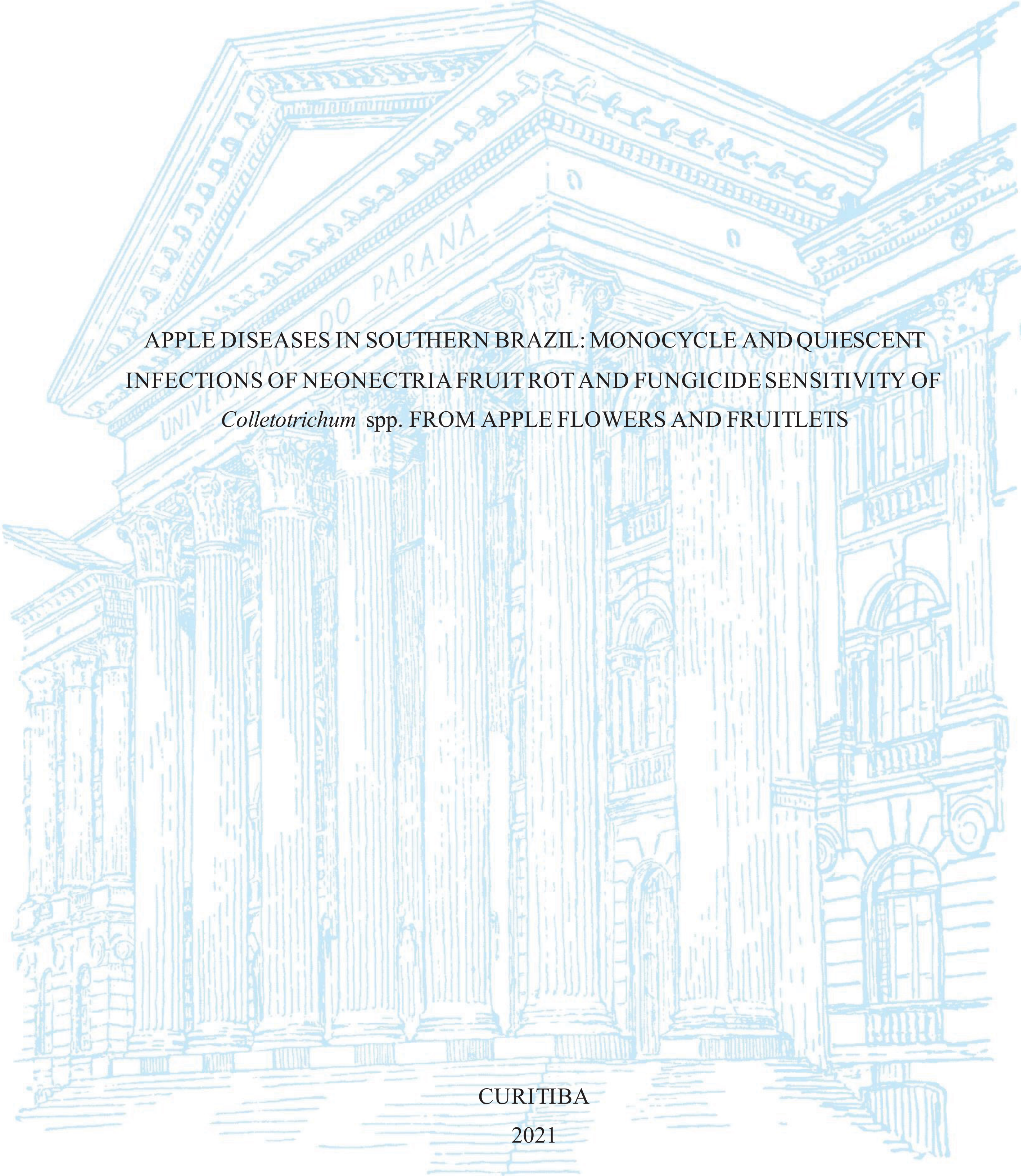
UNIVERSIDADE FEDERAL DO PARANÁ

JHULIA GELAIN



APPLE DISEASES IN SOUTHERN BRAZIL: MONOCYCLE AND QUIESCENT INFECTIONS OF NEONECTRIA FRUIT ROT AND FUNGICIDE SENSITIVITY OF *Colletotrichum* spp. FROM APPLE FLOWERS AND FRUITLETS

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Tese apresentada ao Programa de Pós-Graduação em Agronomia, Área de Concentração em Produção Vegetal, Departamento de Fitossanidade e Fitotecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutora em Ciências.

Orientadora: Prof. PhD Louise Larissa May De Mio
Co-orientador: Prof. PhD Guido Schnabel
Co-orientador: Prof. PhD Walmes M. Zeviani

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JHULIA GELAIN** intitulada: **APPLE DISEASES IN SOUTHERN BRAZIL: MONOCYCLE AND QUIESCENT INFECTIONS OF NEONECTRIA FRUIT ROT AND FUNGICIDE SENSITIVITY OF *Colletotrichum* spp. FROM APPLE FLOWERS AND FRUITLETS**, sob orientação da Profa. Dra. LOUISE LARISSA MAY-DE-MIO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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“Fatti non foste a vivere come bruti,
ma per seguir virtute e canoscenza.” (Dante Alighieri)

RESUMO

O presente estudo teve como objetivo preencher algumas lacunas no conhecimento sobre diversos aspectos das principais doenças do sul do Brasil, Cancro Europeu e podridão de Neonectria em frutos causada por *Neonectria ditissima*, e mancha foliar de Glomerella e podridão amarga causada por *Colletotrichum* spp. Os objetivos específicos de cada capítulo desta tese foram: i) avaliar se infecções quiescentes por infecção natural nos pomares estão ocorrendo em maçãs comercializadas e caracterizar infecções quiescentes por *N. ditissima* nesses frutos; ii) avaliar a germinação e infecção de ascósporos de *N. ditissima* e os componentes monocíclicos da podridão de Neonectria em frutos de maçã e pera; iii) identificar espécies de *Colletotrichum* de flores e frutos de raleio de maçã 'Eva' em pomares do estado do Paraná, avaliar sua sensibilidade a ciprodinil, tebuconazol, iprodiona e fluazinam e determinar a sensibilidade de linha de base desses isolados a benzovindiflupir e natamicina; iv) avaliar e comparar a sensibilidade *in vitro* com estimativa de EC_{50} de isolados de *Colletotrichum melonis* e *C. nymphaeae* de macieiras no estado do Paraná ao fungicida QoI piraclostrobina, bem como caracterizar fenotipicamente os isolados. Nossos resultados indicam que *Neonectria ditissima* está presente em infecções quiescentes em frutos comercializados, e que o fungo é capaz de colonizar e formar estruturas reprodutivas após permanecer quiescente por meses sob condições controladas em câmaras frias. Em relação à germinação *in vitro* de *N. ditissima*, com o mesmo período de molhamento, a germinação de conídios foi sempre superior à de ascósporos em qualquer temperatura. A temperatura ótima para a germinação de ambos os esporos varia em diferentes períodos de umidade, e a faixa é menor para ascósporos do que para conídios. Em relação à inoculação de esporos sexuais e assexuais em frutos de maçã com ferimento, os ascósporos foram capazes de causar podridão de Neonectria nos frutos, mas a doença desenvolveu-se mais lentamente do que as infecções por conídios, comprovado por período de incubação mais longo, período de latência não observado e menor taxa de crescimento da lesão. Conídios e ascósporos inoculados na abertura calicinar de frutos 'Gala' maduros não causaram doença. Em relação à inoculação de dois isolados de *N. ditissima* em maçãs e peras, a doença na pera 'Rocha' tem períodos de incubação e latência mais curtos do que os da maçã 'Gala' e maior AACPD, o que representa um desenvolvimento de monociclo mais rápido. *Colletotrichum melonis* e *C. nymphaeae* pertencentes ao complexo *C. acutatum* foram as espécies predominantemente isoladas de flores e frutos de raleio em pomares 'Eva' do Paraná. Ciprodinil, fluazinam e benzovindiflupir mostraram boa eficácia *in vitro* na inibição do crescimento micelial de *C. melonis* e *C. nymphaeae*. Tebuconazol é um dos poucos produtos já registrados para controle de *Colletotrichum* spp. em maçãs e ainda pode ser usado no manejo, uma vez que não foi encontrada falta de sensibilidade. Natamicina apresentou efeito intermediário no crescimento micelial das espécies de *Colletotrichum* testadas. Iprodiona não apresentou boa eficácia *in vitro* contra o patógeno. A maioria dos isolados testados de *Colletotrichum* spp. apresentaram fenótipo de baixa sensibilidade à piraclostrobina, devendo ser investigada a presença de mutações.

Palavras-chave: *Neonectria ditissima*; podridão amarga; mancha foliar de Glomerella; componentes monocíclicos; controle químico.

ABSTRACT

The present study aimed to fill some gaps in knowledge on several aspects of the main diseases of southern Brazil, European canker and Neonectria fruit rot caused by *Neonectria ditissima*, and Glomerella leaf spot and bitter rot caused by *Colletotrichum* spp. The specific objectives of each chapter of this thesis were: i) assess if quiescent infections from natural infection on the orchards are occurring in commercial apple fruit and characterize *N. ditissima* quiescent infections in Brazilian commercial fruit; ii) evaluate *N. ditissima* ascospore germination and monocyclic components of Neonectria fruit rot on apple and pear; iii) identify *Colletotrichum* species from 'Eva' apple flowers and fruitlets in Paraná state orchards, evaluate their sensitivity to cyprodinil, tebuconazole, iprodione, and fluazinam and determine the baseline sensitivity of these isolates to benzovindiflupyr and natamycin; iv) evaluate and compare the *in vitro* sensitivity with EC_{50} estimation of *Colletotrichum melonis* and *C. nymphaeae* isolates from apple trees in Paraná state to the QoI fungicide pyraclostrobin, as well as to characterize the isolates phenotypically. Our results indicate that *Neonectria ditissima* is present in quiescent infections in commercial fruit, and that the fungus is able to colonize and form reproductive structures after remaining quiescent for months under controlled conditions in cold chambers. Regarding *in vitro* germination of *Neonectria ditissima*, with the same wetness period, conidia germination was always higher than ascospores at any temperature. The optimal temperature for germination of both spores varies in different wetness periods, and the range is lower for ascospores than for conidia. In relation to the inoculation of sexual and asexual spores in detached wounded apple fruit, ascospores were able to cause Neonectria fruit rot but the disease developed slower than conidia infections, proven by longer incubation period, no observed latent period, and lower lesion growth rate. Conidia and ascospores inoculated at the calyx-end of ripe 'Gala' fruit did not cause disease. Regarding inoculation of two *Neonectria ditissima* isolates in apples and pears, the disease on 'Rocha' pear fruit has shorter incubation and latent periods than those of 'Gala' apple and higher AUDPC, which represent a faster monocycle development. *Colletotrichum melonis* and *C. nymphaeae* from the *C. acutatum* species complex were predominantly recovered from flowers and fruitlets in 'Eva' orchards from Paraná. Cyprodinil, fluazinam and benzovindiflupyr showed good *in vitro* efficacy in the mycelial growth inhibition of *C. melonis* and *C. nymphaeae*. Tebuconazole is one of the few products already registered for *Colletotrichum* spp. control in apples and can still be used in the management since no isolate with reduced sensitivity was found. Natamycin showed intermediate effect on the mycelial growth of the *Colletotrichum* species tested. Iprodione did not present good *in vitro* efficacy against the pathogen. Most *Colletotrichum* spp. isolates tested presented low sensitive phenotype to pyraclostrobin, and the presence of mutations should be investigated.

Keywords: *Neonectria ditissima*; bitter rot; Glomerella leaf spot; monocyclic components; chemical control.

SUMMARY

1 GENERAL INTRODUCTION

Apple tree (*Malus domestica* Borkh.) is among the temperate climate fruit species of greatest economic importance worldwide and in Brazil. Apple production is mainly in the three southern states of the country, with Santa Catarina and Rio Grande do Sul accounting for more than 95% of national production, while the state of Paraná occupies the third position.

The main producing cities in Paraná are Palmas, Lapa, Porto Amazonas, and Campo do Tenente. Among the cultivars planted in the state, Gala and Eva stand out, the latter being adapted to regions with little accumulation of cold (PETRI et al., 2011), and consequently one of the most planted in Paraná. Despite the advances that apple orchards have undergone with the development of cultivars with better adaptability and production, dwarfing rootstocks that improved management systems (ROBINSON, 2011), and advances in pest control methods with insecticides and fungicides, phytosanitary problems are still major limitations for apple culture. Among these, Glomerella leaf spot and bitter rot (*Colletotrichum* spp.) and European canker and Neonectria fruit rot (*Neonectria ditissima*) stand out in southern Brazil orchards.

Neonectria ditissima is present in some of the main cultivation areas in the world (KIM; BERESFORD, 2012), including in Brazil. The colonization by the fungus can result in death of woody tissues of the plant (SWINBURNE, 1975; ZELLER, 1926), characterizing European canker disease, and can also cause rots on fruit. Neonectria fruit rot in apples is common in Brazilian orchards, and pear is majorly cultivated near apple orchards and is a known host of *N. ditissima* (WEBER, 2014), but the monocycle of the disease for both fruits is not well elucidated. The production areas in Brazil are located mostly in the south states. The weather conditions, mainly temperature, vary among regions and can be an important variable for the disease establishment and for the epidemic to take place. The occurrence of the sexual stage of the pathogen is common in the orchards; however, the role of ascospores in fruit infections, as the temperature effect in the germination of the pathogen, is also still unclear. In addition, it is not known if the pathogen spores can infect ripe fruit through the calyx opening, being a problem during the storage period. *Neonectria ditissima* is a quarantine apple pathogen in Brazil, restricted to some production areas in Rio Grande do Sul, Santa Catarina, and Paraná states; however, the quarantine certification is based on the absence of symptoms, but a study regarding presence of the pathogen in quiescent infections has never been performed in commercial apple fruit.

Glomerella leaf spot and bitter rot are of great importance for fruit producing regions in Brazil due to the severity of symptoms in the leaves and fruit that has gradually increased over the years (BECKER; KATSURAYAMA; BONETI, 2000; DENARDI et al., 2019; MOREIRA; PERES; MAY DE MIO, 2019). Flowers and fruitlets are potential sources of inoculum for bitter rot infections and for Glomerella leaf spot secondary infections, but a broad sampling in orchards has never been performed. Furthermore, *Colletotrichum* species have different sensitivities to fungicides, so an accurate identification of local populations is crucial for disease management. Chemical is the main control method for apple diseases, and mancozeb, the most used fungicide in Brazilian orchards, is being banned from the European Union, which could lead to import restrictions of apples with residues of this product. In that sense, evaluating sensitivity of already registered and other fungicides that hold promise to Glomerella leaf spot and bitter rot management is a priority.

The present study aimed to fill some gaps in knowledge on several aspects of the main diseases of southern Brazil, European canker and Neonectria fruit rot caused by *Neonectria ditissima*, and Glomerella leaf spot and bitter rot caused by *Colletotrichum* spp., such as studies on physiological traits and monocycle of Neonectria fruit rot of both sexual and asexual spores, the possibility of quiescent *Neonectria ditissima* infections in commercial apple fruit, identification of *Colletotrichum* species in 'Eva' flowers and fruitlets in Paraná orchards and their sensitivity to registered and promising fungicides.

In view of the above, the specific objectives of each chapter of this thesis were: i) assess if quiescent infections from natural infection on the orchards are occurring in commercial apple fruit and characterize *N. ditissima* quiescent infections in Brazilian commercial fruit.; ii) evaluate *N. ditissima* ascospore germination and infection and monocyclic components of Neonectria fruit rot on apple and pear; iii) identify *Colletotrichum* species from 'Eva' apple flowers and fruitlets in Paraná state orchards, evaluate their sensitivity to cyprodinil, tebuconazole, iprodione, and fluazinam and determine the baseline sensitivity of these isolates to benzovindiflupyr and natamycin; iv) evaluate and compare the *in vitro* sensitivity with EC_{50} estimation of *Colletotrichum melonis* and *C. nymphaeae* isolates from apple trees in Paraná state to the QoI fungicide pyraclostrobin, as well as to characterize the isolates phenotypically.

2 LITERATURE REVIEW

2.1 APPLE TREE CULTIVATION

The domesticated apple (Malus × domestica Borkh., family Rosaceae, tribe Pyreae) is a temperate climate fruit tree. Apple trees produce flowers and leaves in the same bud, called mixed buds. They are borne terminally or laterally on fruiting structure classified as brindle, and terminally on spurs. Brindles (10-40cm) are formed in the previous year and spurs are shorter, two years old or older buds (PETRI, 2006). The flowers are hermaphroditic inflorescences with predominantly allogamous reproduction, with cross-fertilization occurring. Due to self-incompatibility, there is a need for cross-pollination, carried out by insects. Thus, commercial orchards must contain a portion of the pollinating cultivar and the presence of pollinating insects to guarantee fruit production. Apple fruit is a pome, i.e., a pseudofruit derived from an inferior ovary with a fleshy receptacle tissue, that can vary in size, color of the epidermis, shape, texture, and flavor of the pulp (PETRI et al., 2006).

Apple tree is one the main fruit crop of temperate regions of the world (VELASCO et al., 2010). World apple production in 2019 corresponded to 87.2 mi tons, and China accounted for around 49%, with a production of 42.4 million tons (“FAOSTAT”, 2019). In Brazil, apple is one of the most important temperate fruit species, and the production is concentrated in the states of Santa Catarina, Rio Grande do Sul and Paraná, which account for more than 99% of national production, with a planted area of approximately 32,420 ha and a production of around 1.2 mi tons (“FAOSTAT”, 2019). Paraná state accounted for 3.6% of apple production between 2008 and 2018 (IBGE, 2019), whose main producing regions are Palmas and cities in the metropolitan region of Curitiba (PETRI et al., 2011).

The cultivars Gala and Fuji and their clones, such as Royal Gala, Imperial Gala, Brookfield, Fuji Suprema, represent more than 90% of apple production in Brazil. However, these apple varieties are insufficiently adapted to the regional climate, requiring the use of chemical agents as hydrogen cyanamide for budbreak to compensate for the lack of chilling, increasing the apple production costs and being associated with harmful effects (PETRI et al., 2014). Moreover, ‘Gala’ and its mutations are also highly susceptible to Glomerella leaf spot, currently the main apple disease in Brazil (DENARDI et al., 2019a). To overcome this problem, ‘Eva’ cultivar was developed by the agronomic institute of Paraná (IAPAR), as a result of ‘Gala’ and ‘Anna’ crossing. The precocity on the production, low chilling requirements, good acceptance by the consumer due to similarity to ‘Gala’ fruit, high productivity and low susceptibility to Glomerella leaf spot (HAUAGGE; TSUNETA, 1999), made the cultivar a suitable and very advantageous option for this region. However, the cultivar is susceptible to bitter rot, and growers have been reporting incidence of Glomerella leaf spot on ‘Eva’ leaves in the last seasons.

Besides Glomerella leaf spot and bitter rot, caused by several fungal species of the genus *Colletotrichum*, other phytosanitary problems limit apple production in southern Brazil and concern growers, such as European Canker and Neonectria fruit rot, caused by the fungus *Neonectria ditissima*. The main aspects of the diseases covered by this study are described in the following items.

