, K.; WONG, F.P. Occurrence and molecular identification of azoxystrobin-resistant *Colletotrichum cereale* isolates from golf course putting greens in the southern United States. **Plant Disease**, Saint Paul, v. 94, p. 751–757, 2010a.

YOUNG, J.R.; TOMASO-PETERSON, M.; TREDWAY, L.P.; CERDA, K. Two mutations in β-tubulin 2 gene associated with thiophanate-methyl resistance in *Colletotrichum cereale* isolates from creeping bentgrass in Mississippi and Alabama. **Plant Disease**, Saint Paul, v. 94, p. 207–212, 2010b.

ZAMBOLIM, L.; COSTA, H. Manejo intgrado de doenças do morangueiro. In: CARVALHO, S.P. (Ed.). **Boletim do morango:** cultivo convencional, segurança alimentar, cultivo orgânico. Belo Horizonte: FAEMG, 2006. p. 55-80.

ZHANG, C.Q.; LIU, Y.H.; DING, L.; ZHU, G.N. Shift of sensitivity of *Botrytis cinerea* to azoxystrobin in greenhouse vegetables before and after exposure to the fungicide. **Phytoparasitica**, Bet Dagan, v. 39, n. 3, p. 293–302, 2011.

2 FUNGICIDE SENSITIVITY OF *Botrytis cinerea* AND *Colletotrichum acutatum* ISOLATES FROM BRAZILIAN CONVENTIONAL AND ORGANIC STRAWBERRY FIELDS

Abstract

Botrytis and Anthracnose fruit rots, caused by *Botrytis cinerea* and *Colletotrichum* spp., respectively, are major strawberry diseases. Chemical control is widely used, and can cause selection of resistant pathogens. This study aimed to compare organic and conventional strawberry production systems by evaluating the sensitivity of *B. cinerea* and *C. acutatum* isolates to the main single-site fungicides used in Brazil. Isolates were collected from conventional and organic fields in four different Brazilian states from 2013 to 2015. Resistance to azoxystrobin, iprodione and thiophanate-methyl was found in *B. cinerea* isolates with EC_{50} values higher than 71.9, 688 and 1.2 µg/ml, respectively. Resistance to the aforementioned fungicides was observed in 87.5, 76.6 and 92.2 % of isolates from conventional fields, and 31.4, 22.9 and 51.4 % of isolates from organic fields, respectively. Moreover, frequencies of populations with multiple fungicide resistance to the three active ingredients were 75 and 8.6 %, with no resistance to any of the fungicides were 6.25 and 34.3 % for isolates collected from conventional and organic areas, respectively. Molecular analyses of the *cytochrome b*, beta-tubulin and *bos1* genes revealed, respectively, the presence of G143A, E198A and I365N/S, Q369P or N373S mutations in resistant populations of *B. cinerea*. Fungicides sprayed preventively on strawberry fruit inoculated with *B. cinerea* failed to control resistant isolates. Isolates of *C. acutatum* resistant to azoxystrobin and difenoconazole were not observed. Mean EC_{50} values for isolates collected from organic fields were 0.44 and 0.95 µg/ml, and from conventional areas were 0.629 and 0.107 µg/ml for azoxystrobin and difenoconazole, respectively. Populations of *C. acutatum* showed insensitivity to thiophanate-methyl rather than resistance and EC_{50} values could not be determined. None of the isolates contained the most commonly mutations associated with fungicide resistance. This study reinforces the importance of continuous research and monitoring of risks associated with fungicide resistance occurrence; the urgency for the implementation of resistance management programs; and the need for an integrated approach between strawberry nurseries and production fields for disease control.

Keywords: *Fragaria* x *ananassa*; Gray mold; Fungicide resistance; Gene mutation; Production systems

2.1 Introduction

Botrytis and Anthracnose fruit rots are major strawberry diseases in Brazil and many other growing areas worldwide, and are responsible for severe pre- and postharvest losses due to infections of flowers and fruit (FERNÁNDEZ-ORTUÑO et al., 2016; MAAS, 1998; MACKENZIE; MERTELY; PERES, 2009; TANAKA; BETTI; KIMATI, 2005). Botrytis fruit rot, also known as gray mold, is caused by the fungus

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana*), which can affect several other hosts and cultivated crops (WILLIAMSON et al., 2007). *Botrytis cinerea* has a necrotrophic life cycle and can overwinter as mycelia, spores or sclerotia in crop debris (BRAUN; SUTTON, 1987; MERTELY et al., 2000; WILLIAMSON et al., 2007). Spores are usually spread by air and water from rain or overhead irrigation, and may infect strawberry plants especially via flowers, but also fruit through surface injuries (BRISTOW; MCNICOL; WILLIAMSON, 1986; DROBY; LICHTER, 2007). Rot symptoms can appear right after pathogen infection or remain quiescent until fruit maturity or harvesting (JARVIS, 1962; MCNICOL; WILLIAMSON; DOLAN, 1985). Gray mold symptoms appear as brown lesions that can evolve from small to large sizes and become covered with the pathogen structures, making the fruit dry and tough with little or no leakage of fruit content (DROBY; LICHTER, 2007; SUTTON, 1990). Infection, sporulation, spore release and germination of *B. cinerea*, symptoms development and increase of disease incidence may occur under favorable conditions, such as temperatures about 20 to 23 °C and prolonged periods of high humidity and rain (BRAUN; SUTTON, 1988; BROOME et al., 1995; BULGER; ELLIS; MADDEN, 1987; WILCOX; SEEM, 1994).

Anthracnose diseases in strawberries are caused by three species complex of *Colletotrichum*: *C. acutatum* Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. & Sacc (DOMINGUES et al., 2001; HOWARD et al., 1992). *C. acutatum* (teleomorph *Glomerella acutata*) is the causal agent of Anthracnose fruit rot, and along with *C. fragarie* and *C. gloeosporioides* (teleomorph *Glomerella cingulata*) can also cause lesions in petioles, stolons, and crown. Moreover, all of them may produce similar symptoms and can be found on the same plant (HOWARD et al., 1992). The main symptoms of Anthracnose fruit rot are flower blight and fruit rot, however root and crown necrosis can occur (PERES et al., 2005). Light-brown water-soaked lesions develop into dark brown sunken lesions on affected tissues, such as petioles and fruit, and under high humidity conditions, spores are produced in a salmon-pink or orange mucilage (MERTELY; PERES; CHANDLER, 2009; SEIJO et al., 2008). *Colletotrichum* spp. may survive on and in strawberry plants, soil, crop debris, weeds and other crops (FRARE et al., 2016; LEANDRO et al., 2003a; MACKENZIE; MERTELY; PERES, 2009; NORMAN; STRANDBERG, 1997). Dissemination occurs mainly at short distances by water splash from rainfall and overhead irrigation, but also by field equipment and harvest operations (FREEMAN;

HOROWITZ; SHARON, 2001; HOWARD et al., 1992; MADDEN; WILSON; ELLIS, 1993). Temperatures from 17 to 28 °C and long periods of wetness are favorable for plant infection, spore production and germination and appressorium formation (LEANDRO et al., 2003a), which leads to high levels of disease in the field (MADDEN et al., 1992).

Botrytis and Anthracnose fruit rots are managed by using a combination of cultural practices, resistance and, mainly, chemical control. The first control measurement that should be adopted by growers is the use of healthy propagation material, which could be responsible for the introduction of pathogens in the area, acting as primary source of inoculum (FREEMAN, 2008; OLIVEIRA, 2015). Although there are no fully resistant strawberry cultivars, some cultivars are less susceptible to the diseases and can be used (LEGARD et al., 2000; SEIJO et al., 2008). Cultural practices such as the use of drip irrigation instead of overhead-irrigation, frequent harvest of fruit, removal of diseased and unmarketable fruit, crop debris and senescent foliage and use of low tunnels are also recommended for control of the diseases caused by *B. cinerea* and *Colletotrichum* spp. (COSTA; VENTURA; LOPES, 2011; DROBY; LICHTER, 2007; FREEMAN, 2008; MERTELY et al., 2000; SMITH, 2008). Fungicide application is traditionally the standard and most popular control method for Botrytis and Anthracnose fruit rots (DOMINGUES et al., 2001; MERTELY; MACKENZIE; LEGARD, 2002; MERTELY; PERES; CHANDLER, 2009).

Chemical control relies on application of single- and multi-site fungicides throughout the strawberry season, especially during blooming and fruitification phases that occur simultaneously (AMIRI; HEATH; PERES, 2013; MERTELY; MACKENZIE; LEGARD, 2002; XIAO et al., 2001). Besides the inorganic and multi-site compounds, and active ingredients with unknown mode of action, such as dodine, compounds from different chemical groups, i.e., quinone-outside inhibitors (QoI), dicarboximides (DC), demethylation inhibitors (DMI), anilinopyrimidines (AP), methyl benzimidazole carbamates (MBC) are registered for strawberries in Brazil (AGROFIT, 2016). The active ingredients, thiophanate-methyl (MBC), iprodione and procimidone (DC) and pyrimethanil (AP) are labeled for gray mold control. However, azoxystrobin (QoI), labeled for Mycosphaerella Leaf Spot management, also suppresses Botrytis fruit rot, and it is on the list of pesticides allowed to be used on the ‘Integrated Production of Strawberry’ program (IDAF, 2012). Besides chemical fungicides, *Bacillus pumillus* is also used as biological control of Botrytis fruit rot in

Brazil (AGROFIT, 2016). There are no active ingredients labeled for controlling *Colletotrichum* species in strawberries in Brazil, however azoxystrobin, difenoconazole (DMI) and thiophanate-methyl are used by growers for anthracnose control, especially preventively (DOMINGUES et al., 2001; D. JULIATO[2], *personal information*). In the organic production system, strawberry diseases are usually managed by using plant extracts, biological control, lime and sulfur compounds and Bordeuax and Viçosa mixtures, that are composed of inorganic and multi-site fungicides (ANDRADE; NUNES, 2001).

As chemical control has been widely and regularly used in strawberry production, development of pathogen resistance with the selection of mutants that are no longer controlled, may put at risk the efficiency of the method (GHINI; KIMATI, 2000). Site-specific fungicides, such as QoIs, DMIs, DCs and MBCs, are classified as having medium to high risk of resistance development (FRAC, 2013). Reduced sensitivity of pathogens to fungicides as a result of a point mutation at the target site is one of the most common mechanisms of fungicide resistance. In fact, emergence of *B. cinerea* and *Colletotrichum* spp. resistance to site-specific fungicides has been extensively reported in several countries (AMIRI; HEATH; PERES, 2013; BANNO et al., 2008; FERNÁNDEZ-ORTUÑO et al., 2016; FORCELINI et al., 2016; GRABKE et al., 2014; ISHII et al., 2009).

The fungicide activity of methyl-benzimidazole carbamates consists in the inhibition of the nuclear division, by binding the beta-tubulin assembly and disrupting chromosome segregation and migration (DAVIDSE, 1986). Thus, resistance to these fungicides can occur as a result of point mutations in the beta-tubulin (*β-tub*) gene, leading to amino acids substitutions at the binding sites, such as F167Y, E198A/K and F200Y (MA; MICHAILIDES, 2005). Resistance to MBCs has already been reported in *Venturia inaequalis* (KOENRAADT; SOMERVILLE; JONES, 1992), *Monilinia laxa* (THOMIDIS; MICHAILIDES; EXADAKTYLOU, 2009), *B. cinerea* (BANNO et al., 2008) and *Colletotrichum* spp. (CHUNG et al., 2010; PERES et al., 2004). Demethylation inhibitor fungicides act as inhibitors of sterol biosynthesis in fungi and resistance is usually related to mutations in the *cyp51* gene or overexpression of the target gene (HEWITT, 1998; MA; MICHAILIDES, 2005). Sensitivity reduction to DMIs was reported in *Monilinia fructicola* (SCHNABEL et al.,

[2] D. JULIATO. Personal communication during visit to the grower farm on July 11, 2013.

2004) SCHNABEL et al., 2014), *Uncinula necator* (DÉLYE; LAIGRET; CORIO-COSTET, 1997). *Colletotrichum gloeosporioides* (XU et al., 2014) and *B. cinerea* (LEROUX et al., 1999). Quinone outside inhibitor fungicides act by inhibiting ATP production during mitochondrial respiration by binding at the *cytochrome b* and blocking electron transfer (BARTLETT et al., 2002). Resistance to QoIs may occur as mutations in the *cytochrome b* (*cytb*) gene, with amino acid replacements at codons 129, 137 and 143 (GISI et al., 2002), and has been reported in pathogens of different crops and in different countries, including *B. cinerea* (AMIRI; HEATH; PERES, 2013; FERNÁNDEZ-ORTUÑO et al., 2016) and *Colletotrichum acutatum* (FORCELINI et al., 2016) on strawberries. The G143A mutation confers high levels of resistance and is the most commonly found in field isolates (GISI et al., 2002). The mode of action of dicarboximide fungicides consists of interference with the osmotic signal transduction pathway (YAMAGUCHI; FUJIMURA, 2005). Resistance to this class of fungicides is usually related to point mutations in the *bos1* gene and the most common substitutions in reduced-sensitive or resistant isolates are found at codons 127, 365, 369, 373 and 1136 (GRABKE et al., 2014; MA et al., 2007). Emergence of resistance to DCs has already been reported in *Alternaria alternata* (DRY; YUAN; HUTTON, 2004), *Monilinia fructicola* (ELMER; GAUNT; FRAMPTON, 1998) and *B. cinerea* in several hosts (FERNÁNDEZ-ORTUÑO et al., 2014; LAMONDIA; DOUGLAS, 1997; WEBER, 2011)

In addition to the risk of fungicide resistance emergence, the presence of pesticide residues on food and environment has raised concern about the indiscriminate use of pesticides in agriculture. Moreover, recent reports from the Brazilian National Health Surveillance Agency (ANVISA) have shown that 59 % of strawberry samples analyzed had residues of pesticides not registered for strawberries or with levels above the permitted limit (ANVISA, 2013). Thus, public concern about sustainable food production has led to an increased demand for organic products and, as a result, the growth of the organic market and, consequently, multiplication of farms that adopt organic practices (DE MOURA; NOGUEIRA; GOUVÊA, 2012).

Considering the historic context of resistance worldwide, monitoring of conventional and organic strawberry fields is important to develop a fungicide-resistance management program for growers in Brazil. Therefore, the specific objective of this study was to compare organic and conventional strawberry systems

by evaluating the *in vitro* and *in vivo* sensitivities of *Botrytis cinerea* isolates to the fungicides azoxystrobin, iprodione and thiophanate-methyl and *Colletotrichum* spp. isolates to azoxystrobin, difenoconazole and thiophanate-methyl; and to molecularly characterize isolates with different levels of sensitivity to these fungicides and to recognize the mechanisms related to resistant strains.

2.2 Material and Methods

2.2.1 Fungal isolates and culture

A total of 99 *Botrytis cinerea* isolates and 79 *Colletotrichum acutatum* isolates were collected from conventional and organic strawberry fields in 2013, 2014 and 2015 in four different Brazilian States: São Paulo, Minas Gerais, Espírito Santo and Bahia (Tables 2.1 and 2.2). Isolates from Espírito Santo and Bahia were provided by Dr. Helcio Costa, from INCAPER (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural).

Symptomatic flowers and fruit were collected and the pathogens were directly isolated by transferring spores from the lesions to water-agar (WA) medium. After two to three days, *B. cinerea* single-spore isolates were transferred to malt-yeast-agar (MYA - 20 g of malt extract, 5 g of yeast extract, 12 g of agar, and water to 1 L) medium and *C. acutatum* single-spore isolates were grown on potato dextrose agar (PDA, Difco®). The pathogens were grown for 7 to 15 days at 23 °C under constant light. The isolates were preserved on filter papers and stored in envelopes at - 20 °C.

Table 2.1 – Origin, codes and total number of *Botrytis cinerea* isolates collected from conventional and organic strawberry fields in 2013, 2014 and 2015

Year	Location (System)[a]	Grower/Farm	Isolates code[b]	Number of Isolates	Location code
2013	Valinhos-SP (C)	Daniel Juliato	C13-01	1	SP1C13
2013	Jarinu-SP (C)	Jurandir	C13-02 to C13-09	8	SP2C13
2013	Jarinu-SP (C)	Eduardo Mingoti	C13-10 to C13-17	8	SP3C13
2013	Piedade-SP (C)	Daniel Juliato	C13-18 to C13-21	4	SP4C13
2013	Piedade-SP (C)	Michel	C13-22	1	SP5C13
2013	São Paulo State (C)	Supermarket	C13-23	1	SP6C13
2013	Minas Gerais State (C)	Wholesale Market	C13-24	1	MG1C13
2013	V.N. Imigrantes-ES (C)	Caxixe Farm	C13-25 to C13-26	2	ES1C13
2013	D. Martins-ES (C)	Carmo Farm	C13-27	1	ES2C13
2013	Atibaia-SP (O)	Alberi Farm	O13-28 to O13-44	17	SP1O13
2013	Jarinu-SP (O)	Márcio	O13-45 to O13-46	2	SP2O13
2014	Valinhos-SP (C)	Daniel Juliato	C14-01 to C14-08	8	SP1C14
2014	Jarinu-SP (C)	Jurandir	C14-09 to C14-24	16	SP2C14
2014	Piedade-SP (C)	Michel	C14-25 to C14-29	5	SP5C14
2014	Ibicoara-BA (C)	Cascavel Farm	C14-30	1	BA1C14
2014	M. Floriano-ES (C)	Vitor Hugo	C14-31	1	ES3C14
2014	V.N. Imigrantes-ES (C)	Braço do Sul Farm	C14-32	1	ES4C14
2014	D. Martins-ES (C)	Sérgio Ronchi	C14-33	1	ES5C14
2014	D. Martins-ES (C)	Sator/Bioagro	C14-34	1	ES6C14
2014	Jarinu-SP (O)	Márcio	O14-35 to O14-40	6	SP2O14
2014	D. Martins-ES (O)	Pedra Azul Farm	O14-41	1	ES1O14
2014	Guaçui-ES (O)	São Pedro Farm	O14-42 to O14-43	2	ES2O14
2014	Guaçui-ES (O)	Isabel Louzada	O14-44	1	ES3O14
2014	Irupi-ES (O)	Irupi Farm	O14-45	1	ES4O14
2015	Valinhos-SP (C)	Daniel Juliato	C15-01 to C15-03	3	SP1C15
2015	Jarinu-SP (O)	Márcio	O15-04 to O15-08	5	SP2O15
Total				99	

[a] BA, ES, MG and SP represent Bahia, Espírito Santo, Minas Gerais and São Paulo States, respectively; V.N. is Venda Nova, D. is Domingos and M is Marechal. C and O represent the strawberry production system: conventional and organic, respectively

[b] C and O represent *Botrytis cinerea* isolates coming from conventional and organic fields, respectively

Table 2.2 – Origin, codes and total number of *Colletotrichum acutatum* isolates collected from conventional and organic strawberry fields in 2013, 2014 and 2015

Year	Location (System)[a]	Grower/Farm	Isolates code[b]	Number of isolates	Local code
2013	Jarinu-SP (C)	Antonio	C13-47 to C13-80	34	SP7C13
2013	Valinhos-SP (C)	Daniel Juliato	C13-81	1	SP1C13
2013	Piedade-SP (C)	Daniel Juliato	C13-82	1	SP4C13
2013	Piedade-SP (C)	Michel	C13-83 to C13-90	8	SP5C13
2013	D. Martins-ES (C)	Pedra Azul Farm	C13-91	1	ES7C13
2013	D. Martins-ES (C)	State Farm	C13-92	1	ES8C13
2013	Atibaia-SP (O)	Alberi Farm	O13-93 to O13-110	18	SP1O13
2013	D. Martins-ES (O)	Pedra Azul Farm	O13-111 to O13-112	2	ES1O13
2013	Irupi-ES (O)	Santa Clara Farm	O13-113	1	ES5O14
2014	S.M. Jetiba-ES (C)	Recreio Farm	C14-46	1	ES9C14
2014	D. Martins-ES (C)	Carmo Farm	C14-47	1	ES10C14
2014	D. Martins-ES (C)	Barcelos Farm	C14-48	1	ES11C14
2014	D. Martins-ES (O)	União Farm	O14-49	1	ES6O14
2015	Valinhos-SP (C)	Daniel Juliato	C15-09 to 15-16	8	SP1C15
Total				79	

[a] ES and SP represent Espírito Santo and São Paulo States, respectively. D. is Domingos and S.M. is Santa Maria. C and O represent the strawberry production system: conventional and organic, respectively

[b] C and O represent *Colletotrichum acutatum* isolates coming from conventional and organic fields, respectively

2.2.2 Fungicide sensitivity assays

2.2.2.1 Fungicides

B. cinerea isolates were tested for their sensitivity to azoxystrobin (Abound Flowable and Amistar 500 WG, both from Syngenta Crop Protection), iprodione (Rovral Brand 4 Flowable, Bayer Crop Science and Rovral SC, FMC) and thiophanate-methyl (Cercobin 700 WP, Ihara and Topsin 4.5 FL, UPI). *Colletotrichum* spp. isolates were tested for their sensitivity to azoxystrobin, thiophanate-methyl and difenoconazole (Score 250 EC, Sygenta Crop Protection). Fungicide stock solutions were prepared in sterile distilled water. Sensitivity to fungicides were tested by determining the EC_{50} values of the isolates, using mycelial growth (spiral gradient

dilution and plate methods), spore germination and fruit assays. EC_{50} represents the effective concentration that inhibit 50 % of a pathogen development.

For *in vitro* tests, salicylhydroxamic acid (SHAM, 99% a.i.; Sigma-Aldrich), which inhibits the fungal alternative respiration pathway, was dissolved in methanol and added to autoclaved medium at 100 µg/ml to test sensitivity of *Botrytis cinerea* isolates to azoxystrobin (AMIRI; HEATH; PERES, 2013). However, other studies evaluating QoI-fungicide sensitivity of *C. acutatum* from strawberry (FORCELINI et al., 2016), *C. graminicola* from turfgrass (AVILA-ADAME; OLAYO; WOLFRAM, 2003) and *C. acutatum* and *Alternaria alternata* from citrus (MONDAL et al., 2005) have shown SHAM does not have any effect on fungal development in the presence of strobilurins, suggesting these fungi do not utilize alternative respiration. Preliminary experiments were performed with some *C. acutatum* isolates with or without SHAM in PDA amended with azoxystrobin and it was observed that the use of SHAM did not affect mycelial growth nor spore germination of the isolates. Therefore, SHAM was not included in the fungicide-amended medium to test *C. acutatum* sensitivity.

2.2.2.2 Mycelial growth: Spiral gradient dilution method

Mycelial growth sensitivity of the isolates was evaluated using the spiral gradient dilution method (AMIRI; HEATH; PERES, 2013; FÖRSTER; KANETIS; ADASKAVEG, 2004). The EC_{50} values of *B. cinerea* isolates to azoxystrobin, iprodione and thiophanate-methyl, and *C. acutatum* isolates to azoxystrobin and difenoconazole were determined.

The characteristics of the fungicides and the concentration ranges are summarized in Table 2.3. The concentration ranges used for each combination pathogen-fungicide were based on the literature and preliminary tests.

Table 2.3 – Characteristics, concentration, and ranges of fungicides used to evaluate the sensitivity of *Botrytis cinerea* and *Colletotrichum acutatum* collected from strawberry fields

Active ingredient (a.i.)	Group Name (FRAC)	Stock Solutions a.i. (µg/ml)[a]	Range (µg/ml)[b]	Recommended field rates (g/L)
Azoxystrobin	QoI - Quinone Outside Inhibitors	930.41 [d]	0.035 to 7.19	0.080
		9304.10 [c, d]	0.350 to 71.9	
Difenoconazole	DMI - DeMethylation Inhibitors	1344.35 [d]	0.050 to 10.4	0.100
Iprodione	Dicarboximides	3476.19 [c]	0.150 to 27.22	0.075
Thiophanate-methyl	MBC - Methyl Benzimidazole Carbamates	1760.10 [c]	0.073 to 13.76	0.490
		5280.30 [c]	0.220 to 41.27	

[a] Stock solution calculated by the Spiral Gradient Endpoint (SGE)
[b] Radial concentration ranges from the center to the perimeter of the 150 mm Petri dish
[c] Concentration ranges used to test *B. cinerea* sensitivity
[d] Concentration ranges used to test *Colletotrichum* spp. sensitivity

In the original spiral gradient dilution method (FÖRSTER; KANETIS; ADASKAVEG, 2004), sterile cellophane strips (50 x 6 mm) are inoculated by submerging them in the spore suspension in a Petri dish, and placed on PDA for one or two days at 23 °C to allow spores to germinate on cellophane. However, the cellophane strips were replaced with agar strips, according to the method described by Amiri et al. (2013). To produce the agar strips, 50 ml of MYA containing 20 g/L of agar ($MYAA^+$) for *B. cinerea*, and PDA containing an additional 7 g/L of agar ($PDAA^+$) for *C. acutatum* were poured into 150-mm-diameter Petri dishes to obtain a 3.3-mm thick layer of agar. The additional amount of agar was used to harden the medium and facilitate strip manipulation. One milliliter of a spore suspension (10^6 spores/ml) of each isolate was spread onto the medium using a Drigalski spatula (Figure 2.1A). The spore suspensions were prepared by adding sterile distilled water to the fungi colonies and scraping the spores from the surface. The Petri dishes were sealed and incubated at 23 °C under constant light for 48 h for homogeneous growth of the pathogen (Figure 2.1B). For production of inoculum strips, an agar slicer was used to cut strips with the dimensions required for the spiral gradient dilution method. Fifteen agar strips (9 cm long and 6 mm wide), obtained by pressing the agar slicer into the media, were cut perpendicularly into 30 strips (4.5 cm long) (Figure 2.1C).

Figure 2.1 - One milliliter of spore suspension (10^6 spores/ml) was added and spread onto the hardened agar medium using a Drigalski spatula (A); homogeneous growth of *Botrytis cinerea* on the medium after incubation at 23 °C under constant light for 48 h (B); use of the agar slicer on the medium with the pathogen isolate to produce the inoculum strips (C)

For fungicide tests, 50 ml of PDA were poured into 150-mm-diameter Petri dishes at least 24 h prior to the addition of fungicide. Fifty microliters of each fungicide stock solution at the concentration indicated in Table 2.3 were applied exponentially on the plates using the spiral plater (Autoplate 4000 and 5000 models, Spiral Biotech), resulting in a radial gradient of 1:100. Four or six inoculum agar strips were applied on the 150-mm-diameter Petri dishes (two inoculums strips per isolate), using a SGE template (Figure 2.2A), placing them perpendicularly to the center of the plate (Figure 2.2B). Each isolate was replicated on three plates for each fungicide. A control treatment without fungicide was prepared identically except that four isolates were tested on each plate and three replications were used.

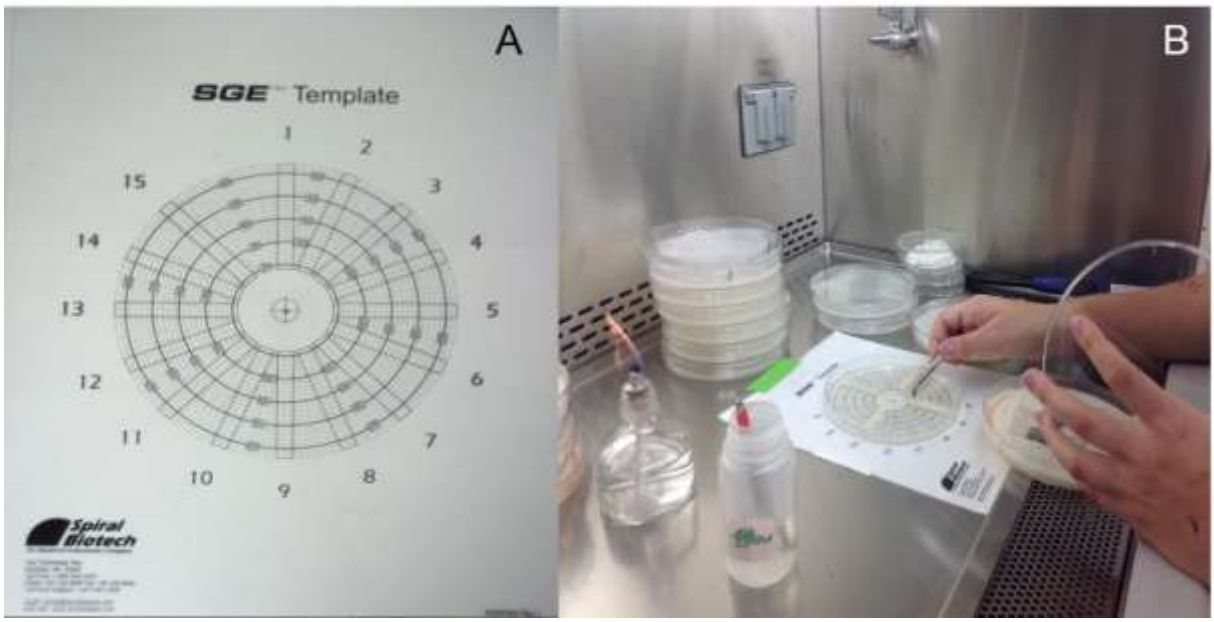

Figure 2.2 – Spiral Gradient Endpoint (SGE) template (A), and application of the inoculum agar strips on the 150-mm-diameter Petri dishes containing PDA with fungicide applied by the spiral plater (B)

Plates were incubated at 23 °C for two or three days for *B. cinerea* and *Co. acutatum*, respectively. The mycelial growth from isolates in the control was evaluated by measuring the radial growth, in centimeters (from the edge of the agar strips) (Figure 2.3A), and the average of three repetitions was divided by two, representing 50 % of the pathogen growth. In the fungicide treatments, the point where radial growth was half the average growth of the control was marked and the distance from this point to the center of the plate was measured in millimeters (Figure 2.3B). Finally, using the SGE software, the fungicide concentration at the point with the red line (Figure 2.3B) was calculated and the EC_{50} of each isolate for each fungicide was determined. The experiments were performed twice.

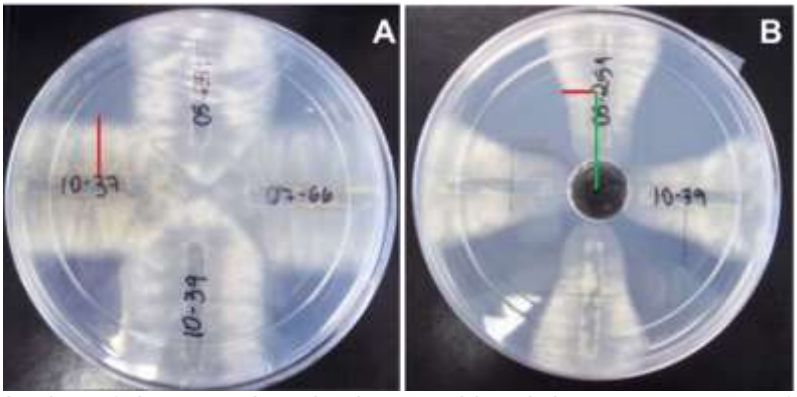

Figure 2.3 – Evaluation of the control evaluation considered the measurement of the radial growth (from the edge of the agar strips) (red line) (A). Fungicide treatment evaluation demonstrating the point where radial growth is half the average growth of the control (red line) and the distance from this point to the center of the plate (green line) (B)

2.2.2.3 Mycelial growth: Plate method

Sensitivity of *C. acutatum* isolates to thiophanate-methyl was tested using the plate method, since the EC_{50} values could not be determined by the spiral gradient dilution method. Isolates mycelial discs (5-mm-diameter), grown on PDA for 7 days, were transferred to plates containing PDA amended with thiophanate-methyl at 1, 10 and 100 µg/ml. Control treatments did not receive fungicide. Three replications per treatment were used and experiments were performed twice. Plates were incubated at 23 °C under constant light for five days. The average colony diameter (two perpendicular measurements) was calculated and the mycelium growth reduction was expressed as MGR = ((C-FT)/C)*100), in which MGR is the mycelial growth reduction, C is the colony diameter of the control treatment and FT is the colony

diameter of the fungicide treatment. The results were expressed as percentage of mycelial growth reduction.