2.2 NEONECTRIA FRUIT ROT

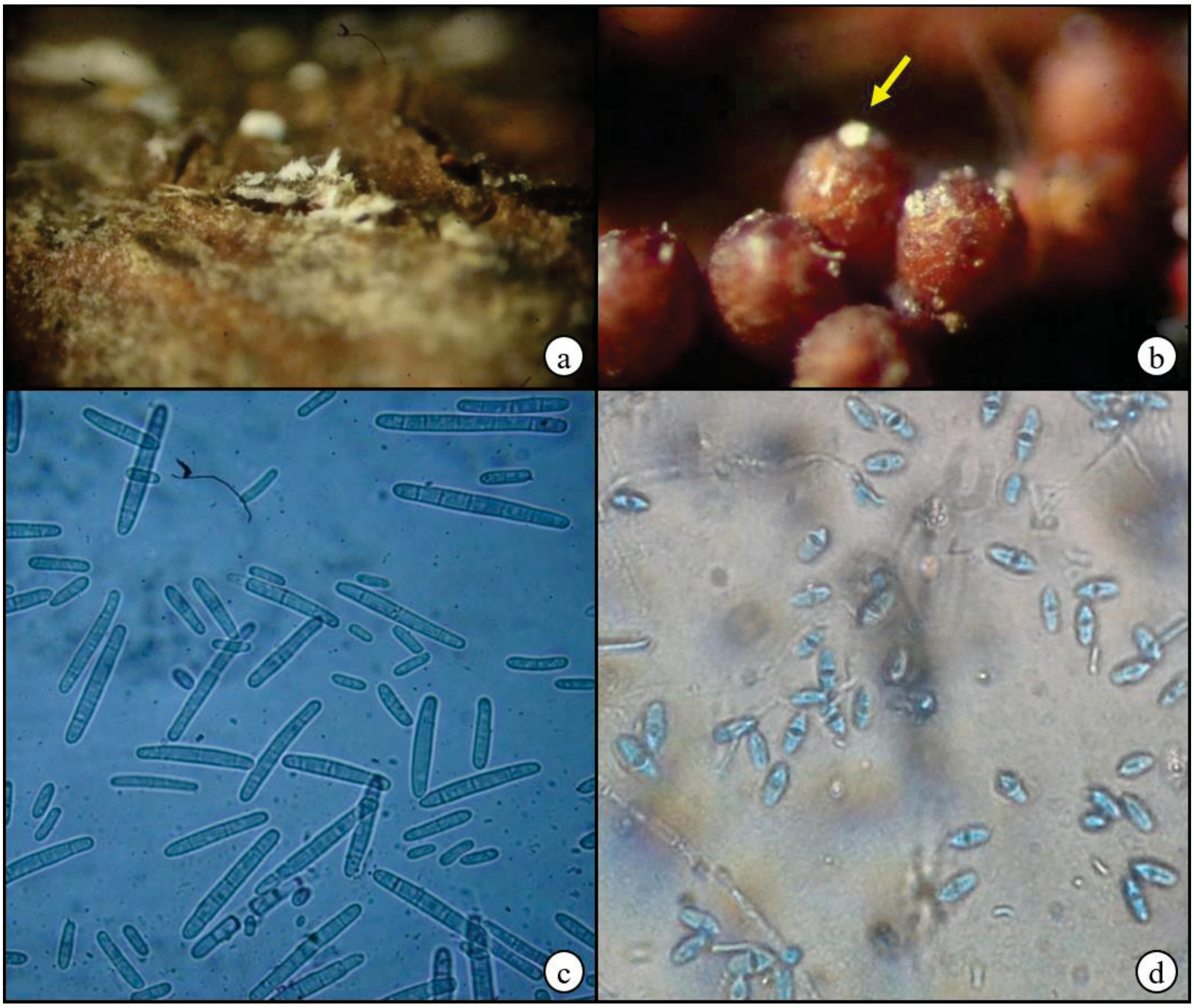
2.2.1 ETIOLOGY

Based on the characteristics of its asexual and sexual reproduction, as well as on evidence from phylogenetic studies, the fungus that causes European canker and Neonectria fruit rot in apples belongs to a well-defined taxonomic group, called *Neonectria ditissima* (Tul. & C Tul.) (CASTLEBURY; ROSSMAN; HYTEN, 2006; CHAVERRI et al., 2011). The fungus produces ascospores from perithecia and conidia from sporodochia that are both produced from cankers on infected wood (WEBER, 2014); apples with Neonectria fruit rot can produce sporodochia on lesions (GELAIN et al., 2020).

Conidia are formed in sporodochia (Figure 1a) mainly on twigs with young cankers. Two types of conidia are produced by *N. ditissima* in nature and in laboratory culture, macroconidia and microconidia. Macroconidia are straight or slightly curved and often 5-septate. Microconidia are short-cylindrical or ellipsoidal and aseptate or 1-septate (Figure 1c).

Perithecia are found clustered on wood, generally on an erumpent stroma, red, subglobose to broadly obpyriform (Figure 1b). Ripe perithecia may be identified by a whitish cirrhus of ascospores (Figure 1b, yellow arrow) especially if sporulating lesions are kept for a few days in a humid chamber. Such ascospore aggregations at the perithecial ostiole are dispersed by rain-splash. Ascospores of *N. ditissima* are ellipsoidal, one-septate and slightly constricted at the septum (Figure 1d) sometimes becoming pale brown at maturity. They are produced in asci which discharge their contents explosively at the perithecial ostiole. Ascospores released in this manner are distributed by wind (CHAVERRI et al., 2011).

Figure 1. Reproduction structures of *Neonectria ditissima*. (a) sporodochia; (b) perithecia with white cirrhi formed on the ostiole (yellow arrow); (c) macro (multiseptated) and microconidia (no septa); (d) one-septate ascospores.

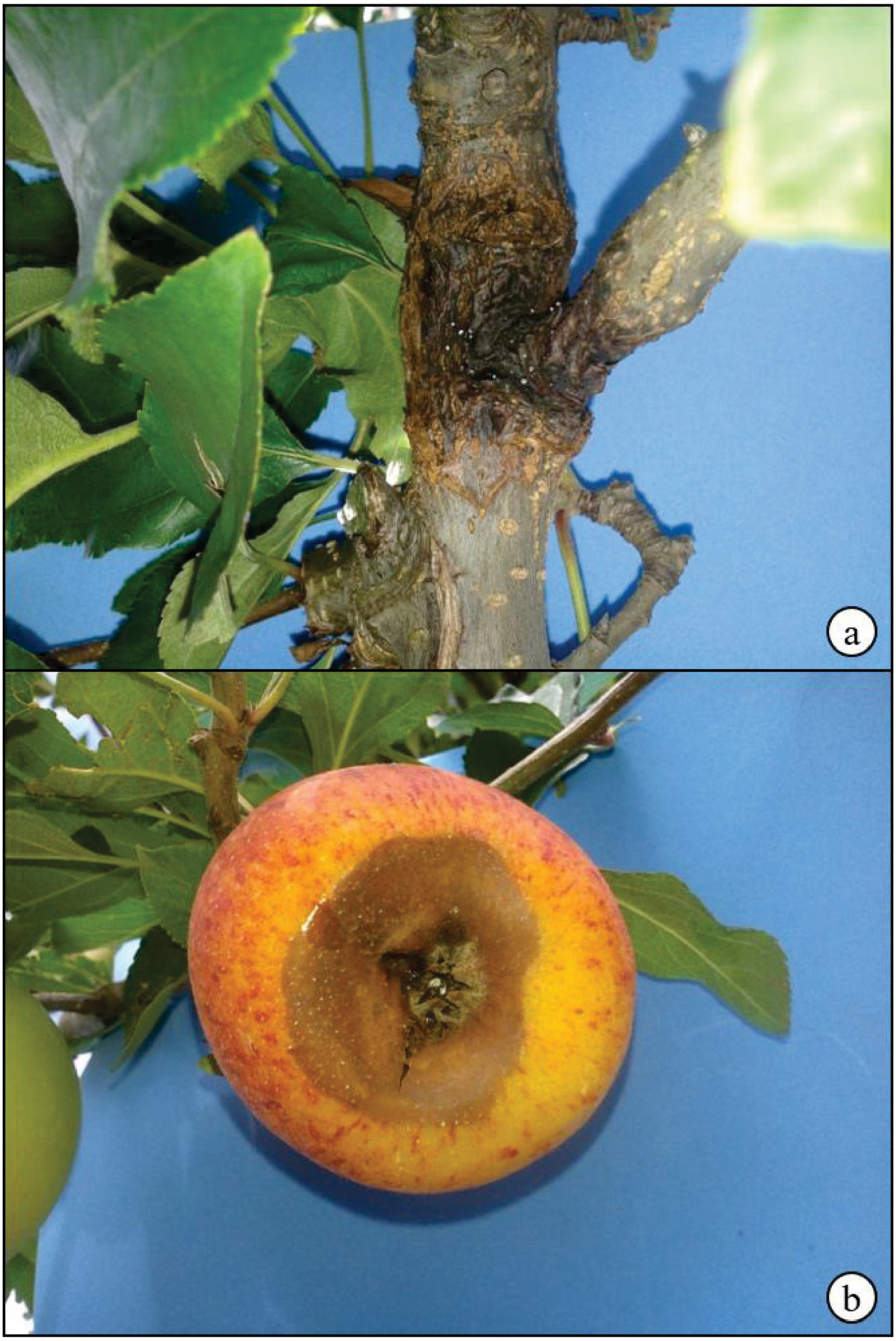


Source: The author (2016)

2.2.2 SYMPTOMS

The fungus causes canker-like lesions in woody tissues, killing part or even the whole tree by ringbarking of branches and tree stems or by killing the rootstock (BUS et al., 2019). European canker is first seen as a reddish-brown spot around a leaf scar, spur or pruning wound. The canker develops and forms ring shaped cracks in the bark, which may appear swollen. If the canker girdles the trunk or branch, any shoots above the canker will die. Within a few weeks, white or pale yellow superficial conidial pustules (sporodochia) develop on the surface of the dead bark (WEBER, 2014) (Figure 2a).

Figure 2. Symptoms of (a) European canker on twig and (b) Neonectria fruit rot on the calyx-end, caused by the fungus *Neonectria ditissima* in 'Gala' apples.



Source: The author (2014)

N. ditissima can also cause severe losses in apple fruits (SWINBURNE, 1975), with a symptom of soft rot (XU; ROBINSON, 2010) (Figure 2b) and the disease can cause serious damage due to fruit rot in orchards with high inoculum on twigs and trunks. In the southern region of Brazil, a higher incidence of *Neonectria* fruit rot has been observed than in other producing countries, probably due to the more favorable climatic conditions and/or the greater amount of inoculum in the orchards, because of the lack of experience in the disease management (ALVES; CZERMAINSKI, 2015). While European canker in branches has generally been well elucidated in the literature, there are few recent studies about infection of *N. ditissima* in fruit (GELAIN et al., 2020).

2.2.3 EPIDEMIOLOGY

Neonectria fruit rot is a serious problem in Northern Ireland (COOKE, 1999), northern Europe, England (BERRIE, 1989), and in Brazil (ALVES; CZERMAINSKI, 2015). Surveys of post-harvest rot of apples in southeastern England showed that the incidence of Neonectria fruit rot in the stored fruits varied from 0.2 to 4.2% for cv. Cox's Orange Pippin (BERRIE, 1989).

Neonectria fruit rot occurs in places with mild temperatures and high humidity in the summer, being common in producing areas located at high latitudes (> 50°) (KIM; BERESFORD, 2012). There is evidence that the susceptibility of apples to *N. ditissima* is initially low and increases with the fruit's maturity (XU; ROBINSON, 2010). The fruits are more susceptible to infection up to 4 weeks after pollination and more resistant to infection when they reach about 2 months of age, becoming more susceptible again the more mature the fruit becomes. *N. ditissima* conidia probably enter apple fruits only through lenticels (BROWN; SWINBURNE, 1971); however, lenticels may not have formed yet in young fruits at the time of early inoculation in experiments (XU; RIDOUT, 1998), suggesting other places of entry for conidia. A possible type of entry site is through stomata, since they are formed very early in the development of the fruit and later become lenticels (TETLEY, 1930).

The infection of fruits by *N. ditissima* in orchards is probably restricted to the fruit closest to sporulating cankers on branches, since conidia and ascospores are available throughout the year, although their production and release depend on precipitation (SWINBURNE, 1975). Both spore types are dispersed, and can cause significant infection, during prolonged periods of rainy weather (XU; BUTT, 1996). Therefore, inoculum of *N. ditissima* as conidia and/or ascospores is available all year except for very dry periods in summer and freezing conditions in winter (WEBER, 2014). The need for a close physical proximity between canker lesions and fruit infections as well as the timing of infections in spring and summer are factors that strongly point to conidia rather than ascospores as the main inoculum for pre- and post-harvest fruit rots (WEBER; DRALLE, 2013), but the role of ascospores in fruit infections is still unclear.

It is known that conidia can germinate (>20% germination) at temperatures from 10-30°C if 24 hours of wetness period is available (GELAIN et al., 2020). Data on ascospore germination rates in different temperatures are to an eight-hour period only and maximum temperature of 20°C (LATORRE et al., 2002) and should be complemented.

Regarding the amount of inoculum for disease expression, only studies referring to European Canker were found in the literature. For freshly made wounds in woody tissues, such as pruning cuts or rasp wounds, only three conidia were required for infection initiation in the greenhouse under optimal conditions, 12 conidia in the laboratory on detached shoots, and 10 to 30 conidia in the field (WALTER et al., 2016).

2.3 GLOMERELLA LEAF SPOT AND BITTER ROT

2.3.1 ETIOLOGY

Due to its complexity, the genus *Colletotrichum* is classified into species complexes using a combination of molecular sequencing information and morphology for species identification (CANNON et al., 2012; DAMM et al., 2012; DOWLING et al., 2020; KHODADADI et al., 2020; WEIR; JOHNSTON; DAMM, 2012). Among the nine major *Colletotrichum* species complexes, *C. gloeosporioides* (CGSC) and *C. acutatum* (CASC) are the most commonly associated with Glomerella leaf spot and bitter rot (DOWLING et al., 2020; JAYAWARDENA et al., 2019; KHODADADI et al., 2020; MUNIR et al., 2016; PERES et al., 2005).

The species known for causing bitter rot of apple trees worldwide from CASC are *C. fioriniae*, *C. nymphaeae* and *C. godetiae* (MOREIRA; PERES; MAY DE MIO, 2019; MUNIR et al., 2016; SATO; MORIWAKI; MISAWA, 2013; VELHO; STADNIK; WALLHEAD, 2019); from CGSC, *C. fructicola, C. aenigma, C. siamense* and *C. theobromicola* (MOREIRA; PERES; MAY DE MIO, 2019; MUNIR et al., 2016; VELHO; STADNIK; WALLHEAD, 2019; WEIR; JOHNSTON; DAMM, 2012; YOKOSAWA et al., 2017). Besides causing bitter rot, *C.*

limetticola, C. paranaense, C. melonis in CASC and *C. fructicola* in CGSC have been identified as causing agents of Glomerella leaf spot (MOREIRA; PERES; MAY DE MIO, 2019; VELHO et al., 2015; VELHO; STADNIK; WALLHEAD, 2019). *Colletotrichum karstii*, a species within the *C. boninense* species complex (CBSC), has also been detected causing Glomerella leaf spot in apples in Brazil (VELHO et al., 2013).

2.3.2 SYMPTOMS

Symptoms of Glomerella leaf spot include severe defoliation that could reduce productivity in subsequent seasons, while bitter rot is the most common symptom of fruit infections (BECKER; KATSURAYAMA; BONETI, 2000; MOREIRA et al., 2021). However, Glomerella leaf spot in fruit can also occur (LEITE JÚNIOR; TSUNETA; KISHINO, 1988).

Glomerella leaf spot occurs with greater intensity in apple producing regions with humid subtropical climate, including the southeast of the United States (GONZÁLEZ; SUTTON; CORRELL, 2006) and southern Brazil (BECKER; KATSURAYAMA; BONETI, 2000). In Brazil, Glomerella leaf spot was first reported in 1983 in the municipality of Porto Amazonas, Paraná state (LEITE JÚNIOR; TSUNETA; KISHINO, 1988). Especially in this state, the rainy summers and the cultivation of the highly susceptible cultivar Gala contributed to the pathogen’s development and increased the inoculum to the point of preventing efficient control. Glomerella leaf spot is associated with severe necrotic, irregular sized, brown-reddish lesions on leaves, which can exceed 70% of leaf area (MOREIRA; PERES; MAY DE MIO, 2019). Severely affected leaves turn brown and abscise; less severely affected leaves become chlorotic and abscise in 2 to 4 weeks. The fungus can also become established in tissues of less susceptible cultivars, resulting in a dwarfing and crinkling of the leaves (LEITE JÚNIOR; TSUNETA; KISHINO, 1988; SUTTON; SANHUEZA, 1998).

Bitter rot disease is present in almost all apple producing regions worldwide. In temperate growing regions with high rainfall, it can cause crop losses as great as 50% (SUTTON et al. 1990). Symptoms on apple fruit initially appear as small circular lesions that are light to dark brown in color. They evolve into circular, sunken, and light brown to brown lesions formed on the fruit surface; in humid conditions, erumpent, mucilaginous, pink conidia masses are formed (GONZÁLEZ; SUTTON; CORRELL, 2006).

Symptoms of Glomerella leaf spot on fruit begin as faint, light brown specks which become dark brown. The lesions do not enlarge and only a few conidia are produced on the surface of these lesions, which distinguishes the disease from bitter rot caused by *Colletotrichum* spp. (SUTTON; SANHUEZA, 1998).

2.3.3 CHEMICAL CONTROL

Multisite protectant fungicides, including mancozeb, captan, and chlorothalonil, among others (Table 1), are registered and have been extensively used in Brazil, according to growers. However, increasingly stringent export regulations for these fungicides (DOWLING et al., 2020), insufficient control in rainy weather and restriction on application on pre-harvest because of the re-entrance periods have made the incorporation of modern systemic fungicides into *Colletotrichum* management programs necessary.

There are currently seven single-site fungicide chemical groups registered for disease control in apples in Brazil (Table 1): methyl benzimidazole carbamates (MBCs – FRAC 1), dicarboximides (FRAC 2), demethylation inhibitors (DMIs – FRAC 3), succinate-dehydrogenase inhibitors (SDHIs – FRAC 7), anilino-pyrimidines (FRAC 9), quinone – outside inhibitors (QoIs – FRAC 11), and 2,6-dinitro-anilines (FRAC 29). Among these, only 4 single-site chemical groups (2 a.i. from MBCs, 1 a.i. from DMIs, 1 a.i. from SDHI, and 2 a.i. from QoIs) are registered against *Colletotrichum* spp. in apples (Table 1, in bold). Moreover, *Colletotrichum acutatum* species complex have inherent resistance to MBCs (CHUNG et al., 2006; PERES et al., 2004) and to the SDHI fluxapyroxad (ISHII et al., 2016), and *Colletotrichum* spp. have high risk of resistance to QoIs with many reports of resistance worldwide (DOWLING et al., 2020), leaving growers with only one DMI registered for the culture as a single-site option with good efficacy and medium risk for resistance in orchards where *C. acutatum* species complex is predominant.

The susceptibility profiles of *Colletotrichum* species against fungicides in apple orchards across Brazil are very limited. One study tested the sensitivity of *Colletotrichum acutatum* species complex isolates to thiophanate-methyl, azoxystrobin and mancozeb (MOREIRA et al., 2019), and the other to benomyl (HAMADA; KATSURAYAMA; DANTAS, 2009). These only two studies available were performed with isolates from the 2010-2011 season or before, leading to a lack of information and scientific basis for decision-making regarding the management of diseases in the field, showing the urgent need for experiments in this subject.

The historic background, mode of action, main target pathogens, resistance mechanisms and efficacy against *Colletotrichum* of the chemical groups registered in Brazil for apple tree culture are described in the next topics.

Table 1. Active ingredients from different chemical groups and FRAC codes registered for apple disease control in Brazil. Active ingredients in bold are registered for *Colletotrichum* spp. control in apples.

Chemical group/group name	FRAC code	Active Ingredient
MBC	1	**carbendazim** **thiophanate-methyl**
Dicarboximides	2	iprodione procymidone
DMI	3	cyproconazole difenoconazole flutriafol imazalil imibenconazole **tebuconazole** tetraconazole
SDHI	7	**fluxapyroxad**
Anilino-pyrimidines	9	cyprodinil pyrimethanil
QoI	11	**trifloxystrobin** kresoxim-methyl **pyraclostrobin**
2,6-dinitro-anilines	29	fluazinam
Multisite (organic)	M	**captan** **chlorothalonil** **dithianon** folpet **mancozeb** **metiram** **propineb**

MBC = Methyl Benzimidazole Carbamates
DMI = Demethylation Inhibitors
SDHI = Succinate Dehydrogenase Inhibitors
QoI = Quinone-outside Inhibitors
FONTE: Agrofit - MAPA (2021).

2.3.3.1 Multisite fungicides (organic)

In favorable climates, such as in Parana State, spraying for Glomerella leaf spot is extended until the postharvest period in mid-autumn to avoid early defoliation of the plant and to ensure growth and production during the following year. This regimen results in >20 multisite fungicide sprays per season of protective fungicides such as mancozeb, metiram, propineb, captan (HAMADA; MAY DE MIO, 2017).

The use of multisite fungicides results in a low risk of selection for pathogen resistance because they have a broad spectrum of action that does not interfere with a specific metabolic process of the fungus (BRENT; HOLLOMON, 2007). Therefore, they can be used in mixtures or alternating with single site and other medium to high resistance risk fungicides. Over the past decades, no cases of field resistance against multisite fungicides have been reported.

Some multisite fungicides such as chlorothalonil and mancozeb are being banned from European Union due to their toxicity to mammals (AXELSTAD et al., 2011; BELPOGGI et al., 2006; CECCONI et al., 2007; DOMICO et al., 2006; LEBAILLY et al., 1997; PENAGOS, 2002). However, restricting the use of multisite fungicides from use in important crops could result in faster development of resistance to single site mode of action fungicides, leading to epidemics, serious crop losses, and leaving growers without highly effective fungicides for a sustainable disease management.

2.3.3.2 Methyl Benzimidazole Carbamates (MBCs – FRAC 1)

Methyl Benzimidazole Carbamates (MBCs) comprise the benzimidazoles (benomyl, carbendazim, thiabendazole) and the thiophanates (thiophanate and thiophanate-methyl). Benzimidazole fungicides were first introduced in the late 1960s as highly effective, systemic fungicides, controlling a broad spectrum of ascomycete pathogens and some Basidiomycetes but inactive against Oomycetes (YOUNG, 2015). These compounds block nuclear division by binding to β-tubulin and preventing microtubule assembly (DAVIDSE; FLACH, 1978; YOUNG, 2015).

Benzimidazoles are used primarily to control ascomycete pathogens and have encountered serious resistance problems and loss of efficacy in the field. The mechanism of resistance to benzimidazoles is based on a decrease in its binding affinity to ß-tubulin (SISLER, 1988; DAVIDSE; ISHII 1995), which is associated with single nucleotide mutations at specific codon positions of the ß-tubulin gene that cause qualitative changes in several amino acids at the benzimidazole binding site (KOENRAADT; SOMERVILLE; JONES, 1992). Molecular characterization of the ß-tubulin gene sequence in benzimidazole-resistant strains of various fungi revealed the existence of several different mutations that confer resistance. Point mutations in the ß-tubulin gene that cause various levels of benzimidazole-resistance have been detected at codon positions 6, 50, 167, 198, 200 and 240 in various phytopathogenic fungi species (ALBERTINI; GREDT; LEROUX, 1999; BARALDI et al., 2003; DAVIDSON et al., 2006; KOENRAADT; SOMERVILLE; JONES, 1992; MA et al., 2005; MA; YOSHIMURA; MICHAILIDES, 2003; MCKAY et al., 1998; QIU et al., 2011).

In the past, MBC fungicides provided good efficacy against *C. gloeosporioides* complex (MACKENZIE; MERTELY; PERES, 2009). On the other hand, *C. acutatum* complex have inherent resistance or insensitivity to MBC fungicides (CHUNG et al., 2006; PERES et al., 2004). However, *C. gloeosporioides* can become resistant to MBCs, threatening to make this chemical class ineffective as a chemical control option (DOWLING et al., 2020). Resistant *C. gloeosporioides* isolates were reported from several fruit crops, including mango (SPALDING, 1982), avocado (SANDERS; KORSTEN; WEHNER, 2000), citrus (PERES et al., 2004), and strawberry (CHUNG et al., 2010). In these isolates, mutations E198A and F200Y in the ß-tubulin gene were reported to be responsible for high and moderate levels of resistance, respectively (CHEN et al., 2013; CHUNG et al., 2006, 2010; KONGTRAGOUL, 2020; MA; MICHAILIDES, 2005).

In Brazil, a study with apple isolates (2010-2011 season) from the *Colletotrichum acutatum* species complex reported lack of sensitivity to thiophanate methyl. The majority of the isolates were highly resistant and no sensitive isolate was found (MOREIRA et al., 2019), which corroborates the inefficacy of the MBCs in controlling Glomerella leaf spot and bitter rot especially in regions where *C. acutatum* species complex is prevalent. Nevertheless, thiophanate-methyl is still one of the most used single site fungicides according to apple growers; in ‘Eva’ commercial orchards located in Palmeira and Porto Amazonas municipalities, in Paraná state, during only one season (2017-2018), this fungicide was applied 6 times.

2.3.3.3 Dicarboximides (FRAC 2)

Dicarboximide fungicides are one of the chemical groups that belong to Histidine Kinase Inhibitors, which also include the phenylpyrroles (fenpiclonil and fludioxonil). Dicarboximide fungicides were discovered in the early 1970s by Sumitomo Chemical Co., Ltd. (FUJINAMI; OZAKI; YAMAMOTO, 1971). In the mid-1970s, iprodione, procymidone, vinclozolin, and chlozolinate, were commercially available and widely used to control diseases caused by the taxonomically related pathogens belonging to the genera *Botrytis, Sclerotinia,* *Monilinia*, and *Alternaria* (CORREIA et al., 2016). The dicarboximides overactivate Hog-like mitogen activated protein kinases in the osmotic signal transduction pathway and result in cell death.

Three main mechanisms have been associated with sensitivity reduction to dicarboximides: i) Point mutations; ii) Efflux pumps; iii) Overexpression of transporters. Point mutations have been reported for *Botrytis cinerea* in a putative osmosensor histidine kinase BcOS1/Daf1. In addition, several types of multidrug resistance strains, in which efflux pumps are activated, have emerged. Gain-of-function mutations of the transcription factor Mrr1, which leads to an overexpression of the ATP-binding cassette transporter AtrB, confers reduced sensitivities to some fungicides, including fludioxonil and cyprodinil. In addition, strains that overexpress the major facilitator superfamily transporter mfsM2 by promoter rearrangements lead to reduced sensitivities to iprodione (FUJIMURA et al., 2015). The discontinuance of dicarboximide applications in the field led to a decrease in populations of the resistant strains, suggesting a fitness penalty of dicarboximide-resistant strains (RAPOSO et al., 2000; WALKER et al., 2013).

The re-entry period of iprodione is <48 h, which would make it a very interesting option to control bitter rot in the harvest period, when the Brazilian growers do not have reliable options for disease management. However, only one study was found regarding *in vitro* efficacy of iprodione against *Colletotrichum* species from Connecticut, USA, and the results were not satisfactory (LAMONDIA, 2001). Studies about Brazilian isolates sensitivity to this dicarboximide are recommended.

2.3.3.4 Demethylation Inhibitors (DMIs - FRAC 3)

Demethylation Inhibitor (DMI) fungicides are the largest member of the group of Sterol Biosynthesis Inhibitors (SBIs), comprising an important group of commercially successful compounds registered for the control of plant diseases. The imidazole and triazole derivatives, known as azoles, are the most important DMI fungicides based on the number of compounds used in agricultural practice. Since 1970, more than 40 DMI molecules have been introduced in agriculture to control many plant pathogens, dominating the agricultural fungicide market (ZIOGAS; MALANDRAKIS, 2015).

DMIs inhibit the demethylation of the precursor sterols by binding to CYP51 enzymes, leading to inhibition of fungal biosynthesis of ergosterol, which is a major component of the plasma membrane of most fungi and needed for fungal growth (CHEN et al., 2020; PARKER et al., 2008; WARRILOW et al., 2010). The DMIs are particularly useful for disease control due to their pre- and post-infection activity against fungal plant pathogens (SZKOLNIK, 1981).

Solo products show poor to good efficacy against *Colletotrichum* spp., though they do not typically perform as well as QoI fungicides (DEFRANCESCO; PSCHEIDT; YANG, 2018). Most of the mixtures available are combinations with QoI fungicides and perform well against diseases on registered crops (DOWLING et al., 2020). Differences in sensitivity within *Colletotrichum* species for different DMI fungicides have been reported (CHEN et al., 2016), which highlights the importance of identification to the species level for an efficient management. Tebuconazole is the only DMI active ingredient registered against *Colletotrichum* spp. in apple trees in Brazil; however, no study regarding sensitivity of Brazilian isolates or efficacy of the molecule has been performed to date.

With intensive use of DMIs in the past decades, reduced sensitivity or even resistance to DMI fungicides have been reported. DMI resistance is a complex phenomenon and four main mechanisms have been described for filamentous fungi, including (i) target-site modifications in the CYP51 gene (DÉLYE; BOUSSET; CORIO-COSTET, 1998; DÉLYE; LAIGRET; CORIO-COSTET, 1997; FENG et al., 2020; MUELLENDER et al., 2021; SCHMITZ et al., 2014), (ii) overexpression of the CYP51 gene (HAMAMOTO et al., 2000; MA et al., 2006; MUELLENDER et al., 2021; SCHNABEL; JONES, 2001); (iii) and increased efflux pump activity including ATP binding cassette (ABC) transporters and major facilitator systems (MFS), in plant pathogens (HAYASHI; SCHOONBEEK; DE WAARD, 2003; KRETSCHMER et al., 2009; LUO; SCHNABEL, 2008) and in *Candida albicans* treated with DMIs in humans (PEREA et al., 2001; SANGLARD et al., 1995); and (iv) multiple CYP51 genes, which can cause overexpression of encoding gene or reduce the fitness cost associated with the CYP51 structure change (ZIOGAS; MALANDRAKIS, 2015). The available data from a number of biochemical and molecular studies on DMI resistance support the hypothesis that a combination of all the resistance mechanisms mentioned above could contribute to the decrease in field efficacy of DMIs (ZIOGAS; MALANDRAKIS, 2015). Specifically for *Colletotrichum* spp., sensitivity changes have been associated with target-site modifications on cyp51 gene (WEI et al., 2020; ZHANG et al., 2017), overexpression of CYP51 gene (WEI et al., 2020) and multiple CYP51 genes (CHEN et al., 2020).

Unlike qualitative resistance, typical for benzimidazoles (KOENRAADT; SOMERVILLE; JONES, 1992) and QoIs (BARTLETT et al., 2002) where resistant isolates are immune to the fungicide, resistance of fungus to DMIs is manifested as quantitative resistance, i.e., the isolates become tolerant to these fungicides but may still be controlled if application of high labeled rate of the fungicide or shortened application intervals are performed (ERICKSON; WILCOX, 1997; STAUB, 1991). This happens because resistance by fungi to the DMI fungicides is generally known to be controlled by the accumulation of several independent mutations, or what is known as 'continuous selection' or 'shifting', in the fungus. Therefore, in a field population of the fungus the sensitivity to the DMI fungicide may range from extremely high to moderate or low.

2.3.3.5 Succinate Dehydrogenase Inhibitors (SDHI – FRAC 7)

Succinate Dehydrogenase Inhibitors (SDHIs), previously called carboxamides, were released in 1969 with restricted activity spectrum to Basidiomycetes, until the release of Boscalid in 2003, which was the first SDHI to control a broad range of pathogens (STAMMLER et al., 2008, 2015). The mode of action of SDHIs is by interfering in the complex II of the mitochondrial respiration chain. The target of SDHI fungicides is the succinate dehydrogenase (SDH) complex in the respiratory chain also referred to as complex II or succinate:ubiquinone oxidoreductase (SQR) (AVENOT; MICHAILIDES, 2010; HÄGERHÄLL, 1997). In other words, the transport of electrons along the respiratory chain in mitochondria is responsible to produce Adenosine triphosphate (ATP), and SDHI fungicides bind to the SDH complex and block the transport of these electrons, stopping energy production in fungal cells.

Benzovindiflupyr is registered for apple bitter rot control in the USA, having excellent efficacy against members of the *C. acutatum* species complex (BECKERMAN et al., 2020), but showed only moderate efficacy against bitter rot and Glomerella leaf spot on apple caused by *C. fructicola* in the southeast (JOHNSON, 2018; RITCHIE et al., 2016). In laboratory studies, *C. gloeosporioides* s.l. and *C. acutatum* s.l. isolates were naturally resistant to boscalid, fluxapyroxad, and fluopyram (ISHII et al., 2016). However, in field trials, a mixture of pyraclostrobin and fluxapyroxad was consistently one of the most effective compounds against bitter rot and Glomerella leaf spot on apple, performing better than the solo products (VILLANI et al., 2018), suggesting a synergistic effect between the two fungicides (DOWLING et al., 2020). Penthiopyrad demonstrated moderate to good efficacy *in vitro* and in field trials for management of Glomerella leaf spot and bitter rot caused by *C. fructicola*, and was granted a special registration for apple in certain states (DOWLING et al., 2020; VILLANI et al., 2019). In Brazil, fluxapyroxad is the only SDHI registered for apple culture against *Colletotrichum* spp. and *Venturia inaequalis*, but to our knowledge no sensitivity study has been performed with Brazilian isolates so far.

Studies show that single-site mutations were responsible for the loss of fungicide efficacy. Several mutations in the target protein at different positions in three SDH subunits B, C, and D were detected in field isolates of some plant pathogens such as *Botrytis cinerea* (VELOUKAS; MARKOGLOU; KARAOGLANIDIS, 2012), *Corynespora cassiicola* (MIYAMOTO et al., 2009; MIYAMOTO; ISHII; TOMITA, 2010), *Alternaria alternata* (AVENOT; MICHAILIDES, 2007). In total, >27 mutations conferring resistance to SDHIs have been reported to date in field populations of different pathogens, and thus far, no mechanism other than mutations in sdh genes has been reported to be linked with resistance in field strains (SIEROTZKI; SCALLIET, 2013). No studies reporting *Colletotrichum* spp. resistance to SDHI fungicides were found, probably because of its more recent insertion in management programs against this fungus.

2.3.3.6 Anilino-pyrimidines (FRAC 9)

In 1993, cyprodinil was released under the code name “CGA 219417” for the protection of cereals, pomefruits, grapes and other crops (HEYE et al., 1994; MASNER; MUSTER; SCHMID, 1994). The anilinopyrimidine fungicides mode of action is not fully understood, but it is believed that they inhibit the biosynthesis of methionine and other amino acids and the secretion of hydrolytic enzymes involved in the infection process of the fungus (HEYE et al., 1994; MASNER; MUSTER; SCHMID, 1994).

In many crops, including peach, anilinopyrimidines are recommended for use early in season to increase their curative effect and limit their volatility (CREEMERS, 1994; CREEMERS; VANMECHELEN, 1999).

Cyprodinil has showed limited *in vitro* inhibitory activity of cyprodinil against isolates of *Colletotrichum acutatum* and especially *C. gloeosporioides* species complexes from strawberries (SCHNABEL et al., 2021). It was observed a strong initial inhibitory action but lack of continued concentration response, a phenomenon also reported when *C. acutatum* and *C. gloeosporioides* isolates were tested in microtiter assays at 3 μM and 30 μM cyprodinil (ABRIL ET AL., 2008; SMITH ET AL., 2013). Cyprodinil did not reduce severity or incidence of Glomerella leaf spot of apple caused by *Colletotrichum* species (not molecularly characterized) compared to the control (KOWATA et al., 2010). This fungicide is currently registered only for *Venturia inaequalis* control in apples.

2.3.3.7 Quinone-outside Inhibitors (QoIs – FRAC 11)

Quinone-outside Inhibitor (QoI) fungicides are derived from natural substances called strobilurins, isolated mainly from mushrooms from the *Strobilurus* genera (basidiomycetes). However, natural strobilurins are unstable under light and not reliable for disease control. Therefore, similar chemicals were synthesized, suitable photo-stable compounds with fungicidal activities were developed (BALBA, 2007). Azoxystrobin was the first QoI product to be announced in 1992 as a commercial fungicide by Syngenta, followed by kresoxim-methyl in 1998, and trifloxystrobin and pyraclostrobin in 2000 (BALBA, 2007; BARTLETT et al., 2002). The QoIs quickly became one of the most important agricultural fungicides, accounting for over 20% of the global fungicide market within the first ten years of their commercial offering (FERNÁNDEZ-ORTUÑO et al., 2016), being widely used against several diseases in different crops to the present day.

These fungicides specifically bind to the quinol oxidation (Qo) site of cytochrome b to inhibit mitochondrial respiration, blocking electron transfer between cytochrome b and cytochrome c1 and inhibiting the synthesis of nicotinamide adenine dinucleotide (NADH) and the mitochondrial membrane protein adenosine triphosphate (ATP) (BALBA, 2007; BARTLETT et al., 2002; FENG et al., 2020; FERNÁNDEZ-ORTUÑO et al., 2016), leading to stopping of the energy production and eventual death of the fungus (BALBA, 2007).

On fruit crops, QoI fungicides are the most effective mode of action against *Colletotrichum* spp. (DOWLING et al., 2020). However, the highly specific mode of action of the QoIs led to resistance in a significant number of target pathogens, usually due to modification of the target site (FERNÁNDEZ-ORTUÑO et al., 2016). In *Colletotrichum* species, resistance has been reported to QoI's in several fruit crops, such as mango (KONGTRAGOUL, 2020), strawberry (ALI et al., 2020; CHECHI et al., 2020; FORCELINI; PERES, 2018), peach and blueberry (HU et al., 2015). In apples, differences in sensitivities among *Colletotrichum* species occur (MUNIR et al., 2016; YOKOSAWA et al., 2017), and resistance has been reported in isolates with point mutations on cytb gene (YOKOSAWA et al.,

2017) and in isolates that did not present mutations on cytb gene (CHECHI et al., 2020; MOREIRA et al., 2019), indicating that other resistance mechanisms may be involved .