2.2.2.4 Spore germination assay

Sensitivity to azoxystrobin of some isolates of *B. cinerea* and *C. acutatum*, respectively, was tested in a spore germination assay. Spore suspensions of the isolates of both pathogens were prepared by adding sterile distilled water to 7 to 14-days-old colonies and scraping the spores from the surface. Spore concentration was determined using a hemocytometer and adjusted to 1 x 10^5 and 5 x 10^4 spores/ml for *B. cinerea* and *C. acutatum*, respectively. Three aliquots (30 µL each) of spore suspensions were placed on plates containing media amended with the fungicide. For *B. cinerea*, malt-extract-agar (MEA - 10 g of malt extract, 15 g of agar, and water to 1 L) medium amended with SHAM (100 µg/ml) and azoxystrobin at 0.1, 0.5, 1, 5, 10, and 100 µg/ml was used. For *C. acutatum*, water-agar medium amended with azoxystrobin at 0.01, 0.1, 1, 10 and 100 µg/ml was tested. Control did not receive fungicide. Three plates (replications) per treatment were used and experiments were performed twice. Plates were incubated at 23 °C under constant light inside a plastic box with water, to provide high humidity. Spore germination was evaluated after 16 to 24 h and 9 h incubation for *B. cinerea* and *C. acutatum*, respectively. Spores with a germ tube length greater than their diameter were considered germinated. The germination percentage was determined by counting the first 100 spores observed under a microscope. Results were expressed as the percentage of germinated spores for the different treatments and the EC_{50} was calculated by linear regressions of spore germination on the $\log_{10}$-transformed fungicide doses.

2.2.2.5 Fruit assay

To evaluate the occurrence of practical resistance, isolates with different levels of sensitivity were inoculated on strawberry fruit treated with fungicides. Nine *B. cinerea* isolates and 12 *C. acutatum* isolates with different EC_{50} values were tested. Fruit that have never been sprayed with systemic fungicides were harvested at the green-white stage (before commercial ripeness) from a commercial organic grower in Jarinu, SP, Brazil and from experimental plots at the Gulf Coast Research and

Education Center, in the University of Florida, Wimauma, FL, USA. All the experiments with *B. cinerea* isolates were performed in USA with the strawberry cultivar 'Radiance', and the experiments with *C. acutatum* were conducted in Brazil with cultivar 'Camarosa'. The first experiments were conducted with *C. acutatum* and due to the end of the strawberry season in Brazil, the other trials with *B. cinerea* were carried out in USA.

After harvest, fruit had sepals removed and were washed in 0.05 % sodium hypochlorite for 2 min, then rinsed twice in distilled water, and allowed to dry at room temperature. After that, strawberries were placed in egg trays (Figure 2.4A) inside plastic boxes and sprayed with the fungicide solutions prepared in sterile distilled water: azoxystrobin (0.08 g/L), iprodione (0.075 g/L) and thiophanate-methyl (0.49 g/L) for *B. cinerea* trials, and azoxystrobin (0.08 g/L), difenoconazole (0.1 g/L) and thiophanate-methyl (0.49 g/L) for *C. acutatum* experiments at recommended field rates in Brazil, according to Table 2.3 using a spray gun (Figure 2.4B). Non-treated controls were sprayed with sterile distilled water. Fruit were incubated for 24 h at room temperature, then inoculated with 30 μL of the spore suspensions at 10^5 or 10^6 spores/ml for *B. cinerea* or *C. acutatum*, respectively. For *B. cinerea* trials, fruit were wounded (1 mm in diameter by 3 mm in depth) with a hypodermic needle at the location of the droplet after pathogen inoculation. Inoculated fruit were kept in sealed moisture chambers at 23 °C and disease incidence and severity were assessed 4 to 7 days after inoculation. For each isolate, three replications per treatment and five fruit per replicate were used and the experiments were conducted twice. Disease incidence was determined as the frequency of fruit showing symptoms compared to the total of inoculated fruit, and disease severity was calculated as the area (proportion) of the visible part of each fruit that became infected.

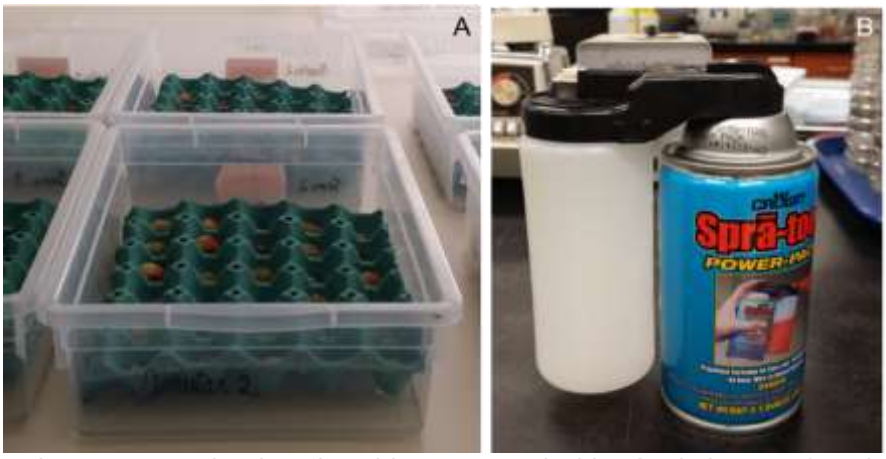

Figure 2.4 – Fruit assay: strawberries placed in egg trays inside plastic boxes after cleaning (A) and ready to be sprayed with sterile distilled water or fungicide solutions with a spray gun (B) according to the treatments of *B. cinerea* or *Colletotrichum* spp. trials

2.2.3 Data analysis

For the spiral gradient dilution method experiment, EC_{50} data from isolates from repeated experiments were compared by ANOVA and the interaction between experiments, and isolates and experiments were combined after tests for homogeneity indicated that variances were similar. Percentage of mycelial growth reduction from isolates from repeated experiments were combined for analysis for the discriminatory doses assay. Means of mycelial growth reduction were compared by Tukey's test. For the spore germination assay, data from repeated experiments were combined after ANOVA and linear regressions of spore germination on the log_{10}-transformed fungicide doses were used to estimate EC_{50} values. Disease incidence and severity of treated fruit were estimated in comparison to the non-treated control in the fruit assay. Data were compared by ANOVA and Tukey's test. Statistical analyses were performed using the Statistica 7.0 (Statsoft, Tulsa, USA) and the SAS (Version 9.2, SAS Institute Inc.) softwares.

2.2.4 Molecular characterization of *Botrytis cinerea* and *Colletotrichum acutatum* isolate mutations linked to resistance to fungicides

2.2.4.1 DNA extraction and molecular identification of the isolates

DNA extraction and molecular characterization of *B. cinerea* and *C. acutatum* isolates were conducted at the Gulf Coast Research and Education Center (GCREC)

in the University of Florida, USA. *B. cinerea* and *C. acutatum* isolates were cultured on MYA and PDA media, respectively, for 7 days and mycelium and spores were collected to perform DNA extraction. Genomic DNA was extracted using the FastDNA®Kit (MP Biomedicals, LLC), according to manufacturer´s protocol. DNA concentration of the samples was measured using a spectrophotometer (Nanodrop, ND 1000, Version 3.8.1). The samples were stored at – 20 °C.

C. acutatum isolates were subjected to polymerase chain reaction (PCR) to confirm the identity of the species as belonging to the *C. acutatum* complex. The primers used were ITS4-Universal (5'-TCCTCCGCTTATTGATATGC-3') and Cacut-Int2 (5'-GGGGAAGCCTCTCGCGG-3'). The primers ITS4-Universal and ITS1 – C.g./C.f. (5'-GACCCTCCCGGCCTCCCGCC-3'), usually used for *C. gloeosporioides* complex, were also tested. PCR was performed in a 20-µL volume containing 2 µL of 20 Mm $MgCl_2$ 10X buffer (Thermo Scientific), 0.4 µL of 10 mM dNTPs (Promega Corp.), 1 µL of each 10 µM primer, 0.4 µL of 5 U/ µL of Taq polymerase (Thermo Scientific) and 2 µL of DNA. PCR was performed in a PTC-200 thermal cycler (MJ Research) using the following parameters: an initial denaturation at 95 °C for 5 min; 32 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. For *C. gloeosporioides*, 26 cycles were used. PCR products were visualized under UV-light on a 1 % agarose gel in 1X Tris-acetate-EDTA buffer (0.04 M Tris-Acetate, 0.0001 M EDTA) with ethidium-bromide staining at 100 V for 25 min. Water was used as negative control and one isolate of each *Colletotrichum* group was used as positive control.

2.2.4.2 Molecular characterization of mutations linked to resistance to QoI fungicides (azoxystrobin): cytochrome *b* gene (*cytb*)

2.2.4.2.1 *Botrytis cinerea*

To identify potential mutations in the *cytochrome b* gene (*cytb*) of *B. cinerea* isolates, especially related to point mutations at codon 143 (G143A) known to confer high levels of resistance to QoI fungicide, the partial *cytb* gene was amplified using the primers Qo13ext (5'-GGTATAACCCGACGGGGTTATAGAATAG-3') and Qo14ext (5'-AACCATCTCCATCCACCATACCTACAAA-3') (FERNÁNDEZ-ORTUÑO; CHEN; SCHNABEL, 2012). PCR was performed in a final volume of 25 µL containing 3 µL of

7.5 mM $MgCl_2$ 5X Buffer (Phire Hot Start II - Thermo Scientific), 2 µL of 25 mM $MgCl_2$, 1 µL of 5 M betaine (Sigma-Aldrich®), 1 µL of 10 mM dNTPs (Promega Corp.), 1.5 µL of each 10 µM primer, 0.1 µL of 0.5 unit of Taq polymerase (Phire Hot Start II - Thermo Scientific) and 2 µL of DNA at 75 ng/ml. PCR was performed using the following parameters: an initial denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were separated in ethidium-bromide-stained 1 % agarose gel as previously described for *Colletotrichum* spp. identification.

The *cytb* gene PCR products obtained were digested using the restriction enzyme *Fnu*4HI (Thermo Scientific). A mix was prepared using 2 µL of the enzyme SatI, 1 µL of the 10X buffer G and 7 µL of ultrapure water. One microliter of this mix was added to 9 µL of ultrapure water, 1 µL of 10X buffer G and 5 µL of an unpurified *cytb* PCR product of each isolate. The digestion was carried out at 37 °C for 2 h and the digested fragments were visualized by electrophoresis in 2 % agarose gel. The presence of a mutation at the codon 143 was identified by the presence of a two-band profile with fragments of 318 and 242 bp long; and the 560 bp fragment remained undigested in the absence of mutation. The *cytb* gene PCR products of some isolates were also purified using the ExoSAP-IT PCR purification kit (Affymetrix, Inc.) by using 17 µL of the PCR products and 3 µL of the kit followed by incubation at 37 °C for 15 min and 80 °C for 15 min. Purified PCR product of each sample was divided in two tubes and 2.5 µL of ultrapure water and 2.5 µL of each primer (10 µM) were added to each one of the tubes. Some selected samples were sent to Genewiz Incorporation (South Plainfield, NJ) for sequencing in both directions to identify possible mutations at the partial *cytb* gene, including G137R and G143A. Sequences were assembled, translated and aligned using the MEGA 6 Version and BioEdit version 7.2.5., and analyzed by BlastN against the GenBank database.

2.2.4.2.2 *Colletotrichum acutatum*

To investigate potential mutations in the *cytochrome b* gene (*cytb*) of *C. acutatum* isolates, especially related to point mutations at codons 129, 137 and 143, the partial *cytb* gene was amplified using the primers gramiF1 (5'-GAAGAGGTATGTACTACGGTTCATATAG-3') and gramiR1 (5'-TAGCAGCTGGAGTTTGCATAG-3') (FORCELINI et al., 2016). PCR was performed

in a final volume of 25 µL containing 1.9 µL of 10X Buffer (Thermo Scientific), 1.6 µL of 25 mM $MgCl_2$, 0.5 µL of 5 M betaine (Sigma-Aldrich®), 0.5 µL of 10 mM dNTPs (Promega Corp.), 0.25 µL of each 10 µM primer, 0.3 µL of Taq polymerase (Thermo Scientific) and 2 µL of DNA at 75 ng/ml. PCR was performed using the following parameters: an initial denaturation at 95 °C for 3 min; 34 cycles of 95 °C for 40 s, 60 °C for 50 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min (FORCELINI et al., 2016). PCR products were separated in ethidium-bromide-stained 1 % agarose gel as previously described.

The *cytb* gene PCR products of nine isolates with different EC_{50} values were purified using the ExoSAP-IT PCR purification kit (Affymetrix, Inc.) as previously described for *B. cinerea cytochrome b* gene. Some selected samples were sent for sequencing in both directions to identify possible mutations at the partial *cytb* gene, including F129L, G137R and G143A. Sequences were assembled, translated, aligned and analyzed as previously described.

2.2.4.3 Molecular characterization of mutations linked to resistance to benzimidazole fungicides (thiophanate-methyl): Beta-tubulin gene (*β-tub*)

2.2.4.3.1 *Botrytis cinerea*

Some *B. cinerea* isolates were investigated to determine potential mutations in the beta-tubulin gene (*β-tub*), where substitutions at positions 167 (F167Y), 198 (E198A/K/V) and 200 (F200Y) may be present (BANNO et al., 2008). A portion of the β-tubulin gene was amplified using primers 155 (5'-CAACCTTCAAAATGCGTGAG-3') and 1174 (5'-AGATGGGTTGCTGAGCTTCA-3') (FEKETE et al., 2012; FOURNIER et al., 2005). PCR was performed as described for the *B. cinerea cytochrome b* gene except for 55 °C annealing temperature. PCR products were visualized under UV-light on a 0.75 % agarose gel in 1X Tris-acetate-EDTA buffer with ethidium-bromide staining. For some isolates, a two-band profile was observed and extracted using QIAquick® Gel Extraction Kit (QIAGEN®), following the manufacturer's protocol. The *β-tub* gene PCR and extraction products were purified using the ExoSAP-IT PCR purification kit, and some isolates with different EC_{50} values were sent for sequencing to identify possible mutations at key points of the

gene. Sequences were assembled, translated, aligned and analyzed as previously described.

2.2.4.3.1 *Colletotrichum acutatum*

To identify potential mutations in the beta-tubulin gene, where point mutations at positions 167, 198 and 200 usually occur (WONG et al., 2008; YOUNG et al., 2010b), some *C. acutatum* isolates were selected. A portion of the beta-tubulin gene was amplified using primers TB2L (5'-GYTTCCAGATYACCCACTCC-3') and TB2R (5'-TGAGCTCAGGAACRCTGACG-3') (PERES et al., 2004). PCR was performed in a final volume of 25 µL containing 1.9 µL of 10X Buffer (Thermo Scientific), 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of 5 M betaine (Sigma-Aldrich®), 0.5 µL of 10 mM dNTPs (Promega Corp.), 0.5 µL of each 10 µM primer, 0.1 µL of Taq polymerase (Thermo Scientific) and 2 µL of DNA at 75 ng/ml. PCR was performed using the following parameters: an initial denaturation at 95 °C for 2 min; 40 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were visualized under UV-light on a 1 % agarose gel in 1X Tris-acetate-EDTA buffer with ethidium-bromide staining at 100 V for 25 min. The *β-tub* gene PCR products were purified using the ExoSAP-IT PCR purification kit, and some isolates were sequenced to identify possible mutations at key points of the gene. Sequences were assembled, translated, aligned and analyzed as previously described.

2.2.4.4 Molecular characterization of mutations linked to resistance to dicarboximide fungicides (iprodione) in *Botrytis cinerea*: *bos1* gene

To investigate molecular mechanisms of iprodione resistance, some *B. cinerea* isolates were sequenced to identify potential mutations in the *bos1* gene associated with iprodione (histidine kinase – dicarboximide fungicides) resistance, especially at the amino acid codons 127 (F127S), 365 (I365N/R/S), 369 (Q369H/P), and 373 (N373S) (GRABKE et al., 2014). Analyses of position 127 of *bos1* gene could be possible when PCR was performed using the primer pair BF1 (5'-TACCGATCGAAAAACCCAAC-3') and BR1 (5'-TGGGCTGGTCTCTCAATCTT-3'), and the other positions could be investigated when the primer pair BF2 (5'-

CAACGTTATGGCACAAAATCTCA-3') and BR2 (5'-AAGTTTCTGGCCATGGTGTTCA-3') were used (MA et al., 2007).

For the primer pair BF1/BR1, PCR was performed as described for *B. cinerea cytb* gene, however 0.5 µL of 25 mM $MgCl_2$ was used and an initial denaturation at 95 °C for 3 min; 30 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min were performed. For the primer pair BF2/BR2, PCR was conducted in a final volume of 25 µL containing 2.5 µL of Thermo Pol Buffer (BioLabs), 0.5 µL of 10 mM dNTPs (Promega Corp.), 1 µL of each 10 µM primer, 0.125 µL of Taq polymerase (Thermo Pol Buffer - BioLabs) and 2 µL of DNA at 25 ng/ml. The following parameters were used: an initial denaturation at 95 °C for 3 min; 40 cycles of 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min. PCR products were separated in ethidium-bromide-stained 1 % agarose gel as previously described for *cytb* gene, purified using the ExoSAP-IT PCR purification kit, sent for sequencing at Genewiz Incorporation, and the sequences were analyzed as previously described.

2.3 Results

2.3.1 Fungicide sensitivity assays

2.3.1.1 Mycelial growth: Spiral gradient dilution method – *Botrytis cinerea*

Overall, EC_{50}s of 67 isolates (67.7 %) were higher than 71.9 µg/ml and those were considered resistant to azoxystrobin (Table 2.4). The growth of these isolates was not inhibited when azoxystrobin was applied at a radial concentration range from the center to the perimeter of the plate of 0.035 to 71.9 µg/ml, showing that their EC_{50} values were higher than 71.9 µg/ml.

Table 2.4 – Sensitivity of *Botrytis cinerea* isolates collected from organic and conventional strawberry fields to azoxystrobin, iprodione and thiophanate-methyl fungicides and their EC_{50} values range, in µg/ml, using the spiral gradient dilution method

(continue)

Location[a]	Azoxystrobin				Iprodione				Thiophanate-methyl			
	Range EC_{50} (µg/ml)[b]		*n* isolates[c]		Range EC_{50} (µg/ml)[b]		*n* isolates[c]		Range EC_{50} (µg/ml)[b]		*n* isolates[c]	
	S	R	S	R	S	R	S	R	S	R	S	R
SP1C13	..	>71.9	0	1	..	3.07	0	1	..	>688	0	1
SP2C13	..	>71.9	0	8	0.90	1.46-5.70	1	7	..	>688	0	8
SP3C13	0.66	>71.9	1	7	0.38	1.65-3.5	1	7	1.25	>688	1	7
SP4C13	..	>71.9	0	4	0.60-0.70	2.39-5.35	2	2	0.89	>688	1	3
SP5C13	1.19	..	1	0	..	2.31	0	1	..	>688	0	1
SP6C13	..	>71.9	0	1	..	1.87	0	1	..	>688	0	1
MG1C13	..	>71.9	0	1	..	4.28	0	1	..	>688	0	1
ES1C13	0.81-1.06	..	2	0	0.42-0.58	..	2	0	0.75-2.13	..	2	0
ES2C13	..	>71.9	0	1	..	2.31	0	1	..	>688	0	1
SP1O13	0.74-2.85	>71.9	11	6	0.76-1.1	1.74-4.49	14	3	0.48-6.34	>688	8	9
SP2O13	0.79-1.22	..	2	0	0.57-0.73	..	2	0	0.51-0.88	..	2	0

Table 2.4 – Sensitivity of *Botrytis cinerea* isolates collected from organic and conventional strawberry fields to azoxystrobin, iprodione and thiophanate-methyl fungicides and their EC_{50} values range, in µg/ml, using the spiral gradient dilution method

(conclusion)

Location[a]	Azoxystrobin				Iprodione				Thiophanate-methyl			
	Range EC_{50} (µg/ml)[b]		*n* isolates[c]		Range EC_{50} (µg/ml)[b]		*n* isolates[c]		Range EC_{50} (µg/ml)[b]		*n* isolates[c]	
	S	R	S	R	S	R	S	R	S	R	S	R
SP1C14	..	>71.9	0	8	..	2.14-3.82	0	8	..	>688	0	8
SP2C14	..	>71.9	0	16	0.70-1.06	1.43-5.03	3	13	..	>688	0	16
SP5C14	..	>71.9	0	5	0.55	1.46-4.70	1	4	..	>688	0	5
BA1C14	1.02	..	1	0	0.83	..	1	0	..	>688	0	1
ES3C14	1.19	..	1	0	0.33	..	1	0	..	0.87	1	0
ES4C14	0.96	..	1	0	0.50	..	1	0	..	>688	0	1
ES5C14	1.42	..	1	0	0.36	..	1	0	..	>688	0	1
ES6C14	..	>71.9	0	1	0.42	..	1	0	..	>688	0	1
SP2O14	0.70-4.34	>71.9	5	1	0.42-0.59	2.08 - 2.12	4	2	..	>688	0	6
ES1O14	3.85	..	1	0	0.81	..	1	0	0.51	..	1	0
ES2O14	0.87	>71.9	1	1	0.38-0.52	..	2	0	0.77-0.86	..	2	0
ES3O14	2.35	..	1	0	0.43	..	1	0	1.44	..	1	0
ES4O14	3.4	..	1	0	..	5.96	0	1	..	2.87	1	0
SP1C15	..	>71.9	0	3	..	2.35-4.3	0	3	..	>688	0	3
SP2O15	0.72-2.38	>71.9	2	3	0.45-0.65	1.80-4.36	3	2	1.23-7.50	>688	2	3
Total/Mean[d]	1.47	>71.9	32	67	0.61	2.97	42	57	1.71	>688	22	77

[a] Location codes according to Table 2.1
[b] Effective concentration range, in µg/ml, that inhibited mycelial growth of *B. cinerea* isolates by 50 % using the spiral gradient dilution method
[c] S and R represent the number of sensitive and resistant isolates, respectively
[d] Total and mean represent the total number of isolates in each class and the mean EC_{50} values of isolates, respectively

Among the isolates from organic fields, 31.4 % showed resistance to the fungicide (Figure 2.5A) and 68.6 % had EC_{50} values lower than 4.5 µg/ml and were considered sensitive (Figure 2.5B and Table 2.4). Two organic growers from São Paulo State had isolates resistant to azoxystrobin: six of 17 isolates from the location SP1O13 (Alberi Farm in Atibaia, SP) and four of 11 isolates from the locations SP2O14 and SP2O15 (Marcio in Jarinu, SP at 2014 and 2015, respectively). One organic grower from Espírito Santo State (ES2O14: São Pedro Farm in Guaçaí, ES) had one of two isolates resistant to the fungicide (Table 2.4). Among isolates from conventional fields, 87.5 % were considered resistant to azoxystrobin (Figure 2.5A). Sensitive isolates had with EC_{50} values lower than 2.5 µg/ml (Figure 2.5B and Table 2.4). The EC_{50} values of sensitive isolates collected from organic and conventional fields varied from 0.66 to 4.34 µg/ml (Table 2.4).

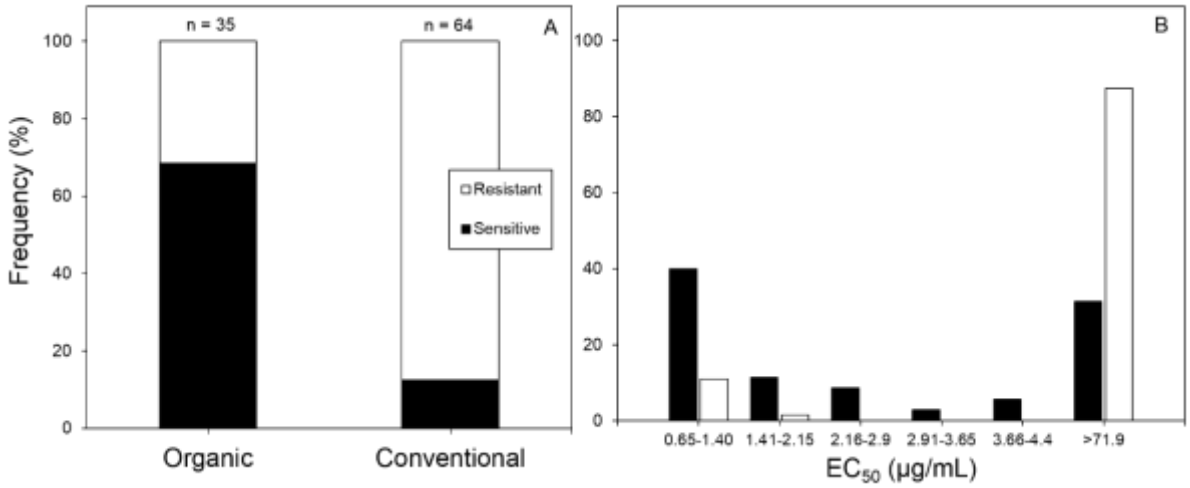

Figure 2.5 – Characterization of *Botrytis cinerea* isolates sensitivity to azoxystrobin: (A) frequency of sensitive (black) and resistant (white) isolates collected from organic (n = 35) and conventional (n = 64) strawberry fields; (B) frequency of isolates from organic (black bars) and conventional fields (white bars) based on their EC_{50} values, in µg/ml. Isolates were considered resistant if their EC_{50} values were higher than 71.9 µg/ml in the mycelial growth assay

All *B. cinerea* isolates were tested for their sensitivity to iprodione. Overall, EC_{50}s of 57 isolates (57.6 %) were higher than 1.2 µg/ml and those were considered resistant to iprodione (Table 2.4). Isolates from organic fields had EC_{50} values ranging from 0.38 to 5.96 µg/ml (Table 2.4); and 77.1 % of them had mean EC_{50} values lower than 1.2 µg/ml and were considered iprodione-sensitive (Figure 2.6A). Two organic growers from São Paulo State and one organic grower from Espírito Santo State presented resistant isolates to iprodione: three of 17 isolates from the

location SP1O13 (Alberi Farm in Atibaia, SP), two of six and two of five from the locals SP2O14 and SP2O15, respectively (Marcio in Jarinu, SP at 2014 and 2015, respectively), and the only isolate from local ES4O14 (Irupi Farm in Irupi, ES) (Table 2.4). The mean EC_{50} for isolates from conventional fields varied from 0.33 to 3.82 µg/ml (Table 2.4 and Figure 2.6B). Among these isolates, 76.6 % were considered resistant to iprodione and their EC_{50} values were higher than 1.2 µg/ml (Figure 2.6A).

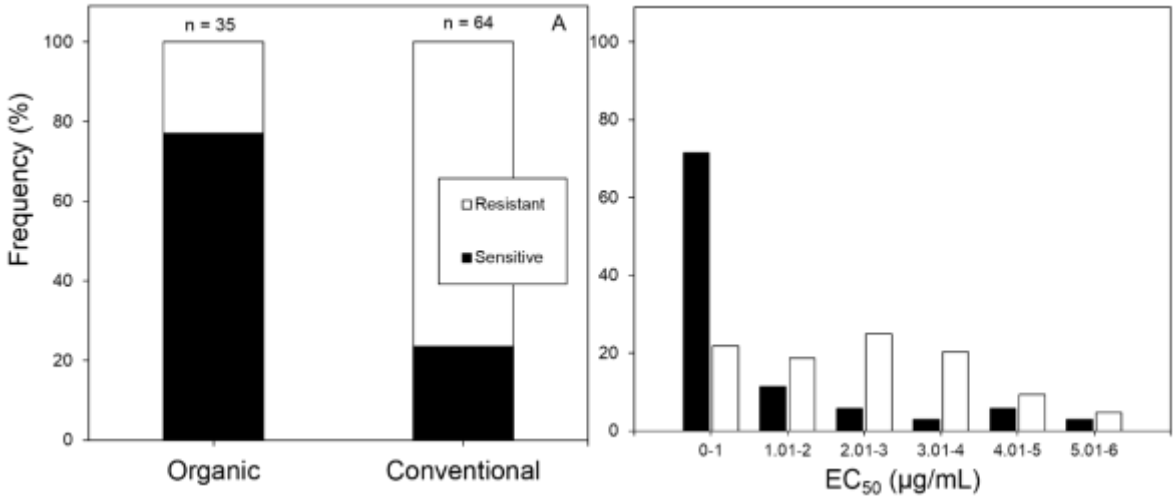

Figure 2.6 – Characterization of *Botrytis cinerea* isolates sensitivity to iprodione: (A) frequency of sensitive (black) and resistant (white) isolates collected from organic (n = 35) and conventional (n = 64) strawberry fields; (B) frequency of isolates from organic (black bars) and conventional fields (white bars) based on their EC_{50} values, in µg/ml. Isolates were considered resistant when their EC_{50} values were higher than 1.2 µg/ml in the mycelial growth assay

Overall, EC_{50}s of 77 isolates (77.8 %) were higher than 688 µg/ml and those were considered resistant to thiophanate-methyl (Table 2.4). The growth of these isolates was not inhibited on PDA amended with thiophanate-methyl applied at a radial concentration range from the center to the perimeter of the plate of 3.63 to 688 µg/ml, showing that their EC_{50} values were higher than 688 µg/ml. Among the isolates from organic fields, 51.4 % showed resistance to the fungicide (Figure 2.7A) and 48.6 % had EC_{50} values lower than 7.5 µg/ml and were considered sensitive (Figure 2.7B and Table 2.4). Two organic growers from São Paulo State had resistant isolates to thiophanate methyl: nine of 17 isolates from the location SP1O13 (Alberi Farm in Atibaia, SP) and nine of 11 isolates from the localtions SP2O14 and SP2O15 (Marcio in Jarinu, SP at 2014 and 2015, respectively) (Table 2.4). Among isolates from conventional fields, 92.2 % were considered resistant to thiophanate-methyl and 7.8 % were considered sensitive (Figure 2.7A) with EC_{50} values lower

than 3 µg/ml (Figure 2.7B and Table 2.4). The EC_{50} values of sensitive isolates collected from organic and conventional fields varied from 0.4 to 7.5 µg/ml (Table 2.4).

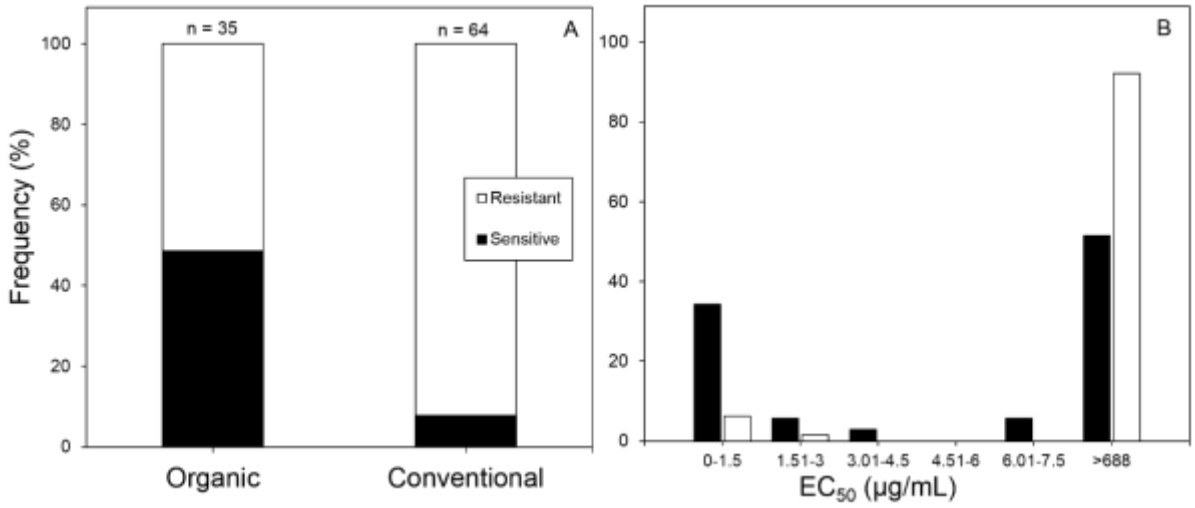

Figure 2.7 – Characterization of *Botrytis cinerea* isolates sensitivity to thiophanate-methyl: (A) frequency of sensitive (black) and resistant (white) isolates collected from organic (n = 35) and conventional (n = 64) strawberry fields; (B) frequency of isolates from organic (black bars) and conventional fields (white bars) based on their EC_{50} values, in µg/ml. Isolates were considered resistant when their EC_{50} values were higher than 688 µg/ml in the mycelial growth assay

Among all *B. cinerea* isolates, 16 isolates, 12 from organic (34.3 %) and 4 from conventional (6.25 %) fields were sensitive to all the fungicides tested; and 51 isolates had reduced sensitivity to all of them: 8.6 % and 75 % of isolates collected from organic and conventional fields, respectively, were considered resistant to azoxystrobin, iprodione and thiophanate-methyl (Figure 2.8).