2.3.3.8 2,6-dinitro-anilines (FRAC 29)

Fluazinam is a broad-spectrum fungicide registered for use on apple, blueberry, and highbush cranberry in the USA (DOWLING et al., 2020). It exhibits a relatively broad antifungal spectrum to various economically important plant diseases caused by *Botrytis, Alternaria, Monilinia, Venturia, Sclerotinia* and *Colletotrichum*, and it also has effect against oomycetes such as *Phytophthora, Pseuporenospora* and *Plasmopara* (MITANI et al., 1996; SMITH et al., 2008). Fluazinam has little curative or systemic activity but has a good persistent effect and rain fastness, being considered a protectant fungicide (BUTZLER; BAILEY; BEUTE, 1998). Although the mode of action is not fully understood, fluazinam is believed to interrupt the fungal cell's energy production by uncoupling mitochondrial oxidative phosphorylation (BRANDT et al., 1992; GUO et al., 1991). Moreover, fluazinam has shown improvement of physiological status of leaves via promoting metabolites, antioxidants, better membrane integrity, and adjustment of the redox status in eggplants (YOUNES; DAWOOD; WARDANY, 2020).

Fluazinam has shown good efficacy against *C. gloeosporioides* and *C. acutatum* species complexes *in vitro* (GANG et al., 2015; LEROUX, 1996). On apple, it performed well in field trials, providing similar levels of control to the commercial mixture of pyraclostrobin and fluxapyroxad (RITCHIE et al., 2016). Despite its proven efficacy against *Colletotrichum* spp., registration for apple culture and low risk of resistance development, no study with fluazinam has been performed to date with apple Brazilian isolates.

2.3.4 VARIETAL RESISTANCE

The main apple clones grown in Brazil are ‘Gala’ and ‘Fuji’, picked in February and April, respectively. Even though these clones are well known references for flavor and have wide acceptance among Brazilian consumers, they also have important limitations for the apple production chain in southern Brazil, especially regarding climate adaptation and phytosanitary issues (DENARDI et al., 2019a).

Gala and Fuji clones do not present satisfactory climatic adaptation in most Brazilian apple growing regions (PETRI et al., 2011), leading to the use of potential harmful sprouting inducers to overcome the dormancy of buds (HAWERROTH et al., 2010). Moreover, Gala and Fuji cultivars are also overly susceptible to many serious pathogens. ‘Gala’ clones are susceptible to Glomerella leaf spot, with high rates of fungicides used for their control (GONZÁLEZ; SUTTON; CORRELL, 2006; VELHO et al., 2015). Chemical control of these pathogens is one of the main production costs for Brazilian apples and has potential damaging effects on the agroecosystem and consumers’ health (PETRI et al., 2011). Thus, the incorporation of genetic resistance to local pathogens reduces production costs, provide greater food security and has been the cheapest and the most efficient approach for control of diseases and pests (DENARDI et al., 2019b).

Glomerella leaf spot has been one of the main targets of the apple breeding programs. ‘Eva’ cultivar was developed by the agronomic institute of Paraná (IAPAR), resulting from ‘Gala’ and ‘Anna’ crossing. The characteristics presented by this cultivar, as precocity on the production, low chilling requirements, good acceptance by the consumer due to similarity to ‘Gala’ fruit, high productivity, and low susceptibility to Glomerella leaf spot (HAUAGGE; TSUNETA, 1999) made the cultivar a suitable and very advantageous option for Paraná orchards, where it has been replacing Gala orchards. Besides ‘Eva’, several other varieties have been released by the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI), such as ‘SCS427 Elenise’, ‘SCS425 Luiza’ and ‘SCS426 Venice’, all resistant to Glomerella leaf spot (DENARDI et al., 2019a, 2019b, 2020).

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3 CHAPTER 1: DETECTION AND CHARACTERIZATION OF QUIESCENT INFECTIONS OF *Neonectria ditissima* IN BRAZILIAN COMMERCIAL APPLE FRUIT[1]

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Abstract

Neonectria ditissima, the causal agent of European Canker and *Neonectria* fruit rot, is a quarantine apple pathogen in Brazil, restricted to some production areas in Rio Grande do Sul, Santa Catarina, and Paraná states. The quarantine rests on a product certification based on the absence of symptoms but it was not known whether the pathogen could be moving around in quiescent infections. In this work, we detected *N. ditissima* after incubation of asymptomatic commercial fruit. The identity of the pathogen was confirmed using morphological and molecular tools, and Koch's postulates were fulfilled on 'Gala' apples. We demonstrated that fruit are weak links in quarantine efforts. Fruit stored at low temperatures can have quiescent *N. ditissima* infections, which may develop rapidly with abundant sporulation when exposed to more suitable conditions, as those of commercialization. Revised management strategies for flower and fruit treatment, as well as studies on efficient detection methods, are strongly recommended. This is the first report of *N. ditissima* quiescent infections in commercial apple fruit in Brazil.

Keywords: *Cylindrocarpon heteronema; Malus domestica; Nectria galligena;* postharvest disease.

Apple fruit (*Malus domestica* Borkh.) is one of the main crops produced in southern Brazil. However, since 2013 the total area harvested has been decreasing (FAO 2019). One of the factors contributing to this reduction is the occurrence of European Canker (EC), a serious disease caused by the fungus *Neonectria ditissima* (Tul. & C Tul.) (Alves and Czermainski 2015). *Neonectria ditissima* can infect and colonize the woody tissue, causing canker lesions in twigs and/or trunks, leading to plant death. The fungus can also cause disease in apple fruit, with symptom of soft brown rot that can be easily separated from the healthy tissue by using a

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spatula or a spoon (Weber, 2014). In the southern region of Brazil, a high incidence of *Neonectria* fruit rot (NFR) has been observed in the orchards, probably due to the more favorable climatic conditions and/or the greater amount of inoculum (Alves and Czermainski 2015).

The disease is confined to some areas of the southern region of Brazil, as most municipalities of the two largest apple producing states Rio Grande do Sul and Santa Catarina. In Paraná state, the third largest apple producer, the disease is confined to few orchards. In other Brazilian areas, the pathogen is considered absent. In 2013, the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) disclosed a Normative Instruction that instituted the National Program for Prevention and Control of this disease. In this instruction, the main disease containment strategy is exclusion, and all plant material must be inspected and certified prior to commercialization, based on the absence of symptoms (Brasil, 2013). There are no standards for pathogen surveillance in asymptomatic fruits due to the lack of evidence of quiescent infections in fruit.

Latent infections in woody tissues of apple plants in nurseries have been reported and a method for detection has been validated by Wenneker et al. (2016). On the other hand, there is few information about infections in apple fruit. In Brazil, no study about quiescent *N. ditissima* is available and only one with NFR with artificial inoculations has been performed to date (Gelain et al. 2020). Therefore, this study aimed to i) know if quiescent infections from natural infection on the orchards are occurring in commercial apple fruit and ii) characterize *N. ditissima* quiescent infections in Brazilian commercial fruit.

To achieve this objective, asymptomatic commercial apple fruit of the cultivars Gala and Fuji were collected from orchards infected with EC in Vacaria city, Rio Grande do Sul state, in 2018 and 2019. The samplings were performed at seven different times (collection codes v0 to v6). The characterization of the samplings is described in Table 1. 'Gala' fruit were harvested in February and 'Fuji' fruit in March, and then stored for periods ranging from three to nine months in cold chambers with controlled temperature (0 ± 1 °C) and atmosphere (1-1.5 kPa O_2, 0.5-2 kPa CO_2) until samples were collected from studies. Fruit samples were surface disinfected in 70% ethanol for 1 minute, 1% sodium hypochlorite solution for 1 minute, rinsed three times in distilled water then dried on paper towel and placed in disinfected transparent plastic pots with moistened cotton to maintain humidity. Disinfected fruit samples were incubated for about a month in Conviron® chambers at 20±2 °C and evaluated daily for possible NFR symptoms and signs.

Structures were scraped from the symptomatic fruit epidermis with a sterilized blade and placed on glass slides for observation under light microscope. Direct isolation was performed for each symptomatic fruit, by gently scraping the sporodochia signs and placing the harvested spores in 5-mL flaks containing sterilized distilled water. Spore concentration of the suspension was evaluated in a Neubauer chamber and the suspension was diluted to 10^2 conidia.mL^{-1}. Aliquots of 50 µL of each suspension was pipetted on petri dishes with WA media and spread with Drigalski spatula. Monosporic isolation was performed by collecting a single spore germinated after 6-8 hours in the WA media under a light microscope. A single isolate was retained for each infected fruit, after their fruit from which they were isolated. Purified cultures were grown in Potato Dextrose Agar (PDA) medium in glass Petri dishes and maintained in BOD chambers at 23 °C for 14 days with 12-hour photoperiod. After this period, the cultural, morphological, molecular, and pathogenic characteristics were analysed.

The isolates were submitted to DNA sequencing and phylogenetic analysis. For this purpose, genomic DNA of putative *N. ditissima* isolates obtained was extracted from 7-day-old cultures, using the modified CTAB protocol (Pereira et al. 2019). The PCR was performed for the regions ITS, *tef1* and *tub2*, and the pairs of primers used for each region were ITS1 and ITS4 (White et al. 1990), tef1-728F and tef1-986R (Carbone and Kohn 1999), and T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), respectively. The PCR products were purified and sequenced by Go Genetic, Curitiba city, Paraná state. The generated forward and reverse sequences were assembled using the BioEdit software (Hall 1999). Comparisons of the sequences from the three genes were made by BLAST search. For phylogenetic analyses, the DNA sequences of species belonging to the Nectriaceae family and the outgroup *Thelonectria gongylodes* (CBS12511), published by Jayawardena et al. 2019, plus more sequences from previous works were added to the dataset and aligned using the MEGA7 (Kumar et al. 2016). *Neonectria austroradiciola*, which has only the ITS sequence available and is not similar to any of our sequences, was not included in the analysis. All sequences used are described in Table 2. The multigenic sequences were assembled using the software SequenceMatrix.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model in MEGA7 (Kumar et al. 2016). The tree with the highest log likelihood (-2051.55) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 1054 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Pathogenicity of the five isolates was confirmed using mature 'Gala' apples, and four fruit per isolate were used. The fruit were surface disinfected and wounded with a sterile needle (2 mm diameter and 4 mm deep). The wound on each fruit was inoculated with a 40-µL conidial suspension of the isolate (10^5 conidia/mL). Four control fruit received only sterile water in the wounded area. Fruit were placed in plastic pots with moistened cotton and incubated in Conviron® chambers for 15 days at 20 °C and a photoperiod of 12 h.

Two 'Gala' fruit from the v0 collection code presented symptoms after incubation in Conviron® chambers. One fruit (f1) was found after 10 days of incubation with a soft dark brown rot symptom and sporodochia structures (Figure 1a) that started in the stylar-end and rapidly advanced towards the entire surface of the fruit. Then, within 14 days of incubation, a second fruit (f2) was found in the Conviron® chamber with the same previously described symptoms (Figure 1b). In the following collections v1-v4, no symptomatic fruit were found. However, in v5 one 'Gala' fruit (f3) and in v6 two 'Fuji' fruit (f4 and f5) presented symptoms and signs. Again, the colonization and sporulation of the fruit were fast and developed similarly to the symptoms described above. The f3 and f4 diseased fruit presented symptoms and signs at 18 days after incubation, and f5 at 19 days after incubation, with less sporulation.

The two types of conidia produced by the assexuate form of *N. ditissima* (=*Cylindrocarpon heteronema*), macro and microconidia, were observed in light optical microscope and matched the description reported by Chaverri et al. (2011). The colonies growth after monosporic isolation on PDA were white with tones of beige. The isolate NF1 presented yellow pigmentation, and the isolates NF2 to NF5 presented light beige pigmentation (Figure 1). Macroconidia were straight or slightly curved, often 5-septate and with rounded ends. Microconidia were short-cylindrical or ellipsoidal and aseptate or 1-septate, for all five isolates.

Using BLAST searches all sequences obtained in this study from all three genes were >99% identical to sequences deposited in GenBank as *Neonectria ditissima*. All five isolates found in fruit grouped into the *Neonectria ditissima* clade, being confirmed as this species (Figure 2). The sequences were deposited in GenBank and the accession numbers for the 3 genes sequenced for each isolate are described in Table 2. The alignment derived in this study have been lodged in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S27048).

Fifteen to eighteen days after inoculation of the isolates on 'Gala' apple fruit wounded fruit for confirmation of pathogenicity, typical symptoms of *Neonectria* fruit rot were observed. The fungus was reisolated and presented the same cultural, morphological, and molecular characteristics as the colonies used for inoculation.

We confirmed *Neonectria ditissima* in commercial apple fruit after remaining quiescent for a long period. The short incubation and latent periods and the abundant sporulation after transferring fruit to higher temperatures than those of cold chambers show that NFR can develop after long-term storage. Therefore, quarantine measures based on visual observation of symptoms and signs on fruit is not enough to confine the pathogen. Quiescent infections of *N. ditissima* in apple fruit have never been described before in Brazil, and there are few studies worldwide, only with artificial inoculation (Xu and Robinson 2010).

Although low incidence of NFR infections after long-term storage (2.56% in v0, 0.55% in v5 and 1.11% in v6) was found in our work, the importance of the pathogen once it has been stablished in an apple orchard and the fact that the disease is confined to some regions of the country lights an alert to the importance of avoiding fruit infections on the field. Quiescent infections are difficult to detect with conventional tools since there is little activity of the pathogen. Brazilian law for disease contention based solely on NFR symptoms should be reviewed. In addition, our study showed that symptoms were observed only after long storage periods (>7 months), so monitoring should be focused on fruit commercialized near the next growing season.

The spores of *N. ditissima* can travel only limited distances, but some agents such as humans, that can carry spores on clothing, pruning shears, transport boxes or machinery, infected seedlings or, as we proved with this work, fruit with quiescent infections, can difficult disease containment. Although commercial fruit are not typically source of inoculum for field infections, fruit transported and commercialized with quiescent *N. ditissima* can start infections on apple trees or other hosts, which include several (at least 67) cultivated and non-cultivated species (Flack and Swinburne 1977). It is important to note that even if unlikely, quiescent fruit infections have proven to be relevant. One example is from a study that detected *Monilinia laxa* in commercial imported fruits in Brazil, a species never found before in our country (Pereira et al 2018); the species was found 4 years later in commercial orchards (personal communication, to be published).

Our results indicate that *Neonectria ditissima* is present in quiescent infections in commercial fruit, and that the fungus is able to colonize and form reproductive structures after remaining quiescent for months under controlled conditions in cold chambers. This fact is of extreme relevance since Brazilian quarantine laws prohibit the transit of contaminated material but does not consider infections that are not visible at the time of harvest. We recommend quarantine control measures to avoid propagation of the disease to apple and other hosts, as well as management strategies to avoid flower and fruit infections on the orchards.

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Author's contributions: all authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

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Table 1 Characterization of the collection of apple fruit samples from the city of Vacaria, Rio Grande do Sul state, for *Neonectria ditissima* quiescent infection detection assay

collection code	cultivar	month/year	storage period (months)	n° sampled fruit
v0	Gala	dez/18	9	78
v1	Fuji	jun/19	3	100
v2	Gala	jun/19	4	100
v3	Gala	jun/19	4	100
v4	Fuji	aug/2019	5	100
v5	Gala	oct/2019	8	180
v6	Fuji	oct/2019	7	180
total				838

Table 2 Species, strain numbers and Genbank accession numbers from ITS, TUB and *tef1* of the *Neonectria* isolates used in the phylogenetic analyses

species	strain number	genbank accession numbers		
		ITS	TUB	tef1
Neonectria candida	CBS 151 29 IMI 113894/MUCL 28083	AY677291	DQ789863	DQ789723
N. coccinea	CBS 119158/GJS 98-114	JF268759	KC660727	JF268734
N. confusa	CBS 127485/HMAS 99197*	FJ560437	FJ860054	n.d.
N. confusa	CBS 127484/HMAS 99198	KM515889	KM515886	n.d.
N. ditissima	CBS 100318	KM515890	DQ789858	KM515944
N. ditissima	CBS 100317	KM515891	KM515887	KM515945
N. ditissima	CBS 835.97	JF735310	DQ789880	JF735784
N. ditimopsis	HMAS 98329*	JF268764	JF268729	JF268745
N. faginata	CBS 217.67/IMI 105738/ATCC 16547*	HQ840385	JF268730	JF268746
N. faginata	CBS 119160/GJS 04-159	HQ840384	DQ789883	DQ789740
N. fuckeliana	CBS 239.29/IMI 039700	HQ840386	DQ789871	JF268748
N. hederae	IMI 058770a/ATCC 16543*	n.d.	DQ789895	DQ789752
N. hederae	CBS 714.97/PD 97/1932	n.d.	DQ789878	KC660461
N. lugdunensis	CBS 125485/DAOM 235831/TG 2008-07	KM231762	KM232019	KM231887
N. major	CBS 240.29/IMI 113909*	JF735308	DQ789872	JF735782
N. microconidia	MAFF 241518	KC660534	KC660756	KC660476
N. microconidia	MAFF 241570	KC660549	KC660751	KC660494
N. neomacrospora	CBS 198.62/BBA 9628/IMI 113890	AJ009255	HM352865	HM364351
N. neomacrospora	CBS 324.61/DSM 62489/IMB 9628	JF735312	DQ789875	n.d.
N. obtusispora	CBS 183.36/IMI 113895	AM419061	AM419085	JF735796
N. punicea	CBS 242.29	KC660522	DQ789873	DQ789730
N. shennongjiana	HMAS 183185	FJ560440	FJ860057	n.d.
N. tsugae	CBS 788.69*	KM231763	KM232020	n.d.
Thelonectria gongylodes	CBS 12511/GJS 90-48	JQ403330	HM352870	HM364357
N. ditissima (NF1)**	LEMIDRSMd 19-01	MW116073	MW118608	MW118604
N. ditissima (NF2)**	LEMIDRSMd 19-02	MW136939	MW118609	MW118605
N. ditissima (NF3)**	LEMIDRSMd 19-03	MW136945	MW118610	MW118606
N. ditissima (NF4)**	LEMIDRSMd 19-04	MW136944	MW125664	MW125663
N. ditissima (NF5)**	LEMIDRSMd 19-05	MW136940	MW125665	MW118607

Ex-type (or ex-epitype) strains are in bold and marked with an asterisk* and voucher strains are in bold

** Sequences from the isolates found in the present study

n.d. = no data available

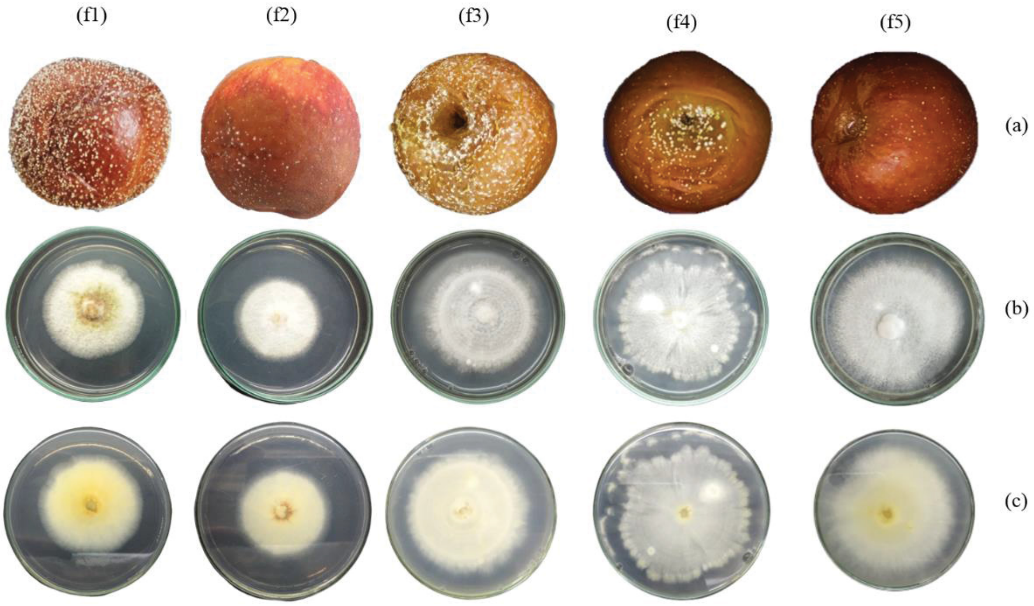

Fig 1 Commercial 'Gala' apple fruit after 10-19 days of incubation, from f1 (left) to f5 (right) (a), upper side (b) and lower side of 14-day colonies of monosporic isolates from NF1 (left) to NF5 (right) on Potato Dextrose Agar (PDA) incubated at 23 °C in BOD chambers (c).