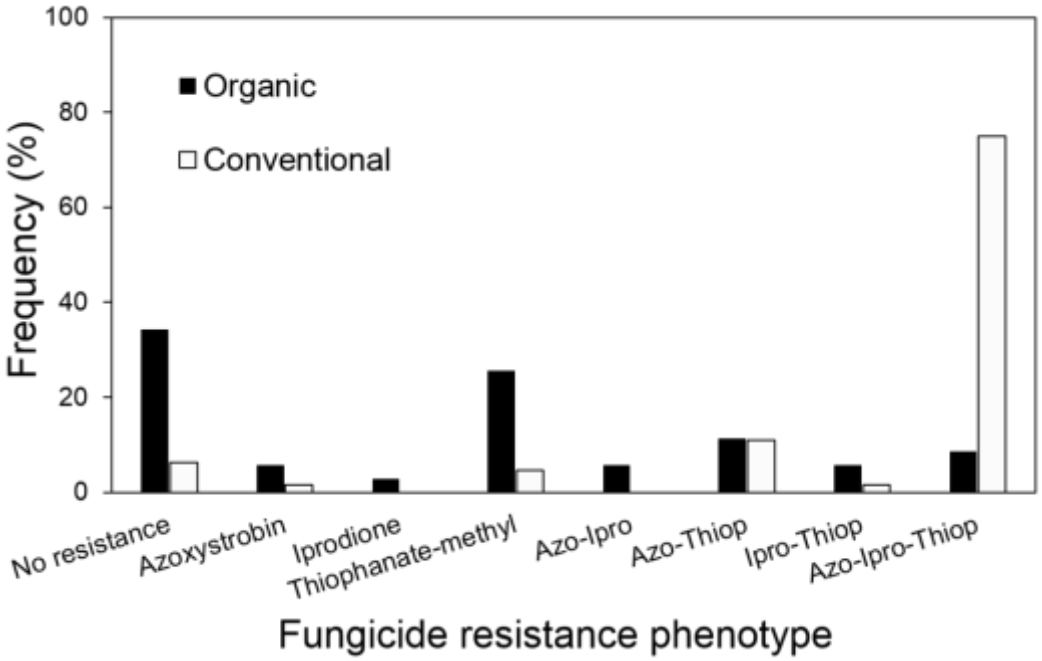

Figure 2.8 – Overall frequency of fungicide resistance phenotype observed in *Botrytis cinerea* populations across strawberry organic (black bars) and conventional (white bars) fields. Azo-Ipro, Azo-Thiop, Ipro-Thiop and Azo-Ipro-Thiop indicate resistance to azoxystrobin and iprodione, azoxystrobin and thiophanate-methyl, iprodione and thiophanate-methyl and azoxystrobin, iprodione and thiophanate-methyl, respectively

Only three isolates, two (5.7 %) and one (1.6 %) from organic and conventional fields, respectively, were resistant only to azoxystrobin; one isolate, from an organic grower, was moderately resistant to iprodione (2.85 %); and 12 isolates, 9 from organic (25.7 %) and 3 from conventional (4.7 %) fields were resistant to thiophanate-methyl only (Figure 2.8). Two isolates, from organic fields, had reduced sensitivity to both azoxystrobin and iprodione (5.7 %); 11 isolates, four from organic (11.4 %) and seven from conventional (10.9 %) growers, showed resistance to both azoxystrobin and thiophanate-methyl; and three isolates, two from organic (5.7 %) and one from conventional (1.6 %) areas had reduced sensitivity to both iprodione and thiophanate-methyl (Figure 2.8).

2.3.1.2 Mycelial growth: Spiral gradient dilution method – *Colletotrichum acutatum*

EC_{50} values of *C. acutatum* isolates to azoxystrobin ranged from 0.074 to 4.17 µg/ml and the mean value was 0.581 µg/ml (Table 2.5). EC_{50} values of isolates collected from organic fields varied from 0.14 to 1.25 µg/ml and the average value

was 0.44 µg/ml; and from conventional fields from 0.08 to 4.17 µg/ml, mean 0.636 µg/ml (Figure 2.8A). Among all the isolates, 77.2 % of the isolates from organic fields and 80.7 % from conventional growers had EC_{50} values ranging from 0.074 to 0.6 µg/ml and 12.3 % of the isolates from conventional growers had EC_{50} values higher than the range of isolates from organic farms.

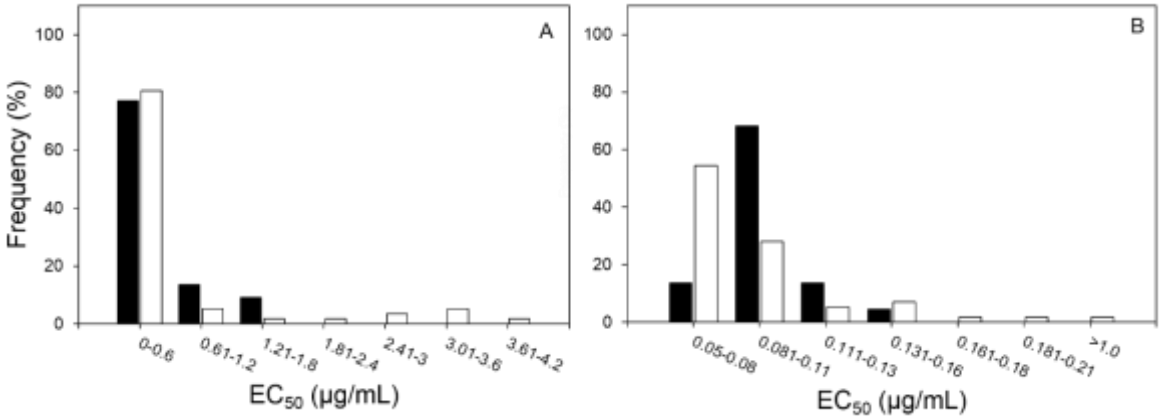

Figure 2.8 – EC_{50} (µg/ml) distribution of *Colletotrichum* spp. isolates collected from organic (black bars) and conventional (white bars) strawberry fields to azoxystrobin (A) and difenoconazole (B), using the spiral gradient dilution method (mycelial growth assay)

EC_{50} values of *C. acutatum* isolates to difenoconazole varied from 0.05 to 1.21 µg/ml and the average value was 0.104 µg/ml (Table 2.5). EC_{50} values of isolates from organic fields varied from 0.06 to 0.14 µg/ml, mean of 0.095 µg/ml; and from conventional growers from 0.05 to 1.21 µg/ml and mean value of 0.107 µg/ml (Figure 2.8B). Among all the isolates, 95.5 % of the isolates from organic fields and 87.7 % from conventional growers had EC_{50} values ranging from 0.05 to 0.13 µg/ml (Figure 2.8B) and 5.3 % of the isolates from conventional growers had EC_{50} values higher than the range of isolates from organic farms.

EC_{50} values of *C. acutatum* isolates for the fungicide thiophanate-methyl could not be determined using the spiral gradient dilution method. Forty-four isolates were tested, however the results varied among the experiments. Isolates collected from conventional and organic fields behaved the same way in the presence of the fungicide. For this reason, the discriminatory doses assay was performed.

Table 2.5 – Sensitivity of *Colletotrichum acutatum* isolates collected from organic and conventional strawberry fields to azoxystrobin, difenoconazole and thiophanate-methyl fungicides using the spiral gradient dilution or discriminatory dose assay methods

Location[b]	Number of isolates	Azoxystrobin[a]	Difenoconazole[a]	Thiophanate-methyl[a]	
		Range EC_{50} (µg/ml)[c]		EC_{50} (µg/ml)[d]	
				<100	>100
SP7C13	34	0.095 - 4.164	0.054 - 0.207	0	34
SP1C13	1	0.498	0.108	0	1
SP4C13	1	0.394	0.390	0	1
SP5C13	8	0.091 - 0.496	0.059 - 1.21	0	8
ES7C13	1	1.772	0.064	0	1
ES8C13	1	0.588	0.093	0	1
SP1O13	18	0.140 - 1.254	0.060 - 0.114	16	2
ES1O13	2	0.563 - 1.204	0.081 - 0.130	0	2
ES5O14	1	0.157	0.069	0	1
ES9C14	1	0.185	0.177	1	0
ES10C14	1	0.280	0.070	0	1
ES11C14	1	0.226	0.144	0	1
ES6O14	1	0.258	0.081	0	1
SP1C15	8	0.074 - 0.177	0.079 - 0.154	0	8
Total/Mean[e]	79	0.577	0.103	17	62

[a] Azoxystrobin and difenoconazole were tested using the spiral gradient dilution method and thiophanate-methyl using the discriminatory dose assay
[b] Location codes according to Table 2.2
[c] Effective concentration range, in µg/ml, that inhibited mycelial growth of *C. acutatum* isolates by 50 % using the spiral gradient dilution method
[d] Effective concentration average, in µg/ml, that inhibited mycelial growth of *C. acutatum* isolates by 50 % using the plate assay method
[e] Total and mean represent the total number of isolates in each class and the mean EC_{50} values of isolates, respectively

2.3.1.3 Mycelial growth: Plate method

Of the 79 *Colletotrichum acutatum* isolates tested for sensitivity to thiophanate-methyl using the discriminatory dose assay, 62 isolates (78.5 %) had EC_{50} values higher than 100 µg/ml and 17 isolates (21.5 %) had EC_{50} values lower than 100 µg/ml (Table 2.5).

Of the isolates from conventional growers, 98 % had EC_{50} values higher than 100 µg/ml and 36 % of these isolates had the same mycelial growth inhibition at doses 1, 10 and 100 µg/ml. Of the isolates from organic growers, 72.7 % had EC_{50}

values lower than 100 µg/ml, and in half of them, mycelial growth inhibition was the same at 10 and 100 µg/ml.

2.3.1.4 Spore germination assay

Spore germination of nine *B. cinerea* isolates was not inhibited by azoxystrobin at concentrations up to 100 µg/ml. The same isolates did not have mycelial growth inhibition at 71.9 µg/ml in the spiral gradient dilution assay. EC_{50} values of the other six isolates ranged from 0.25 to 0.91 µg/ml and from 0.70 to 1.39 µg/ml in the spore germination and spiral gradient dilution assays, respectively.

Azoxystrobin was able to control spore germination of all the *C. acutatum* isolates tested, and EC_{50} values varied from 0.01 to 0.98 µg/ml. These values were lower than the values for mycelial growth, using the spiral gradient dilution method, in which the same isolates had EC_{50} values ranging from 0.07 to 4.16 µg/ml. However, the relationship between EC_{50} values of spore germination and mycelial growth of isolates were significant and data were assessed by a linear regression ($R^2 = 0.87$, $p < 0.0001$). Isolates collected from conventional strawberry fields had EC_{50} values varying from 0.01 to 0.98 µg/ml, whereas EC_{50} values of isolates from organic fields ranged from 0.01 to 0.11 µg/ml.

2.3.1.5 Fruit assay

Typical Botrytis and Anthracnose fruit rot symptoms and signs were seen on non-treated control and on some fungicide-sprayed fruit. Disease incidence and severity of fungicide-treated fruit were estimated in comparison to the nontreated control (water-sprayed).

Field rates of azoxystrobin, iprodione and thiophanate-methyl failed to control Botrytis fruit rot symptoms on fruit inoculated with resistant isolates (Table 2.6). Incidence and severity values of fungicide-treated fruit inoculated with azoxystrobin-resistant isolates were not different from nontreated control for both experiments, whereas they differed for azoxystrobin-sensitive isolates. In the first trial, disease incidence and severity on fruit treated with azoxystrobin varied, respectively, from 0.0 to 78.6 % and 0.0 to 20.3 % for sensitive isolates, and from 93.3 to 100 % and 79.2 to 100 % for resistant isolates. In the second trial, disease incidence and severity

ranged, respectively, from 0.0 to 86.7 % and 0.0 to 61.8 % (sensitive isolates), and from 87.9 to 100 % and 47.1 to 100 % (resistant isolates). Disease severity on fruit treated with azoxystrobin and inoculated with sensitive isolates differed from fruit inoculated with resistant isolates in both experiments (Table 2.6).

Disease incidence of iprodione-treated fruit inoculated with resistant isolates were not different from the non-treated control for both experiments, except for one isolate (isolate 14-17, EC_{50} = 1.51 µg/ml, in the mycelial growth assay), and ranged from 95.2 to 100 % and 80.0 to 100 % in the first and second experiments, respectively (Table 2.6). Disease severity of fruit sprayed with iprodione and inoculated with resistant isolates did not differ from nontreated control in the first experiment (79.2 to 100 %), and in the second experiment, values were different for some isolates (29.2 to 47.8 %), but not for others (82.1 to 99.1 %). Disease incidence and severity of iprodione-sprayed fruit inoculated with sensitive isolates differed from nontreated control for both trials, and varied, respectively, from 14.3 to 35.3 % and 2.3 to 43.5 % for the first experiment, and from 20.0 to 40.0 % and 1.8 to 32.5 % for the second experiment (Table 2.6).

Disease incidence of fruit treated with thiophanate-methyl and inoculated with resistant isolates were not different from nontreated control for both trials, except for one isolate in second trial (isolate 13-37, incidence = 28.6 %), and ranged from 86.7 to 100 and from 74.5 to 100 % for first and second trials, respectively. Disease severity of fungicide-treated fruit was similar or not to the non-treated control, and varied from 56.5 to 100 % in the first experiment, and from 30.1 to 94.7 % in the second experiment. Disease severity on fruit treated with azoxystrobin and inoculated with sensitive isolates differed from fruit inoculated with resistant isolates in both experiments. Disease incidence on fruit treated with thiophanate-methyl were not different among the tested isolates (Table 2.6).

Table 2.6 – Efficacy of azoxystrobin, iprodione and thiophanate-methyl in controlling Botrytis fruit rot on strawberry fruit inoculated with *Botrytis cinerea* isolates with different levels of sensitivity

(continue)

Fungicide[x]	Isolate	EC_{50}[y] (µg/ml)	Phenotype[z]	Botrytis fruit rot[w] (%)			
				Experiment 1		Experiment 2	
				Incidence	Severity	Incidence	Severity
Azoxystrobin	C13-01	>71.9	R	100.0 a	100.0 a	100.0 a	71.7 abc
(0.08 g/L)	C13-16	>71.9	R	100.0 a	97.2 ab	100.0 a	91.4 ab
	C13-28	>71.9	R	100.0 a	79.2 b	90.5 a	47.1* cd
	C13-37	1.39 (0.91)	S	0.0* c	0.0* d	0.0 c	0.0* e
	C14-08	>71.9	R	93.3 ab	100.0 a	100.0 a	97.6 a
	C14-17	>71.9	R	100.0 a	93.8 ab	87.9 a	98.0 a
	C14-30	1.02 (0.19)	S	78.6* ab	16.3* cd	50.0* b	61.8* bc
	O14-38	0.70 (0.61)	S	66.7 b	20.3* c	86.7 a	24.6* de
	O15-07	>71.9	R	100.0 a	100.0 a	100.0 a	100.0 a
Iprodione	C13-01	3.07	R	100.0 a	95.5 a	100.0 a	82.1 ab
(0.075 g/L)	C13-16	3.25	R	95.2 a	83.3 ab	80.0 ab	29.2* bc
	C13-28	4.49	R	100.0 a	79.2 ab	100.0 a	47.8* abc
	C13-37	0.46	S	35.3* b	43.5* bc	35.7* b	19.1* c
	C14-08	2.21	R	100.0 a	87.1 a	100.0 a	93.9 a
	C14-17	1.51	R	100.0 a	66.9 ab	65.5* ab	39.8* bc
	C14-30	0.83	S	28.6* bc	15.5* c	20.0* b	1.8* c
	O14-38	2.12	R	100.0 a	100.0 a	100.0 a	99.1 a
	O15-07	0.46	S	14.3* c	2.3* c	40.0* ab	32.5* bc

Table 2.6 – Efficacy of azoxystrobin, iprodione and thiophanate-methyl in controlling Botrytis fruit rot on strawberry fruit inoculated with *Botrytis cinerea* isolates with different levels of sensitivity

(conclusion)

Fungicide[x]	Isolate	EC_{50}[y] (µg/ml)	Phenotype[z]	Botrytis fruit rot[w] (%)			
				Experiment 1		Experiment 2	
				Incidence	Severity	Incidence	Severity
Thiophanate-methyl	C13-01	>688	R	86.7 a	75.2 ab	100.0 a	75.7 ab
(0.49 g/L)	C13-16	>688	R	90.5 a	92.4 a	86.7 a	78.7 ab
	C13-28	>688	R	93.3 a	91.2 a	100.0 a	76.7* ab
	C13-37	>688	R	93.3 a	56.5* b	28.6* b	36.2* b
	C14-08	>688	R	100.0 a	100.0 a	85.7 a	66.9* ab
	C14-17	>688	R	100.0 a	81.5 ab	74.5 a	30.1* b
	C14-30	>688	R	90.5 a	69.1* ab	100.0 a	35.3* b
	O14-38	>688	R	93.3 a	98.7 a	93.3 a	94.7 a
	O15-07	>688	R	100.0 a	92.4 a	100.0 a	78.6 ab

[w] Botrytis fruit rot incidence and severity on fungicide-sprayed fruit were estimated in comparison to the non-treated control (water-sprayed). Data are the means of three repetitions for each experiment. Values, in the column, followed by the same letters for each fungicide separately are not significantly different at $p = 0.05$, as determined by analysis of variance and Tukey´s test. Numbers followed by (*) are significantly different from the nontreated control, as determined by ANOVA at $p = 0.05$

[x] Fruit not sprayed with any fungicide (nontreated control) or sprayed preventively with azoxystrobin (0.08 g/L), iprodione (0.075 g/L) and thiophanate-methyl (0.49 g/L) were previously surface-sterilized. Strawberries were incubated for 24 hours at room temperature, wounded and inoculated with 30 µL of a *B. cinerea* spore suspension at 10^5 spores/ml for each isolate. Botrytis fruit rot incidence and severity were evaluated after 4 to 5 days of incubation at 23 °C

[y] Effective concentration range (EC_{50}), in µg/ml, that inhibited mycelial growth and spore germination (in parentheses) of *B. cinerea* isolates by 50 %

[z] S and R indicate sensitive and resistant, respectively

Twelve *C. acutatum* isolates were inoculated on fruit treated with azoxystrobin and thiophanate-methyl in two experiments, whereas, for difenoconazole, some of these isolates were not tested and others were tested once.

Field rates of azoxystrobin were able to completely control anthracnose symptoms on fruit inoculated with some *C*. *acutatum* isolates (incidence and severity = 0.0 %), whereas for other isolates, the rate exhibited slight control on disease development. However, for all the isolates except for one (15-09), disease incidence and severity of fruit treated with azoxystrobin were different from nontreated control for both experiments. Disease incidence on treated fruit varied from 0.0 to 79.8 % in the first trial, and from 0.0 to 42.9 % in the second trial, whereas disease severity ranged from 0.0 to 14.1 % and from 0.0 to 13.6 % in the first and second trials, respectively (Table 2.7). Disease incidence and severity on fruit treated with azoxystrobin and inoculated with the pathogen were different among the isolates used, except for disease severity in the second trial, where there was no difference among the isolates (Table 2.7). Disease incidence and severity on fruit treated with azoxystrobin and inoculated with *C*. *acutatum* are not related to the EC_{50} values of the isolates.

Field rates of thiophanate-methyl and difenoconazole failed to control anthracnose on fruit inoculated with *C. acutatum* isolates. Disease incidence and severity of fruit treated with thiophanate-methyl and iprodione were different or not from non-treated control depending on the isolate and experiment. Similarly, disease incidence and severity showed great variation among the isolates, except for the second experiment, in which disease incidence on fruit treated with thiophanate-methyl was the same for the different inoculated isolates (Table 2.7).

Table 2.7 – Efficacy of azoxystrobin, difenoconazole and thiophanate-methyl in controlling Anthracnose fruit rot on strawberry fruit inoculated with *Colletotrichum acutatum* isolates with different levels of sensitivity

(continue)

Fungicide[z]	Isolate	EC_{50} (µg/ml)	Anthracnose fruit rot[y] (%)							
			Experiment 1				Experiment 2			
			Incidence		Severity		Incidence		Severity	
Azoxystrobin	C13-67	3.33	22.2*	b	3.6*	b	0.0*	b	0.0*	a
	C13-79	0.45	0.0*	b	0.0*	b	7.1*	ab	0.3*	a
	C13-80	0.50	6.7*	b	1.0*	b	0.0*	b	0.0*	a
	C13-90	0.20	0.0*	b	0.0*	b	7.1*	ab	8.3*	a
	C13-91	1.77	0.0*	b	0.0*	b	0.0*	b	0.0*	a
	O13-95	0.67	0.0*	b	0.0*	b	0.0*	b	0.0*	a
	O13-104	0.21	0.0*	b	0.0*	b	6.7*	ab	2.5*	a
	O13-111	1.20	13.3*	b	1.1*	b	6.7*	ab	0.5*	a
	O13-113	0.16	78.6*	a	14.1*	a	33.3*	ab	3.8*	a
	C14-48	0.23	8.3*	b	0.6*	b	6.7*	ab	5.4*	a
	O14-49	0.26	26.7*	b	2.6*	b	42.9*	a	10.8*	a
	C15-09	0.07	79.8	a	14.1*	a	30.0*	ab	13.6*	a
Thiophanate-methyl	C13-67	>100	88.9	a	70.0	ab	64.9	a	98.0	a
	C13-79	>100	77.3	ab	58.1	abcd	88.1	a	35.7*	cd
	C13-80	>100	100.0	a	78.2*	a	86.7	a	73.6	abc
	C13-90	>100	14.3*	c	3.7*	de	100.0	a	66.4	abc
	C13-91	>100	15.0*	c	2.4*	e	80.0	a	47.9*	bcd
	O13-95	<100	53.3*	abc	16.5*	bcde	100.0	a	6.9*	d
	O13-104	<100	26.7*	bc	9.9*	cde	73.3*	a	44.4*	bcd
	O13-111	>100	93.3	a	61.8	abc	93.3	a	49.1*	bcd
	O13-113	>100	91.7	a	59.7*	abc	93.3	a	63.1	abc
	C14-48	>100	100.0	a	64.7	abc	86.7	a	85.1	ab
	O14-49	>100	100.0	a	57.5*	abcde	95.2	a	83.3	ab
	C15-09	>100	95.2	a	44.4*	abcde	100.0	a	100.0	a

Table 2.7 – Efficacy of azoxystrobin, difenoconazole and thiophanate-methyl in controlling Anthracnose fruit rot on strawberry fruit inoculated with *Colletotrichum acutatum* isolates with different levels of sensitivity

(conclusion)

Difenoconazole	C13-67	0.073	…		…		…		…	
	C13-79	0.141	100.0	a	81.1*	ab	…		…	
	C13-80	0.129	93.3	ab	66.5*	b	63.3*	b	41.4*	b
	C13-90	0.059	69.6*	b	100.0	a	…		…	
	C13-91	0.064	…		…		…		…	
	O13-95	0.091	90.0	ab	99.7	a	…		…	
	O13-104	0.097	93.3	ab	97.0	a	100.0	a	92.1	a
	O13-111	0.130	…		…		…		…	
	O13-113	0.069	…		…		100.0	a	83.7*	a
	C14-48	0.144	…		…		…		…	
	O14-49	0.081	…		…		95.2	a	69.9*	ab
	C15-09	0.154	100.0	a	77.9	ab	100.0	a	90.7	a

[y] Anthracnose fruit rot incidence and severity of fungicide-sprayed fruit were compared to the nontreated control (water-sprayed). Data are the mean of three repetitions for each experiment. Values, in columns, followed by the same letters for each fungicide separately are not significantly different at $p = 0.05$, as determined by analysis of variance and Tukey´s test. Numbers followed by (*) are significantly different from the nontreated control, as determined by ANOVA at $p = 0.05$. Data not available is represented by (…)

[z] Fruit not sprayed with any fungicide (nontreated control) or sprayed preventively with azoxystrobin (0.08 g/L), difenoconazole (0.1 g/L) and thiophanate-methyl (0.49 g/L) were previously surface-sterilized. Strawberries were incubated for 24 hours at room temperature and inoculated with 30 µL of a *Colletotrichum acutatum* spore suspension at 10^6 spores/ml from each isolate. Anthracnose fruit rot incidence and severity were evaluated after 7 days of incubation at 23 °C

2.3.2 Molecular characterization of *Botrytis cinerea* and *Colletotrichum acutatum* isolate mutations linked to resistance to fungicides

2.3.2.1 Molecular identification of *Colletotrichum acutatum* isolates

All 79 isolates were identified as belonging to the *Colletotrichum acutatum* species complex.

2.3.2.2 Molecular characterization of mutations linked to resistance to QoI fungicides (azoxystrobin): cytochrome *b* gene (*cytb*)

2.3.2.2.1 *Botrytis cinerea*

The DNA of 99 *B. cinerea* isolates collected from conventional and organic fields were extracted and the *cytb* gene was amplified by polymerase chain reaction and a 560-bp fragment could be visualized by UV light after gel electrophoresis. For rapid detection of the G143A mutation, the *cytb* gene PCR products of 88 isolates were digested using the restriction enzyme *Fnu*4HI, which recognizes the 5′-GCNGC-3′ nucleotide pattern. The presence of a mutation at the codon 143 was identified by the presence of a two-band profile with fragments of 318 and 242 bp; and the fragment of 560 bp remained undigested in the absence of mutation (Figure 2.9).

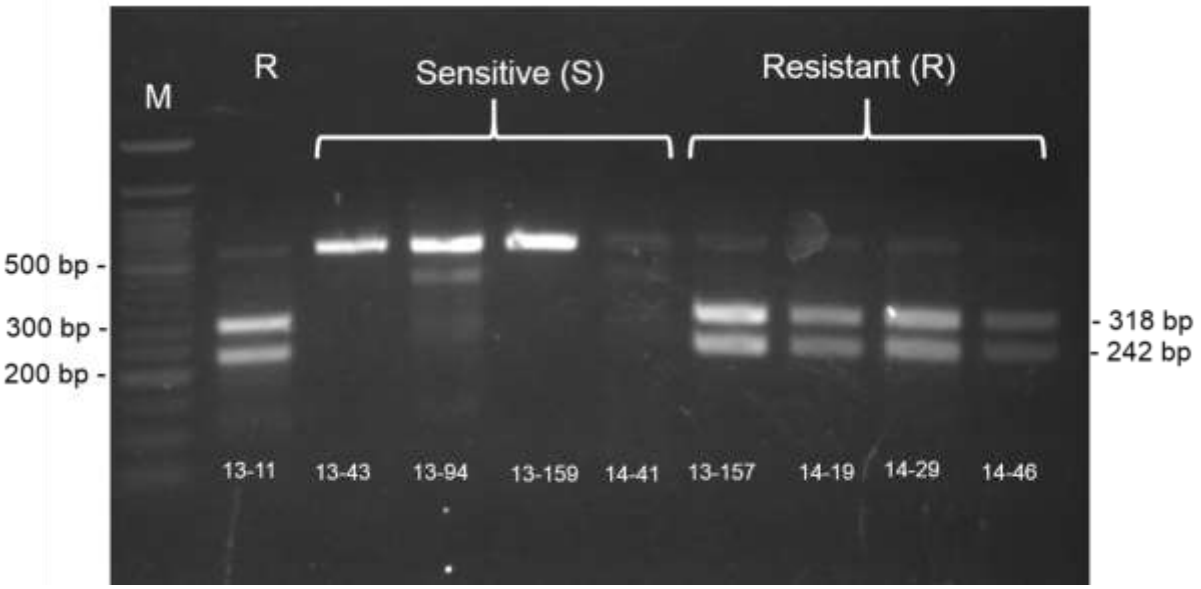

Figure 2.9 – Restriction fragment length polymorphisms (RFLP) of the *cytochrome b* (*cytb*) gene from *Botrytis cinerea* isolates sensitive (S) and resistant (R) to azoxystrobin. The *Fnu*4HI restriction enzyme was unable to digest the 560-bp fragment of the sensitive isolates at position 143 but digested the mutant resistant isolates. The presence and absence of the mutation was identified by the digestion of the *cytb* PCR products, followed by electrophoresis on 2 % agarose gel. Isolates identification is shown in the respective lanes. M = 50-bo ladder marker

The fragments amplified from 19 isolates remained undigested, whereas the fragments from 56 isolates revealed the two-band profile; fragments of six isolates were not amplified and seven isolates showed a larger band profile (approximately 1500 bp). All of the 19 undigested samples had EC_{50} values for azoxystrobin ranging from 0.66 to 4.34 µg/ml and all the 55 isolates with the two-band profile showed EC_{50} values higher than 71.9 µg/ml and were considered azoxystrobin-resistant (Table 2.6).

The *cytb* gene PCR products of 22 isolates were sent for sequencing: the six isolates that were not amplified, the seven isolates with approximately 1500 bp band profile, three isolates that were not subjected to restriction fragment length polymorphisms (RFLP) analysis and eight that were subjected to RFLP. The samples were compared to two sequences from GenBank: *Botryotinia fuckeliana* mitochondrial *cytb* gene for cytochrome *b* complete cds (accession no. AB262969.1) and *Botryotinia fuckeliana* mitochondrial *cytb* gene for *cytochrome b* complete cds cytb gene with Bcbi-143/144 intron strain: Bc-o-26 (accession no. AB428335.1). None of the sequences has the mutation at the codon 143, and the second sequence contains an intron between the codons 143 and 144 (Bcbi 143-144).

Three *cytb* gene profiles were observed among *B. cinerea* isolates: isolates without the Bcbi-143/144 intron and without the point mutation at position 143, considered azoxystrobin-sensitive (Figure 2.10A); isolates without the Bcbi-143/144 intron and with the point mutation G143A, considered azoxystrobin-resistant (Figure 2.10B); and isolates with the Bcbi-143/144 intron and without the point mutation at 143, considered azoxystrobin-sensitive (Figure 2.10C).