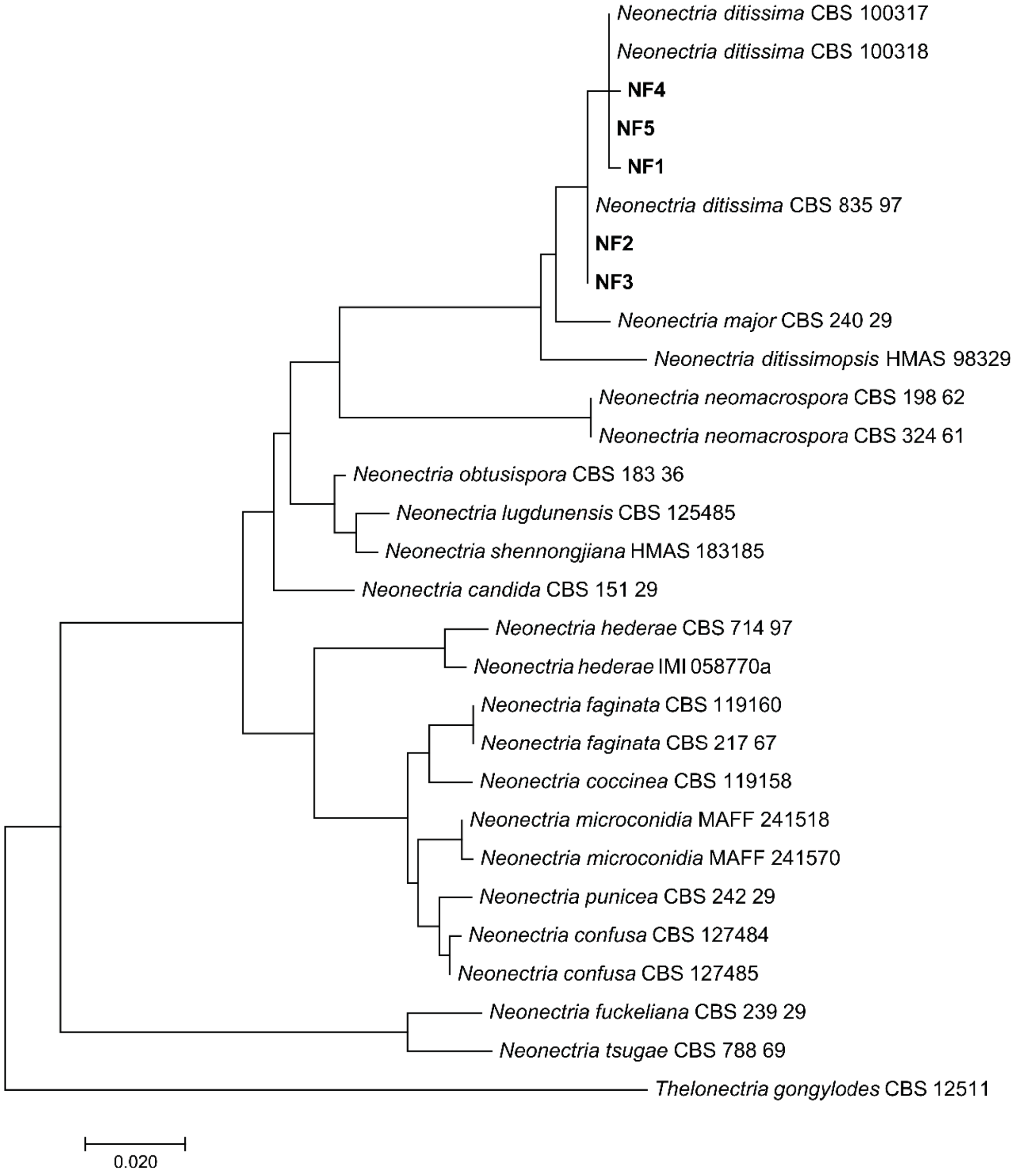

Fig 2 Molecular Phylogenetic analysis by Maximum Likelihood method of combined ITS, *tef1* and TUB sequence data of *Neonectria* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. Isolates from this study are shown in bold. The tree is rooted in *Thelonectria gongylodes.*

4 CHAPTER 2: ASCOSPORE GERMINATION AND MONOCYCLIC COMPONENTS OF NEONECTRIA FRUIT ROT ON APPLE AND PEAR[2]

ABSTRACT

European Canker caused by the fungus *Neonectria ditissima* (Tul. & C. Tul) is a serious disease of apple trees (*Malus domestica* Borkh) in Brazil. Neonectria fruit rot (NFR) is common in Brazilian orchards, but several aspects of its monocycle are not well elucidated. Therefore, the objectives of this study were to: i) test *in vitro* germination of ascospores at different temperatures and wetness periods and compare with conidia germination, ii) evaluate and compare the monocyclic components of NFR caused by conidia and ascospore infection in wounded and unwounded ripe detached 'Gala' apple and iii) evaluate and compare the monocyclic components of NFR caused by conidia infection in 'Rocha' pear fruit under different temperatures. Germination of conidia and ascospores occurred in the range of 12–28°C. At 35°C no germination occurred, for both ascospores and conidia, regardless of the wetness period (3-60 hours). With the same wetness period, conidia germination was always higher than ascospores at any temperature. For ascospores, the optimal temperature for germination ranged from 18-21°C, lower than the estimated for conidia, which ranged from 21 to 24°C. Regarding inoculation of ascospores and conidia in apple fruit, incubation period varied from 35 to 49 days after ascospore inoculation except at temperature of 22°C in which it was not observed, and for conidia from 14 to 42 days, being observed at all tested temperatures. When comparing the same temperatures, mean lesion growth rate was always higher for fruits inoculated with conidia than with ascospores. Unwounded fruits inoculated in the calyx-end did not present Neonectria fruit rot symptoms at the last day of evaluation. The disease on 'Rocha' pear fruit has shorter incubation and latent periods than those of 'Gala' apple and higher AUDPC at 25°C. The fast monocycle development represents risk if the disease spread to pear orchards but also sets pear fruit as a good option for future NFR control studies.

Keywords: *Cylindrocarpon heteronema*; *Nectria galligena*; *Malus domestica; Pyrus communis;* Postharvest disease; monocyclic components.

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[2] Prepared in accordance with the standards of European Journal of Plant Pathology

INTRODUCTION

The cultivation of apple tree (*Malus domestica* Borkh) presents several limitations, as many diseases that affect the culture. Among them, European Canker, caused by the fungus *Neonectria ditissima* (Tul. & C. Tul.), anamorph *Cylindrocarpon heteronema* (Berk. & Broome), is a worldwide spread disease that causes damage in apple trees in Europe, North America, Chile, Australia, New Zealand, Japan and South Africa (Kim and Beresford 2012). In Brazil, the pathogen is recognized as a quarantine pest A2 in Brazil, i.e., already present in the country, but with localized dissemination and submitted to an official control program (MAPA 2017).

In twigs, branches and trunks, the fungus causes canker-like lesions, while in fruits it causes a symptom of soft rot (Weber 2014). The disease can cause serious damage due to fruit rot in orchards with sporulation on twigs and trunks. In the southern region of Brazil, a higher incidence of *Neonectria* fruit rot has been observed than in other producing countries, probably due to the more favorable climatic conditions and/or the inoculum present in the orchards, because of the lack of experience in the disease management (Alves and Czermainski 2015). While European canker in woody tissues has generally been well elucidated in the literature, there are few recent studies about infection of *N. ditissima* in fruit.

The fungus produces ascospores from perithecia and conidia from sporodochia that are both produced from cankers on infected wood. Two types of conidia are produced by *N. ditissima* in nature and in laboratory culture: macroconidia (straight or slightly curved and often 5-septate) and microconidia (short-cylindrical or ellipsoidal and aseptate or 1-septate) (Booth 1966). Ascospores of *N. ditissima* are ellipsoidal, one-septate, and slightly constricted at the septum. Ripe perithecia may be identified by a whitish cirrus of ascospores (Weber 2014). Both spore types are dispersed, and can cause significant infection, during prolonged periods of rainy weather (Xu and Butt 1996). In general, inoculum of *N. ditissima* as conidia and/or ascospores is available all year round except for very dry periods in summer and freezing conditions in winter (Weber 2014) but the role of ascospores in fruit infections is still unclear. Data on ascospore germination rates in different temperatures are to an eight-hour wetness period only (Latorre et al. 2002) and should be complemented.

Environmental conditions, mainly temperature and moisture, influence the development of an epidemic by affecting various physiological processes of the pathogen (Campbell and Madden 1990). Ripe fruit are susceptible to *Neonectria* infection (Xu and Robinson 2010), and, although there are some studies about the influence of climatic characteristics on Neonectria fruit rot (Brown and Swinburne 1971b; Xu and Robinson 2010), the effect of the temperature on monocyclic components of the disease on fruits has not been extensively studied. Monocyclic components can be useful resources on predicting how the disease is going to behave under different temperatures and is also relevant for understanding the most suitable conditions for the development of the pathogen.

Although *N. ditissima* is most commonly described as an apple tree pathogen, pear (*Pyrus communis* L.) is also a host (Flack and Swinburne 1977) and occasionally pear orchards have high incidence of the disease (Weber 2016). Pears can be a complementary alternative to the cultivation of apples, because while the latter is already produced in large national scale, Brazil produced only 22,108 tons on 2017 and imported 146,778 tons of pears in 2016 (FAOSTAT 2019). However, pear orchards are commonly near apple orchards due to the similarity of chilling requirements and cultural treatments, which may represent a risk if *N. ditissima* is dispersed from apple to pear orchards. There is no recent scientific report on European Canker and Neonectria fruit rot on pear, and the disease monocycle in this fruit has never been described.

In view of the above, this study aimed to: i) assess *in vitro* germination rates of conidia and ascospores at different temperatures and wetness period; ii) evaluate and compare the monocyclic components of Neonectria fruit rot caused by conidia and ascospores in wounded and unwounded ripe detached 'Gala' apple at different temperatures; and iii) evaluate and compare the monocyclic components of Neonectria fruit rot caused by conidia in wounded and unwounded ripe detached 'Rocha' pear fruit and 'Gala' apple fruit under different temperatures.

MATERIAL AND METHODS

Effect of temperature and wetness period on conidia and ascospore germination of *Neonectria ditissima in vitro*

To produce the ascospore suspension, 'Gala' apple tree twigs with typical European Canker symptoms and perithecia formed on their surface were collected from orchards in Vacaria (Rio Grande do Sul state, southern Brazil). The twigs were placed in humid chamber until perithecia formed whitish cirrhi (Supplementary 1). Under a stereoscopic microscope, using a pin, sterilized, and dipped into sterile water, 20 to 25 cirrhi were touched lightly with the tip of the pin, sterilizing the pin before touching the next cirrhus. The tip of the pin with the cirrhus on it was rinsed in 1 ml sterile water in a 5 mL penicillin flask to wash the spores off.

The flask containing suspended cirrhi were briefly shaken on a vortex. The ascospore concentration was counted on hemocytometer, and the suspension was adjusted to 10^5 ascospore/ml.

To produce the conidial suspension, several twigs presenting European Canker symptoms were surface disinfested by immersion in 70% alcohol solution, had their external tissues removed and small pieces (5 mm approximately) were placed in plates containing water agar (WA) medium, about 5 pieces from the same twig in each plate. The plates were incubated in BOD chamber at 20°C with 12-hour photoperiod for 3 days. Growing mycelium from plates were transferred to plates with Spezieller Nährstoffarmer Agar (SNA) medium (Leslie and Summerell 2006) to induce sporulation. For making 1 liter of SNA medium, the ingredients consist of: KH_2PO_4 (1 g), KNO_3 (1 g), $MgSO_4$* $7H_20$ (0.5 g), KCl (0.5 g), glucose (0.2 g), sucrose (0.2 g) and agar (20 g). The plates were then incubated in BOD chamber at 20°C with 12-hour photoperiod for 10 days. After this period, with the aid of a pipette, 2 mL of sterilized distilled water was added to the plates. The conidia produced were gently scraped with the aid of a Drigalsky spatula, and the suspension was transferred to a 5-ml penicillin flask and adjusted on hemocytometer to a concentration of 10^5 conidia/ml.

Spore germination was assessed by placing 40-µl drops of a 10^5 spores/ml conidia and ascospore suspensions into Petri dishes with water agar media. The suspension was spread with a Drigalsky spatula. The dishes were kept in growth chambers at temperatures of 12, 16, 20, 26 and 35°C (first experiment repetition – block 1) and 14, 18, 22, and 28°C (second experiment repetition – block 2) and wetness periods of 3, 6, 12, 24, 36, 48 and 60 h in the dark. Spore germination was halted with Lactophenol-blue solution after the completion of the wetness periods. Conidia and ascospore germination were assessed by counting the number of germinated spores out of 100 spores under a light microscope (400 ×) in each of the 4 quadrants of each petri dish. A spore was considered germinated only if the germ tube length was equal to or greater than the width of the spore. The sample germination estimate was calculated by the germinated spores/400.

A randomized block design was used to take account the different inoculum used. To determine the effect of the temperature and wetness period on conidia and ascospore germination, third-degree polynomial models were fitted to the data. The optimal temperature for germination, at a given wetness period, was determined by numerical optimization routines using Brent's method. The data were analyzed using the R software (R Core Team 2020).

Effect of temperature on monocyclic components of Neonectria fruit rot in 'Gala' apples infected by *Neonectria ditissima* ascospores and conidia

The spore suspensions adjusted to 1 x 10^5 spores/ml used in this assay were the same as the ones used in the ascospore and conidia germination assay for both experiment repetitions.

Commercial 'Gala' fruit were disinfested by immersion in 70% alcohol for 1 minute, immersion in 1% sodium hypochlorite for 1 minute and rinsed three times in distilled water. Then, they were dried on paper towel and placed individually in disinfested transparent plastic pots. Apples were inoculated with 40µl of the suspension in 5-mm-deep wounds, made with autoclaved toothpicks on the equatorial region. The plastic pots were sprayed with sterilized distilled water to ensure enough moisture for spore germination on the fruit. A group of 4 fruit at each temperature was used as control, in which 40µl of sterile distilled water was pipetted at the wounds. The inoculated and control fruit in plastic pots were then placed in Conviron® chambers, with 12h photoperiod at 14, 22 and 26°C (first experiment repetition) and at 12, 16 and 20°C (second experiment repetition).

The experimental design was in 2x6 (ascospore or conidia x 6 temperatures) full factorial, with 20 fruit (replicates) per treatment for ascospore inoculation and 30 fruit (replicates) per treatment for conidia inoculation. Incubation period (day in which 50% of the inoculated fruits presented symptoms of the disease), latent period (day in which 50% of the inoculated fruits presented signs of the pathogen) and lesion diameter (measured with the aid of a digital caliper) (mm) were assessed every three days for 63 days. Contaminated fruit were removed throughout the experiment. Mean lesion growth rate (LGR) (mm/day) of each fruit with disease (fruits that did not show lesions were not considered) was calculated by the diameter values recorded on the last day of evaluation divided by the number of days of evaluation (n = 63). Then, the mean LGR of each temperature and inoculated spore was calculated. Sample estimation was performed for the determination of IP and LP and for the obtention of the LGR of NFR on fruit inoculated with ascospores and conidia.

To assess if the calyx opening would be a suitable entrance of *N. ditissima* in non-wounded fruit, inoculation of ascospores and conidia were carried out in this portion of apples. The experiment was conducted and evaluated in the same way as described previously for the wounded apples, but with 3 replicates per treatment.

Effect of temperature on monocyclic components of Neonectria fruit rot of apples and pears infected by *Neonectria ditissima* conidia

Ripe apple cv. Gala and pear cv. Rocha fruits were disinfested by immersion into a 70% alcohol solution for 1 min, 0.5 % hypochlorite solution for 2 min, rinse in sterile distilled water and air dried. Conidia production of two *N. ditissima* isolates, MdNd18-16 and MdNd18-20 from LEMID collection, was by transferring mycelial discs stored in penicillin flasks with water (Castellani 1964) to Petri dishes with SNA medium. The spore suspensions were made by the addition of 2 ml of sterile distilled water to the plates and gentle scraping with a Drigalski spatula. The spore suspension scraped from each isolate was transferred to one 5-ml penicillin flask, shaken in a vortex, and calibrated to 10^5 conidia/ml. Spore viability was assessed by pipetting 40 µl of each suspension in Petri dishes with water agar medium, spreading with a Drigalski spatula, incubating the plates for 24 hours at 25°C in BOD chambers and halting spore germination by the addition of Lactophenol Blue solution. The % germination was assessed as described in the previous experiments.

Inoculations were performed by placing 40 µl of the conidia suspensions (10^5 conidia/ml) on the equatorial portion of the fruits, on a 5-mm-deep wound made with a sterile needle. Immediately after the inoculation, fruit were kept under temperatures of 5, 10, 12, 16, 20, 26 and 35°C. The experimental design was a 2x7 (2 fruit species x 7 temperatures) full factorial, with 6 fruit per treatment. A group of 3 apples and 3 pears for each temperature was wounded and 40 µl of sterile distilled water was pipetted on the wound as the control treatment. Incubation period, latent period, and lesion diameter (mean of 2 perpendicular measurements with the aid of a digital caliper) (mm) were assessed daily for 13 days.

A parallel experiment was conducted by inoculation on the intact epidermis of the fruits (unwounded apples and pears). The experiment was conducted and evaluated in the same way as described previously for the wounded apples and pears.

Differences between the incubation periods of each isolate on ‘Gala’ apples and ‘Rocha’ pears were verified by survival analysis with Weibull distribution. To determine the effect of the temperature and fruit species on the development of the disease, the area under disease progress curve (AUDPC) was calculated from the lesion diameter data collected throughout the evaluation dates. AUDPC data was transformed to $\sqrt{AUDPC}$ for the analysis. The AUDPC data from the disease caused by both isolates at different temperatures was fitted to a 3rd degree polynomial model.

Analysis of variance (ANOVA) was performed on AUDPC because these data satisfied the normality and homogeneity of variance requirements of ANOVA. AUDPC means were compared using Tukey's honestly significant difference (HSD) test at P = 0.05. The data were analyzed using the R software (R Core Team 2020).

RESULTS

Effect of temperature and wetness period on conidia and ascospore germination of *Neonectria ditissima in vitro*

The germination data at different temperatures and wetness periods were fitted to third-degree polynomial models and are presented as contour lines (Figure 1). Germination of conidia and ascospore occurred in the range of 12–28°C. At 35°C no germination occurred, for both ascospores and conidia, regardless of the wetness period. With the same wetness period, conidia germination was always higher than ascospores at any temperature. Values above 60% germination were obtained for conidia at 26°C under wetness periods longer than 12 h. On the other hand, ascospores at this temperature reached 60% germination after only 24 hours or more of wetness. 12 hours of wetness period at 14°C provided 50% of conidia germination, but less than 32% of ascospore germination. This pattern is also present with longer wetness periods and higher temperatures. The maximum ascospore germination reached 81% at 22°C after 60 hours of wetness period, while conidia after 60 hours germinated more than 90% at all tested temperatures from 12 to 28°C (Figure 1).

The estimate optimal temperatures for germination varied across different wetness periods and can be observed by the dashed lines in Figure 1. It can be observed that the optimal temperature decreases when the wetness period increases for both spores. For ascospores, the optimal temperature ranged from 18-21°C, lower than those estimated for conidia, which ranged from 21 to 24°C (Figure 1).

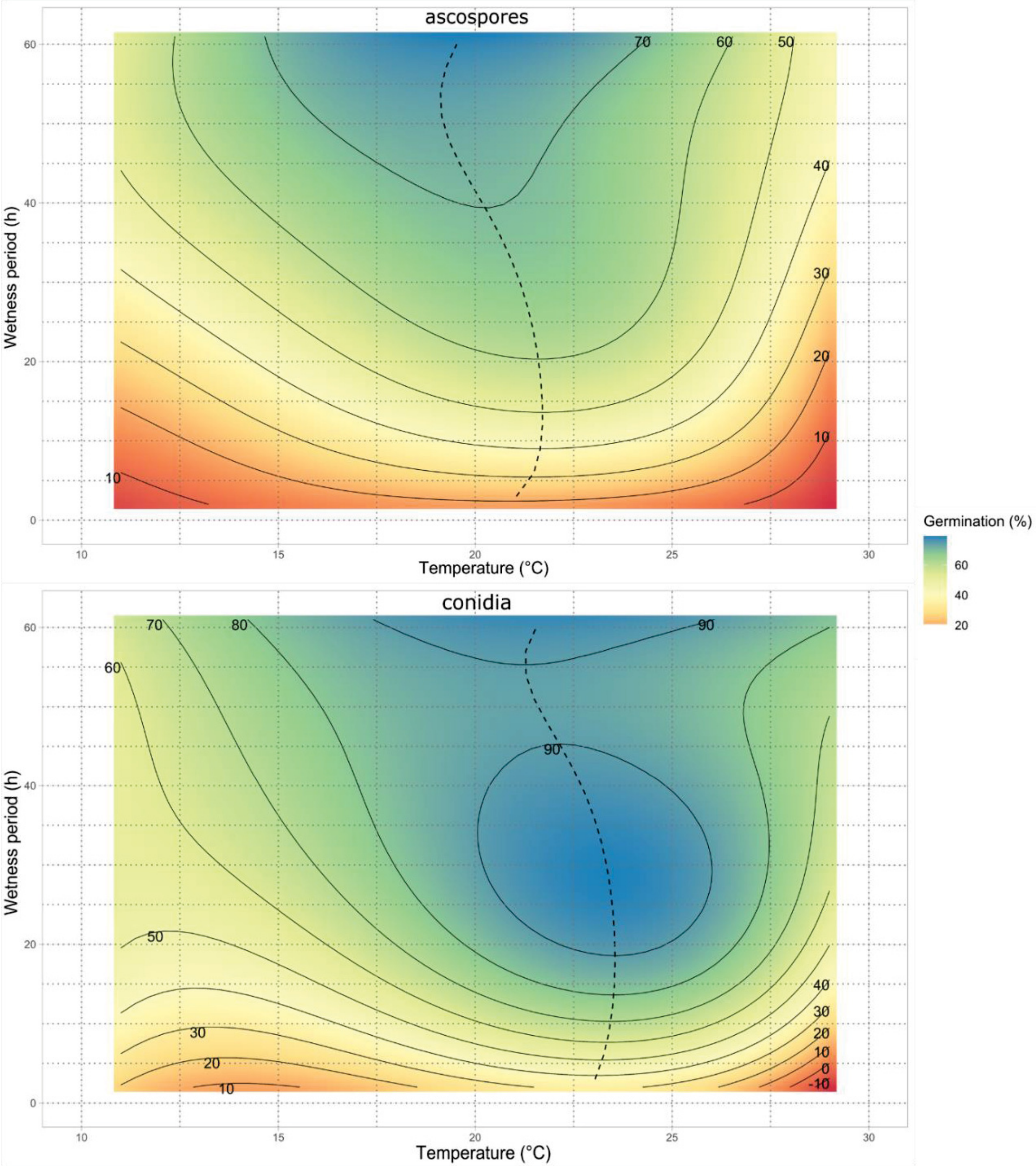

Figure 1. Sample statistics of germination percentages of *Neonectria ditissima* ascospores and conidia at different temperatures (°C) and wetness periods (hours). Dashed lines represent the optimal temperatures for germination on different incubation periods.

Effect of temperature on monocyclic components of Neonectria fruit rot in 'Gala' apples infected by *Neonectria ditissima* ascospores and conidia

Incubation period varied from 35 to 49 days after ascospore inoculation. The shortest incubation period for apples infected by ascospores was 35 days after inoculation at 16°C, followed by 39 days at 20°C, 42 days at 26°C and 49 days at 14°C. Incubation period was not observed at temperatures of 12 and 22°C. Latent period was not observed even at 63 days after inoculation of ascospores on apple fruit, regardless of the temperature (Table 1).

Regarding conidia inoculation, incubation period was between 14 to 42 days, being observed at all temperatures. The shortest incubation period was 14 days at 16°C, followed by 16 days at 22 and 26°C, 18 days at 20°C, 30 days at 12°C, and 42 days at 14°C. Latent period varied from 51 to 63 days after inoculation. The shortest latent period was observed at 51 days after inoculation in fruits incubated at 26°C, followed by 56 days at 16 and 22°C, and 63 days in fruits incubated at 20°C (Table 1).

Table 1. Sample estimates of incubation period - IP (days after inoculation), latent period - LP (days after inoculation) and incidence (%) of *Neonectria* fruit rot in ripe wounded 'Gala' fruit inoculated with ascospore and conidia suspensions.

Type of spore	Temperature (°C)	PI[1]	PL[2]	Incidence (%)[3]
Ascospore	12	56	no[4]	50.0
	14	49	no	62.5
	16	35	no	75.0
	20	39	no	71.4
	22	no	no	40.0
	26	42	no	83.33
Conidia	12	30	no	100.0
	14	42	no	58.8
	16	14	56	93.1
	20	18	63	100.0
	22	16	56	94.1
	26	16	51	100.0

[1] IP = incubation period (day after inoculation in which 50% of the inoculated fruit showed symptoms of the disease).

[2] LP = latent period (day after inoculation in which 50% of the inoculated fruit showed signs of the pathogen).

[3] Incidence (%) = percentage of fruit with lesions at the last day of evaluation (63 days after inoculation).

[4] Not observed.

Regarding the lesion growth rates of Neonectria fruit rot in apples inoculated with ascospores and conidia at different temperatures, at 12°C the mean lesion growth rate was lower for both spores inoculated. A growth pattern from 14°C to 22°C and a decrease in LGR at 26°C was observed for the fruit inoculated with ascospores. On the other hand, for fruit inoculated with conidia the LGR increasing pattern persists at 26°C. When comparing the same temperatures, mean LGR was always higher for fruits inoculated with conidia than with ascospores (Figure 2).

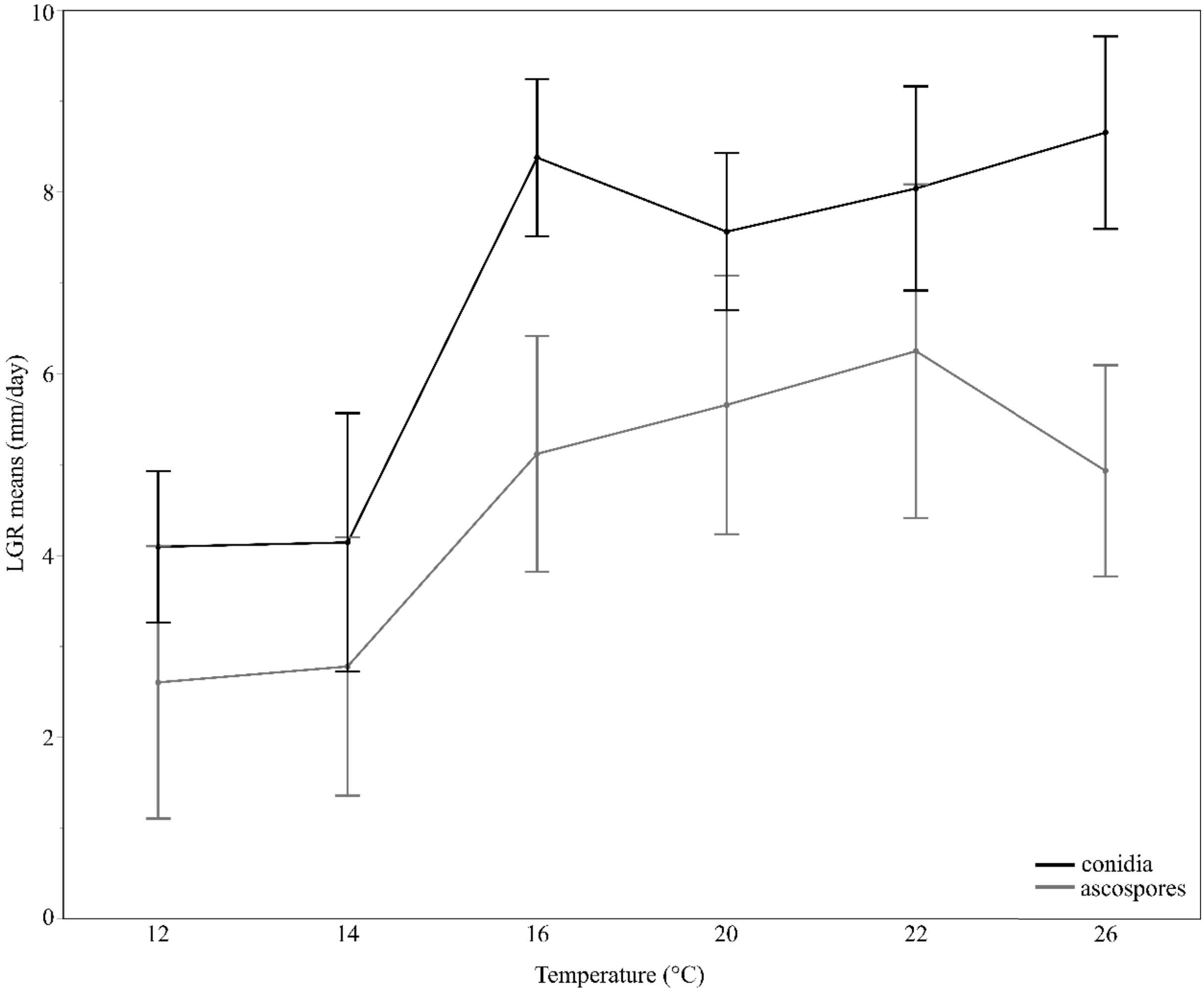

Figure 2. Sample estimates of mean lesion growth rate (LGR) (mm/day) of Neonectria fruit rot in ripe wounded 'Gala' apples inoculated with *Neonectria ditissima* ascospores and conidia and incubated at different temperatures. Bars represent the confidence intervals.

Effect of temperature on monocyclic components of Neonectria fruit rot of apples and pears infected by *Neonectria ditissima* conidia

Both MdNd18-16 and MdNd18-20 isolates presented germination above 90% after 24 hours of incubation at 25°C.

Weibull survival analysis was performed for time between inoculation and appearance of symptoms (incubation period). For both isolates (MdNd18-16 and MdNd18-20) there was a significant effect of temperature ($p < 0.05$). NFR in 'Rocha' pear maintained at 25°C had shorter incubation period when compared to the other evaluated temperatures, regardless of the isolate. NFR in pears incubated at temperatures of 10 and 12°C did not differ on incubation period, as the temperatures of 16 and 20°C (Figure 3a, b).

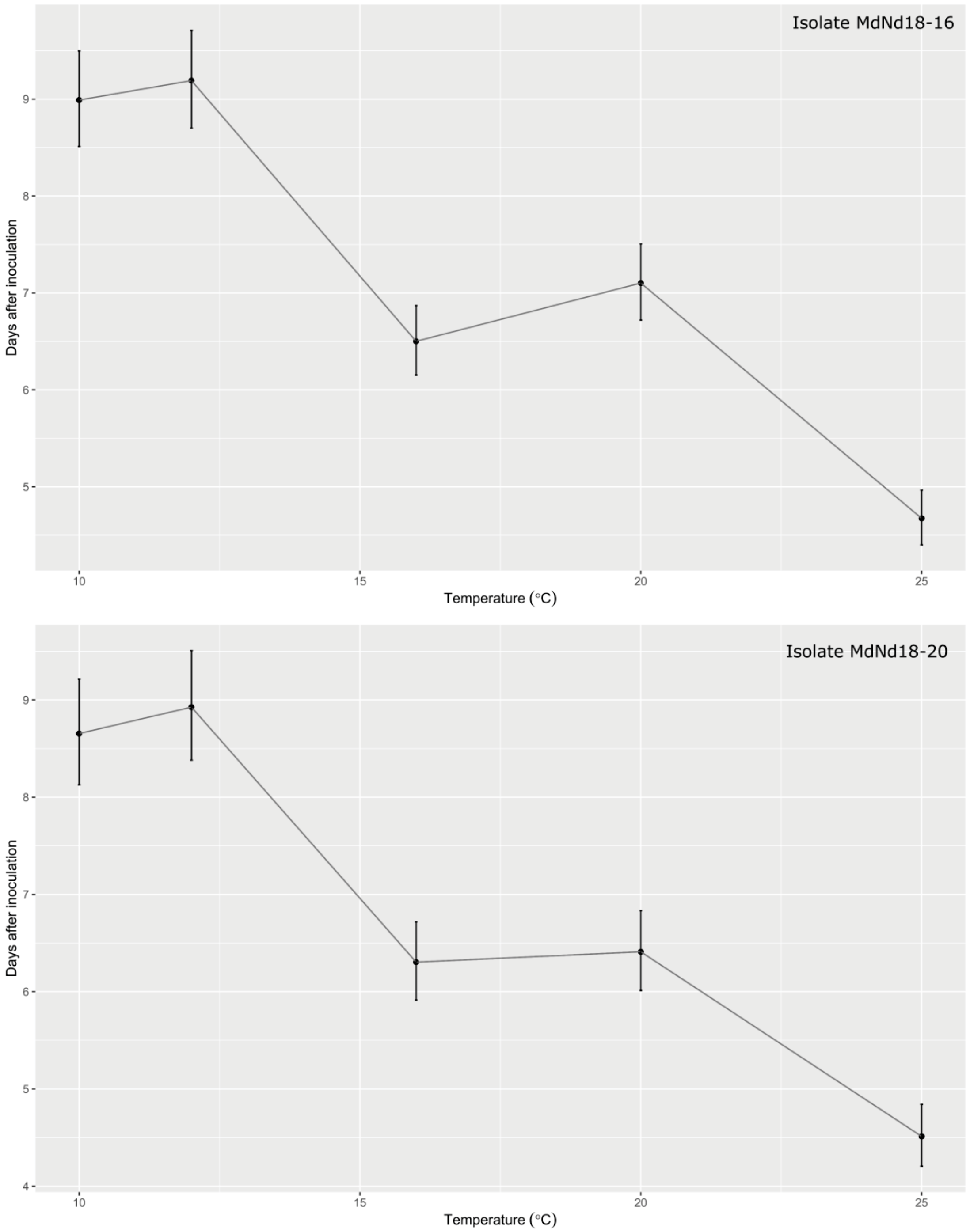

Figure 3. Estimated incubation periods (time between inoculation and appearance of symptoms) of Neonectria fruit rot on 'Rocha' pears inoculated with suspension adjusted to 1 x 10^5 conidia/ml of the isolates MdNd18-16 (a) and MdNd18-20 (b), incubated at 10, 12, 16, 20 and 25°C temperatures in Conviron chambers. Bars represent the lower and upper confidence intervals.

Disease was not observed in apple or pear fruits incubated at 5 and 35°C. At 20, 16, 12, and 10°C only pear fruit had developed symptoms until the 13th day after inoculation. Disease incidence was 100 % when 'Rocha' pear fruit were incubated at 10, 12, 16, 20 and 25°C for both isolates 13 days after inoculation. The inoculation of MdNd18-16 and MdNd18-20 isolates caused disease on 'Gala' apple fruit only at 25°C until the last day of evaluation (Table 2).

Table 2. Incidence (%) of Neonectria fruit rot on 'Rocha' pear and 'Gala' apple inoculated with isolates MdNd18-16 and MdNd18-20, incubated at different temperatures, evaluated daily until 13 days after inoculation.

temp[1]	Incidence (%)			
	Apple		Pear	
	MdNd18-16	MdNd18-20	MdNd18-16	MdNd18-20
5	0	0	0	0
10	0	0	100	100
12	0	0	100	100
16	0	0	100	100
20	0	0	100	100
25	100	100	100	100
35	0	0	0	0

[1] Incubation temperature of the fruits (°C)
[2] Days until the appearance of signs of the pathogen on >50% of the fruit repetitions.
[3] Not observed until the last day of evaluation (13 days after inoculation)

Latent period was only recorded in pear fruit incubated at 25°C, on the 8th day after inoculation of MdNd18-16 and after 9 days of MdNd18-20 inoculation. It was characterized by the formation of sparse white mycelium and sporodochia with conidia on the lesion surface (Figure 4) that spread through the entire surface of the lesion as it grew.

No disease was observed in the equatorial portion of the fruit that were inoculated with sterile distilled water.