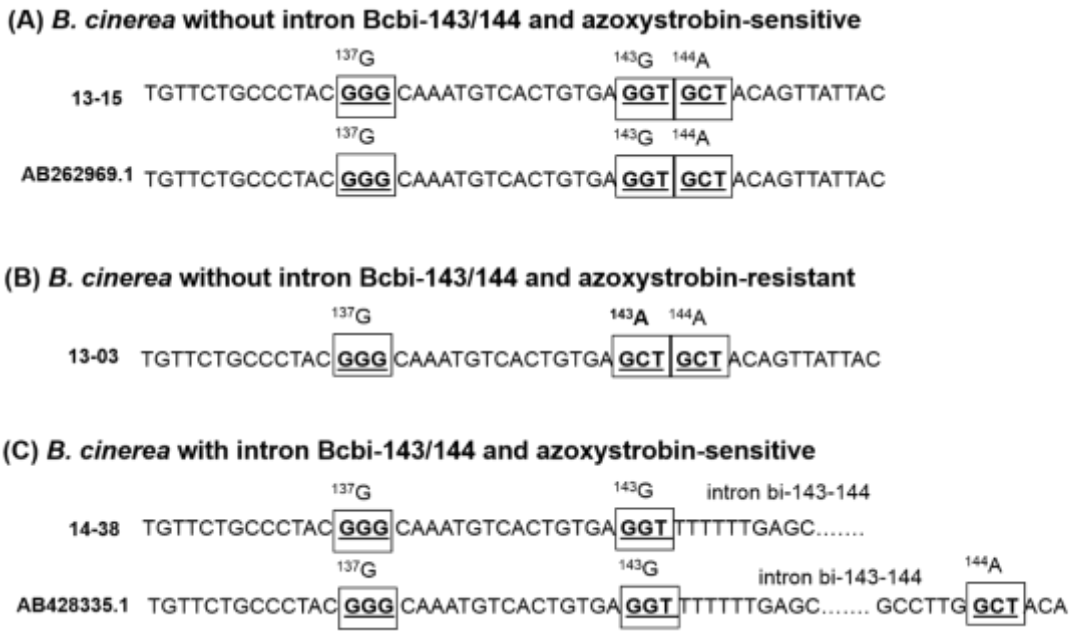

Figure 2.10 – Partial nucleotide sequences of *cytochrome b* (*cytb*) of *Botrytis cinerea* isolates: (A) azoxystrobin-sensitive isolates without the Bcbi-143/144 intron; (B) azoxystrobin-resistant isolates without the Bcbi-143/144 intron; and (C) azoxystrobin-sensitive isolates with the Bcbi-143/144 intron. Nucleotides encoding amino acids 137 (glycine), 143 (glycine or alanine) and 144 (alanine) are boxed. No mutation was observed at codon 137 in any of the sequenced isolates. Isolate marked with ^{143}Ala (B) indicate amino acid substitution at position 143. Sequences were compared to *B. cinerea* sequences from GenBank without (accession no. AB262969.1) or with (accession no AB428335.1) the Bcbi-143/144 intron

Of the 22 isolates sent for sequencing, six did not contain the intron nor the mutation at position 143; nine did not have the intron but possessed the mutation G143A; and seven (the ones with upper band at the gel agarose) contained the intron but did not show the mutation at codon 143 (Table 2.8). All azoxystrobin-resistant isolates (EC_{50} > 71.9 µg/ml) had the mutation G143A and no mutation was observed in any of the sensitive isolates. No mutation at position 137 was observed in any of the sequenced isolates. Isolates with or without a mutation at position 143

(Table 2.8), had their *cytb* gene PCR products digested or not digested by restriction enzyme *Fnu*4HI, respectively.

Table 2.8 – Characterization of the partial cytochrome *b* (*cytb*) gene of *Botrytis cinerea* isolates collected from organic and conventional strawberry fields and EC_{50} mean values (µg/ml) to azoxystrobin

Isolate	Azoxystrobin EC_{50} (µg/ml)[a]	*cytb* gene			
		G143A mutation[b]	RFLP[c]	Bcbi-143/144 intron	Phenotype[d]
C13-03	>71.9	Present	Two-band	Absent	R
C13-06	>71.9	Present	Two-band	Absent	R
C13-07	>71.9	Present	-	Absent	R
C13-12	>71.9	Present	Two-band	Absent	R
C13-15	0.774	Absent	One-band	Absent	S
C13-16	>71.9	Present	-	Absent	R
C13-22	1.19	Absent	Upper band	Present	S
C13-26	0.81	Absent	Upper band	Present	S
O13-35	1.71	Absent	-	Absent	S
O13-37	1.39	Absent	Upper band	Present	S
O13-38	>71.9	Present	Two-band	Absent	R
C14-18	>71.9	Present	-	Absent	R
C14-31	1.19	Absent	Upper band	Present	S
C14-32	0.960	Absent	-	Absent	S
C14-33	1.42	Absent	-	Absent	S
O14-37	1.62	Absent	Upper band	Present	S
O14-38	0.70	Absent	Upper band	Present	S
O14-39	1.13	Absent	Upper band	Present	S
O14-41	3.85	Absent	-	Absent	S
O14-42	>71.9	Present	Two-band	Absent	R
O14-44	2.35	Absent	-	Absent	S
O15-07	>71.9	Present	-	Absent	R

[a] Effective concentration average of the fungicide azoxystrobin, in µg/ml, that inhibited 50 % of the mycelial growth using the spiral gradient dilution method
[b] Point mutation in the *cytochrome b* (*cytb*) gene at position 143: G143A, GGT to GCT
[c] Restriction fragment length polymorphism (RFLP) of the *cytochrome b* (*cytb*) gene visualized at 2 % gel agarose: two-band profile indicates presence of mutation at codon 143; one-band profile represents absence of mutation; upper band corresponds a band of a different size; and – indicates no results
[d] S and R = sensitive and resistant *B. cinerea* isolates without and with mutation in *cytb* gene, respectively

2.3.2.2.1 *Colletotrichum acutatum*

Nucleotide sequencing of part of the cytochrome *b* (*cytb*) of nine *Colletotrichum acutatum* isolates did not reveal any amino acids substitutions at codons 129, 137 and 143 (Figure 2.11). The isolates were compared to a *C. acutatum* sequence from GenBank (accession no. KR349346.1). EC_{50} values of the nine sequenced isolates varied from 0.074 to 4.17 µg/ml for the mycelial growth assay and from 0.01 to 0.98 µg/ml for the spore germination assay. Even the isolates with the highest EC_{50} mean values to azoxystrobin did not contain a mutation. Despite some differences among the isolates on nucleotide sequences from codons 130 and 144, there were no substitutions of the amino acids leucine (L) and alanine (A), respectively (Figure 2.11).

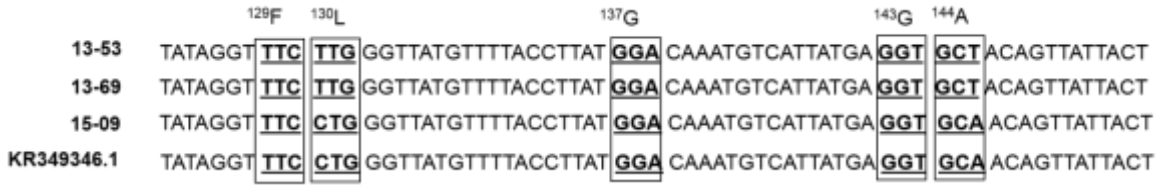

Figure 2.11 – Partial nucleotide sequences of *cytochrome b* (*cytb*) of *Colletotrichum acutatum* isolates. Nucleotides encoding amino acids 129 (phenylalanine), 130 (leucine), 137 (glycine), 143 (glycine) and 144 (alanine) are boxed. No mutation was observed at codons 129, 137 and 143 in any of the sequenced isolates. Changes of nucleotides were detected at codons 130 and 144 of some isolates, but without amino acid changes. Sequences were compared to the *Colletotrichum acutatum* sequence from GenBank (accession no. KR349346.1)

2.3.2.3 Molecular characterization of mutations linked to resistance to benzimidazole fungicides (thiophanate-methyl): Beta-tubulin gene (*β-tub*)

2.3.2.3.1 *Botrytis cinerea*

A portion of the beta-tubulin gene of *B. cinerea* isolates was amplified by polymerase chain reaction and the fragments were visualized under UV light. Some isolates showed a two-band profile and other isolates one fragment of 642 bp (Figure 2.12).

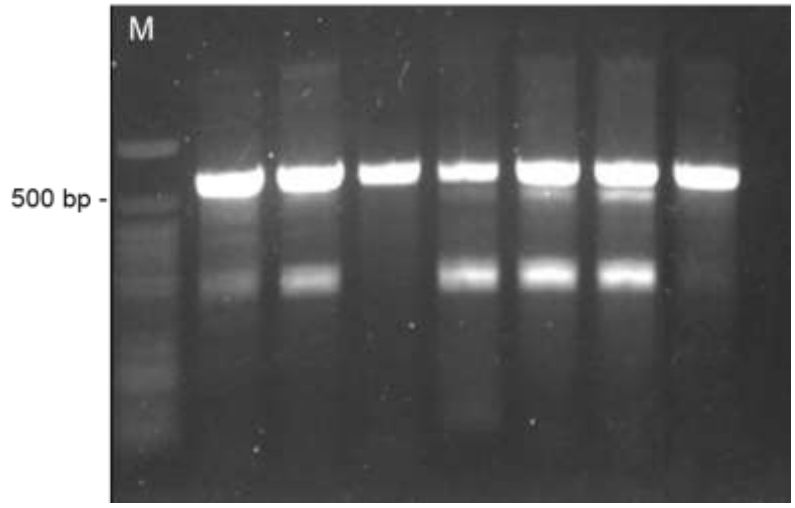

Figure 2.12 – Gel electrophoresis (0.75 % agarose) of partial beta-tubulin (*β-tub*) gene polymerase chain reaction fragments amplified using the primer pairs 155/1174. Some isolates of *Botrytis cinerea* showed one band fragment of 642 bp and other isolates revealed a two-band profile. M = 50 bp molecular marker

Twelve isolates with different profiles were chosen to be sequenced: seven with a one-band and five with two-band profiles; four having EC_{50} means 0.89, 1.05, 1.23 and 1.25 µg/ml (sensitive) and eight with an EC_{50} mean higher than 688 µg/ml (resistant). The bands of the isolates with two-band profiles were extracted from the gel and both were submitted for sequencing. The sample sequences were compared to a sequence from GenBank (accession no. Z69263.2). The lower bands of the two-band profile isolates did not show similarity to the upper bands, to the unique band of the other isolates, or to the sequence from GenBank. The thiophanate-methyl-sensitive isolates did not contain a mutation at position 198; and the resistant isolates had a substitution from glutamic acid (E) to alanine (A) at position 198 (E198A). No mutations at positions 167 and 200 were revealed in any of the sequenced isolates (Figure 2.13).

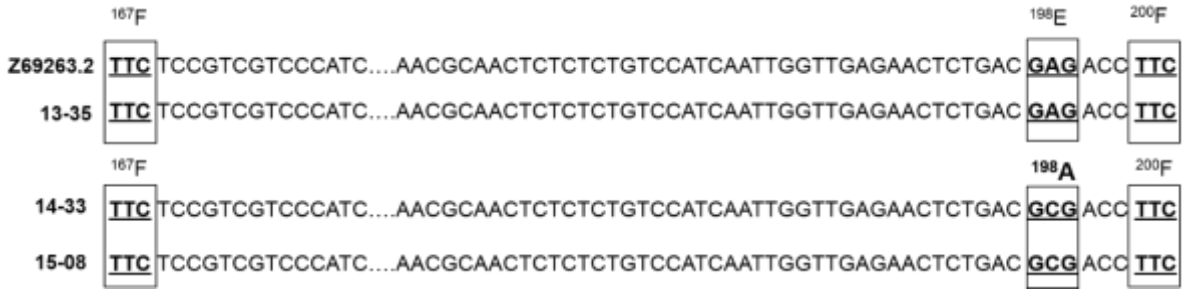

Figure 2.13 – Partial nucleotide sequences of beta-tubulin (*β-tub*) gene in sensitive and resistant *Botrytis cinerea* isolates and an isolate from GenBank (accession no. Z69263.2). Nucleotides encoding amino acids 167 (phenylalanine), 198 (glutamic acid or alanine) and 200 (phenylalanine) are boxed. Mutations were not observed at codons 167 and 200 of neither sensitive nor resistant isolates. Isolates marked with ^{198}A indicate amino acid substitution at codon 198 from glutamic acid (E) to alanine (A) in resistant isolates

2.3.2.3.2 *Colletotrichum acutatum*

Nucleotide sequencing of a portion of the beta-tubulin (*ß-tub*) gene of seven *C. acutatum* isolates with different sensitivity responses to thiophanate-methyl did not reveal any substitutions at codons 167, 198 and 200 (Figure 2.14). Isolate sequences were compared to *C. fioriniae* (*C. acutatum* group) sequence from GenBank (accession no. XM_007601271.1).

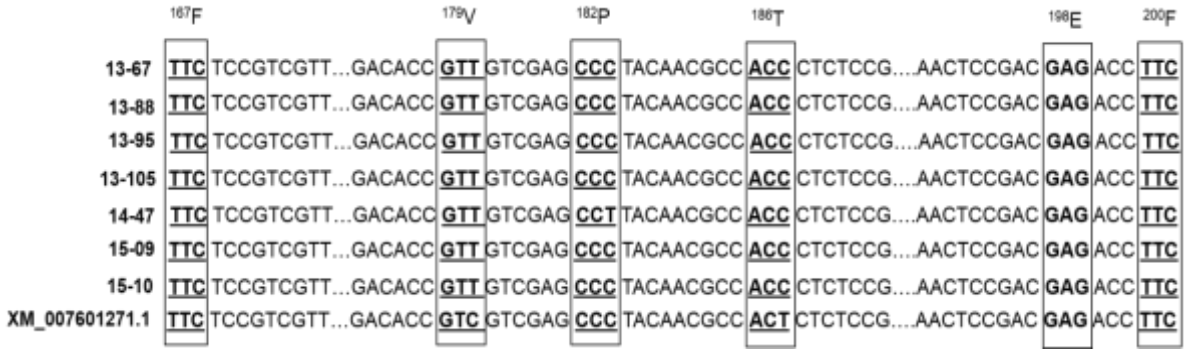

Figure 2.14 – Partial nucleotide sequences of beta-tubulin (*ß-tub*) of *Colletotrichum* spp. isolates. Nucleotides encoding amino acids 167 (phenylalanine), 179 (valine), 182 (proline), 186 (theorine), 198 (glutamic acid) and 200 (phenylalanine) are boxed. No mutation was observed at codons 129, 137 and 143 in any of the sequenced isolates. Change of nucleotides were detected at codons 179, 182 and 186 of some isolates, but without amino acids substitution. Sequences were compared to *Colletotrichum fioriniae* sequence from GenBank (accession no. XM_007601271.1)

Despite some differences among the isolates in nucleotide sequences from codons 179, 182 and 186, there were no substitutions of the amino acids valine (V), proline (P) and threonine (T), respectively (Figure 2.14). Therefore, *Colletotrichum* spp. isolates sensitivity response to thiophanate-methyl may be related to modifications occurring at other regions of beta-tubulin gene.

2.3.2.4 Molecular characterization of mutations linked to resistance to dicarboximide fungicides (iprodione) in *Botrytis cinerea*: *bos1* gene

The *bos1* gene of *B. cinerea* isolates were amplified by polymerase chain reaction using the sets of primers pairs BF1/BR1 and BF2/BR2 and the fragments could be visualized by UV light after gel electrophoresis. The PCR products of nine isolates (two sensitive and seven resistant to iprodione) with EC_{50} values ranging from 0.52 to 4.49 µg/ml, were sent for sequencing (Table 2.9). The samples were compared to a *B. cinerea* sequence from GenBank (accession no. JX192631.1).

Sequences of five of the nine isolates were amplified by primer pair BF1/BR1 and did not show any point mutations at position 127 of *bos1* gene (Table 2.9). The two iprodione-sensitive isolates did not have any amino acids substitutions at positions 365, 369 and 373. The resistant isolates possessed the mutations at codons 365 (I365S and I365N) or codons 369 (Q369P) and 373 (N373S). Two isolates, with EC_{50} mean values of 3.07 and 4.49 µg/ml, had the I365S mutation, whereas one isolate (EC_{50} = 2.12 µg/ml) presented the mutation I365N. Four isolates, having EC_{50} mean values ranging from 2.25 to 3.94, had the Q369P and the N373S mutations (Table 2.9).

Table 2.9 – Characterization of the partial *bos1* gene of *Botrytis cinerea* isolates collected from organic and conventional strawberry fields and EC_{50} mean values (µg/ml) to iprodione

Isolates	EC_{50} (µg/ml)[b]	Phenotype[c]	Amino acid at codons of *bos1* gene[a]			
			127	365	369	373
C13-01	3.07	MR	…	S	Q	N
O13-28	4.49	MR	F	S	Q	N
O13-29	0.76	S	**F**	**I**	**Q**	**N**
C14-02	3.37	MR	F	I	P	S
C14-05	3.82	MR	…	I	P	S
C14-14	3.94	MR	F	I	P	S
O14-38	2.12	MR	F	N	Q	N
O14-42	0.52	S	…	**I**	**Q**	**N**
C15-01	2.25	MR	…	I	P	S
JX192631.1			F	I	Q	N

[a] Amino acids phenylalanine (F), serine (S), isoleucine (I), glutamine (Q), proline (P), and asparagine (N) coded at positions 127, 365, 369 and 373 of *bos1* gene
[a] Effective concentration average of iprodione, in µg/ml, that inhibited 50 % of pathogen mycelial growth using the spiral gradient dilution method
[c] S and R = sensitive and resistant *B. cinerea* isolates without and with mutations in the *bos1* gene, respectively
Amino acids of isolates with no mutations are marked in bold

2.4 Discussion

In Brazilian strawberry conventional fields, azoxystrobin, iprodione and thiophanate-methyl are frequently used to control Botrytis fruit rot, whereas azoxystrobin, difenoconazole and thiophanate-methyl are used for Anthracnose fruit rot. Some of the active ingredients are registered as single fungicides, whereas other compounds are combined pre-packaged mixtures. Azoxystrobin is not specifically registered for Botrytis fruit rot control and there are no active ingredients officially

registered to Anthracnose fruit rot control, although they are widely used by growers (AGROFIT, 2016; D. JULIATO[3], *personal information*).

This study provides information on the occurrence, frequency distribution and molecular characterization of resistance to single-site fungicides in *Botrytis cinerea* and *Colletotrichum* spp. populations collected from conventional and organic Brazilian strawberry fields. Fungicide resistance in *B. cinerea* isolates was found in almost every conventional commercial field and 75 % of the collected isolates were resistant to all the tested fungicides. Although at lower frequencies, resistant isolates were also recovered from organic strawberry fields: 34.3 % of them were sensitive to all fungicides and 8.6 % were resistant to the three tested active ingredients. Fungicide resistance was not found in *Colletotrichum* spp. isolates for azoxystrobin and difenoconazole fungicides, and the pathogen has shown insensitivity rather than resistance to thiophanate-methyl.

Populations of *B. cinerea* resistant to azoxystrobin (EC_{50} > 71.9 µg/ml) were found in conventional (87.5 %) and organic (31.4 %) strawberry fields. Overall, 67.7 % of the isolates were azoxystrobin-resistant. Isolates with EC_{50} values for mycelial growth ranging from 5 to 500 µg/ml (AMIRI; HEATH; PERES, 2013) were considered highly resistant to pyraclostrobin fungicides, whereas isolates with EC_{50} higher than 100 µg/ml were considered resistant to azoxystrobin (CHATZIDIMOPOULOS; PAPAEVAGGELOU; PAPPAS, 2013; JIANG et al., 2009). High resistance frequencies to QoI fungicides in conventional strawberry fields were also reported in the USA, Europe and Japan (AMIRI; HEATH; PERES, 2013; FERNANDEZ-ORTUNO et al., 2016; ISHII et al., 2009). Overall frequency of QoI-resistant populations of *B. cinerea* agree with previous reports in the USA (FERNÁNDEZ-ORTUÑO; CHEN; SCHNABEL, 2012), Spain (FERNÁNDEZ-ORTUÑO et al., 2016), Germany (WEBER, 2011), and Italy (DE MICCOLIS ANGELINI et al., 2014), resistance frequencies of 66.7, 66 to 79, 76.8, and 66.7 % were reported, respectively.

Many plant pathogens, including *B. cinerea*, develop an alternative respiration pathway *in vitro* which allow them to avoid the toxic effects of QoI fungicides, since their activity is based on inhibition of ATP production during mitochondrial respiration by binding at the quinol-oxidase (Qo) site of cytochrome *b* (*cytb*). However, this

[3] D. JULIATO. Personal communication during visit to the grower farm on July 11, 2013.

alternative pathway does not decrease field efficiency of QoIs (BARTLETT et al., 2002). Thereby, the addition of salicylhydroxamic acid (SHAM) into the media to inhibit the fungal alternative respiration pathway is very common (AMIRI; HEATH; PERES, 2013; FERNÁNDEZ-ORTUÑO et al., 2016; LEROUX et al., 2010). Studies comparing the effect of the addition or not of SHAM to fungicide-amended media have already been conducted for *B. cinerea* (BANNO et al., 2009; WEBER; HAHN, 2011) and other pathogens, such as *Monilinia fructicola* (PRIMIANO, 2015) showing that EC_{50} values of isolates were usually higher when SHAM was not included into the medium, giving a false positive result on sensitivity evaluations.

Mean EC_{50} values of sensitive isolates, regardless their origin, based upon mycelial growth was 1.47 µg/ml, varying from 0.66 to 4.34 µg/ml. Similar findings have been reported for isolates with no previous azoxystrobin exposure in *B. cinerea* on greenhouse vegetables of China, where EC_{50} values ranged from 0.04 to 5.25 µg/ml (ZHANG et al., 2011). In other studies, the mean EC_{50} values of azoxystrobin for *B. cinerea* were 0.09 µg/ml (WEBER; HAHN, 2011) and 0.329 µg/ml (JIN et al., 2009). Other authors have reported lower EC_{50} values for *B. cinerea* from strawberries, ranging from 0.07 to 0.64 µg/ml (BANNO et al., 2009) and from 0.12 to 0.33 µg/ml (LEROUX et al., 2010), and from lettuce, ranging from 0.06 to 0.25 µg/ml (CHATZIDIMOPOULOS; PAPAEVAGGELOU; PAPPAS, 2013). EC_{50} values for sensitive *B. cinerea* isolates varied from 0.01 to 0.02 µg/ml and from 0.08 to 0.013 µg/ml to pyraclostrobin (CHATZIDIMOPOULOS; PAPAEVAGGELOU; PAPPAS, 2013) and trifloxystrobin (WEBER, 2011), respectively. Lower EC_{50} values for pyraclostrobin than azoxystrobin was already reported for *C. acutatum* from strawberries (FORCELINI et al., 2016) and citrus (MONDAL et al., 2005) and for *Guignardia citricarpa* from citrus (HINCAPIE et al., 2014). Isolates considered azoxystrobin-resistant based upon mycelial growth had mean EC_{50} values higher than 100 µg/ml in the spore germination assay. Sensitive isolates showed lower EC_{50} values for spore germination than mycelial growth, which was also observed in other studies for *B. cinerea* isolates from different hosts (CHATZIDIMOPOULOS; PAPAEVAGGELOU; PAPPAS, 2013; JIN et al., 2009; ZHANG et al., 2011).

However, it is important to mention that all the isolates considered sensitive to azoxystrobin did not have the G143A mutation that causes a substitution from glycine to alanine at codon 143 of cytochrome *b* gene, whereas all the resistant isolates with EC_{50} higher than 71.9 µg/ml possessed this mutation. The occurrence of the G143A

point mutation has been linked to the expression of high or complete resistance of isolates, leading to failure of field control of the disease, even with higher fungicide concentrations (BANNO et al., 2009; GISI et al., 2002). This mutation is the most frequently associated with QoI fungicides and has been extensively documented in resistant isolates from several pathogens worldwide, including *B. cinerea* from strawberries and other small fruits (AMIRI; HEATH; PERES, 2013; BANNO et al., 2009; FERNÁNDEZ-ORTUÑO et al., 2016; ISHII et al., 2009; JIANG et al., 2009; LEROUX et al., 2010). As observed in our studies, *B. cinerea* isolates possessing the additional intron (Bcbi-143/144) inserted between the codons 143 and 144 of the *cytochrome b* gene have been already reported (BANNO et al., 2009; JIANG et al., 2009; LEROUX et al., 2010). All the isolates having this intron did not have the mutation at the codon 143 and were considered azoxystrobin-sensitive with EC_{50} values less than 5 µg/ml. All azoxystrobin-resistant isolates did not have the intron inserted between the codons. It is suggested that the mutation at codon 143 in cytochrome *b* gene prevents self-splicing of the 143/144 intron, which may lead to cytochrome deficiency and lethal mutation (BANNO et al., 2009; GRASSO et al., 2006).

Field rates of commercial formulations of azoxystrobin failed to control gray mold on detached fruit inoculated with QoI-resistant *B. cinerea* isolates. Symptoms on fungicide-treated fruit inoculated with a sensitive isolate (13-37) were not expressed, whereas disease was expressed on fruit inoculated with the other two azoxystrobin-sensitive isolates (14-30 and 14-38). Isolate 14-38 possesses the 143/144 intron and, although the influence of the occurrence of the intron on field resistance is still not well understood. It was verified that peach fruit treated with azoxystrobin and inoculated with *Monilinia fructicola* isolates, that do not have the G143A mutation but do have the 143/144 intron and with EC_{50} values higher than 1 µg/ml, showed brown rot symptoms (CHEN et al., 2014; PRIMIANO, 2015). This could explain the suppression effect of azoxystrobin on isolate 14-38 even though it was not completely controlled by field rate of the fungicide. Isolate 14-30 did not have the mutation nor the intron and expressed symptoms. However, incidence and severity levels were different from the non-treated control and from fungicide-treated fruit inoculated with resistant isolates. Another reason for disease expression in sensitive isolates is that maybe the time elapsed between the inoculation and evaluation days was too long. However, the same 4- to 5-day period for Botrytis fruit

rot assessment was used by other authors (AMIRI; HEATH; PERES, 2013; FERNÁNDEZ-ORTUÑO et al., 2014). Injuries and wounds made on fruit to enable pathogen infection may also contribute for disease occurrence even on fruit treated with fungicides.

Azoxystrobin is registered for gray mold control as suppressor only in the USA and, for this reason, has probably not completely controlled disease development (MERTELY; PERES, 2006). This could be the reason why this active ingredient is not officially registered for Botrytis fruit rot control in strawberries in Brazil. However since azoxystrobin has been sprayed for controlling other strawberry diseases, the fungicide may be responsible for selection pressure on *B. cinerea* QoI-resistant strains.

Resistance of *B. cinerea* populations to dicarboximide fungicides can be classified as low, moderate, or high. Low to moderate levels are typically found in isolates collected from fields, whereas high resistance is rarely seen in the field or just reported on laboratory mutant strains (GRABKE et al., 2014; LEROUX et al., 1999). In our studies, we consider populations of *Botrytis cinerea* with EC_{50} values higher than 1.2 µg/ml resistant to iprodione, based on studies of Ma et al. (2007), where isolates with EC_{50} values for mycelial growth ranging from 0.20 to 0.41 µg/ml were considered iprodione-sensitive, whereas resistant isolates had EC_{50} values varying from 1.12 to 2.99 µg/ml. Leroux et al. (1999) and Raposo et al. (1996) also reported dicarboximide-resistant strains had EC_{50} values higher than 1 and 1.6 µg/ml, respectively, and EC_{50} values ranging from 1.4 to 5.1 µg/ml were reported on resistant *B. cinerea* isolates from Japan (OSHIMA et al., 2002). Isolates resistant to procymidone, another DC fungicide, showed EC_{50} values ranging from 1.72 to 4.02 µg/ml (SUN et al., 2010). Some authors, have reported that isolates able to grown on 0.4 µg/ml were already considered resistant (BANNO et al., 2009). On the other hand, isolates having EC_{50} values lower than 2.58 µg/ml were still considered sensitive (JIANG et al., 2009). In another study, isolates having EC_{50} ranging from 9.2 to 24 µg/ml were considered moderately resistant (WEBER, 2011). Fernández-Ortuño et al. (2014) have classified isolates with absence of growth or 20 % of nontreated control growth at 10 µg/ml of iprodione as sensitive and low resistant isolates, respectively.

Overall, 57.6 % of the isolates, regardless their origin, showed EC_{50} values ranging from 1.2 to 4.34 µg/ml. Classification of *B. cinerea* isolates into highly

sensitive, sensitive, low, moderately and highly resistant to iprodione based upon spore germination assay was done for populations from strawberries and blackberries in the USA. Low to moderately resistant population had 20 to 50 % of their spores inhibited at 5 µg/ml (FERNÁNDEZ-ORTUÑO et al., 2016; GRABKE et al., 2014). Isolates with reduced sensitivity to iprodione were found in conventional (76.6 %) and organic (22.9 %) strawberry fields. The frequency of reduced sensitive isolates found on organic farm can be compared to the results previously reported in conventional fields in New Zealand (23 %), South Carolina, USA (23 %) and Spain (18 %) (BEEVER; BRIEN, 1983; FERNÁNDEZ-ORTUÑO et al., 2016; GRABKE et al., 2014). Moreover, the overall frequency of dicarboximides-resistant isolates of *B. cinerea* (57.6 %), regardless their origin, was similar to frequencies reported in Australia (54.2 %) and Spain (44 %) (DIÁNEZ et al., 2002; WASHINGTON; SHANMUGANATHAN; FORBES, 1992). The high resistance frequency observed in conventional fields was also reported on small fruits in Germany (64 %) (WEBER, 2011) and may be due to relatively high exposure of *B. cinerea* isolates to iprodione in recent years.

Studies have shown that dicarboximide-resistant strains usually have point mutations in the *bos1* gene, as observed in our studies. Isolates possessing the I365N mutation (isoleucine to asparagine) usually have an additional mutation at codon 127, causing a substitution of phenylalanine to serine (F127S) (GRABKE et al., 2014). However, the isolate possessing the I365N mutation (EC_{50} = 2.12 µg/ml) did not have the mutation F127S. Iprodione-resistant isolates of *B. cinerea* from strawberry fields from Spain and the USA also had the I365N mutation and, in addition, the I365S (isoleucine to serine) mutation (FERNÁNDEZ-ORTUÑO et al., 2015, 2016). Isolates having the I365N mutation with lower EC_{50} values than the isolates with the I365S mutation were also observed in other studies (FERNÁNDEZ-ORTUÑO et al., 2016; MA et al., 2007). Sequencing of isolates with EC_{50} values varying from 2.25 to 3.94 µg/ml indicated they had mutations at codons 369 (Q369P) and 373 (N373S), resulting in substitution of glutamine to proline and asparagine to serine, respectively, as previously observed in *B. cinerea* isolates with EC_{50} ranging from 2.48 to 2.68 µg/ml (MA et al., 2007). Only mutations at codon 365 were observed in resistant isolates collected from vineyards in France, but not in the others (LEROUX et al., 2002). Procymidone-resistant *B.* cinerea isolates from cucumbers also possessed the Q369P and the N373S mutations (SUN et al., 2010).

A similar result was obtained in field strains collected in Japan (OSHIMA et al., 2002).

Field rates of the commercial formulation of iprodione failed to control gray mold on detached fruit inoculated with dicarboximide-resistant *B. cinerea* isolates. Symptoms on fungicide-treated fruit inoculated with sensitive isolates were also expressed, however disease incidence and severity were lower and differed from the non-treated control and primarily from fungicide-treated fruit inoculated with resistant isolates. In other studies, iprodione-treated fruit inoculated with sensitive isolates did not develop any lesions (FERNÁNDEZ-ORTUÑO et al., 2014). The isolates used in our fruit assay study, with EC_{50} values ranging from 0.46 to 0.83 µg/ml, and classified as sensitive can be considered low resistant to iprodione, according to previous reports (BANNO et al., 2009). However, it was shown that sensitive isolates with EC_{50} values lower than 0.76 µg/ml did not have any of the most common mutations in the *bos1* gene mentioned by other authors. In fact, differences on gray mold lesion sizes were also observed on iprodione-treated fruit inoculated with low and moderately resistant isolates, in which low resistant isolates had significantly smaller lesion sizes (GRABKE et al., 2014). Therefore, considering all the variation in the classification of sensitive and resistant isolates to dicarboximides, separating *B. cinerea* strains and interpreting *in vivo* results may lead to incorrect answers.