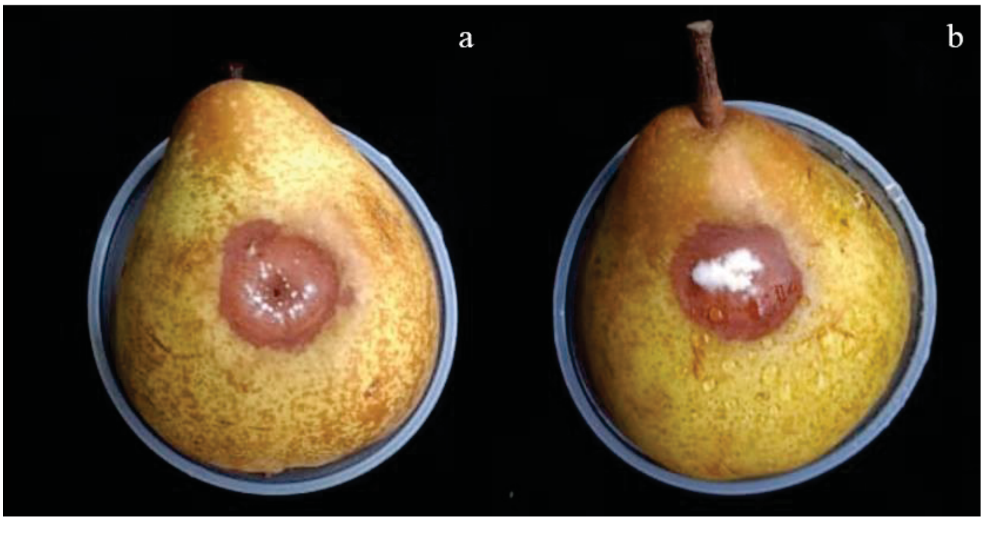

Figure 4. 'Rocha' pear inoculated with MdNd18-16 (a) and with MdNd18-20 (b) *Neonectria ditissima* isolates showing symptoms and signs of *Neonectria* fruit rot, with sparse white mycelium and sporodochia with conidia.

Regarding AUDPC of NFR in apples, as disease only appeared at apples incubated at 25°C until the last day of evaluation (13 days after inoculation), only the effect of isolate was compared. The transformed AUDPC data differed significantly for the isolates, being significantly higher for the isolate MdNd18-16 isolate ($p < 0.05$) (Figure 5).

Regarding AUDPC of NFR in pears, the transformed data at different temperatures was fitted to a grade 3 polynomial model, and the arbitrary weight of 0.1 was given to the data from 5 and 35°C which had variance 0 (no fruit presented lesion) not to eliminate extremity temperatures because, despite the 0 variance, they are useful to adjust data to the regression curve. Comparison of means by ANOVA did not find evidence of differences of the interaction temperature x isolate ($p > 0.05$); in that way, we considered the effects additive. The differences between the isolates were also not significant, but AUDPC was significant at different temperatures ($p < 0.05$).

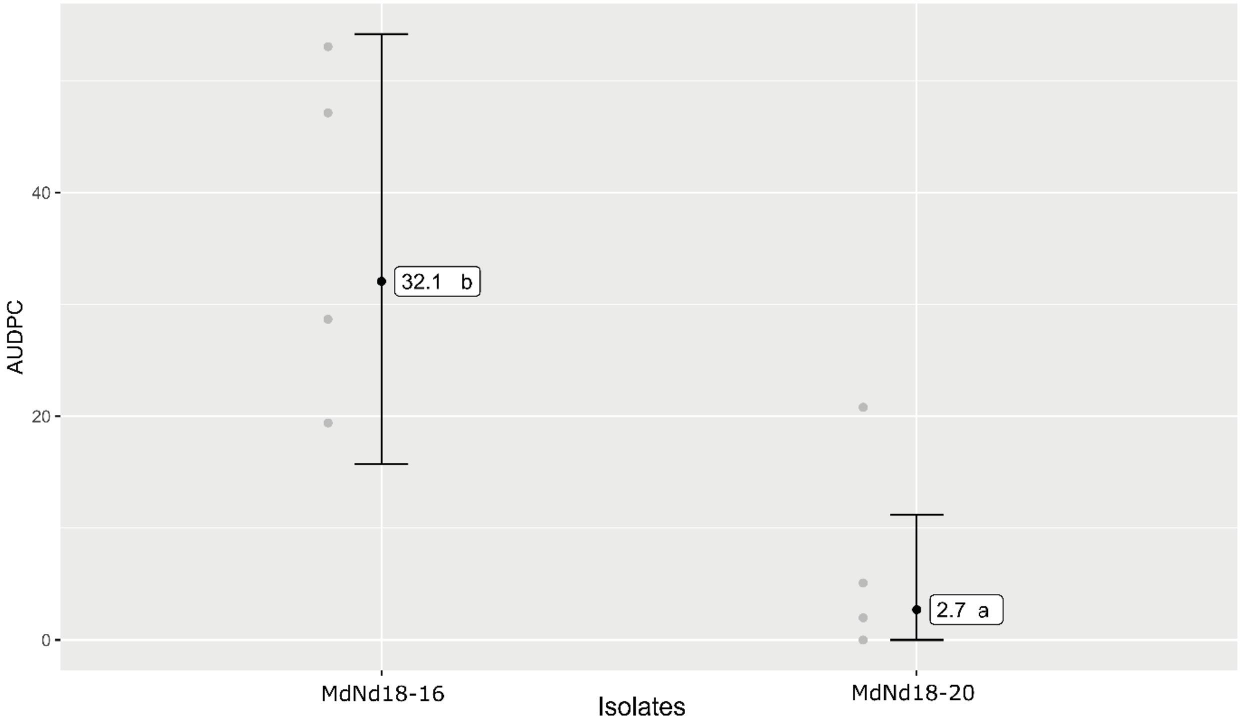

Figure 5. Average of area under disease progress curves (AUDPC) of Neonectria fruit rot lesions caused by two isolates of *Neonectria ditissima*, MdNd18-16 and MdNd18-20, in detached 'Gala' fruits incubated for 13 days at 25°C. The bars represent the confidence interval (95%). Means followed by the same letters do not differ at 5% significance.

The equation of the curves that describe AUDPC of Neonectria fruit rot in pears at different temperatures is:

$$f(x) = z - 0.18t + 0.069t^2 - 0.0018t^3$$

Where: z = constant value (1.51 for isolate MdNd18-16 and 1.608 for isolate MdNd18-20); and t is the temperature (°C).

The curves that describe AUDPC of Neonectria fruit rot disease from artificial conidia inoculation of isolates MdNd18-16 (orange curve) and MdNd18-20 (blue curve) in pears at different temperatures are shown in Figure 6. Because the effects of the factors are additive, the optimal temperature estimated was the same for both isolates, being of 23.65°C (Figure 6).

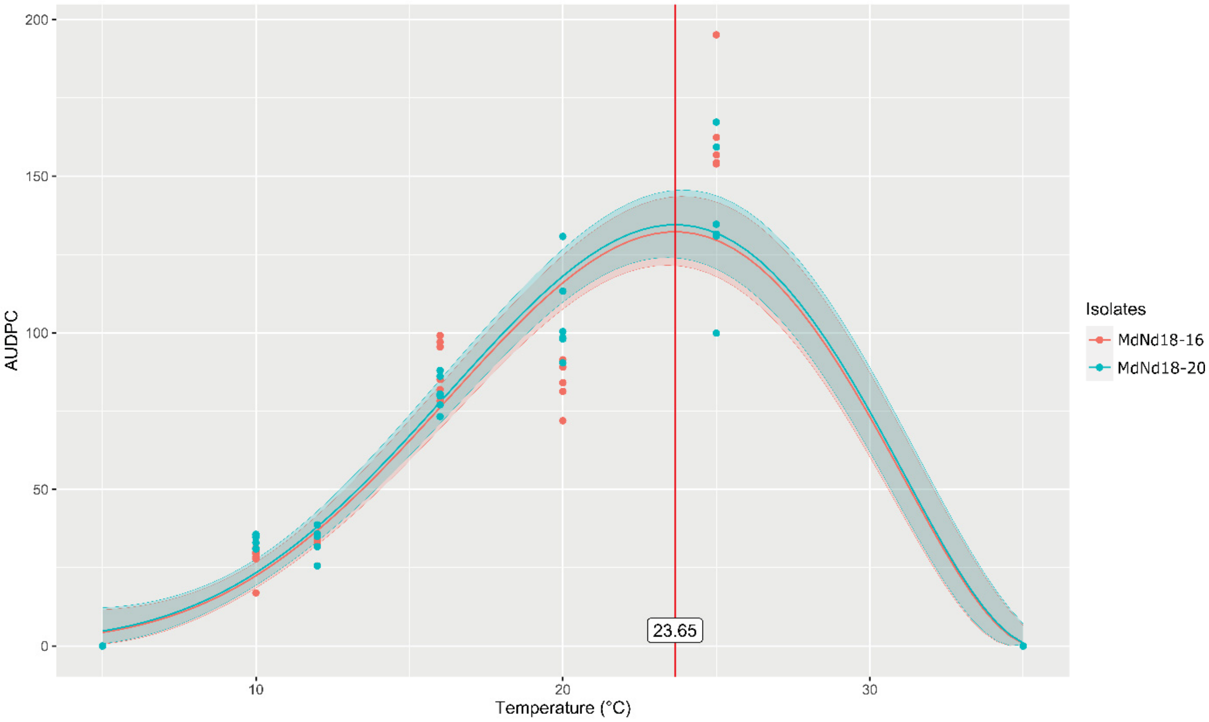

Figure 6. Area under disease progress curves (AUDPC) of the lesion diameters of Neonectria fruit rot in 'Rocha' pears inoculated with conidia suspensions of the isolates MdNd18-16 and MdNd18-20 at different temperatures (5-35°C) estimated by grade 3 polynomial model.

Discussion

The present study elucidated physiological traits of the sexual spores of *Neonectria ditissima*, as well as monocyclic components of Neonectria fruit rot (NFR) in apple fruit infected by ascospores and pear fruit infected by conidia. The information obtained helped to better understand the role of ascospores in fruit infections and confirmed the higher susceptibility of pear fruit in comparison to apple fruit to NFR.

In the present study, the wetness period has shown to influence the germination of *N. ditissima* spores, in agreement with a previous study with germination of different isolates of *Neonectria ditissima in vitro*. In a previous *in vitro* experiment, conidial germination of *N. ditissima* in WA medium (1.5%) with dextrose (1.5%) was low at 6°C and 32°C (Latorre et al. 2002). The lowest temperature tested in our study was 12°C and the conidial germination reached up to 63% with 60 hours of wetness periods; the highest temperature tested was 35°C and no conidia germinated regardless of the wetness period. Optimum germination temperature of the previous study was estimated between 20°C and 25°C, with 2 to 24 hours of wetness period, and at this temperature germination was always higher than 80% within 24 h of incubation, which is consistent with the present study. Ascospore germination was low at 5°C but rapidly increased until temperatures reached 20°C. Germination rate, calculated as germination frequency over time at 20°C, was 2.3 times faster for ascospores than conidia in that study, suggesting that European Canker could be more aggressive in areas where abundant ascospores are produced (Latorre et al. 2002), which could be extended to Neonectria fruit rot. However, in the present *in vitro* study with only WA medium we found a lower germination % for ascospore when compared to conidia at any temperature after the same wetness period. The results are consistent with the *ex vivo* experiment of ascospores and conidia inoculation in detached wounded apple fruit, where lesion growth rate and disease incidence were lower for fruit inoculated with ascospores at all temperatures than disease caused by conidia inoculation. As the ascospore inoculum was obtained from field cankers in Latorre et al. 2002 and at the present study, we could infer that the ascospore germination was higher in the previous study because of the addition of dextrose which can be an additional source of nutrient for ascospore germination and would suggest a higher nutritional demand of ascospores when compared to conidia. However, as the inoculated fruit from our study were wounded, they should have been the nutrient source for germination of ascospores in our assay, which may rule out this hypothesis. An experiment with and without additional nutrient in medium and visualization of germination in fruit under scanning electron microscopy is recommended.

Regarding the method of obtention of spores, conidia was produced in laboratory and ascospores were obtained from apple twigs with perithecia, very similarly to a previous study (Latorre et al. 2002). To avoid discrepancies on the comparison of conidia and ascospore germination, conidia were produced in SNA medium, which, differently from PDA and other nutritive medium, produces almost only macroconidia (Leslie and Summerell 2006). Since this is the predominant spore found in field and the suspension was calibrated to 10^5 conidia/ml counting only macroconidia, we assured germination rates of the *in vitro* assay are highly reliable and similar to field conditions. In the present work we used freshly collected ascospores and conidia; however, conidia produced in media and on cankers could be frozen at -20°C for 3 days without having an effect on disease incidence or lesion size after inoculation on apple twigs (Scheper et al. 2015), but it still unknown if frozen ascospore suspensions would also present this behavior and it should be tested for facilitating further experiments using ascospores.

This is the first study with *N. ditissima* ascospore inoculation on fruit. Ascospores were able to cause disease in ripe wounded apple fruit, even though the lower lesion growth rates, lower incidences, and longer wetness periods than the conidia infections. This suggests an ascospore disadvantage in the colonization process in fruits when compared to conidia. The results are in agreement with a previous study, which reported that even the most susceptible apple cultivar inoculated with *N. ditissima* ascospores under optimal conditions had low infection rates on twigs (Dubin and English 1974). The lower germination rates of ascospores at any temperature tested is the first thought on the slower monocycle development of NFR caused by ascospores than by conidia. However, in apple fruits, artificial wounds inoculated with 10–100 conidia gave rise to limited rot lesions which became arrested in their development, whereas higher inoculum doses gave rise to spreading fruit rots (Swinburne 1971). In that sense, 40 microliters inoculated of a suspension at 10^5 spore/ml would provide about 4000 spores, so even if the viability of ascospores is lower than the conidia, it is not likely to have interfered with the differences in NFR monocyclic components on wounded apples in the present study.

Ascospore production in perithecia peaks in spring but continues throughout summer until fall (Dubin and English 1974), therefore the sexual spores are available in the maturation period of apples. This combined to incidences of up to 83% obtained in the present study shows that if ascospore inoculum is not removed from the orchard and several hours under high wetness periods occur during fruit maturation period, especially with constant rain that induces ascospore discharge by the perithecia (Dubin and English 1974), producers may have postharvest problems with Neonectria fruit rot. Flower and immature fruit infections should be assessed in future studies.

There is some information that point to conidia rather than ascospores as the main inoculum for pre- and post-harvest Neonectria fruit rot (Weber and Dralle 2013; Weber 2014). However, no previous studies with ascospore as source of inoculum for fruit infections are available. As this sexual spore can be explosively discharged by perithecia (Weber 2014), can be wind transported to long distances, and is commonly found in Brazilian orchards, this is a relevant information that could aid on disease management. A factor that can contribute with the rate of field infections in woody tissues and fruit is the management of the orchard, as older cankers left in trees are known to most likely produce perithecia instead of sporodochia (Gómez-Cortecero et al. 2016), so if the cankers are not being constantly removed the incidence of old cankers, and consequently of perithecia production and ascospore infections, are more likely.

Summer dispersal of both conidia and ascospores could be linked to NFR field infections in the calyx-end of the apple fruit (Dubin and English 1974). In the present study with artificial inoculation in this part of the fruit, no disease was observed even at 63 days after the inoculation. Direct penetration without any natural opening or wound does not occur (Zeller 1926; Dubin and English 1974; Weber 2014). In a previous study with artificial inoculation, large differences in the disease incidence was found, with some years presenting high incidence of NFR in the calyx-end of apples and no fruit with symptom in other evaluated year with the same methodology of inoculation (Xu and Robinson 2010). The authors attributed this oscillation in NFR incidence to possible histological differences in fruit due to differences in the climatic conditions, rendering fruit more or less susceptible to colonization by *N. ditissima* (Xu and Robinson 2010). The calyx-end of mature ‘Gala’ apples do not seem like a suitable entrance for this pathogen, but histological and physiological experiments are necessary to better understand pre-infection process.

Temperatures of 26°C caused a reduction in lesion growth rate when compared to 22°C in apple fruits inoculated with ascospore suspensions, which was not observed for conidia inoculation, that caused a higher lesion growth rate than all the other tested temperatures. This is consistent and could be explained by the germination experiment from this study, that showed a lower germination of ascospores at 26°C than in lower temperatures from 16 - 22°C after 12 hours wetness periods, while for conidia the germination at 26°C reached more than 80% with 12 hours or more of wetness period.

A previous study observed symptoms in wounded apple fruit inoculated with *N. ditissima* conidia suspension and stored at approximately 15°C only after 2 months (Brown and

Swinburne 1971a), which is consistent with the results obtained in the present work, where incubation periods of fruit incubated at some temperatures such as 12 and 14°C also reached almost 2 months. Apple fruit have a long shelf life, so the evaluation of the fruits for such a long time (63 days in our study) is not only possible but may represent what will happen with infected apples during commercialization.

Neonectria fruit rot monocyclic components evaluated in the present study point to a much higher susceptibility of pear than apple fruit. This is the first experiment regarding NFR in pear fruit, but the results are in agreement with a previous report of a much greater European Canker disease severity in pear orchards than in apples (Zeller 1926). Both isolates inoculated were pathogenic to apple and pear fruit. However, for apples the AUDPC of NFR disease caused by different isolates (MdNd18-16 and MdNd18-20) were statistically different at the same temperature, while for pears AUDPC from different isolates at the same temperature were very similar. Previous studies reported differences in monocyclic components between *N. ditissima* isolates inoculated on detached apples (Gelain et al. 2020), which indicates that it may be related to a host-isolate specificity as there is evidence for *Colletotrichum nymphaeae* in unwounded 'Gala' apple fruit and detached leaves (Hamada et al. 2020). On the other hand, the very similar disease development regarding colonization of pear fruit indicates the lack of this specificity in this host.

Even temperatures of 10°C were enough for disease occurrence in pear, which is consistent with > 12% *in vitro* germination of both isolates provided with 24 hours of incubation period. As wounded fruit serve as source of nutrients and humidity, the germination rates could have been even higher, and, although the incubation period was longer for this temperature, the disease still occurs.

Temperatures of 5 and 35°C were limiting for *N. ditissima* colonization, as we did not see any symptom development at these temperatures for neither isolate nor fruit species. This is explained by the lack of conidia germination, as we could see in the *in vitro* assessments. While quiescent infections from commercial apple fruit stored in temperatures even below 5°C have been confirmed (Gelain et al. 2021), we do not know if it is possible for the pathogen to remain quiescent on temperatures of about 35°C in the fruit and for how long, or if it is lethal to the pathogen's conidia. This can be directly related to hot water treatments on fruit, that if tested and proved to be efficient could be used not only to prevent the development of the disease and reduce losses due to fruit rot, but also as a measure to avoid dissemination of the quiescent pathogen. This also can be a good alternative for a clean treatment and is a suitable option for organic apple and pear production.

Colonization of *N. ditissima* in pear fruit is favored by temperatures of 25°C, indicated by the higher AUDPC and shortest incubation period presented in this work. The slow development of *Neonectria* fruit rot on ripe inoculated apples, even when incubated at temperatures of 16 and 20°C, contrasts with the fast outbreak of symptoms and signs of the quiescent Neonectria fruit rot disease from a previous study (Gelain et al. 2021). A possible explanation for this behaviour is the long exposition to low temperatures in cold chamber storage at which apple fruit from the previous experiment, that could have worked as a variation of the Overnight Freezing Incubation Technique (ONFIT), partially degrading superficial tissues of the fruit, and facilitating the development of symptoms and signs of the disease once the fruit are transferred to suitable environmental conditions.