In Brazil, there is a restricted amount of active ingredients registered for strawberries and complete resistance (pathogens are not controlled despite the application of higher field doses of the fungicide) was observed for at least two in our studies: azoxystrobin and thiophanate-methyl. Despite the emergence of resistant isolates, the disease can still be controlled with higher iprodione concentrations than that used to control sensitive isolates. Therefore, strawberry growers choose to spray iprodione in their farms.

In our studies, it was considered that *B. cinerea* isolates having EC_{50} values higher than 688 µg/ml were resistant to thiophanate-methyl. In fact, other studies have reported highly benzimidazole resistant isolates with EC_{50} values higher than 200, 436, 500 and 1000 µg/ml (FERNÁNDEZ-ORTUÑO; SCHNABEL, 2012; LAMONDIA; DOUGLAS, 1997; LUCK; GILLINGS, 1995; MERCIER; KONG; COOK, 2010; WEBER; HAHN, 2011; WEBER, 2011; YOURMAN; JEFFERS; DEAN, 2000).

B. cinerea isolates sensitive to thiophanate-methyl had EC_{50} values ranging from 0.4 to 7.5 µg/ml, with a mean of 1.71 µg/ml. Isolates collected from small-fruit

crops production in Germany had EC_{50} values varying from 0.05 to 0.126 µg/ml and were considered sensitive to thiophanate-methyl, whereas isolates with EC_{50} values from 6.1 to 16.1 µg/ml were considered moderatly resistant to the fungicide (WEBER, 2011). In other studies, isolates with EC_{50} values ranging from 0.05 to 0.17 µg/ml and from 4.67 to 16.1 µg/ml were also classified as sensitive and moderately resistant to thiophanate-methyl, respectively (WEBER; HAHN, 2011). *B. cinerea* isolates collected from ornamental crops had EC_{50} values ranging from 0.05 to 2.35 µg/ml and were considered sensitive to thiophanate-methyl (YOURMAN; JEFFERS; DEAN, 2000).

Populations of *Botrytis cinerea* resistant to thiophanate-methyl were found in conventional (92.2 %) and organic (51.4 %) strawberry fields. Overall, 77.8 % of the isolates were thiophanate-methyl resistant. High frequencies of resistance to benzimidazole fungicides, have already been reported: in Spain, 94 % of the collected isolates in 1997 were resistant to benomyl, another benzimidazole fungicide (DIÁNEZ et al., 2002); in South Carolina (USA), more than 81 % of the isolates were resistant to thiophanate-methyl (FERNÁNDEZ-ORTUÑO; SCHNABEL, 2012; YOURMAN; JEFFERS, 1999); in Connecticut (USA), 74.4 % of isolates collected from greenhouses were resistant to the fungicide (LAMONDIA; DOUGLAS, 1997); in strawberry fields of Southern States of the USA, this frequency was 76 and 85 % in 2012 and 2013, respectively (FERNÁNDEZ-ORTUÑO et al., 2014); in California, resistance was found in 92 % of the isolates (MERCIER; KONG; COOK, 2010), in Greece, 62 % of isolates collected from vegetable crops showed resistance to carbendazim, another benzimidazole fungicide (MYRESIOTIS; KARAOGLANIDIS; TZAVELLA-KLONARIA, 2007).

Consistent with previously published results, resistance to benzimidazole fungicides, as well as QoI and dicarboximide fungicides, is associated with target site mutations. All the sequenced isolates resistant to thiophanate-methyl had the mutation in the beta-tubulin (*ß-tub*) gene at the codon 198 (E198A), resulting in a replacement of glutamic acid (E) to alanine (A), but did not have a mutation at position 200. The occurrence of the E198A point mutation is usually found on *B. cinerea* highly resistant to benzimidazoles, and despite increases in fungicide concentration, a failure in field control of gray mold occurred (CHATZIDIMOPOULOS et al., 2014; FERNÁNDEZ-ORTUÑO; SCHNABEL, 2012; LEROUX et al., 2002). A mutation at position 200 (F200Y), leading to substitution of phenylalanine (F) by

tyrosine (Y), is frequently related to moderately resistant strains (BANNO et al., 2008; CHATZIDIMOPOULOS et al., 2014; LEROUX et al., 2002; YARDEN; KATAN, 1993). The same was observed for *Venturia inaequalis* isolates, where substitutions at codons 198 and 200 lead to high and medium resistance levels, respectively, to benzimidazoles. However, highly resistant isolates can possess amino acid substitutions at both codons 198 and 200 (FERNÁNDEZ-ORTUÑO et al., 2015). Two types of mutation at codon 198, E198A and E198K, resulting in replacement from glutamic acid (E) to lysine (K), were reported on *B. cinerea* isolates highly-resistant to benzimidazole fungicides in Japan (BANNO et al., 2008).

All benzimidazole-resistant isolates of *B. cinerea* inoculated on detached fruit treated with thiophanate-methyl produced gray mold. Field rates of the commercial formulation of thiophanate-methyl failed to control symptom expression. Most of the isolates had the same incidence and severity as the non-treated control and the fungicide-treated fruit. Similar results were observed for benzimidazole-resistant isolates collected from South Carolina (USA) strawberry fields (FERNÁNDEZ-ORTUÑO; SCHNABEL, 2012) and ornamental crops (YOURMAN; JEFFERS, 1999); and strawberry fields from the Southern States of the USA (FERNÁNDEZ-ORTUÑO et al., 2014). Two thiophanate-methyl-sensitive isolates of *B. cinerea* collected from ornamental crops produced disease symptoms on fungicide-treated geranium seedlings, however disease incidence was very low (YOURMAN; JEFFERS, 1999).

The high frequencies of resistant isolates to thiophanate-methyl can be related to the intensive use of benzimidazole fungicides since their introduction in the 1960s in Brazil, the high risk character of *B. cinerea* for resistance development and the absence of fitness cost associated with benzimidazole-resistant isolates in the field (FRAC, 2013; LEROUX et al., 2002; RAPOSO et al., 1996).

Most of the isolates from conventional fields were resistant to azoxystrobin, iprodione and thiophanate-methyl at the same time (75 %). However, only 8.6 % of the isolates from organic fields had resistance to the three tested active ingredients, whereas 34.3 % of them had no resistance to any of the fungicides tested. The highest and the lowest frequencies of the resistant isolates from organic areas were found to thiophanate-methyl (51.4 %) and iprodione (22.9 %), respectively. However, it should be noted that the frequency of isolates from organic farms with lower EC_{50} values was greater than isolates from conventional areas for all the fungicides (Figures 2.5, 2.6 and 2.7). Considering that site-specific fungicides are not permitted

in organic strawberry production, the presence of resistant *B. cinerea* populations in organic farms is intriguing, despite the lower frequencies observed. This fact could be explained by the risk of cross contamination and gene flow between fields, since strawberry production takes place in concentrated regions within the Brazilian States. Another reason is the introduction of new populations of *B. cinerea* every year via transplants from different nurseries, inasmuch as strawberries are grown annually in Brazil. Additionally, *B. cinerea* populations resistant to the main single-site fungicides used in Florida (USA) strawberry production were found in transplants from Northern America nurseries prior to transferring to the fields (OLIVEIRA, 2015). This study could explain the introduction of *B. cinerea* resistant isolates to organic fields, considering the absence of organic production of strawberry transplants in Brazil (M. BARBOSA[4], *personal information*) and other countries. Moreover, many conventional and organic growers acquire strawberry transplants from Brazilian nurseries, due to low prices, however they do not achieve the demanded standard for nursery certification (OLIVEIRA; SCIVITTARO, 2006), which can also lead to the overall emergence of fungicide resistance observed in our studies.

The occurrence and widespread multiple resistance to fungicides require modification in gray mold management, including changes in fungicides regulations and recommendations. Considering the high frequencies of isolates with multiple resistance to the most commonly used fungicides, as well the restricted number available of active ingredients with different mode of actions registered for strawberries, future perspectives for successful gray mold management in Brazil are uncertain.

Isolates of *C. acutatum* resistant to azoxystrobin were not found in this study and the average EC_{50} value based upon mycelial growth was 0.577 µg/ml. In fact, it has been reported that *C. acutatum* not exposed to QoI fungicides populations collected from strawberries in Florida had a mean EC_{50} of 0.3 µg/ml (FORCELINI et al., 2016). Similar observations were reported for never-exposed-isolates of *C. acutatum* from citrus with an EC_{50} average 0.4 µg/ml (MONDAL et al., 2005). However, *C. graminicola* from turfgrass had lower EC_{50} values to azoxystrobin (0.05 to 0.12 µg/ml) (AVILA-ADAME; OLAYO; WOLFRAM, 2003). EC_{50} values of isolates collected from organic fields were lower than 1.25 µg/ml; in fact, it was observed that

[4] BARBOSA, M. Personal communication during visit to the grower farm on June 2, 2016.

sensitive isolates of *C. acutatum* had EC_{50} values not higher than 1.625 µg/ml (FORCELINI et al., 2016). Isolates resistant to azoxystrobin had EC_{50} values higher than 10 µg/ml for *C. graminicola* from turfgrass and 100 µg/ml for *C. acutatum* (AVILA-ADAME; KÖLLER, 2003; FORCELINI et al., 2016). Moderately resistant isolates of *C. acutatum* had EC_{50} values ranging from 31.4 and 37 µg/ml (FORCELINI et al., 2016). None of our isolates showed the aforementioned EC_{50} values, and only 10 % of them, all from conventional fields, had values above 1.7 µg/ml, but still were considered sensitive.

Azoxystrobin was able to control spore germination of *C. acutatum* isolates and EC_{50} values could be determined. Previous authors reported that these assays were inadequate for evaluating *C. acutatum* sensitivity to QoI fungicides, claiming spore germination of sensitive isolates was not inhibited even at high fungicide doses (FORCELINI et al., 2016). However, this study differs from ours in the assessment method: single colonies originated from germinated spores were evaluated 2 to 3 days after spore deposition onto fungicide-amended media, whereas the percentage of spores producing germ tubes after nine hours of incubation were evaluated in our studies. Since strobilurins are known by having better effect on inhibiting spore germination, the longer waiting period for evaluation of colonies development may have led to the idea of lack of inhibition by the fungicide.

EC_{50} values determined upon spore germination assay were lower than for mycelial growth assay and were similar to the results found for *Plasmopara viticola* on grapes (WONG; WILCOX, 2000). This difference has already been observed for other pathogens such as *Colletotrichum capsici* (JIN et al., 2009) and *Alternaria alternata* (MONDAL et al., 2005). Since fungicidal activity of QoI fungicides is the inhibition of ATP production during mitochondrial respiration, spore germination could be more sensitive than mycelial growth to the effects of these fungicides. In fact, spore germination assays are the only ones used for determining fungi sensitivity to QoI fungicides in some studies (PASCHE; WHARAM; GUDMESTAD, 2004).

The nine isolates analyzed molecularly (EC_{50} ranging from 0.074 to 4.16 µg/ml) did not have any of the three mostly common found mutations in the cytochrome *b* gene: F129L, G137R and G143A. The occurrence of the G143A mutation, conferring complete or total resistance of pathogen populations to QoI fungicides, has already been described in several fungi, such as *B. cinerea*, *C. gloeosporioides* and *C. acutatum* from strawberries (AMIRI; HEATH; PERES, 2013;

FORCELINI et al., 2016; ISHII et al., 2009); and *Colletotrichum graminicola* and *C. cereale* from turfgrass (AVILA-ADAME; KÖLLER, 2003; YOUNG et al., 2010a). The presence of this mutation can lead to practical field resistance and serious control failures of diseases worldwide (BANNO et al., 2008; FERNÁNDEZ-ORTUÑO et al., 2016; FORCELINI et al., 2016; GISI et al., 2002). The F129L mutation, usually related to intermediate or moderate resistance, has been found in *C. acutatum* from strawberries (FORCELINI et al., 2016) and *C. cereale* in turfgrass.(YOUNG et al., 2010a), but not in our isolates. Although the mean EC_{50} value is 0.577 µg/ml, higher values were observed for isolates collected from conventional fields, despite the absence of mutation in the cytochrome *b* gene. Other mutations of nitrogenous bases at specific codons were observed in the sequenced portion of the cytochrome *b*, but none of them led to amino acid substitutions.

Field rates of commercial formulations of azoxystrobin failed to control Anthracnose fruit rot on detached strawberry fruit inoculated with some *C. acutatum* isolates and completely controlled other isolates. The presence or absence of symptoms were not related to the EC_{50} values nor to the absence of point mutations in the cytochrome *b* leading to QoI fungicide resistance, since isolates having the lowest EC_{50} mean values expressed disease symptoms and did not have the mutation in the gene. The occurrence of disease symptoms may be related to the period of seven days between fruit inoculation and disease evaluation, considering that disease assessment was made five days after inoculation in studies where azoxystrobin-treated fruit inoculated with sensitive isolates of *C. acutatum* did not develop symptoms (FORCELINI et al., 2016). Another explanation would be the use of few repetitions per treatment, meaning one diseased fruit would contribute for a higher level of disease incidence in the final data. Azoxystrobin must have completely inhibited disease development on fruit inoculated with sensitive isolates; however, disease severity on fungicide-treated fruit significantly differed from non-treated fruit.

In this study, difenoconazole-resistant isolates of *C. acutatum* were not found and the mean EC_{50} value based upon mycelial growth was 0.103 µg/ml. DMI fungicides have a significantly lower risk of resistance development than QoI fungicides (XU et al., 2014). Similar results have been reported for not exposed populations of *C. acutatum* in citrus, with EC_{50} mean value of 0.10 µg/ml (SILVA JUNIOR, 2011), and for *C. acutatum* in strawberries, with average of 0.156 µg/ml (FREEMAN et al., 1997). As observed for isolates collected from organic fields,

exposed and not exposed *C. acutatum* populations from citrus had EC_{50} values ranging from 0.07 to 0.14 µg/ml (SILVA JUNIOR, 2011). Propiconazole and tebuconazole also controlled *C. acutatum* mycelial growth by 50% at 0.1 µg/ml in strawberries (KOSOSKI et al., 2001). Only one isolate, from a conventional grower, had an EC_{50} value higher than 1 µg/ml, whereas the maximum EC_{50} mean value for all the other isolates was 0.2 µg/ml. Mondal et al. (2005) have also observed an EC_{50} mean value of 1.01 µg/ml in *C. acutatum* isolates from citrus to fenbuconazole, another DMI fungicide and *C. gloeosporioides* populations from strawberries had EC_{50} values ranging from 0.27 to 1.33 µg/ml to tebuconazole (XU et al., 2014).

Field rates of commercial formulations of difenoconazole, however, did not completely inhibited disease development on fungicide-treated fruit inoculated with *C. acutatum* isolates with different levels of difenoconazole sensitivity. In fact, it has already been observed that strawberry plants treated with difenoconazole and inoculated with *C. acutatum* isolates presented average mortality higher than 50 %, whereas nontreated plants had 70 to 80 % of mortality (FREEMAN et al., 1997). Failure in controlling symptoms expression may be due to the same reasons presented for azoxystrobin trials.

C. acutatum isolates showed a divergent response regarding sensitivity to thiophanate-methyl, with great variation of EC_{50} values. EC_{50} values could not be determined using the spiral gradient dilution method and mycelial growth was tested using discriminatory doses of thiophanate-methyl. In fact, 30.4 % of the tested isolates showed the same mycelial growth inhibition values for the three fungicide doses used (1, 10 and 100 µg/ml), which was not higher than 50 % and 11.4 % of them had the same mycelial inhibition at 10 and 100 µg/ml. Similar results were found for *C. acutatum* from citrus tested against benomyl, another benzimidazole fungicide, where colony area was reduced to about 25 % of the control at 1 µg/ml, but higher concentrations did not further reduce growth (PERES et al., 2004) and even at 1000 µg/ml, mycelial growth was not completely inhibited. Mycelial growth of *C. acutatum* from citrus was similar at carbendazim concentrations from 1 to 1000 µg/ml and the EC_{50} could not be determined due to the similar growth inhibition of 50 to 60 % at the different concentrations tested (SILVA JUNIOR, 2011). This behavior was also observed for *C. acutatum* from citrus that had mycelial growth at a benomyl concentration of 2500 µg/ml and was considered insensitive (GOES; KIMATI, 1998). Similar observations occurred in the fruit assay, where isolates with different levels of

sensitivity showed varied responses on the expression of symptoms on fruit treated with field rates of commercial formulations of thiophanate-methyl.

Despite the varied sensitivity responses to thiophanate-methyl, 98.2 and 27.3 % of the isolates collected from conventional and organic growers had EC_{50} values higher than 100 µg/ml. Populations of *B. cinerea* and *C. gloeosporioides* with the aforementioned EC_{50} values were considered resistant to MBC fungicides (CHUNG et al., 2006; MERCIER; KONG; COOK, 2010), whereas resistant populations of *C. gloeosporioides* from citrus and *C. cereale* from turfgrass were considered resistant at 10 µg/ml (PERES et al., 2004; WONG et al., 2008; YOUNG et al., 2010b). Several studies report the *C. acutatum* response to benzimidazole fungicides as less sensitive, since none of the analyzed isolates showed mutations at codons 167 (F167Y), 198 (E198A/K) or 200 (F200Y) in the beta-tubulin gene, responsible for conferring resistance to MBC fungicides (MA; MICHAILIDES, 2005). The E198A mutation has already been reported for resistant isolates of *C. gloeosporioides* from citrus and blueberries and *C. cereale* from turfgrass (PERES et al., 2004; WONG et al., 2008). In bentgrass, *Colletotrichum cereale* resistant populations to thiophanate-methyl presented the E198A and F200Y mutations (YOUNG et al., 2010b) and *C. acutatum* from strawberries and grapevines had the E198K mutation (CHUNG et al., 2006).

Although substitutions at other codons in the beta-tubulin gene of our isolates were observed, none of them caused amino acid replacement. *Colletotrichum* spp. have great genetic variability and studies of the beta-tubulin gene, among others, are used to molecularly classify *Colletotrichum*, which can represent several species. Just the *C. acutatum* complex is comprised of 30 species (BRAGANÇA et al., 2016). In our studies, isolates were simply classified as belonging to the complex *C. acutatum*. However, further molecular studies would be needed to differentiate these species, which may explain the variations observed in the beta-tubulin gene.

Considering the absence of the most commonly found mutations in the beta-tubulin gene that confer resistance to MBC fungicides, the low inhibition levels of *C. acutatum* by thiophanate-methyl might be related to the findings of Nakaune and Nakano (2007). They have shown that benzimidazole resistance of *C. acutatum* is not associated with amino acid substitutions in the beta-tubulin gene responsible for high resistance in other pathogens, but resistance is caused by enhanced expression of beta-tubulin 1 gene (*CaTUB1*) regulated by the protein *CaBEN1*. Laboratory

mutants, with interrupted expression of CaTUB1 and without any alteration on beta-tubulin gene, had their mycelial growth completely inhibited at 0.5 and 10 µg/ml of benomyl and thiophanate-methyl, respectively, whereas wild-type isolates were not inhibited at 100 µg/ml of thiophanate-methyl (NAKAUNE; NAKANO, 2007). However, the mechanism by which *CaBEN1* enhances the expression of *CaTUB1* gene is still unclear.

Despite the fact that resistance to single-site fungicides was not detected for *C. acutatum* populations of Brazilian strawberry fields, higher frequencies of isolates from conventional fields with higher EC_{50} values than from organic areas were observed. Considering the little information and few studies about fungicide sensitivity of *Colletotrichum* spp. in strawberries in Brazil, the report of resistance occurrence in other countries and to other pathogens and the selection pressure of restricted number of active ingredients registered for strawberry production, this study is important for future search for resistance emergence.

Therefore, disease control in general on strawberries will have to be achieved by integrating chemical and cultural control methods, such as with reduced fungicide application programs accompanied by monitoring of the resistance, the use of less susceptible cultivars, control of irrigation, the use of tunnels, the acquisition of healthy propagation material, among others. Constant inspections and monitoring for disease symptoms and fungicide resistance development in nurseries as well as their certification should be crucial for disease management of strawberry production systems. The development of systems that monitor the weather and recommend fungicide sprays only with favorable conditions for disease development can also be employed (PAVAN; FRAISSE; PERES, 2011). The use of these systems rather than fungicide sprays based upon a weekly calendar helps to reduce pesticide usage and, consequently, selection pressure of resistant populations of pathogens.

2.5 Conclusion

Botrytis cinerea populations collected from conventional and organic strawberry fields showed resistance to azoxystrobin, iprodione and thiophanate-methyl. Moreover, 75 and 8.6 % of isolates collected from conventional and organic areas, respectively, had multiple fungicide resistance to the three active ingredients

tested. Only 6.25 and 34.3 % of isolates from conventional and organic farms, respectively, did not show resistance to any of the fungicides tested.

C. acutatum populations collected from organic and conventional strawberry fields did not have resistance to the fungicides azoxystrobin and difenoconazole. However, isolates from conventional growers had higher EC_{50} values than isolates from organic areas. *C. acutatum* populations, regardless their origin, showed insensitivity to thiophanate-methyl rather than resistance.

This study reinforces the importance of continuous research and monitoring of risks associated with fungicide resistance occurrence; the urgency for the implementation of resistance management programs; and the need for an integrated approach between strawberry nurseries and production fields for disease control.

References

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA. **Programa de Análise de Resíduos de Agrotóxicos em Alimentos (PARA):** relatório de atividades de 2011 e 2012. Brasília, 2013. 45 p.

AGROFIT - SISTEMA DE AGROTÓXICOS FITOSSANITÁRIOS. 2016. Disponível em: <http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons>. Acesso em 05 maio 2016.

AMIRI, A.; HEATH, S.M.; PERES, N.A. Phenotypic characterization of multifungicide resistance in *Botrytis cinerea* isolates from strawberry fields in Florida. **Plant Disease**, Saint Paul, v. 97, n. 3, p. 393–401, 2013.

ANDRADE, L.N.T.; NUNES. M.U.C. **Produtos alternativos para controle de doenças e pragas em agricultura orgânica**. Aracaju: Embrapa-Tabuleiros Costeiros, 2001. 20 p. (Documentos, 281).

AVILA-ADAME, C.; KÖLLER, W. Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. **Pest Management Science**, Sussex, v. 59, n. 3, p. 303–309, 2003.

AVILA-ADAME, C.; OLAYO, G.; WOLFRAM, K. Characterization of Colletotrichum graminicola isolates resistant to strobilurin-related QoI fungicides. **Plant Disease**, Saint Paul, v. 87, n. 12, p. 1426–1432, 2003.

BANNO, S.; FUKOMORI, F.; ICHIISHI, A.; OKADA, K.; UEKUSA, H.; KIMURA, M.; FUJIMURA, M. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. **Phytopathology**, Saint Paul, v. 98, n. 4, p. 397–404, 2008.

BANNO, S.; YAMASHITA, K.; FUKUMORI, F.; OKADA, K.; UEKUSA, H.;

TAKAGAKI, M.; KIMURA, M.; FUJIMURA, M. Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. **Plant Pathology**, Oxford, v. 58, n. 1, p. 120–129, 2009.

BARTLETT, D.W.; CLOUGH, J.M.; GODWIN, J.R.; HALL, A.A.; HAMER, M.; PARR-DOBRZANSKI, B. The strobilurin fungicides. **Pest Management Science**, Sussex, v. 58, n. 7, p. 649–662, 2002.

BEEVER, R.E.; BRIEN, H.M.R. A survey of resistance to the dicarboximide fungicides in *Botrytis cinerea*. **New Zealand Journal of Agricultural Research**, Wellington, v. 26, n. 3, p. 391–400, 1983.

BRAGANÇA, C.A.D.; DAMM, U.; BARONCELLI, R.; MASSOLA JÚNIOR, N.S.; CROUS, P.W. Species of the *Colletotrichum acutatum* complex associated with anthracnose diseases of fruit in Brazil. **Fungal Biology**, Oxford, v. 120, n. 4, p. 547–561, 2016.

BRAUN, P.G.; SUTTON, J.C. Inoculum sources of *Botrytis cinerea* in fruit rot of strawberries in Ontario. **Canadian Journal of Plant Pathology**, Ontario, v. 10, p. 1–5, 1987.

_____. Infection cycles and population dynamics of *Botrytis cinerea* in strawberry leaves. **Canadian Journal of Plant Pathology**, Ontario, v. 10, n. 2, p. 133–141, 1988.

BRISTOW, P.R.; MCNICOL, R.J.; WILLIAMSON, B. Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. **Annals of Applied Biology**, Warwick, v. 109, n. 3, p. 545–554, 1986.

BROOME, J.C.; ENGLISH, J.T.; MAROIS, J.J.; LATORRE, B.A.; AVILES, J.C. Development of an infection model for Botrytis bunch rot of grapes based on wetness duration and temperature. **Phytopathology**, Saint Paul, v. 85, p. 97–102, 1995.

BULGER, M.A.; ELLIS, M.A.; MADDEN, L.V. Influence of temperature and wetness duration on infection of strawberry flowers by *Botrytis cinerea* and disease incidence of fruit originating from infected flowers. **Phytopathology**, Saint Paul, v. 77, n. 8, p. 1225-1230, 1987.

CHATZIDIMOPOULOS, M.; PAPAEVAGGELOU, D.; PAPPAS, A.C. Detection and characterization of fungicide resistant phenotypes of *Botrytis cinerea* in lettuce crops in Greece. **European Journal of Plant Pathology**, Dordrecht, v. 137, n. 2, p. 363–376, 2013.

CHATZIDIMOPOULOS, M.; GANOPOULOS, I.; VELLIOS, E.; MADESIS, P.; TSAFTARIS, A.; PAPPA, A.C. Development of a two-step high-resolution melting (HRM) analysis for screening sequence variants associated with resistance to the QoIs, benzimidazoles and dicarboximides in airborne inoculum of Botrytis cinerea. **FEMS Microbiology Letters**, Amsterdan, v. 360, n. 2, p. 126–131, 2014.

CHEN, S.; SHANG, Y.; WANG, Y.; SCHNABEL, G.; LIN, Y.; YIN, L. F.; LUO, C. X. Sensitivity of *Monilinia fructicola* from peach farms in China to four fungicides and characterization of isolates resistant to carbendazim and azoxystrobin. **Plant Disease**, Saint Paul, v. 98, n. 11, p. 1555-1560, 2014.

CHUNG, W.H.; ISHII, H.; NISHIMURA, K.; FUKAYA, M.; YANO, K.; KAJITANI, Y. Fungicide sensitivity and phylogenetic relationship of anthracnose fungi isolated from various fruit crops in Japan. **Plant Disease**, Saint Paul, v. 90, n. 4, p. 506–512, 2006.

CHUNG, W.H.; CHUNG, W.C.; PENG, M.T.; YANG, H.R.; HUANG, J.W. Specific detection of benzimidazole resistance in *Colletotrichum gloeosporioides* from fruit crops by PCR-RFLP. **New Biotechnology**, New York, v. 27, n. 1, p. 17–24, 2010.

COSTA, H.; VENTURA, J.A.; LOPES, U.P. Manejo integrado de pragas e doenças do morangueiro. **Horticultura Brasileira,** Brasília, v. 29, S5856-5877, 2011. **Anais...** Apresentado no CONGRESSO BRASILEIRO DE OLERICULTURA, 51., 2011, Viçosa.

DAVIDSE, L.C. Benzimidazole fungicides: mechanisms of action and biological impact. **Annual Review of Phytopathology**, Palo Alto, v. 24, p. 43–65, 1986.

DE MOURA, F.A.; NOGUEIRA, C.M.; GOUVÊA, M.A. Atributos determinantes na decisão de compra de consumidores de alimentos orgânicos. **Agroalimentaria**, Caracas, v. 18, n. 35, p. 75–86, 2012.

DE MICCOLIS ANGELINI, R.M.; ROTOLO, C.; MASIELLO, M.; GERIN, D.; POLLASTRO, S.; FARETRA, F. Occurrence of fungicide resistance in populations of *Botryotinia fuckeliana* (*Botrytis cinerea*) on table grape and strawberry in southern Italy. **Pest Management Science**, Sussex, v. 70, n. 12, p. 1785–1796, 2014.

DÉLYE, C.; LAIGRET, F.; CORIO-COSTET, M.F. A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. **Applied and Environmental Microbiology**, Washington, v. 63, n. 8, p. 2966–2970, 1997.

DIÁNEZ, F.; SANTOS, M.; BLANCO, R.; TELLO, J.C. Fungicide resistance in *Botrytis cinerea* isolates from strawberry crops in Huelva (southwestern Spain). **Phytoparasitica**, Bet Dagan, v. 30, n. 5, p. 529, 2002.

DOMINGUES, R.J.; TÖFOLI, J.G.; OLIVEIRA, S.H.F.; GARCIA JÚNIOR, O. Controle químico da flor preta (*Colletotrichum acutatum* Simmonds) do morangueiro em condições de campo. **Arquivos do Instituto Biológico**, São Paulo, v. 68, n. 2, p. 37–42, 2001.

DROBY, S.; LICHTER, A. Post-harvest *Botrytis* infection: Etiology, development and management. In: ELAD, Y.; WILLIAMSON, B.; TUDZYNSKI, P.; DELEN, N. (Ed.). **Botrytis:** biology, pathology and control. Dordrecht: Springer, 2007. p. 349–367.

DRY, I.B.; YUAN, K. H.; HUTTON, D.G. Dicarboximide resistance in field isolates of *Alternaria alternata* is mediated by a mutation in a two-component histidine kinase gene. **Fungal Genetics and Biology**, Orlando, v. 41, n. 1, p. 102–108, 2004.

ELMER, P.A.G.; GAUNT, R.E.; FRAMPTON, C.M. Spatial and temporal characteristics of dicarboximide-resistant strains of *Monilinia fructicola* and brown rot incidence in stone fruit. **Plant Pathology**, Oxford, v. 47, n. 4, p. 530–536, 1998.

FEKETE, E.; FEKETE, E.; LÁSZLÓ, I.; KARAFFAA, L.; ÁRNYASIC, M.; ASADOLLAHIA, M. SÁNDOR, E. Genetic diversity of a *Botrytis cinerea* cryptic species complex in Hungary. **Microbiological Research**, Jena, v. 167, n. 5, p. 283–291, 2012

FERNÁNDEZ-ORTUÑO, D.; CHEN, F.; SCHNABEL, G. Resistance to pyraclostrobin and boscalid in *Botrytis cinerea* isolates from strawberry fields in the Carolinas. **Plant Disease**, Saint Paul, v. 96, n. 8, p. 1198–1203, 2012.

FERNÁNDEZ-ORTUÑO, D.; GRABKE, A.; LI, X.; SCHNABEL, G. Independent emergence of resistance to seven chemical classes of fungicides in *Botrytis cinerea*. **Phytopathology**, Saint Paul, v. 105, n. 4, p. 424–432, 2015.

FERNÁNDEZ-ORTUÑO, D.; GRABKE, A.; BRYSON, P.K.; AMIRI, A.; PERES, N.A.; SCHNABEL, G. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven southern U.S. states. **Plant Diseae**, Saint Paul, v. 98, n. 6, p. 825–833, 2014.

FERNÁNDEZ-ORTUÑO, D.; SCHNABEL, G. First report of thiophanate-methyl resistance in *Botrytis cinerea* on strawberry from South Carolina. **Phytopathology**, Saint Paul, v. 96, n. 11, p. 1700, 2012.

FERNÁNDEZ-ORTUÑO, D.; TORÉS, J.A.; CHAMORRO, M.; PÉREZ-GARCÍA, A.; VICENTE, A. Characterization of resistance to six chemical classes of site-specific fungicides registered for gray mold control on strawberries in Spain. **Plant Disease**, Saint Paul, 2016. Disponível em: <https://www.researchgate.net/publication/303372062_Characterization_of_Resistance_to_Six_Chemical_Classes_of_Site-Specific_Fungicides_Registered_for_Gray_Mold_Control_on_Strawberries_in_Spain>. Acesso em: 21 maio 2016.

FORCELINI, B.B.; SEIJO, T.E.; AMIRI, A.; PERES, N.A. Resistance in strawberry isolates of *Colletotrichum acutatum* from Florida to quinone-outside inhibitor fungicides. **Plant Disease**, Saint Paul, 2016. doi: http://dx.doi.org/10.1094/PDIS–01–16–0118–RE.

FÖRSTER, H.; KANETIS, L.; ADASKAVEG, J.E. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. **Phytopathology**, Saint Paul, v. 94, n. 2, p. 163–170, 2004.

FOURNIER, E.; GIRAUD, T.; ALLBERTINI, C.; BRYGOO, Y. Partition of the *Botrytis cinerea* complex in France using multiple gene genealogies. **Mycologia**, New York, v. 97, n. 6, p. 1251–1267, 2005.

FRARE, G.F.; COUTO, H.T.Z.; CIAMPI-GUILLARDI, M.; AMORIM, L. The causal agent of citrus postbloom fruit drop, *Colletotrichum acutatum*, can survive on weeds. **Australasian Plant Pathology**, Collingwood, v. 45, n. 4, p. 339–346, 2016.

FREEMAN, S. Management, survival strategies, and host range of Colletotrichum acutatum on strawberry. **HortScience**, Alexandria, v. 43, n. 1, p. 66–68, 2008.

FREEMAN, S.; HOROWITZ, S.; SHARON, A. Pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. **Phytopathology**, Saint Paul, v. 91, p. 986–992, 2001.

FREEMAN, S.; NIZANI, Y.; DOTAN, S.; EVEN, S.; SANDO, T. Control of *Colletotrichum acutatum* in strawberry under laboratory, greenhouse, and field conditions. **Plant Disease**, Saint Paul, v. 81, n. 7, p. 749–752, 1997.

FUNGICIDE RESISTANCE ACTION COMMITTEE. **Pathogen risk list.** 2013. Disponível em: <http://www.frac.info/docs/default-source/publications/pathogen-risk/pathogen-risk-list.pdf> Acesso em: 26 maio 2016.

GHINI, R.; KIMATI, H. **Resistência de fungos a fungicidas**. Jagariúna: EMBRAPA Meio Ambiente, 2000. 78 p.

GISI, U.; SIEROTZKI, H.; COOK, A.; MCCAFFERY, A. Mechanisms influencing the evolution of resistance to Qo-inhibitor fungicides. **Pest Management Science**, Sussex, v. 58, n. 9, p. 859–867, 2002.

GOES, A.; KIMATI, H. Colletotrichum acutatum, agente causal da queda prematura dos frutos citricos: resistente ou insensível a benomyl? **Summa Phytopatholica**, Botucatu, v. 24, p. 246-253, 1998.

GOLEMBIEWSKI, R.C.; VARGAS JR., J.M.; JONES, A.L.; DETWEILER, A.R. Detection of demethylation inhibitor (DMI) resistance in *Sclerotinia homoeocarpa* populations. **Plant Disease**, Saint Paul, v. 79, p. 491–493, 1995.

GRABKE, A.; FERNÁNDEZ-ORTUÑO, D.; AMIRI, A.; LI, X.; PERES, N.A.; SMITH, P. Characterization of iprodione resistance in *Botrytis cinerea* from strawberry and blackberry. **Phytopathology**, Saint Paul, v. 104, p. 396–402, 2014.

GRASSO, V.; SIEROTZKI, H.; GARIBALDI, A.; GISI, U. Characterization of the cytochrome *b* gene fragment of *Puccinia* species responsible for the binding site of QoI fungicides. **Pesticide Biochemistry and Physiology**, San Diego, v. 84, n. 2, p. 72–82, 2006.

HEWITT, H.G. **Fungicides in crop protection**. Wallingford: CAB International, 1998. 221 p.

HINCAPIE, M.; WANG, N.Y.; PERES, N.A.; DEWDNEY, M.M. Baseline sensitivity of *Guignardia citricarpa* isolates from Florida to azoxystrobin and pyraclostrobin. **Plant Disease**, Saint Paul, v. 98, n. 6, p. 780–789, 2014.

HOWARD, C.M.; MAAS, J.L.; CHANDLER, C.K.; ALBREGTS, E.E. Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. **Plant Disease**, Saint Paul, v. 76, n. 10, p. 976–981, 1992.

ISHII, H.; FOUNTAINE, J.; CHUNG, W.H.; KANSAKO, M.; NISHIMURA, K.; TAKAHASHI, K. Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. **Pest Management Science**, Sussex, v. 65, n. 8, p. 916–922, 2009.

JARVIS, W.R. The infection of strawberry and raspberry fruits by *Botrytis cinerea* Fr. **Annals of Applied Biology**, Warwick, v. 50, n. 3, p. 569–575, 1962.

JIANG, J.; DING, L.; MICHAILIDES, T.J.; LI, H.; MA, Z. Molecular characterization of field azoxystrobin-resistant isolates of *Botrytis cinerea*. **Pesticide Biochemistry and Physiology**, San Diego, v. 93, n. 2, p. 72–76, 2009.

JIN, L.; CHEN, Y.; CHEN, C.; WANG, J.; ZHOU, M. Activity of azoxystrobin and SHAM to four phytopathogens. **Agricultural Sciences in China**, Beijing, v. 8, n. 7, p. 835–842, 2009.

KOENRAADT, H.; SOMMERVILLE, S.; JONES, A.L. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. **Phytopathology**, Saint Paul, v. 82, p. 1348–1354, 1992.

KOSOSKI, R.M.; FURLANETTO, C.; TOMITA, C.K.; CAFÉ FILHO, A.C. Antracnose do Morangueiro. **Fitopatologia Brasileira**, Brasília, v. 26, n. 3, p. 662–666, 2001.

LAMONDIA, J.A.; DOUGLAS, S.M. Sensitvity of *Botrytis cinerea* from Connecticut greenhouses to benzimidazole and dicarboximide fungicides. **Plant Disease**, Saint Paul, v. 81, n. 7, p. 729–732, 1997.

LEANDRO, L.F.S.; GLEASON, M.L.; NUTTER, F.W.; WEGULO, S.N.; DIXON, P.M. Influence of temperature and wetness duration on conidia and appressoria of *Colletotrichum acutatum* on symptomless strawberry leaves. **Phytopathology**, Saint Paul, v. 93, n. 4, p. 513–20, 2003a.

LEGARD, D.E.; XIAO, C.L.; MERTELY, J.C.; CHANDLER, C.K. Effects of plant spacing and cultivar on incidence of Botrytis fruit rot in annual strawberry. **Plant Disease**, Saint Paul, v. 84, n. 5, p. 531–538, 2000.

LEROUX, P.; CHAPELAND, F.; DESBROSSES, D.; GREDT, M. Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. **Crop Protection**, Guidford, v. 18, n. 10, p. 687–697, 1999.

LEROUX, P.; GREDT, M.; LEROCH, M.; WALKER, A.S. Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. **Applied and Environmental Microbiology**, Washington, v. 76, n. 19, p. 6615–6630, 2010.

LEROUX, P.; FRITZ, R.; DEBIEU, D.; ALBERTINI, C.; LANEN, C.; BACH, J. Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. **Pest Management Science**, Sussex, v. 58, n. 9, p. 876–888, 2002.

LUCK, J.E.; GILLINGS, M.R. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. **Mycological Research**, Cambridge, v. 99, n. 12, p. 1483–1488, 1995.

MAAS, J.L. **Compendium of strawberry diseases**. 2nd ed. Saint Paul: American Phytopathological Society Press, 1998.128 p.

MA, Z.; MICHAILIDES, T.J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. **Crop Protection**, Guidford, v. 24, n. 10, p. 853–863, 2005.

MA, Z.; YAN, L.; LUO, Y.; MICHAILIDES, T. J. Sequence variation in the two-component histidine kinase gene of *Botrytis cinerea* associated with resistance to dicarboximide fungicides. **Pesticide Biochemistry and Physiology**, San Diego, v. 88, n. 3, p. 300–306, 2007.

MACKENZIE, S.J.; MERTELY, J.C.; PERES, N.A. Curative and protectant activity of fungicides for control of crown rot of strawberry caused by *Colletotrichum gloeosporioides*. **Plant Disease**, Saint Paul, v. 93, n. 8, p. 815–820, 2009.

MADDEN, L.V.; WILSON, L.L.; ELLIS, M.A. Field spread of anthracnose fruit rot of strawberry in relation to ground cover and ambient weather conditions. **Plant Disease**, Saint Paul, v. 77, n. 9, p. 861–866, 1993.

MADDEN, L.V.; WILSON, L.L.; YANG, X.; ELLIS, M.A. Splash dispersal of *Colletotrichum acutatum* and *Phytophthora cactorum* by short-duration simulated rains. **Plant Pathology**, Oxford, v. 41, n. 4, p. 427–436, 1992.

MCNICOL, R.J.; WILLIAMSON, B.; DOLAN, A. Infection of red raspberry styles and carpels by *Botrytis cinerea* and its possible role in post-harvest grey mould. **Annals of Applied Biology**, Warwick, v. 106, n. 1, p. 49–53, 1985.

MERCIER, J.; KONG, M.; COOK, F. Fungicide resistance among Botrytis cinerea isolates from California strawberry fields. **Plant Health Progress**, Saint Paul, 2010. doi:10.1094/PHP–2010–0806–01–RS.

MERTELY, J.C.; PERES, N.A. **Root necrosis of strawberries caused by *Colletotrichum acutatum*.** Gainesville: University of Florida; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences, 2005. (Publication, PP-211).

MERTELY, J.C.; MACKENZIE, S.J.; LEGARD, D.E. Timing of fungicide applications for *Botrytis cinerea* based on development stage of strawberry flowers and fruit. **Plant Disease**, Saint Paul, v. 86, n. 9, p. 1019–1024, 2002.

MERTELY, J.C.; PERES, N.A.; CHANDLER, C.K. **Anthracnose fruit rot of strawberry**. Gainesville: University of Florida, Plant Pathology Department; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences, 2009. 4 p.

MERTELY, J.C.; CHANDLER, C.K.; XIAO, C.L.; LEGARD, D.E. Comparison of sanitation and fungicides for management of Botrytis fruit rot of strawberry. **Plant Disease**, Saint Paul, v. 84, n. 17, p. 1197–1202, 2000.

MONDAL, S.N.; BATHIA, A.; SHILTS, T.; TIMMER, L.W. Baseline sensitivities of fungal pathogens of fruit and foliage of citrus to azoxystrobin, pyraclostrobin, and fenbuconazole. **Plant Disease**, Saint Paul, v. 89, n. 11, p. 1186–1194, 2005.

MYRESIOTIS, C.K.; KARAOGLANIDIS, G.S.; TZAVELLA-KLONARIA, K. Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. **Plant Disease**, Saint Paul, v. 91, n. 4, p. 407–413, 2007.

NAKAUNE, R.; NAKANO, M. Benomyl resistance of *Colletotrichum acutatum* is caused by enhanced expression of beta-tubulin 1 gene regulated by putative leucine zipper protein CaBEN1. **Fungal Genetics and Biology**, Orlando, v. 44, n. 12, p. 1324–1335, 2007.

NORMAN, D.J.; STRANDBERG, J.O. Survival of *Colletotrichum acutatum* in soil and plant debris of leatherleaf fern. **Plant Disease**, Saint Paul, v. 81, n. 10, p. 1177–1180, 1997.

OLIVEIRA, M.S. **Fungicide resistance profile of quiescnet Botrytis cinerea infections on strawberry transplants**. 2015. 100 p. Thesis (Master of Science) - University of Florida, Gainesville, 2015.

OLIVEIRA, R.P.; SCIVITTARO, W.B. Desempenho produtivo de mudas nacionais e importadas de morangueiro. **Revista Brasileira de Fruticultura**, Cruz das Almas, v. 28, p. 520–522, 2006.

OSHIMA, M.; FUJIMURA, M.; BANNO, S.; HASHIMOTO, C.; MOTOYAMA, T.; ICHIISHI, A.; YAMAGUCHI, I. A point mutation in the two-component histidine kinase BcOS-1 gene confers dicarboximide resistance in field isolates of *Botrytis cinerea.* **Phytopathology**, Saint Paul, v. 92, n. 1, p. 75–80, 2002.

PASCHE, J.S.; WHARAM, C.M.; GUDMESTAD, N.C. Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. **Plant Disease**, Saint Paul, v. 88, n. 2, p. 181–187, 2004.

PAVAN, W.; FRAISSE, C.W.; PERES, N.A. Development of a web-based disease forecasting system for strawberries. **Computers and Electronics in Agriculture**, New York, v. 75, n. 1, p. 169–175, 2011.

PERES, N.A.; TIMMER, L.W.; ADASKAVEG, J.E.; CORRELL, J.C. Lifestyles of *Colletotrichum acutatum*. **Plant Disease**, Saint Paul, v. 89, n. 8, p. 784–796, 2005.

PERES, N.A.R.; SOUZA, N.L.; PEEVER, T.L.; TIMMER, L.W. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus. **Plant Disease**, Saint Paul, v. 88, n. 2, p. 125–130, 2004.

PRIMIANO, I.V. **Adaptabilidade de isolados de *Monilinia fructicola* com diferentes níveis de sensibilidade à azoxistrobina.** 2015. 82 p. Dissertação (Mestrado em Fitopatologia) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2015.

RAPOSO, R.; DELCAN, J.; GOMEZ, Z.; MELGAREJO, P. Distribution and fitness of isolates of *Botrytis cinerea* with multiple fungicide resistance in Spanish greenhouses. **Plant Pathology**, Oxford, n. 45, p. 497–505, 1996.

SCHNABEL, G.; BRYSON, P.K.; BRIDGES, W.C.; BRANNEN, P.M. Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. **Plant Disease**, Saint Paul, v. 88, n. 9, p. 1000–1004, 2004.

SEIJO, T.E.; CHANDLER, C.K.; MERTELY, J.C.; MOYER, C.; PERES, N.A. Resistance of strawberry cultivars and advanced selections to Anthracnose and Botrytis fruit rots. **Proceedings of the Florida State Horticultural Society**, Tallahassee, v. 121, p. 246–248, 2008.

SILVA JUNIOR, G.J. **Podridão Floral dos Citros :** dinâmicas temporal e espacial, sensibilidade de *Colletotrichum acutatum* a fungicidas e controle da doença. 2011. 131 p. Tese (Doutorado em Fitopatologia) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2011.

SMITH, B.J. Epidemiology and pathology of strawherry anthracnose: a North American perspective. **HortScience**, Alexandria, v. 43, n. 1, p. 69–73, 2008.

SUN, H.; WANG, H.-C.; CHEN, Y.; LI, H.-X.; CHEN, C.-J.; ZHOU, M.-G. Multiple resistance of *Botrytis cinerea* from vegetable crops to carbendazim, diethofencarb, procymidone, and pyrimethanil in China. **Plant Disease**, Saint Paul, v. 94, n. 5, p. 551–556, 2010.

SUTTON, J.C. Epidemiology and management of Botrytis leaf blight of onion and gray mold of strawberry: a comparative analysis. **Canadian Journal of Plant Pathology**, Ontario, v. 12, n. 1, p. 100–110, 1990.

TANAKA, M.A.S.; BETTI, J.A.; KIMATI, H. Doenças do morangueiro (*Fragaria* x *ananassa*). In: KIMATI, H.; AMORIM, L.; REZENDE, J.A.M.; BERGAMIN FILHO, A.; CAMARGO, L.E.A. (Ed.). **Manual de fitopatologia:** doenças das plantas cultivadas. São Paulo: Ceres, 2005. p. 489-499.

THOMIDIS, T.; MICHAILIDES, T.; EXADAKTYLOU, E. Contribution of pathogens to peach fruit rot in northern Greece and their sensitivity to iprodione, carbendazim, thiophanate-methyl and tebuconazole fungicides. **Journal of Phytopathology**, Berlin, v. 157, n. 3, p. 194–200, 2009.

WASHINGTON, W.S.; SHANMUGANATHAN, N.; FORBES, C. Fungicide control of strawberry fruit rots, and the field occurrence of resistance of *Botrytis cinerea* to iprodione, benomyl and dichlofluanid. **Crop Protection**, Guidford, v. 11, n. 4, p. 355–360, 1992.

WEBER, R.W.S. Resistance of *Botrytis cinerea* to multiple fungicides in Northern German small-fruit production. **Plant Disease**, Saint Paul, v. 95, n. 10, p. 1263–1269, 2011.

WEBER, R.W.S.; HAHN, M. A rapid and simple method for determining fungicide resistance in *Botrytis*. **Journal of Plant Diseases and Protection**, Berlin, v. 118, n. 1, p. 17–25, 2011.

WILCOX, W.F.; SEEM, R.C. Relationship between strawberry gray mold incidence, environmental variables, and fungicide applications during different periods of the fruiting season. **Phytopathology**, Saint Paul, v. 84, p. 264–270, 1994.

WILLIAMSON, B.; TUDZYNSKI, B.; TUDZYNSKI, P.; VAN KAN, J. *Botrytis cinerea*: The cause of grey mould disease. **Molecular Plant Pathology**, London, v. 8, n. 5, p. 561–580, 2007.

WONG, F.P.; CERDA, K.; HERNANDEZ-MARTINEZ, R. Detection and characterization of benzimidazole resistance in California populations of *Colletotrichum cereale*. **Plant Disease**, Saint Paul, v. 92, p. 239–246, 2008.

WONG, F.P.; WILCOX, W.F. Distribution of baseline sensitivities to azoxystrobin among isolates of *Plasmopara viticola*. **Plant Disease**, Saint Paul, v. 84, n. 3, p. 275–281, 2000.

XIAO, C.L.; CHANDLER, C.K.; PRICE, J.F.; DUVAL, J.R.; MERTELY, J.C.; LEGARD, D.E. Comparison of epidemics of Botrytis fruit rot and powdery mildew of strawberry in large plastic tunnel and field production systems. **Plant Disease**, Saint Paul, v. 85, n. 8, p. 901–909, 2001.

XU, X.F.; LIN, T.; YUAN, S.K.; DAI, D.J.; SHI, H.J. Characterization of baseline sensitivity and resistance risk of *Colletotrichum gloeosporioides* complex isolates from strawberry and grape to two demethylation-inhibitor fungicides, prochloraz and tebuconazole. **Australasian Plant Pathology**, Collingwood, v. 43, n. 6, p. 605–613, 2014.

YAMAGUCHI, I.; FUJIMURA, M. Recent topics on action mechanisms of fungicides. **Journal of Pesticide Science**, Tokyo, v. 30, n. 2, p. 67–74, 2005.

YARDEN, O.; KATAN, T. Mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin that correlate with benomyl-resistance phenotypes of field strains of *Botrytis cinerea*. **Phytopathology**, Saint Paul, v. 83, n. 12, p. 1478–1483, 1993.

YOURMAN, L.F.; JEFFERS, S.N.; DEAN, R.A. Genetic analysis of isolates of *Botrytis cinerea* sensitive and resistant to benzimidazole and dicarboximide fungicides. **Phytopathology**, Saint Paul, v. 90, n. 8, p. 851–859, 2000.

YOUNG, J.R.; TOMASO-PETERSON, M.; CERDA, K.; WONG, F.P. Occurrence and molecular identification of azoxystrobin-resistant *Colletotrichum cereale* isolates from golf course putting greens in the southern United States. **Plant Disease**, Saint Paul, v. 94, p. 751–757, 2010a.

YOUNG, J.R.; TOMASO-PETERSON, M.; TREDWAY, L.P.; CERDA, K. Two mutations in β-tubulin 2 gene associated with thiophanate-methyl resistance in *Colletotrichum cereale* isolates from creeping bentgrass in Mississippi and Alabama. **Plant Disease**, Saint Paul, v. 94, p. 207–212, 2010b.

YOURMAN, L.F.; JEFFERS, S.N. Resistance to benzimidazole and dicarboximide fungicides in greenhouse isolates of *Botrytis cinerea*. **Plant Disease**, Saint Paul, v. 83, n. 6, p. 569–575, 1999.

ZHANG, C.Q.; LIU, Y.H.; DING, L.; ZHU, G.N. Shift of sensitivity of *Botrytis cinerea* to azoxystrobin in greenhouse vegetables before and after exposure to the fungicide. **Phytoparasitica**, Bet Dagan, v. 39, n. 3, p. 293–302, 2011.

3 SPATIAL AND TEMPORAL DYNAMICS OF *Botrytis cinerea* AND *Colletotrichum* spp. IN BRAZILIAN CONVENTIONAL AND ORGANIC STRAWBERRY FIELDS

Abstract

Botrytis and Anthracnose fruit rots, caused by *Botrytis cinerea* and *Colletotrichum* spp., respectively, are major diseases in strawberry production worldwide. This study aimed to characterize the spatial and temporal development of these diseases in conventional and organic strawberry fields using epidemiological tools. Experiments were carried out during the 2015 strawberry season in conventional and organic farms in Sao Paulo State. In the conventional farm, two sub-areas, with cultivars 'Camarosa' and 'Oso Grande', were selected and each was composed by five 7-m beds with two lines of plants; and three 5-m beds, with an average of 16 plants per line, were chosen for disease evaluation. In the organic area, one sub-area with 'Camarosa' cultivar was selected, containing four 7-m beds with three lines of plants; and two 5-m beds, with an average of 16 plants per line were evaluated. Disease incidence was assessed weekly for symptomatic fruit and plants from June to October. Temporal progress models were fitted to the cumulative incidence (proportion) of diseased strawberry fruit or plants over time with non-linear regression analysis. Maps with symptomatic fruit or plants were prepared for each assessment date for each area. The spatial pattern of the diseases was analyzed according to the index of dispersion (*D*), the index of aggregation (V/M), and the Taylor's power law. The incidence-diseased fruit density relationship was analyzed by a linear model. Fruit with symptoms of *Colletotrichum* spp. were not observed in the organic area and were found in few assessment dates in the conventional area, with low final cumulative incidence. Cumulative incidence data of Botrytis fruit rot in symptomatic fruit and plants were generally well described by the logistic model. The organic area presented disease incidence and daily progress rate higher than the conventional area. The aggregation of diseased fruit on strawberry plants were observed for both areas. The linear relationship between diseased fruit density and incidence of diseased plants was highly significant ($p < 0.001$) and indicated diseased fruit density to be aggregated on strawberry plants for both areas. Botrytis fruit rot presented similar epidemiological patterns for both conventional and organic areas, however, disease intensity was greater for the organic area; this was likely due to differences in disease control management.

Keywords: *Fragaria* x *ananassa*; Gray mold; Logistic model; Diseased fruit density-incidence relationship; Production system

3.1 Introduction

Strawberries (*Fragaria* x *ananassa* Duch) are perennial herbaceous plants belonging to the order Rosales and family Rosaceae. Most common cultivars originated from the breeding of American and European species that have had their geographic ranges expanded to several temperate areas of the world for production

(RONQUE, 1998; FAO, 2016). In Brazil, strawberries are cultivated mainly as an annual crop in subtropical and temperate small areas using conventional or organic production practices (RADMANN et al., 2006). Temperature and photoperiod affect crop development. Mild temperatures and short days are favorable in stimulating flowering and fruitification, whereas high temperatures stimulate vegetative growth and stolons production. Most of the strawberries planted in Brazil are short-day cultivars sensitive to photoperiod, which are highly favored to flower by photoperiod of 12 hours or less and temperatures of 15 to 18 °C during the day and 8 to 10 °C at night (RONQUE, 1998). Strawberry fruit are manually harvested 2 to 3 times a week depending on grower's schedule and peaks of fruit production, and harvest period depends on the cultivar used. In Brazil, two of the most popular strawberry cultivars planted by growers show different fruitification patterns. The cultivar 'Camarosa' has longer fruitification period than most cultivars, and can be more exposed to the occurrence of diseases (PASSOS, 1999), while 'Oso Grande', a short-day premature cultivar, has been shown to initiate production 60 days after transplanting (BERNARDI et al., 2005).

Considering that blooming and fruitification occur simultaneously, strawberries are heavily affected by diseases (especially by fungi with the ability to infect flowers and fruit), causing yield and economic losses throughout crop-growing season and postharvest. Botrytis fruit rot or gray mold (caused by *Botrytis cinerea*), and Anthracnose fruit rot (caused mainly by *Colletotrichum acutatum* and *C. gloeosporioides*) are major strawberry diseases in Brazil and worldwide (BANNO et al., 2009; FERNANDEZ-ORTUÑO et al., 2016; MAAS, 1998; TANAKA; BETTI; KIMATI, 2005;).

Botrytis cinerea has a wide host range, causes diseases on more than 200 crops worldwide, and can be found in various parts of plants. Symptoms are most destructive on mature or senescent tissues and occur mainly on immature and ripe strawberry fruit. *B. cinerea* can overwinter in crop debris and senescent foliage within plant canopy, serving as important source of primary inoculum (BRAUN; SUTTON, 1987; MERTELY et al., 2000). The pathogen can be introduced within an area from nursery transplants (OLIVEIRA, 2015), but also be spread by wind, rain and overhead irrigation (BRISTOW; MCNICOL; WILLIAMSON, 1986; DROBY; LICHTER, 2007; WILLIAMSON et al., 2007). Infections usually occur through flowers and remain quiescent until weather conditions are favorable or host physiology changes

and symptoms can be expressed still during the crop-growing season but also at postharvest stage (DROBY; LICHTER, 2007; JARVIS, 1962; WILLIAMSON et al., 2007). Once in the plant, the fungus can sporulate with favorable growing conditions, and the diseased parts of plant can become a source of secondary inoculum in strawberry production since flowering and fructification occur simultaneously (DROBY; LICHTER, 2007; WILLIAMSON et al., 2007). Secondary infections can occur via contact of healthy fruit with infected parts of plants or via spore dispersion within the same growing season. Thus, the fungus has been shown to exhibit polycyclic nature with successive cycles of infection (BRAUN; SUTTON, 1988). Spore production and release is regulated by a diurnal cycle that is dependent on fluctuations of temperature and humidity (WILLIAMSON et al., 2007).

Colletotrichum spp. are responsible for causing anthracnose diseases in several types of hosts worldwide, including strawberries (SMITH, 2008). A single *Colletotrichum* species may infect distinct plant hosts, whereas many species can cause symptoms in a single host, such as *C. acutatum*, *C. gloeosporioides* and *C. fragariae* in strawberries (FREEMAN, 2008). Strawberry leaves, petioles, crowns, roots, flowers, and fruit are infected by *Colletotrichum* spp. (MERTELY; PERES; CHANDLER, 2009; UREÑA-PADILLA et al., 2002). Anthracnose fruit rot, caused by *C. acutatum* and *C. gloeosporioides*, produces symptoms on fruit, but lesions can also be found on petioles, stolons and flowers (HOWARD et al., 1992; MAAS, 1998). *Colletotrichum* spp. can be introduced into strawberry growing areas by infected transplants from nurseries, and symptoms may or may not be present on leaves and petioles (HOWARD et al., 1992; PERES et al., 2005). The pathogen can survive on and in strawberry plants, soil, crop debris, weeds, and other crops without showing any symptoms on epiphytic stages or quiescent infections (FRARE et al., 2016; LEANDRO et al., 2003a; MACKENZIE et al., 2009). Dissemination of *Colletotrichum* spp. occurs mainly at short distances by water splash from rainfall and overhead irrigation, but also through field equipment and harvest operations (FREEMAN; HOROWITZ; SHARON, 2001; FREEMAN, 2008; HOWARD et al., 1992).). Infections occur in every part of strawberry plants and are very dependent on wetness duration and temperature during flowering and fruitification periods (LEANDRO et al., 2003a; WILSON; MADDEN; ELLIS, 1990). Very short rains are favorable for *Colletotrichum* spp. infection, leading potentially to high levels of disease incidence in the field (MADDEN et al., 1992). The latent period for Anthracnose is highly dependent on

temperature, varying from 2 to 3 days at 25 °C and from 6 to 17 days at 5 °C (KING et al., 1997).

The temporal and spatial dynamics of plant disease are crucial for interpreting disease occurrence and development, field pathosystem behavior, and selecting different management and production practices. (CAMPBELL; MADDEN, 1990). These analyses are possible with the aid of epidemiological tools like temporal models of disease development over time and methods to analyze the spatial patterns and distribution of infected plants (MADDEN; HUGHES, 1995; NUTTER JR., 1997).

Epidemiology, in plant pathology, is the science studying the dynamics of diseases in populations and has been presented with several definitions by plant pathologists (KRANZ, 1974; MADDEN; HUGHES; VAN DEN BOSCH, 2007; VANDERPLANK, 1963). Plant diseases can be classified as monocyclic and polycyclic given their temporal dynamic. Moreover, depending on spatial distribution and transmission patterns, diseases can spread in either random or aggregate patterns in the field (CAMPBELL; MADDEN, 1990). Studies about the influence of environmental conditions (e.g., temperature, wetness period, rainfall, surface topography, source of inoculum, etc.) on the development of diseases throughout time and space have been conducted on strawberries to characterize the occurrence of different diseases and develop methods of control (BOFF et al., 2001; MADDEN; WILSON; ELLIS, 1993; YANG et al., 1990).

Understanding the dynamics of plant diseases and comparing production systems (i.e., such as conventional and organic), with the aid of epidemiological tools can also help us understand the influence of these practices on disease development and propose methods of control. Since there are currently no cultivars of strawberry that are highly resistant to Botrytis and Anthracnose fruit rots, a combination of cultural and chemical control methods are widely used in Brazil (COSTA; VENTURA; LOPES, 2011; TANAKA; BETTI; KIMATI, 2005). In farms where conventional production practices are employed, fungicide spray during the whole strawberry season is very common in controlling diseases, whereas in organic systems, an integrated management using cultural and biological control, besides Bordeaux and Viçosa mixtures, is more common (ANDRADE; NUNES, 2001). In farms where conventional production practices are employed, fungicide sprays that are applied throughout the entire season are very common for controlling diseases, whereas in

organic systems, an integrated management approach that utilizes a combination of cultural and biological controls (i.e., besides Bordeaux and Viçosa mixtures) is more common (ANDRADE; NUNES, 2011). However, indiscriminate and continuous usage of fungicides can promote the selection of resistant pathogens, and this has been extensively reported with *Botrytis cinerea* and *Colletotrichum* spp. under conventional systems worldwide (AMIRI; HEATH; PERES, 2013; BANNO et al., 2008; FERNÁNDEZ-ORTUÑO et al., 2016; FORCELINI et al., 2016; ISHII et al., 2009; MERCIER; KONG; COOK, 2010). For this reason, epidemiological studies may help in characterize the field development patterns of diseases under different management practices.

Furthermore, little information on temporal and spatial dynamics of Anthracnose and Botrytis fruit rots in conventional and organic strawberry production is available. Therefore, this study was conducted to characterize the spatial and temporal development of Botrytis and Anthracnose fruit rots in conventional and organic strawberry fields using epidemiological tools.

3.2 Material and Methods

In order to study the spatial and temporal progress of the diseases caused by *B. cinerea* and *Colletotrichum* spp. in conventional and organic strawberry fields, experiments were carried out during the 2015 strawberry season in Valinhos and Jarinu, both in São Paulo State.

3.2.1 Description of the experimental areas

A conventional commercial farm in Valinhos, São Paulo, Brazil (23°01'34.1” S; 47°02'16.9” W; altitude: 660 m) was utilized for the conventional portions of the experiments. Strawberry plants were cultivated on black-plastic-mulch covered beds, (which were 1 m wide) that contained two rows of plants spaced 50 cm apart between rows and 20 cm apart within rows. The distance between bed centers was 1.5 m and the bed aisles were covered with straw (Figure 3.1A). The field was overhead irrigated by sprinklers in the beginning of the season to promote establishment, and once plants were established, drip irrigation system was used. Two sub-areas, with cultivars ‘Camarosa’ and ‘Oso Grande’, were selected and each

one of them was composed by five 7-m beds; and three 5-m beds, with an average of 16 plants per line (32 plants/bed), were selected for disease evaluation (Figure 3.1B). The selected areas received the standard fungicide spray treatment adopted by the conventional grower (Appendix A). Senescent foliage and crop debris within the strawberry plants were removed weekly.

Figure 3.1 – Experiments installed in strawberry fields in a conventional area in Valinhos-SP (A, B) and in an organic grower in Jarinu-SP (C, D) to evaluate the spatial and temporal progress of the diseases caused by *B. cinerea* and *Colletotrichum* spp in 2015. Gray rectangles represent beds covered by black plastic with two (B) and three (D) lines of plants (symbolized by X). Black squares represent the areas chosen to be evaluated

The organic portions of the experiments were conducted on an organic farm in Jarinu, São Paulo, Brazil (23°01'13.1" S; 46°41'38.0" W; Altitude: 781 m). Strawberry plants from 'Camarosa' cultivar were grown on black-plastic-mulch covered beds, which were 1 m wide and contained three rows of plants spaced 30 cm apart between rows and 30 cm apart within rows. The distance between bed centers was 1.5 m and the bed aisles were covered with straw (Figure 3.1C). Irrigation regimes were performed the same way as the conventional site. One sub-area, containing

four 7-m plant beds was selected along with and two sub-areas with 5-m beds (each with an average of 16 plants per line – or 48 plants/bed), were selected for disease evaluation (Figure 3.1D). The selected areas were treated with the standard operation management procedures adopted by the organic grower (Appendix B). Senescent foliage and crop debris within the strawberry plants were not frequently removed.

3.2.2 Disease assessment and data collection

From the beginning to the end of the strawberry harvest season, flowers and fruit with disease symptoms caused by *B. cinerea* and *Colletotrichum* spp. in each plant were monitored weekly. If the symptoms had been initial at the field, flowers and fruit were brought to the laboratory and kept in sealed moisture chambers at 23 °C for 24 to 48 hours in order to better visualize the expression of symptoms and pathogens structures.

The conventional area was assessed weekly 19 times from June 2nd to October 20th. The organic farm was visited 14 times and evaluations took place every 15 days in the beginning of the harvest season (from June 2nd to July 30th) and weekly from the middle to the end of the season (from July 30th to October 14th).

Disease incidence was evaluated for symptomatic fruit and plants with symptomatic fruit, and was described as the proportion of diseased fruit per total of harvested fruit during the season and proportion of plants having symptomatic fruit out of total plants evaluated, respectively. Regardless of the strawberry cultivar, each plant was considered to produce an average of 30 fruit throughout the entirety of the harvest season (D. JULIATO, *personal information*[5]). Diseased fruit density was also recorded, and defined by the average of number of symptomatic strawberry fruit in relation to strawberry plants. Each strawberry bed was treated as one replication, so there were three and two replications per area for conventional and organic farms, respectively.

[5] D. JULIATO. Personal communication during visit to the grower farm on October 15, 2013.

3.2.3 Temporal analysis of the diseases

Temporal progress models were fitted to the cumulative incidence (proportion) of diseased strawberry fruit or plants based on the first detection of symptoms, via non-linear regression analysis. Monomolecular ($y(t) = y_{max} - (y_{max} - y_0)*(\exp(-r*t))$, logistic ($y(t) = y_{max} /(1+((y_{max} - y_0)/ y_0)*\exp(-r*t))$ and Gompertz ($y = y_{max}*(\exp(-(-\log(y_{max}/y_0))*\exp(-r*t)))$ models were fitted to the incidence data. Here, y is the cumulative proportion of fruit or plants with disease symptoms, t is time, in days, after appearance of first symptom, y_{max} is the curve asymptote, y_0 is the initial inoculum, and r is the disease progress rate. The model that best fitted to the incidence data was selected based on the coefficient of determination (R^2), the standard error of the parameters estimated by each model fit, and the distribution of residuals (MADDEN; HUGHES; VAN DEN BOSCH, 2007).

The number of non-cumulative symptomatic fruit or plants assessed at each evaluation day over time was analyzed by the area under the disease progress curve (AUPDC), with the equation AUDPC = $\sum ((x_i + x_{i+1})/2)*(t_{i+1} - t_i)$, where x is the number of symptomatic fruit or plants over time t_i and $(t_{i+1} - t_i)$ is the time period between to following assessments (CAMPBELL; MADDEN, 1990). The AUDPC data from conventional and organic areas were compared by ANOVA.

3.2.4 Characterization of the spatial dynamics of the diseases

The spatial pattern of fruit or plants with symptoms of Botrytis and Anthracnose fruit rots was monitored in both strawberry farms. Maps showing the cumulative incidence of symptomatic fruit and diseased plants were prepared for each assessment date for each plot. Data were examined for the presence of aggregation or randomness by partitioning the areas in the conventional and organic farms, respectively, into quadrats of 4 (2 by 2) and 6 (3 by 2) plants. The spatial pattern of the diseased fruit and plants was analyzed according to the index of dispersion (D), the index of aggregation (V/M), and Taylor's power law. The index of dispersion (D) and modified Taylor's power law were used to estimate aggregation of diseased plants (proportion) (MADDEN; HUGHES; VAN DEN BOSCH, 2007). The index of aggregation (V/M) and Taylor's power law was used for the diseased fruit density (number of symptomatic fruit per plant) (TAYLOR, 1984).

The index of dispersion (*D*) was calculated to estimate the degree of aggregation as the proportion between observed variance of plants with symptomatic fruit (Vobs) and the expected binomial variance (Vbin). *D* value was estimated according to equation D = Vobs/Vbin, where Vobs = $\sum(X_i - np)^2 / n^2 (N-1)$ and Vbin = $p(1-p) / n$. Here, X_i is the number of diseased plants within a quadrat, n is the number of plants per quadrat, p is the disease incidence, and N is the number of quadrats. Randomness within each quadrat was determined by chi-square test, with (N-1) degrees of freedom at $p < 0.05$. Index of dispersion values not significantly different from 1 ($D = 1$) indicated the randomness distribution pattern of symptomatic plants. When the observed variance is greater than the expected binomial variance, *D* is significantly greater than 1 ($D > 1$), suggesting aggregation of diseased plants (MADDEN; HUGHES; VAN DEN BOSCH, 2007). The index of aggregation (V/M), defined as the variance-to-mean ratio, was used to estimate the degree of aggregation of diseased fruit density. It was calculated by dividing the variance of the number of diseased fruit by the mean. Ratio values not different and greater than 1, respectively, suggest random or aggregated pattern of disease distribution, according to a chi-square test, with (N-1) degrees of freedom at $p = 0.05$ (MADDEN; HUGHES; VAN DEN BOSCH, 2007; TAYLOR, 1984).

Power law analyses were performed on both the incidence of diseased plants and diseased fruit density. For the incidence of diseased plants data, the modified Taylor's power law was fitted according to the equation log (Vobs) = log (A) + b log (Vbin). Vobs and Vbin are the observed (dependent variable) and expected binomial (independent variable) variances, respectively, and log (A) and b are the intercept and slope parameters, respectively. Linear regression models were fitted to the data and the significance of relationship between the variances was determined by *F* test ($p = 0.05$). Goodness-of-fit for the model was determined with the coefficient of determination (R^2) and the distribution of residuals. Student's *t*-tests ($p < 0.05$) were used to determine the significance of the individual estimated parameters. When $b = 1$ and log (A) = 0, a random condition in the spatial distribution was inferred; when $b = 1$ and log (A) > 0, a constant level of aggregation for all the disease incidence values was implied and aggregation did not depend on disease incidence; and when $b > 1$ and log (A) > 0, the degree of aggregation varied according to disease incidence (MADDEN; HUGHES, 1995; MADDEN; HUGHES; VAN DEN BOSCH, 2007). Analysis for diseased fruit density data used Taylor's power law to describe

the relationship between the mean (*m*) and the observed variance (Vobs) for the number symptomatic fruit, and was fitted according to the equation log (Vobs) = log (*A*) + *b* log (*m*). For the incidence of diseased plants, a goodness-of-fit of the model was determined with the coefficient of determination (R^2) values and the estimated parameters were tested for significance with Student's *t*-tests. When log (*A*) = 0 and *b* = 1, there was no aggregation (CARISSE; MELOCHE; TURECHEK, 2011; LI et al., 2007; TAYLOR, 1961).

3.2.5 Incidence-diseased fruit density relationship

The relationship between the incidence of diseased strawberry plants and the number of symptomatic fruit per plant was evaluated. Aggregated pattern is confirmed when the observed data points lie above the expected Poisson distribution line. The model ln (*m*) = β_0 + β_1 CLL (*p*), where *m* represents the average number of symptomatic fruit per plant, CLL(*p*) is the complementary log-log transformed incidence, CLL = ln(-ln(1-*p*)), where *p* is the incidence of diseased plants, and β_0 and β_1 are the models parameters, was fitted to the data by linear regression. Model fit was evaluated based on coefficient of determination (R^2) and the distribution of residuals, and Student's *t*-tests was used to determine if the slope was greater than 1, based on the estimated β_1 and its standard error (CARISSE; MELOCHE; TURECHEK, 2011; MCROBERTS; HUGHES; MADDEN, 2003).

3.3 Results

The conventional farm site showed Anthracnose fruit rot symptoms for three and four assessment dates for areas planted with the strawberry cultivars 'Camarosa' and 'Oso Grande', respectively (Appendix C). The final incidence of cumulative diseased fruit and plants displaying symptoms of anthracnose were, 0.77 and 9.66 %, respectively, for the 'Camarosa' cultivar, and 1.89 and 23.19 %, respectively, for the 'Oso Grande' cultivar. Epidemiological models of temporal progress and the spatial dynamics of Anthracnose fruit rot could not be fitted to the incidence data. Fruit with anthracnose symptoms were not observed in any of the plants from the organic area. Symptoms of Botrytis fruit rot were observed during six assessment dates for conventional areas planted with 'Oso Grande', however, the final incidence

of cumulative diseased fruit and plants were 0.54 and 14.39 %, respectively (Appendix C). Temporal and spatial analyses of the disease could not be performed because none of the most commonly used epidemiological models could be fitted to the data. Therefore, only data from Botrytis fruit rot incidence on ‘Camarosa’ strawberry cultivar in both areas were analyzed by fitting epidemiological models of temporal and spatial dynamics of diseases.

3.3.1 Temporal analysis of Botrytis fruit rot

For the conventional grower sites, the mean incidence of diseased fruit and plants reached 6 and 49 %, respectively, whereas for the organic site, there were 21.4 and 90.5 % of fruit and plants, respectively, with gray mold symptoms at the end of the strawberry season (Figure 3.2). Incidence of diseased fruit and plants increased from 2.8 to 5.5 % (Figure 3.2A) and 30.2 to 44.5 % (Figure 3.2C), respectively, within 15 days for the conventional site, whereas in the organic site, showed an increase from 2.7 to 15.1 % (Figure 3.2B) and 41.7 to 87.5 % (Figure 3.2D) within the same 15 days and assessment dates. For the conventional grower (Figure 3.1A), incidences of diseased fruit remained low, and basically unchanged over the first 40 days of evaluation; while disease increase was also low over the entire period compared to the organic area (Figure 3.1B).

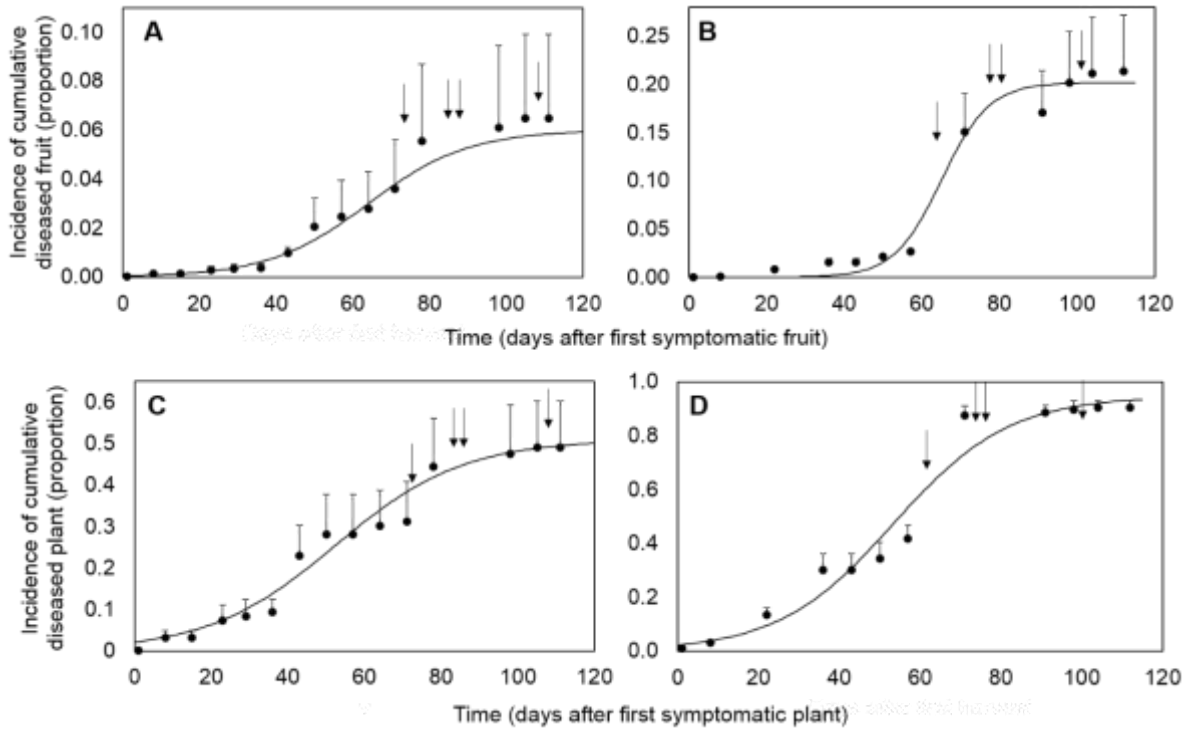

Figure 3.2 – Incidence of cumulative Botrytis fruit rot (proportion) of symptomatic fruit (A, B) and plants with symptomatic fruit (C, D) in conventional (A, C) and organic (B, D) strawberry fields. Solid lines indicate the logistic model ($y(t) = y_{max}/(1+((y_{max} - y_0)/ y_0)*\exp(-r*t)$ fitted to the observed incidence of Botrytis fruit rot. Closed circles and error bars represent the average incidence and standard errors, respectively, of two (B, D) and three (A, C) replications. Arrows indicate rainfall events over 25 mm

The logistic model showed a better fit to the observed data of incidence of diseased fruit and plants for the organic (Figure 3.2B) and conventional (Figure 3.3C) areas, respectively, when compared to the monomolecular and Gompertz models (Table 3.1). Although the logistic model presented the higher values of R^2 and random distribution of residuals when compared to the other models, the y_0 parameter related to the initial inoculum was not significantly adjusted to the data of incidence of diseased fruit and plants for the conventional and organic growers, respectively, by the non-linear model (Table 3.1). The rate of disease increase (r) in the organic area was higher than in the conventional area for diseased fruit, and the same for diseased plants, according to the t test (Table 3.1).

Table 3.1 – Parameters and respective errors estimated by non-linear regression of monomolecular, logistic and Gompertz models fitted to the cumulative incidence (proportion) of strawberry fruit or plants with Botrytis fruit rot symptoms in conventional and organic strawberry fields

Location	Model[a]	Estimated parameters			
		R^2	y_{max} (SE)	y_0 (SE)	r (SE)
Diseased fruit					
Conventional	Monomolecular	0.928	2.196 (30.9)	-0.011 (0.005)	0.0003 (0.005)
	Logistic	0.987	0.067* (0.003)	0.0004 (0.00002)	0.077* (0.009)
	Gompertz	0.854	0.078* (0.034)	1.425 (1.124)	0.019 (0.014)
Organic	Monomolecular	0.870	9.502 (290.04)	-0.046 (0.028)	0.0002 (0.008)
	Logistic	0.982	0.202* (0.007)	0.000005* (0.00)	0.163* (0.033)
	Gompertz	0.752	0.243 (0.191)	3.439 (4.364)	0.018 (0.025)
Diseased Plants					
Conventional	Monomolecular	0.959	2.053 (2.405)	0.038 (0.028)	0.003 (0.004)
	Logistic	0.970	0.507* (0.029)	0.021* (0.007)	0.059* (0.008)
	Gompertz	0.970	0.553* (0.045)	61.297 (55.32)	0.034* (0.006)
Organic	Monomolecular	0.933	6.032 (18.68)	0.056 (0.080)	0.002 (0.006)
	Logistic	0.967	0.947* (0.055)	0.022 (0.015)	0.070* (0.015)
	Gompertz	0.957	1.037* (0.125)	312.8 (768.9)	0.037* (0.012)

[a] Monomolecular, logistic and Gompertz models were fitted to the observed cumulative incidence of strawberry fruit and plants with Botrytis fruit rot symptoms using $y(t) = y_{max} - (y_{max} - y_0)*(\exp(-r*t)$, $y(t) = y_{max}/(1+((y_{max} - y_0)/ y_0)*\exp(-r*t)$ and $y = y_{max}*(\exp(-(-\log(y_{max}/y_0))*\exp(-r*t))$, respectively, where y is the cumulative proportion of fruit or plants with disease symptoms, t is time, in days, after appearance of first symptom, y_{max} is the curve asymptote, y_0 is the initial inoculum, r is the disease progress rate, R^2 is the coefficient of determination and SE is the standard error of the parameter. Values followed by (*) indicate the parameters were significantly fitted to the disease incidence data by the non-linear models ($p < 0.05$)

The increase in the number of symptomatic fruit and plants at the same period in both areas can also be observed in Figure 3.3. This increase occurred after a rainfall event that happened in the last week of August, 2015, about, 50 to 60 days after the appearance of the first symptomatic fruit for both areas. Moreover, the AUDPC values of the organic grower were higher than the conventional area, according to the ANOVA analysis ($p < 0.05$) for both symptomatic fruits and diseased plants. Mean AUDPC values for conventional and organic sites, were 564.2 and 4387.8 units of area for symptomatic fruit, respectively (Figure 3.3A) and 322.7 and 1519 units of area for diseased plants, respectively (Figure 3.3B).

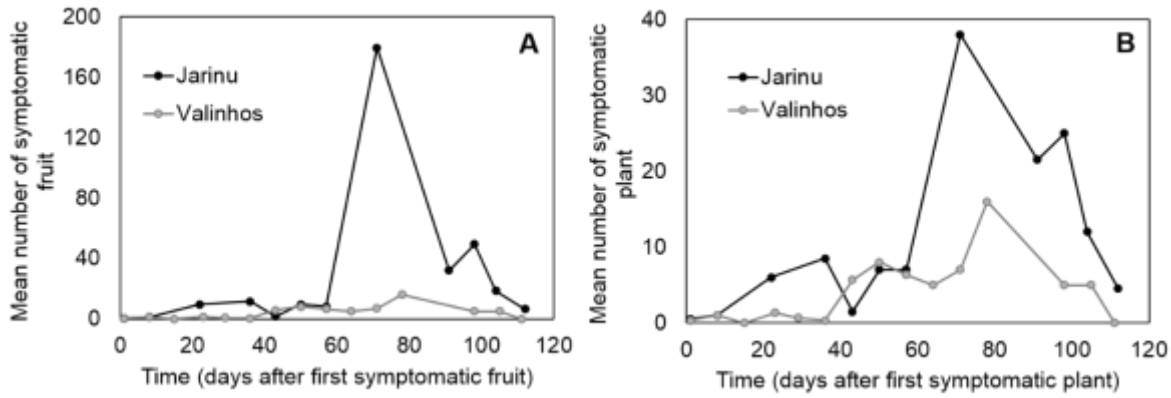

Figure 3.3 – Mean number of symptomatic strawberry fruit (A) and plants (B) registered over time, in days, after the first symptom appearance in conventional and organic strawberry fields, during the 2015 strawberry season

3.3.2 Characterization of the spatial dynamics of Botrytis fruit rot

Maps of disease distribution were produced for conventional and organic areas on each assessment date, however six of them for each cumulative data of diseased plants (Table 3.2) or fruit (Table 3.3) were selected for showing data of incidence of diseased plants or diseased fruit density and index of dispersion or aggregation.

Table 3.2 – Incidence of diseased plants (%) and dispersion index (*D*) of cumulative incidence of strawberry plants with fruit having Botrytis fruit rot symptoms over time in conventional and organic strawberry fields

Location	Date[a]	Incidence of diseased plants (%)[b]	Index of Dispersion (*D*)[c]
Conventional[f]	18/06/2015	0.03	1.01
	16/07/2015	8.33	1.04
	07/08/2016	28.13	2.18*
	03/09/2015	44.46	2.02*
	06/10/2015	49.09	1.61*
Organic[e]	25/06/2015	1.04	1.01
	16/07/2015	13.54	0.48
	03/09/2015	87.50	1.07
	30/09/2015	89.58	0.99
	14/10/2015	90.49	1.13

[a] Day/month/year
[b] Average of the proportion of plants with symptomatic fruit in relation to the total plants in the area
[c] Dispersion index (*D*) calculated by dividing the observed variance by the binomial variance. Values followed by (*) were significantly greater than 1 according to a chi-square test indicating that the spatial pattern of symptomatic plants was aggregated
[d] Quadrat size: 2 by 2
[e] Quadrat size: 2 by 3

Aggregation ($D > 1$) of strawberry plants with symptomatic fruit was detected for the conventional site area for diseased plants, especially with incidence levels greater than 10 %. Index of dispersion values were significantly equal to 1 ($D = 1$) for all dates that incidence of diseased plants was evaluated for the organic area – characterizing a random distribution pattern for symptomatic plants (Table 3.2).

The index of aggregation (i.e., the variance-to-mean ratio), for cumulative data of symptomatic fruit, ranged from 1.00 to 5.44 and from 1.00 to 2.89 for the conventional and organic areas, respectively (Table 3.3). Values of index of aggregation greater than 1 (V/M > 1) were observed for diseased fruit density levels higher than 0.1 symptomatic fruit per plant, which indicated an aggregated pattern for the distribution of diseased fruit (Table 3.3). Diseased fruit density was higher for the organic area (0.01 to 6.40 number of symptomatic fruit per plant) than conventional site (0.01 to 1.90 number of symptomatic fruit per plant).

Table 3.3 – Diseased fruit density (number of symptomatic fruit plant^{-1}) and index of aggregation (variance/mean) of cumulative number of strawberry fruit with Botrytis fruit rot symptoms over time in conventional and organic strawberry fields

Location	Date[a]	Diseased fruit density[b] (number of symptomatic fruit plant^{-1})	Index of aggregation[c] (V/M)
Conventional[d]	18/06/2015	0.01	1.00
	16/07/2015	0.10	1.51*
	07/08/2016	0.61	2.82*
	03/09/2015	1.63	5.03*
	06/10/2015	1.90	5.44*
Organic[e]	25/06/2015	0.01	1.00
	16/07/2015	0.20	1.91*
	03/09/2015	4.52	2.62*
	30/09/2015	6.10	2.80*
	14/10/2015	6.40	2.89*

[a] Day/month/year
[b] The average of total number of strawberry fruit with Botrytis fruit rot symptoms in relation to the total of plants in the area
[c] Variance-to-mean ratio (V/M), calculated by dividing the of the number of diseased fruit by the mean. Values followed by (*) were significantly greater than 1 according to a chi-square test indicating that diseased fruit density (number of symptomatic fruit plant^{-1}) was aggregated
[e] Quadrat size: 2 by 2
[f] Quadrat size: 2 by 3

The relationship between log (Vobs) and log (Vbin) was highly significant ($p < 0.01$) for cumulative incidence data of symptomatic fruit and diseased plants for both

conventional and organic areas. Taylor's power law model provided a good fit to the data with coefficient of determination (R^2) values higher than 0.93 (Figures 3.4 and 3.5).

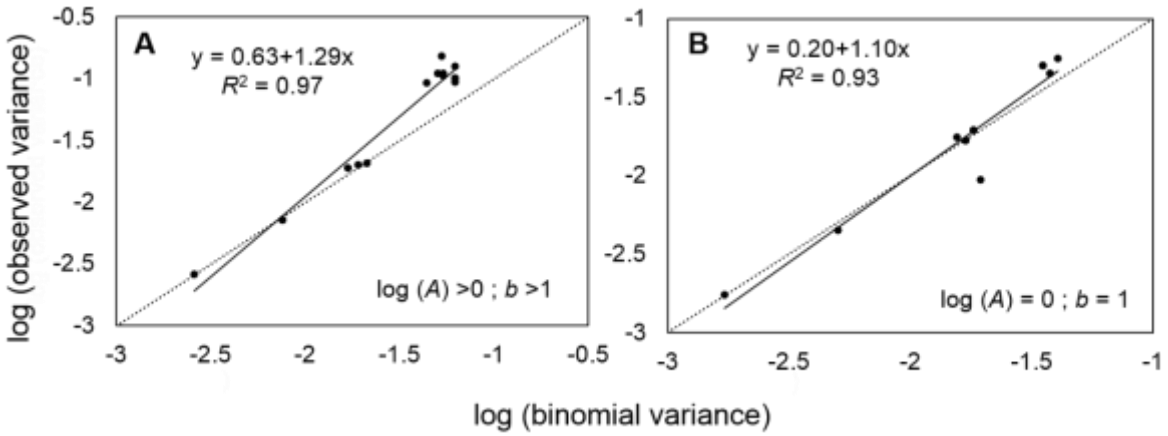

Figure 3.4 – Relationship between the logarithm of the observed variance (y) and logarithm of the expected binomial variance (x) for Botrytis fruit rot incidence in strawberry plants with symptomatic fruit for the data of conventional (A) and organic (B) strawberry fields, respectively. Solid lines represent log (Vobs) = log (*A*) + *b* log (Vbin) fitted to the data of cumulative incidence of plants with diseased fruit by linear regression using quadrat sizes 2 by 2 (A) and 2 by 3 (B). Dashed line represents the binomial fit

The regression parameters applied to the cumulative incidence data of the conventional area (Figure 3.4A), indicated that the aggregation of diseased plants increased with increasing disease incidence (log (*A*) > 0; *b* >1). The parameters applied to the cumulative (Figure 3.4B) incidence data of the organic grower indicated a random condition for the spatial distribution of diseased plants, given that estimates of log (*A*) and *b* were not statistically different from 0 and 1, respectively.

The estimates of log (*A*) and *b* were statistically greater than 0 and 1, respectively, for the cumulative data of symptomatic fruit per plant for both conventional and organic areas (Figure 3.5), suggesting diseased fruit density is aggregated on strawberry plants. Moreover, *b* > 1 indicates the degree of aggregation increased with increasing diseased fruit density; in other words, the greater the number of symptomatic fruit per plant, the higher the degree of aggregation.

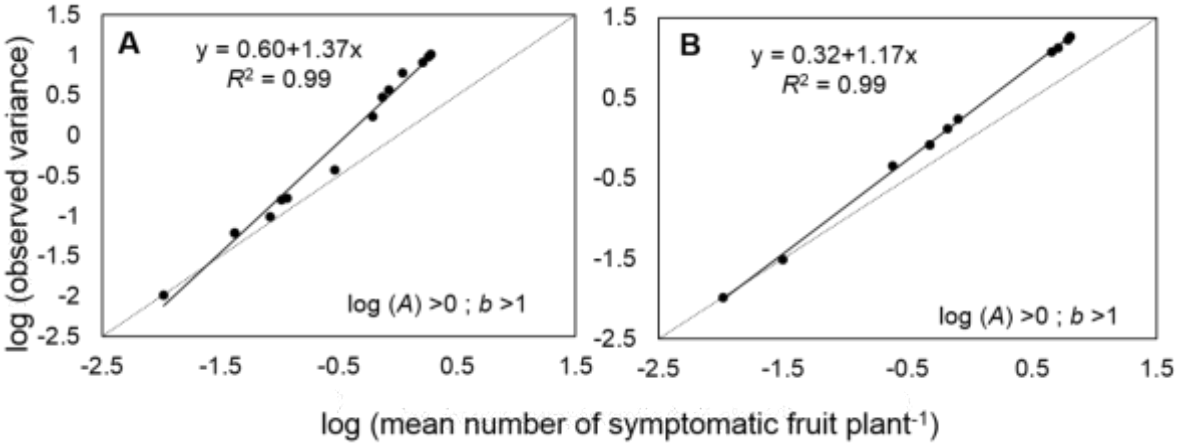

Figure 3.5 – Relationship between the logarithm of the observed variance (y) and logarithm of mean number of symptomatic fruit per plant (x, disease density) for Botrytis fruit rot incidence in strawberry fruit for the data of conventional (A) and organic (B) strawberry fields. Solid lines represent log (Vobs) = log (*A*) + *b* log (mean) fitted to the data of cumulative mean of diseased fruit per plant by linear regression using quadrat sizes 2 by 2 (A) and 2 by 3 (B). Dashed line represents the binomial fit

3.3.3 Incidence-diseased fruit density relationship

The relationship between the incidence of diseased strawberry plants and the number of symptomatic fruit per plant was evaluated. In general, the increase of incidence of diseased plants has led to the increase of diseased fruit density, in other words, the greater the incidence of diseased plants, the higher the number of symptomatic fruit per plant (Figure 3.6). The observed data points in Figure 3.6 lie above the expected Poisson line and the pattern of diseased fruit density over strawberry plants was aggregated than would be expected at random. There were more symptomatic fruit per plant for the organic area (Figure 3.6B) than for the conventionally grown site (Figure 3.6A).

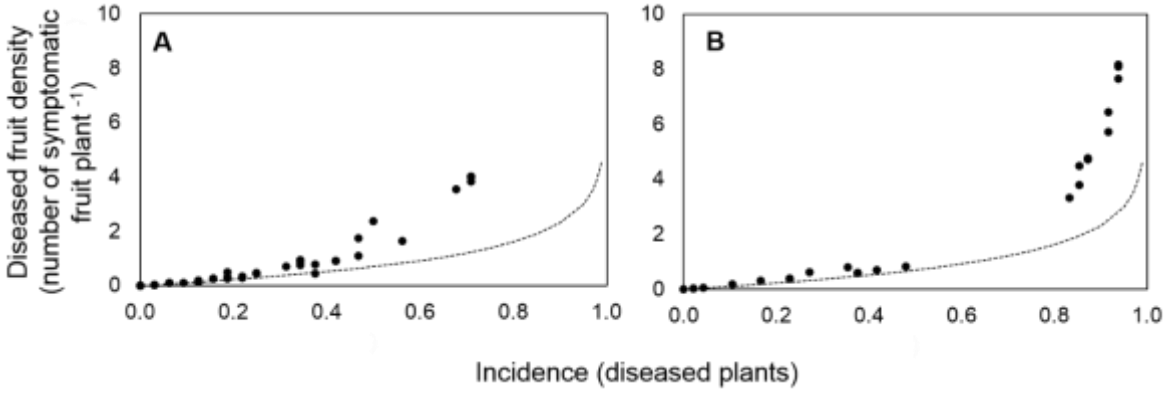

Figure 3.6 – Relationship between diseased fruit density, in number of symptomatic strawberry fruit per plant, and incidence of diseased plants (%) for Botrytis fruit rot in conventional (A) and organic (B) strawberry fields. Observed data for cumulative diseased fruit density and incidence with the expected Poisson relationship (dashed line)

The linear relationship between the logarithm transformation of diseased fruit density, ln (*m*), and the CLL transformation of incidence of diseased plants was highly significant ($p < 0.001$) for Botrytis fruit rot in both conventional and organic areas. The model ln (*m*) = $\beta_0 + \beta_1$ CLL (*p*) provided a good fit to the data with coefficient of determination (R^2) values of 0.97 (Figure 3.7A) and 0.99 (Figure 3.7B) for the conventional and organic areas, respectively. The parameter β_1 was greater than 1 according to the *t* test, indicating diseased fruit density is aggregated on strawberry plants in both areas.

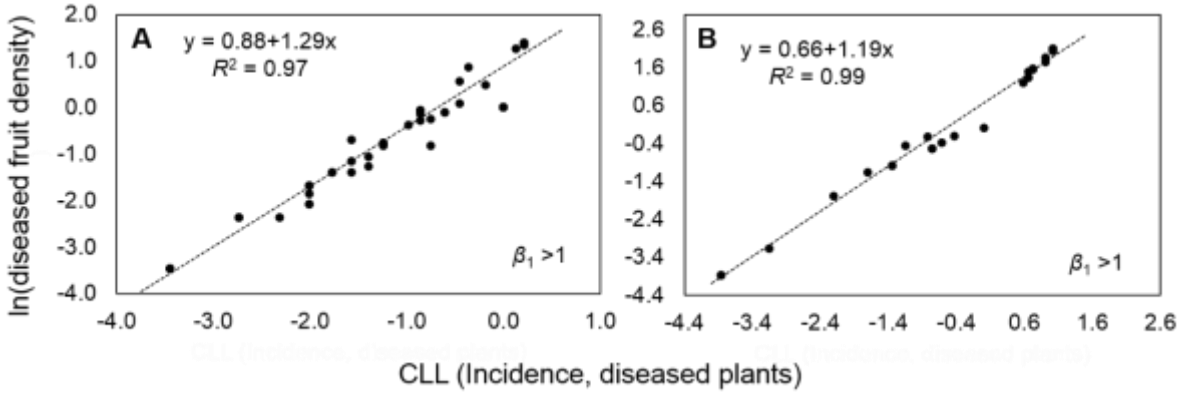

Figure 3.7 – Relationship between the logarithm of diseased fruit density, in number of symptomatic strawberry fruit per plant, and CLL, a function of disease incidence in strawberry plants for Botrytis fruit rot in conventional (A) and organic (B) strawberry fields. Dashed line represents ln (*m*) = $\beta_0 + \beta_1$ CLL (*p*) fitted to cumulative data, in which *m* represents the average number of symptomatic fruit per plant and CLL(*p*) is the complementary log-log transformed incidence, CLL = ln(-ln(1-*p*)), where *p* is the incidence of diseased plants

3.4 Discussion

Fruit with symptoms caused by *Colletotrichum* spp. were not found in the organic area. In the conventional area, symptomatic fruit were found on few assessment dates and final cumulative incidence of anthracnose was considered to be too low for a standard epidemiological analysis. The few symptomatic fruit and plants in strawberry fields may have been due to the absence or occurrence of low primary source of inoculum that is usually carried with infected transplants from nurseries, indicating the propagation material was probably disease-free (HOWARD et al., 1992; MERTELY; LEGARD, 2004). Since *Colletotrichum* spp. are mainly dispersed over short distances by water splash, the use of drip-irrigation instead of overhead-irrigation for both strawberry growers and the absence of intense rains during the harvest period may have contributed to the reduced progression of disease throughout the study site (MADDEN et al., 1992; SMITH, 2008; YANG et al., 1990). The use of straw mulches in the aisles between the beds may have also affected spore dissemination within the field, along with the low or absent occurrence of the disease, similar to results from other studies (MADDEN; WILSON; ELLIS, 1993; YANG et al., 1990). Even if the inoculum was present and dispersed within the strawberry plants, fungicide sprays may have protected plants and fruit from *Colletotrichum* spp. infection. As long as resistance was not reported for *Colletotrichum* spp. isolates to the main single-site fungicides in Brazil (Chapter 2), Anthracnose fruit rot may have been efficiently controlled by chemical application. On the organic area, broad-spectrum protectant products (i.e., Bordeaux mixture and calcium sulfate) applied during the harvest period may have also reduced disease occurrence. Reductions of fruit rot via foliar applications of calcium sulfate have been previously reported from other studies (SMITH; GUPTON, 1993).

On the other hand, strawberry fruit and plants showing symptoms of Botrytis fruit rot were particularly present on the cultivar ‘Camarosa’ throughout conventional and organic sites, despite that this cultivar has been considered less susceptible to this disease (LEGARD et al., 2000).

Cumulative incidence of Botrytis fruit rot in fruit and plants over time were generally well described by the logistic model. Progress of leather rot in strawberry fruit has also been shown to exhibit similar patterns typical of logistic increase (REYNOLDS; MADDEN; ELLIS, 1988). The logistic model is often used to describe

polycyclic diseases, where inoculum is increased and infected plants become sources of inoculum to other plants within the same crop season (CAMPBELL; MADDEN, 1990; ZADOKS; SCHEIM, 1979). Since inoculum can be introduced with propagation material (OLIVEIRA, 2015) and survive on senescent leaves and fruit (MERTELY et al., 2000), spores of *B. cinerea* have been shown to be dispersed by air currents and water from rain (BRISTOW; MCNICOL; WILLIAMSON, 1986). These spores may infect new and healthy leaves and fruit, which, upon favorable conditions, will become source of secondary inoculum, promoting new and several cycles of dissemination and infection within the same strawberry season (BRAUN; SUTTON, 1988). For this reason, Botrytis fruit rot may be classified as polycyclic disease, which is usually described by logistic models.

As long as observed progress curves of non-cumulative incidence data were not typical of the ones that can be described with the commonly used epidemiological models, the area under the disease progress curve (AUDPC) was applied to analyze non-cumulative data of diseased fruit and plant incidence, which was higher in organic than in conventional fields (MADDEN; HUGHES; VAN DEN BOSCH, 2007). The disease increase rate was higher for the organic area, as well as the disease intensity, with larger incidence levels of diseased plants and fruit, which can explain the reduction in yield for the organic production. It has been reported that yields of organic strawberry production represent about 60 to 70 % of conventional production (DONADELLI; KANO; FERNANDES JUNIOR, 2012; SOUZA, 2006).

Intensification of aggregation with increasing diseased fruit density on strawberry plants ($b > 1$) was observed only when the mean number of diseased fruit per plant reached 0.1 and 0.2 symptomatic fruit per plant for the organic and conventional areas, respectively. Aggregation of peach fruit with brown rot symptoms, caused by *Monilinia fructicola*, has also been shown to increase with the disease incidence in organic peach orchards (KESKE; MAY-DE MIO; AMORIM, 2013). However, information about increasing aggregation with increasing diseased fruit within a plant has not often been reported in the literature and it is more related to increasing incidence of plants with symptomatic fruit – for example, citrus black spot [(where $b > 1$ were found only in groves with low disease incidence, under 50 %) (SPÓSITO et al., 2008)], and for citrus postbloom fruit drop, where aggregation was observed with incidence of symptomatic plants above 12.6 % (SILVA-JUNIOR et al., 2014).

Although aggregation has been observed for diseased fruit per plant on both areas, and diseased plants throughout the conventional area, the organic area showed a random distribution pattern of plants showing symptoms, with low values of *D*. Aggregation, which is generally the rule rather than an exception for plant pathogens (MADDEN; HUGHES, 1995), was not observed in any incidence level of diseased plants, low or high, for the organic area In fact, largest values of index of dispersion (*D*) would be expected in the midrange values of disease incidence (MADDEN; HUGHES; ELLIS, 1995), which were not observed for the organic area that had diseased incidence on plants increasing from less than 20 % to more than 80 %.

Random distribution of peach blossom blight infection (i.e., *M. fructicola*) has also been observed in organic orchards, however, incidence levels were shown to be lower than 20 % during the entire season (KESKE; MAY-DE MIO; AMORIM, 2013). A random pattern of infection has also been reported for citrus canker, (i.e., caused by the bacteria *Xanthomonas citri* subsp. *citri*), in Brazil, especially after the introduction of the citrus leaf miner (BERGAMIN FILHO et al., 2000). Random patterns are usually observed at the beginning of certain epidemics like citrus postbloom fruit drop, which has shown an incidence lower than 12 % (SILVA-JUNIOR et al., 2014). In this case, the assessment of diseased fruit is more reliable than the analysis of infected plants, leading to a more appropriate interpretation of the mechanism of pathogen dispersion, and the consequent disease development in the field.

Pathogens dispersed primarily by water-splash over short distances (e.g., *Colletorichum* spp. causing citrus postbloom fruit drop, and the bacteria *Xanthomonas citri* subsp. *citri*, causal agent of citrus canker, before the introduction of the citrus leaf miner), may cause epidemics that present an aggregated distribution pattern for diseased plants, given that the probability of a plant to be infected is higher for plants that are closer to the source of inoculum (BERGAMIN FILHO et al., 2000; SILVA-JUNIOR et al., 2014). Aggregation pattern was also observed in our studies for Botrytis fruit rot with symptomatic fruit within a plant, suggesting that nearby fruit and plants present higher chances of becoming diseased. However, this aggregation, observed for both conventional and organic areas, may be related to harvesting operations and sanitations methods adopted by growers rather than water-splash dispersal.

The relationship between diseased fruit density and incidence of diseased plants indicated an aggregated pattern of diseased fruit within strawberry plants. The relationship between incidence and severity or between incidence and lesion density has been studied for other fruit diseases, such as apple and pear scab and citrus black spot along with many other diseases in general (CARISSE; MELOCHE; TURECHEK, 2011; LI et al., 2007; MCROBERTS; HUGHES; MADDEN, 2003; SPÓSITO et al., 2008; TURECHEK; MADDEN, 2001). However, most of these works attempted to establish a relationship between the two variables to measure disease intensity in order to establish a basic metric for disease management for these particular diseases. Based on this study, it can be inferred that the presence of plants with symptomatic fruit are an indicator of necessary required disease control for Botrytis fruit rot, regardless of diseased fruit density.

Despite the observation of same temporal and spatial patterns of Botrytis fruit rot for conventional and organic areas, disease intensity was higher in the organic area. Given that the production of organic strawberry transplants does not presently occur in Brazil, organic growers must acquire their plants from the same nurseries as conventional growers; and correspondingly, the source of primary inoculum for *B. cinerea* can be inferred to be the same for both. Therefore, differences in disease intensity levels are more likely related to the control measurements adopted by the growers rather than the source of inoculum. Organic growers generally utilize lower numbers of sprays than conventional growers – using products that are not specifically effective in controlling Botrytis fruit rot as the single-site and systemic fungicides used in conventional production systems (AGROFIT, 2016). Moreover, the removal of infected fruit, and mainly, senescent foliage, has been shown to be important in decreasing the incidence of disease in the field by altering the microclimate and reducing the contamination of healthy fruit within the same and nearby plants, especially compared to areas where no control management was used (MERTELY et al., 2000). If the organic grower had used the leaf and fruit sanitation more frequently, lower levels of disease incidence could have been observed. However, the reductions would have been relatively small compared to standard fungicide programs adopted by conventional growers (MERTELY et al., 2000). In fact, leaf and fruit sanitation was more frequently made in the conventional area and was supplemented by fungicide sprays, being responsible for lower levels of disease intensity than in the organic area.

Although reduction of yield is frequently reported in organic production system, comparison of incidence levels and intensity of disease caused by *B. cinerea* in conventional and organic strawberry was never reported. To the best of our knowledge, this is the first time that epidemiological studies are conducted for Botrytis fruit rot on strawberries comparing conventional and organic production systems.

3.5 Conclusion

The temporal progress of Botrytis fruit rot incidence in fruit and plants was generally well described by the logistic model. The organic site showed a disease incidence and daily progress rate higher than the conventional area. It is therefore likely that Botrytis fruit rot can be classified as a polycyclic disease, with secondary cycles within the same strawberry season.

The spatial pattern of disease distribution was found to be aggregated for diseased strawberry fruit for both conventional and organic areas.

Botrytis fruit rot presents the same temporal and spatial patterns for both conventional and organic areas, however disease intensity is lower in conventional growers, which is likely due to the different management practices utilized by the different growing methods.

References

AGROFIT. - SISTEMA DE AGROTÓXICOS FITOSSANITÁRIOS. 2016. Disponível em: <http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons>. Acesso em: 05 maio 2016.

AMIRI, A.; HEATH, S.M.; PERES, N.A. Phenotypic characterization of multifungicide resistance in *Botrytis cinerea* isolates from strawberry fields in Florida. **Plant Disease**, Saint Paul, v. 97, n. 3, p. 393–401, 2013.

ANDRADE, L.N.T.; NUNES. M.U.C. **Produtos alternativos para controle de doenças e pragas em agricultura orgânica**. Aracaju: Embrapa-Tabuleiros Costeiros, 2001. 20 p. (Documentos, 281).

BANNO, S.; FUKOMORI, F.; ICHIISHI, A.; OKADA, K.; UEKUSA, H.; KIMURA, M.; FUJIMURA, M. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. **Phytopathology**, Saint Paul, v. 98, n. 4, p. 397–404, 2008.

BERGAMIN FILHO, A.; AMORIM, L. Epidemiologia de doenças de plantas. In: AMORIM, L.; REZENDE, J.A.M.; BERGAMIN FILHO, A. (Ed.). **Manual de Fitopatologia:** Princípios e conceitos. 4 ed. São Paulo: Ceres, 2011, v. 1, cap. 18, p. 101-118.

BERGAMIN FILHO, A.; AMORIM, L.; LARANJEIRA, F.F.; GOTTWALD, T.R. Epidemiology of citrus canker in Brazil with and without the asian citrus leafminer. In: IINTERNATIONAL CITRUS CANKER RESEARCH WORKSHOP, 1., 2000, Fort Pierce. **Proceedings…** Fort Pierce: International Citrus Canker Research Workshop, 2000. p. 6.

BERNARDI, J.; HOFFMAN, A.; ANTUNES, L. E. C.; FREIRE, J.M. Cultivares. In: EMBRAPA UVA E VINHO. **Sistema de produção de morango para mesa na região da Serra Gaúcha e encosta superior do Nordeste.** Bento Gonçalves, 2005. (Sistema de Produção, 6). Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Morango/MesaSerraGaucha/cultivares.htm>. Acesso em: 03 julho 2016.

BOFF, P.; KASTELEIN, P.; DE KRAKER, J.; GERLAGH, M.; KÖHL, J. Epidemiology of grey mould in annual waiting-bed production of strawberry. **European Journal of Plant Pathology**, Dordrecht, v. 107, n. 6, p. 615–624, 2001.

BRAUN, P.G.; SUTTON, J.C. Inoculum sources of *Botrytis cinerea* in fruit rot of strawberries in Ontario. **Canadian Journal of Plant Pathology**, Ontario, v. 10, p. 1–5, 1987.

_____. Infection cycles and population dynamics of *Botrytis cinerea* in strawberry leaves. **Canadian Journal of Plant Pathology**, Ontario, v. 10, n. 2, p. 133–141, 1988.

BRISTOW, P.R.; MCNICOL, R.J.; WILLIAMSON, B. Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. **Annals of Applied Biology**, Warwick, v. 109, n. 3, p. 545–554, 1986.

CAMPBELL, C.L.; MADDEN, L.V. **Introduction to plant disease epidemiology.** New York: John Wiley, 1990. 532 p.

CARISSE, O.; MELOCHE, C.; TURECHEK, W.W. Spatial heterogeneity, incidence-incidence and incidence-lesion density relationship of apple scab (*Venturia inaequalis*) in managed orchards. **European Journal of Plant Pathology**, Dordrecht, v. 130, n. 3, p. 349–365, 2011.

COSTA, H.; VENTURA, J.A.; LOPES, U.P. Manejo integrado de pragas e doenças do morangueiro. **Horticultura Brasileira,** Brasília, v. 29, S5856-5877, 2011. **Anais...** Apresentado no CONGRESSO BRASILEIRO DE OLERICULTURA, 51., 2011, Viçosa.

DONADELLI, A.; KANO, C.; FERNANDES JUNIOR, F. Estudo de caso: análise econômica entre o custo de produção de morango orgânico e convencional. **Pesquisa & Tecnologia**, Campinas, v. 9, n. 2, p. 1-5, 2012.

DROBY, S.; LICHTER, A. Post-harvest *Botrytis* infection: Etiology, development and management. In: ELAD, Y.; WILLIAMSON, B.; TUDZYNSKI, P.; DELEN, N. (Ed.). **Botrytis:** biology, pathology and control. Dordrecht: Springer, 2007. p. 349–367.

FAO. 2016. Available at: <http://faostat3.fao.org/browse/Q/QC/E>. Accessed: May 25, 2016.

FERNÁNDEZ-ORTUÑO, D.; TORÉS, J.A.; CHAMORRO, M.; PÉREZ-GARCÍA, A.; VICENTE, A. Characterization of resistance to six chemical classes of site-specific fungicides registered for gray mold control on strawberries in Spain. **Plant Disease**, Saint Paul, 2016. Disponível em: <https://www.researchgate.net/publication/303372062_Characterization_of_Resistance_to_Six_Chemical_Classes_of_Site-Specific_Fungicides_Registered_for_Gray_Mold_Control_on_Strawberries_in_Spain>. Acesso em: 21 maio 2016.

FORCELINI, B.B.; SEIJO, T.E.; AMIRI, A.; PERES, N.A. Resistance in strawberry isolates of *Colletotrichum acutatum* from Florida to quinone-outside inhibitor fungicides. **Plant Disease**, Saint Paul, 2016. doi: http://dx.doi.org/10.1094/PDIS–01–16–0118–RE.

FRARE, G.F.; COUTO, H.T.Z.; CIAMPI-GUILLARDI, M.; AMORIM, L. The causal agent of citrus postbloom fruit drop, *Colletotrichum acutatum*, can survive on weeds. **Australasian Plant Pathology**, Collingwood, v. 45, n. 4, p. 339–346, 2016.

FREEMAN, S. Management, survival strategies, and host range of Colletotrichum acutatum on strawberry. **HortScience**, Alexandria, v. 43, n. 1, p. 66–68, 2008.

FREEMAN, S.; HOROWITZ, S.; SHARON, A. Pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. **Phytopathology**, Saint Paul, v. 91, p. 986–992, 2001.

HOWARD, C.M.; MAAS, J.L.; CHANDLER, C.K.; ALBREGTS, E.E. Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. **Plant disease**, Saint Paul, v. 76, n. 10, p. 976–981, 1992.

ISHII, H.; FOUNTAINE, J.; CHUNG, W. H.; KANSAKO, M.; NISHIMURA, K.; TAKAHASHI, K. Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. **Pest Management Science**, Sussex, v. 65, n. 8, p. 916–922, 2009.

JARVIS, W.R. The infection of strawberry and raspberry fruits by *Botrytis cinerea* Fr. **Annals of Applied Biology**, Warwick, v. 50, n. 3, p. 569–575, 1962.

KESKE, C.; MAY-DE-MIO, L.L.; AMORIM, L. Spatial pattern of brown rot within peach trees related to inoculum of *Monilinia fructicola* in organic orchard. **Journal of Plant Pathology**, Dordrecht, v. 95, n. 1, p. 67–73, 2013.

KRANZ, J. **Epidemics of plant diseases:** mathematical analysis and modeling. Berlin: Springer, 1974. 170 p.

LEANDRO, L.F.S.; GLEASON, M.L.; NUTTER, F.W.; WEGULO, S.N.; DIXON, P.M. Influence of temperature and wetness duration on conidia and appressoria of *Colletotrichum acutatum* on symptomless strawberry leaves. **Phytopathology**, Saint Paul, v. 93, n. 4, p. 513–20, 2003a.

LEGARD, D.E.; XIAO, C.L.; MERTELY, J.C.; CHANDLER, C.K. Effects of plant spacing and cultivar on incidence of Botrytis fruit rot in annual strawberry. **Plant Disease**, Saint Paul, v. 84, n. 5, p. 531–538, 2000.

LI, B.H.; YANG, J.R.; LI, B.D.; XU, X.M. Incidence-density relationship of pear scab (*Venturia nashicola*) on fruits and leaves. **Plant Pathology**, Oxford, v. 56, n. 1, p. 120–127, 2007.

MAAS, J.L. **Compendium of strawberry diseases**. 2nd ed. Saint Paul: American Phytopathological Society Press, 1998.128 p.

MACKENZIE, S.J.; MERTELY, J.C.; PERES, N.A. Curative and protectant activity of fungicides for control of crown rot of strawberry caused by *Colletotrichum gloeosporioides*. **Plant Disease**, Saint Paul, v. 93, n. 8, p. 815–820, 2009.

MADDEN, L.V; HUGHES, G. Plant Disease Incidence: Distributions, heterogeneity, and temporal analysis. **Annual Review of Phytopathology**, Palo Alto, v. 33, p. 529–564, 1995.

MADDEN, L.V.; HUGHES, G.; ELLIS, M.A. Spatial heterogeneity of the incidence of grape downy mildew. **Phytopathology**, Saint Paul, v. 85, p. 269–275, 1995.

MADDEN, V.; HUGHES, G., VAN DEN BOSCH, F. **The study of plant disease epidemics**. Saint Paul: APS Press, 2007. 421 p.

MADDEN, L.V.; WILSON, L.L.; ELLIS, M.A. Field spread of anthracnose fruit rot of strawberry in relation to ground cover and ambient weather conditions. **Plant Disease**, Saint Paul, v. 77, n. 9, p. 861–866, 1993.

MADDEN, L.V.; LOUIE, R.; ABT, J.J.; KNOKE, J.K. Evaluation of tests for randomness of infected plants. **Phytopathology**, Saint Paul, v. 72, p. 195–198, 1982.

MADDEN, L.V.; WILSON, L.L.; YANG, X.; ELLIS, M.A. Splash dispersal of *Colletotrichum acutatum* and *Phytophthora cactorum* by short-duration simulated rains. **Plant Pathology**, Oxford, v. 41, n. 4, p. 427–436, 1992.

MCROBERTS, N.; HUGHES, G.; MADDEN, L.V. The theoretical basis and practical application of relationships between different disease intensity measurements in plants. **Annals of Applied Biology**, Warwick, v. 142, n. 2, p. 191–211, 2003.

MERCIER, J.; KONG, M.; COOK, F. Fungicide resistance among *Botrytis cinerea*

isolates from California strawberry fields. **Plant Health Progress**, Saint Paul, 2010. mdoi:10.1094/PHP–2010–0806–01–RS.

MERTELY, J.C.; LEGARD, D.E. Detection, isolation, and pathogenicity of *Colletotrichum* spp. from strawberry petioles. **Plant Disease**, Saint Paul, v. 88, p. 407–412, 2004.

MERTELY, J.C.; PERES, N.A.; CHANDLER, C.K. **Anthracnose fruit rot of strawberry**. Gainesville: University of Florida, Plant Pathology Department; Florida Cooperative Extension Service; Institute of Food and Agricultural Sciences, 2009. 4 p.

MERTELY, J.C.; CHANDLER, C.K.; XIAO, C.L.; LEGARD, D.E. Comparison of sanitation and fungicides for management of Botrytis fruit rot of strawberry. **Plant Disease**, Saint Paul, v. 84, n. 17, p. 1197–1202, 2000.

NUTTER JR., F.W. Quantifying the temporal dynamics of plant virus epidemics: a review. **Crop Protection**, Guidford, v. 16, n. 7, p. 603–618, 1997.

OLIVEIRA, M.S. **Fungicide resistance profile of quiescnet Botrytis cinerea infections on strawberry transplants**. 2015. 100 p. Thesis (Master of Science) - University of Florida, Gainesville, 2015.

PASSOS, F.A. Melhoramento do morangueiro no Instituto Agronômico de Campinas. In: DUARTE FILHO, J.; CANÇADO, G.M.A.; REGINA, M.A.; ANTUNES, L.E.C.; FADINI, M.A.M. (Ed.). **Morango:** tecnologia de produção e processamento. Caldas: EPAMIG, 1999. p. 259-264.

PERES, N.A.; TIMMER, L.W.; ADASKAVEG, J.E.; CORRELL, J.C. Lifestyles of *Colletotrichum acutatum*. **Plant Disease**, Saint Paul, v. 89, n. 8, p. 784–796, 2005.

RADMANN, E.B.; BIANCHI, V.J.; OLIVEIRA, R.P.; FACHIELLO, J.C. Caracterização e diversidade genética de cultivares de morangueiro. **Horticultura Brasileira**, Brasília, v. 24, n. 1, p. 84–87, 2006.

REYNOLDS, K.M.; MADDEN, L.V.; ELLIS, M.A. Spatio-temporal analysis of epidemics development of leather rot. **Phytopathology**, Saint Paul, v. 78, p. 246-252,1988.

RONQUE, E.R.V. **Cultura do morangueiro:** revisão e prática. Curitiba: EMATER, 1998. 206 p.

SILVA-JUNIOR, G.J.; SPÓSITO, M.B.; MARIN, D.R.; RIBEIRO-JUNIOR, P.J.; AMORIM, L. Spatiotemporal characterization of citrus postbloom fruit drop in Brazil and its relationship to pathogen dispersal. **Plant Pathology**, Oxford, v. 63, n. 3, p. 519–529, 2014.

SMITH, B.J. Epidemiology and pathology of strawherry anthracnose: A North

American perspective. **HortScience**, Alexandria, v. 43, n. 1, p. 69–73, 2008.

SMITH, B.J.; GUPTON, C.L. Calcium applications before harvest affect the severity of anthracnose fruit rot of greenhouse-grown strawberries. **Acta Horticulturae,** The Hague, v. 348, n. 1, p. 477-482, 1993.

SOUZA, J.L. Produção orgânica de morango. In: CARVALHO, SP. (Ed.). **Boletim do morango**: cultivo convencional, segurança alimentar, cultivo orgânico. Belo Horizonte: FAEMG, 2006. p. 145-159.

SPÓSITO, M.B.; AMORIM, L.; BASSANEZI, R.B.; BERGAMIN FILHO, A.; HAU, B. Spatial pattern of black spot incidence within citrus trees related to disease severity and pathogen dispersal. **Plant Pathology**, Oxford, v. 57, n. 1, p. 103–108, 2008.

TANAKA, M.A.S.; BETTI, J.A.; KIMATI, H. Doenças do morangueiro (*Fragaria* x *ananassa*). In: KIMATI, H.; AMORIM, L.; REZENDE, J.A.M.; BERGAMIN FILHO, A.; CAMARGO, L.E.A. (Ed.). **Manual de fitopatologia:** doenças das plantas cultivadas. São Paulo: Ceres, 2005. p. 489-499.

TAYLOR, L.R. Aggregation, variance and the mean. **Nature**, London, v. 189, n. 4766, p. 732–735, 1961.

_____. Assessing and interpreting the spatial distributions of insect populations. **Annual Review of Entomology**, Stanford, v. 29, n. 1, p. 321–357, 1984.

TURECHEK, W.W.; MADDEN, L.V. Effect of scale on plant disease incidence and heterogeneity in a spatial hierarchy. **Ecological Modelling**, Amsterdan, v. 144, n. 1, p. 77–95, 2001.

UREÑA-PADILLA, A.R.; MACKENZIE, S.J.; BOWEN, B.W.; LEGARD, D.E. Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. **Phytopathology**, Saint Paul, v. 92, n. 11, p. 1245–1252, 2002.

VANDERPLANK, J.E. **Plant diseases:** epidemics and control. New York: Academic Press, 1963. 349 p.

WILLIAMSON, B.; TUDZYNSKI, B.; TUDZYNSKI, P.; VAN KAN, J. *Botrytis cinerea*: the cause of grey mould disease. **Molecular Plant Pathology**, London, v. 8, n. 5, p. 561–580, 2007.

WILSON, L.; MADDEN, L.; ELLIS, M. Influence of temperature and wetness duration on infection of immature and mature strawberry fruit by *Colletotrichum acutatum*. **Phytopathology**, Saint Paul, v. 80, n. 1, p. 111–116, 1990.

YANG, X.; MADDEN, L.V.; WILSON, L.L.; ELLIS, M.A. Effects os surface topography and rain intensity on splash dispersal of *Colletotrichum acutatum*. **Phytopathology**, Saint Paul, v. 80, p. 1115–1120, 1990.

ZADOKS, J.C.; SCHEIN, R.D. **Epidemiology and plant disease management**. Oxford: University Press, 1979. 428 p.

APPENDIXES

Appendix A – Fungicide sprays made by the conventional grower in the strawberry field in Valinhos (São Paulo) in the 2015 season: date of applications, commercial products used with dose (amount of commercial product per 100 L of water) and their respective active ingredients and reason or event responsible for the fungicide application

Date[a]	Commercial products	Dose (commercial product/100 L water)	Active ingredients	Reason/Event
13/04	Score	30 ml	difenoconazole	Preventive application
14/04	Amistar WG	30 g	azoxystrobin	Soil application
29/05	Cercobin 700 WP	100 g	thiophanate-methyl	Rain
03/06	Amistar Top	30 ml	azoxystrobin + difenoconazole	Rain + low temperatures
12/06	Rovral SC	150 ml	iprodione	-
17/06	Cercobin 700 WP	100 g	thiophanate-methyl	Preventive application
	Frowncide 500 SC	100 ml	fluazinam	Preventive application
23/06	Sumilex 500 WP	100 g	procymidone	Botrytis fruit rot
06/07	Sumilex 500 WP	101 g	procymidone	Botrytis fruit rot
10/07	Amistar Top	30 ml	azoxystrobin + difenoconazole	Botrytis fruit rot
	Mythos	200 ml	pyrimethanil	
14/07	Sumilex 500 WP	100 g	procymidone	Rain + low temperatures + Botrytis fruit rot
17/07	Ridomil Gold MZ	125 g	metalaxyl + mancozeb	Preventive application
	Frowncide 500 SC	100 ml	fluazinam	
27/07	Cercobin 700 WP	100 g	thiophanate-methyl	Rain
	Frowncide 500 SC	100 ml	fluazinam	
26/08	Sumilex 500 WP	125 ml	procymidone	Preventive application
12/09	Sumilex 500 WP	125 ml	procymidone	Rain
15/09	Rovral SC	125 ml	iprodione	Botrytis fruit rot
	Mythos	125 ml	pyrimethanil	

[a] Day/month

Appendix B – Standard management adopted by the organic grower in the strawberry field in Jarinu (São Paulo) in the 2015 season: date of applications, commercial products and mixtures and ingredients used

Date[a]	Products	Ingredients
07/07	Bio ACE®	vitamins, minerals and herbal antioxidants
	Dipel	biological insecticide
	Azamax	insect growth regulator
14/07	Mixture	boron, calcium, potassium sulfate, magnesium
18/07	Bordeaux mixture	copper sulfate and lime
24/07	Bio ACE®	vitamins, minerals and herbal antioxidants
	Dipel	biological insecticide
	Azamax	insect growth regulator
28/07	Mixture	potassium, fertilizers
04/08	Mixture	fertilizers
11/08	Bio ACE®	vitamins, minerals and herbal antioxidants
	Dipel	biological insecticide
15/08	Mixture	sulfur and lime
21/08	Mixture	potassium, fertilizers
25/08	Dipel	biological insecticide
	Azamax	insect growth regulator
29/08	Bordeaux mixture	copper sulfate and lime
01/08	Mixture	potassium, magnesium
08/09	Bio ACE®	vitamins, minerals and herbal antioxidants
	Bordeaux mixture	copper sulfate and lime
18/09	Mixture	sulfur and lime
25/09	Mixture	potassium, fertilizers
01/10	Bio ACE®	vitamins, minerals and herbal antioxidants
	Dipel	biological insecticide
08/10	Bio ACE®	vitamins, minerals and herbal antioxidants
	Dipel	biological insecticide

[a] Day/month

Appendix C – Anthracnose and Botrytis fruit rot incidence (%) of symptomatic fruit and plants with diseased fruit on cultivars ‘Oso Grande’ and ‘Camarosa’ of the conventional strawberry grower from Valinhos (São Paulo State) in 2015 on different evaluation dates and final incidence of cumulative diseased fruit and plants

Date[a]	Incidence (%)					
	Anthracnose Fruit rot				Botrytis Fruit rot	
	Oso Grande		Camarosa		Oso Grande	
	Fruit	Plant	Fruit	Plant	Fruit	Plant
10/07/2015	...[b]	...	...	...	0.03	0.85
07/08/2015	...	...	...	...	0.11	3.42
27/08/2015	...	...	...	...	0.07	1.96
03/09/2015	...	...	...	...	0.09	2.77
23/09/2015	0.08	1.15	...	...	0.06	0.83
30/09/2015	0.40	9.86	0.11	2.15	0.06	2.05
06/10/2015	1.04	16.99	0.31	9.38	...	…
14/10/2015	0.37	6.56	0.35	7.51	...	…
Cumulative	1.89	23.19	0.77	9.66	0.54	14.39

[a] Day/month/year
[b] (...) Absence of diseased fruit or plants