Differently from the inoculation study with ascospores and conidia in apple fruit, this experiment had to be ended at 13 days after inoculation since pear fruit have higher perishability and the fruit incubated at ≥25°C started to deteriorate after this period. In the other experiment from this study, apple fruit incubated at temperatures of 16, 22 and 26°C had the lower incubation periods of NFR (14, 16 and 16 days, respectively), which is not consistent with this experiment in which only apples incubated at 25°C presented symptoms in the 13th day after inoculation. Incubation periods for apple fruit also presented high variation in a previous study, from 15 to 41 days (Gelain et al. 2020) even coming from the same batch as in the present study (same cultivar, same orchard and collected at the same time) and this phenomenon is not well understood.

The most important method to avoid problems with the diseases caused by *Neonectria ditissima* in apple, pear or other host trees and fruit is exclusion, which prevent the introduction of the pathogen into a country, region, or orchard. Although conidia of *N. ditissima* can travel only limited distances, the aid of some other agent such as humans, that can carry spores on clothing, pruning shears, transport boxes or machinery, and infected seedlings or fruit with quiescent infections, can difficult disease containment. Ascospore infection was not evaluated in pear fruit; however, the information on monocyclic components of NFR caused by ascospores on pears would be of great practical interest because airborne ascospores can spread infections to orchards without the disease (Swinburne 1971; Weber and Børve 2021).

The wide range of favorable conditions for the development of the disease makes the use of predictive models of disease difficult because favorable conditions will always occur in the orchards, especially regarding NFR in apple fruit, since in the major apple-producing regions in Brazil, warm and rainy summers predominate. Cultural methods aimed at reducing the initial inoculum, removing diseased tissues from the orchards, should be chosen to reduce the use of fungicides for fruit protection.

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Supplementary 1. Group of red *Neonectria ditissima* perithecia on 'Gala' tree twig from Vacaria, Rio Grande do Sul (a); single perithecia after incubation in humid chamber showing detail of the white cirrhus indicating perithecia maturation (b), and ascospores germinating on water agar medium (c).

5 CHAPTER 3: IDENTIFICATION AND FUNGICIDE SENSITIVITY OF *Colletotrichum* spp. FROM APPLE FLOWERS AND FRUITLETS IN BRAZIL[3]

Abstract

Glomerella leaf spot and bitter rot, caused by *Colletotrichum* spp., are major diseases in southern Brazil, the main apple producing region of the country. The objectives of this study were to identify *Colletotrichum* species from 'Eva' apple flowers and fruitlets in Paraná state orchards, evaluate their sensitivity to cyprodinil, tebuconazole, iprodione, and fluazinam, and determine the baseline sensitivity of these isolates to benzovindiflupyr and natamycin. For that purpose, monosporic isolates were obtained with modified Overnight Freezing Incubation Technique (ONFIT) from 320 flowers and 100 fruitlets sampled from 10 trees from each orchard located in Lapa, Palmeira, and Porto Amazonas municipalities. Phylogenetic trees were built from *GAPDH* and *TUB2* sequences to identify isolates to the species level. After that, the isolates were tested for their sensitivity to fungicides (EC_{50}), *in vitro*, with mycelial growth inhibition assays. *Colletotrichum* spp. were recovered from flowers and fruitlets from the three orchards evaluated. *C. acutatum* species complex was the predominant. *C. melonis* and *C. nymphaeae* presented differences in sensitivity to fluazinam and tebuconazole, but not to the other fungicides. The lowest EC_{50} values of *Colletotrichum* spp. were observed for cyprodinil (mean $EC_{50} < 0.02$) and benzovindiflupyr (mean $EC_{50} < 0.05$); intermediate for fluazinam (mean $EC_{50} < 0.33$) and tebuconazole (mean $EC_{50} < 0.14$); and the highest for natamycin (mean $EC_{50} < 5.56$) and iprodione (mean $EC_{50} > 12$). Cyprodinil and fluazinam are potential fungicides to be incorporated in Glomerella leaf spot and bitter rot management programs since they are already registered for apple culture. Tebuconazole is one of the few products already registered for *Colletotrichum* spp. control in apples and can still be used in the management since no lack of sensitivity was found. Benzovindiflupyr and natamycin registration for *Colletotrichum* spp. in apples should be considered, the first because of its *in vitro* efficacy and the second for being a good post-harvest option.

Keywords: baseline sensitivity; benzovindiflupyr; bitter rot; chemical control; Glomerella leaf spot; natamycin.

Introduction

Glomerella leaf spot and bitter rot, caused by species within the *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* species complexes, are important diseases of

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apple worldwide (Dowling et al. 2020). Symptoms of Glomerella leaf spot include severe defoliation that could diminish productivity in subsequent seasons, while bitter rot is the most common symptom of fruit infections (Becker et al. 2000; Moreira et al. 2021). Glomerella leaf spot is associated with severe necrotic, irregular sized, brown-reddish lesions on leaves, which can exceed 70% of leaf area (Moreira, et al., 2019) . Symptoms on apple fruit initially appear as small circular lesions that are light to dark brown in color. They evolve into circular, sunken, and light brown to brown lesions formed on the fruit surface (Sutton 1990). *Colletotrichum* spp. are not known to cause blossom blight of apple (Peres et al. 2005), which contrasts with other crops such as almonds (Förster and Adaskaveg 1999), strawberry (Howard 1992), and citrus (Fagan 1979). However, *Colletotrichum nymphaeae* was isolated from asymptomatic apple flowers and fruitlets in the 2010-2011 season in one orchard in Paraná state, indicating that the fungus is remaining quiescent in these organs until favorable conditions favor their development and reproduction (Hamada et al. 2020). The same isolates were pathogenic to ripe fruit and leaves (Hamada et al. 2020), suggesting that the flowers and fruitlets may serve as sources of inoculum.

Brazil is consistently among the top 15 apple producing countries in the world (FAO, 2020). In southern Brazil, where most of the country's apples are grown, Glomerella leaf spot and bitter rot are particularly problematic because climate conditions during fruit maturation are conducive to disease development. The highly susceptible cultivar 'Gala' has been grown almost exclusively for many years, making both Glomerella leaf spot and bitter rot the most important apple diseases in this region (Moreira et al. 2019c). Glomerella leaf spot has been so problematic in Paraná that, according to the local growers, the Gala cultivar is being replaced by 'Eva', a cultivar with good agronomic characteristics and lower susceptibility to Glomerella leaf spot (Hauagge and Tsuneta 1999). Unfortunately, this cultivar is susceptible to bitter rot.

Colletotrichum species vary in aggressiveness, host preference, dependence on environmental factors for disease development, and fungicide sensitivity, which makes the diseases even more difficult to manage because many species can have different fungicide resistance profiles (Dowling et al. 2020). Several *Colletotrichum* species were reported in the southern states of Brazil. In one study, multiple species of the *C. acutatum* species complex were found in 'Gala' leaf and bud tissue in Paraná between 2010 and 2011, including *C. nymphaeae* (predominant species), *C. melonis*, *C. paranaense* and *C. limetticola* (Moreira et al. 2019c). Isolates collected during 2010-2011 growing seasons from flowers and leaves of cultivar 'Eva' also belonged to the *C. acutatum* species complex (Moreira et al. 2019c). Studies about incidence and prevalence of *Colletotrichum* species with more isolates from more recent growing seasons from 'Eva' apples could be useful to improve disease management programs to this cultivar.

Fungicide applications are the main method for apple disease control. Single-site fungicides registered in Brazil for apple disease management are from seven FRAC codes: FRAC 1 (carbendazim, thiophanate-methyl), FRAC 2 (procymidone, iprodione), FRAC 3 (difenoconazole, flutriafol, cyproconazole, imazalil, imibenconazole, tebuconazole, tetraconazole), FRAC 7 (fluxapyroxad), FRAC 9 (cyprodinil, pyrimethanil), FRAC 11 (pyraclostrobin, trifloxystrobin, kresoxim-methyl), and FRAC 29 (fluazinam). Some of these fungicides are labelled for use against Glomerella leaf spot and bitter rot (i.e. carbendazim, thiophanate-methyl, pyraclostrobin, fluxapyroxad, and tebuconazole) but for others the activity against *Colletotrichum* species affecting Brazilian apples is not known. The main multi-site fungicides registered and used to control Glomerella leaf spot and bitter rot are mancozeb and captan (Ministério da Agricultura, Pecuária e Abastecimento, 2020). Brazilians apple growers rely mainly on mancozeb to control the diseases caused by *Colletotrichum* spp., but this fungicide was recently classified by the Risk Assessment Committee (RAC) of the European Chemical Agency (ECHA) as "toxic for reproduction 1B" (R1B) substance, for causing severe brain malformations due to ethylene thiourea, one of its metabolites (ECHA, 2019). This resulted in mancozeb being banned from European Union member states (European Commission for Health and Food Safety, 2020). This could soon impact import guidelines for Brazilian growers and lead to a ban for mancozeb for export fruit when the existing Maximum Residue Levels (MRLs) is reviewed (EFSA, 2020). Therefore, exploring all Glomerella leaf spot and bitter rot management options based on already available fungicides is a priority and may aid in management programs.

Chemical control of *Colletotrichum* spp. is further complicated due to reduction or even loss of sensitivity to fungicides. *Colletotrichum* spp. isolates collected from apple orchards in Brazil in the 2010-2011 growing season were resistant to a quinone outside inhibitor (QoI) fungicide (Moreira et al. 2019b) and isolates from the *C. gloeosporioides* species complex collected before 2009 were phenotypically classified as resistant to a methyl benzimidazole carbamate (MBC) fungicide (Hamada et al. 2009). To our knowledge no other studies are available assessing efficacy of fungicides against *Colletotrichum* spp. of Brazilian apple orchards.

Fungicides from other FRAC groups hold promise for management of *Colletotrichum* species. Benzovindiflupyr, for example, is a newly registered succinate dehydrogenase inhibitor – SDHI (FRAC 7) in the USA with *in vitro* inhibitory activity against *C. gloeosporioides* and *C. acutatum* of apple and peach fruit (Ishii et al. 2016). Natamycin (FRAC 48) is an antifungal antibiotic produced by *Streptomyces natalensis* or *S. chattanoogensis.* It binds to ergosterol, an important component of the cell membrane and growing tips of germinating spores and vegetative hyphae (Pedersen 1992; Van Leeuwen et al. 2009, 2010). It has known activity against *C. gloeosporioides* of mango (Liu et al. 2019) and *C. acutatum* of strawberry (Haack et al. 2018) and its more favorable mammalian toxicity profile compared to many other fungicides (Arima et al. 2014) make it an attractive postharvest disease management possibility. However, the sensitivity of Brazilian *Colletotrichum* spp. isolates to benzovindiflupyr or natamycin is unknown.

The objectives of this study were to (i) identify *Colletotrichum* species from 'Eva' apple flowers and fruitlets in Paraná state orchards; (ii) evaluate their sensitivity to cyprodinil, tebuconazole, iprodione, and fluazinam; and (iii) determine the baseline sensitivity of these isolates to benzovindiflupyr and natamycin.

Material and Methods

Sampling and fungal isolation

The isolates were collected from three commercial areas of apple production of 'Eva' cultivar located in the municipalities of Lapa, Porto Amazonas, and Palmeira, in the state of Paraná, southern Brazil. The collections were carried out in the 2017/2018 season. The plants of all three orchards were conducted in central leader, on Maruba rootstock with M9 (Porto Amazonas and Palmeira) or M7 (Lapa) filter. Porto Amazonas apple trees were 4 years old, Palmeira 7 and Lapa 10 years old. The orchards were treated with conventional pesticides during the growing seasons. Lapa orchard received 33 fungicide applications from dormancy until harvest, Porto Amazonas 26 and Palmeira 23. In all three regions the most used fungicide was mancozeb, reaching 18 applications in Lapa. Captan was applied 4 times in Porto Amazonas and Palmeira and was not applied in Lapa. DMI fungicides were used in all three regions, in a maximum of 6 applications. QoI fungicides were used only in the Lapa orchard, 8 times during one productive cycle. The MBC thiophanate-methyl was used 6 times in Porto Amazonas and Palmeira and applied only once in Lapa (Supplementary 1).

Open flowers were sampled at full bloom in 2017 (20-22/08). During bloom 32 asymptomatic flowers of each of ten randomly picked apple trees (experimental trees) of each orchard were collected. The flowers were placed in transparent plastic boxes (11 x 11 x 3.5 cm), 16 flowers per box, on sterile moist filter paper. Sporulation of latent *Colletotrichum* infections in flowers was induced by placing the boxes with the flowers in a freezer at -16°C for one hr and subsequently in a room at 25°C in the dark with 100 % humidity for another 10 days (Hamada et al. 2020; Mertely and Legard 2004). The flowers were evaluated daily for signs of the pathogen. When characteristic orange masses were observed on the flowers, direct isolation was performed by removing the conidia masses from the flower with the aid of a sterilized blade under a stereoscopic microscope (20x) (Olympus, GSWH10X/22) and placing them on petri dishes containing potato dextrose agar (PDA) medium.

Fruitlets with mean diameter of 30 mm were collected approximately three weeks after the beginning of fruitification (14-15/09) at thinning time. A total of 10 fruitlets were collected from each experimental tree previously used for flower collection. The fruitlets were taken to the laboratory and subjected to overnight freezing incubation technique (ONFIT) (Luo and Michailides 2001). Surface disinfestation was carried out by immersing and stirring the fruit for 5 min in a solution of distilled water (2 L) with sodium hypochlorite 0.525% (32 mL), ethanol 92.8% (32 mL) and Tween 80 (1 drop). The fruitlets were then washed in sterilized distilled water and dried on sterilized paper and placed in 50 mL plastic cups with filter paper moistened with 1 mL sterilized distilled water. They were maintained for 15 hrs in a freezer (-16°C) and incubated at 25°C with 100% humidity. The evaluation was performed daily until the 9th day after incubation, and isolation under a stereoscopic microscope was performed when signs of *Colletotrichum* spp. were observed.

To generate single spore isolates from these fungal cultures, *Colletotrichum* isolates growing on PDA were incubated at 25ºC in the dark for 10 days. Conidia were scraped off the plate, suspended in 1 ml of sterile distilled water, and 40 µL of conidia suspension adjusted to 10^4 was spread on 1.5 % water agar (WA) plates. The WA plates were incubated at 25°C for 6 hrs, and one germinated conidium was transferred onto PDA and incubated at 25°C in the dark. The monosporic isolates were preserved on filter paper in Eppendorf tubes with silica gel (Fong et al. 2000).

Molecular identification of Colletotrichum *species*

For identification of the *Colletotrichum* species, 88 isolates were selected, being at least 28 isolates per orchard sampled from different host tissues. DNA was extracted according to Chi et al. (2009). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-tubulin (*TUB2*) sequencing was performed to identify isolates to the species level (Munir et al. 2016;

Vieira et al. 2020). Polymerase chain reaction (PCR) primer pairs GDF1 (GCCGTCAACGACCCCTTCATTGA) and GDR2 (GGGTGGAGTCGTACTTGAGCATGT) were used for *GAPDH* amplification (Templeton et al. 1992); T1 (AACATGCGTGAGATTGTAAGT) and T2 (TAGTGACCCTTGGCCCAGTTG) for *TUB2* amplification (O'Donnell and Cigelnik 1997).

PCR products were purified and sequenced by Genewiz (South Plainfield, New Jersey). The generated forward and reverse sequences were assembled using the BioEdit software (Hall 1999). For phylogenetic analyses, the DNA sequences of species belonging to the *Colletotrichum acutatum* and *C. gloeosporioides* complexes and the outgroups *C. orchidophilum* (CBS632.80) and *Glomerella cingulata* f.sp. *camelliae* (ICMP10646) were added to the dataset and aligned using the Mega software v.7.0.26 (Kumar et al. 2016). The multigenic sequences were assembled using the software SequenceMatrix.

Phylogenetic analyses based on Bayesian inference were performed for the concatenated *GAPDH* + *TUB2* alignments. Evolution model used for both genes was GTR I + G, estimated in JModeltest v.2.0.1 (Darriba et al. 2012) using the Akaike Information Criterion (AIC). The partitioned analysis was performed twice using MrBayes v.3.2 (Ronquist et al. 2012) and the Markov Chain Monte Carlo (MCMC) algorithm to generate phylogenetic trees with Bayesian posterior probabilities (BPP). Four MCMC chains were run simultaneously for random trees for 1×10^6 generations. Samples were taken every 1000 generations. The first 25% of the trees were discarded as the burn-in phase of each analysis, and posterior probabilities were determined from the remaining trees. The relationship between *Colletotrichum* species and host tissue was tested by Contingency Analysis in JMP® Pro software Version 14 (SAS Institute Inc., Cary, NC).

Fungicide sensitivity assays

Of the 88 isolates identified to the species level, 30 *C. nymphaeae* and 30 *C. melonis* isolates from different geographical origin were selected for fungicide sensitivity assays. Concentrations of benzovindiflupyr, cyprodinil, fluazinam, iprodione, natamycin, and tebuconazole were chosen based on published literature or our own preliminary trials. Cyprodinil, fluazinam, iprodione and tebuconazole were chosen because they are registered in Brazil for application in apple orchards or postharvest. Benzovindiflupyr and natamycin were chosen due to documented efficacy against *Colletotrichum* spp. The fungicides, their FRAC code, trade name, medium used, and fungicide concentrations used are described in Table1.

Table 1. Fungicides used to determine *Colletotrichum* spp. sensitivity

Active ingredient	FRAC code	Commercial name	Medium	Fungicide concentrations (µg/ml)						
iprodione	2	Rovral (Bayer CropScience)	PDA[1]	0	1	3	10	30	100	-
tebuconazole	3	Elite (Bayer CropScience)	PDA[2]	0	0.03	0.1	0.3	1	3	-
benzovindiflupyr	7	Aprovia (Syngenta)	YBA[3]	0	0.01	0.05	0.25	1.25	5	-
cyprodinil	9	Vangard (Syngenta)	CZA[4]	0	0.005	0.01	0.05	0.1	1	10
fluazinam	29	Secure (Syngenta)	PDA[5]	0	0.02	0.1	0.5	2.5	12.5	-
natamycin	48	Zivion M (DSM Food Specialties B.V.)	PDA[4]	0	3	5	10	15	30	-

[1] Dutra et al. 2019.
[2] Chen et al. 2016.
[3] Ishii et al. 2016.
[4] Haack et al. 2018.
[5] Gang et al. 2015.

Isolates were cultured on PDA plates at 25°C for 8 days in darkness. Mycelial discs (5 mm in diameter) from the periphery of actively growing colonies on PDA were transferred onto fungicide-amended PDA medium. The commercial formulations of iprodione, tebuconazole, benzovindiflupyr, cyprodinil, fluazinam and natamycin were added to sterile distilled water and adjusted to concentrations of 1,000 or 10,000 µg/ml stock solutions. Different amounts of the stock solutions were added to autoclaved medium cooled to 60°C to obtain the final concentrations of fungicide (Table 1).

Cultures were incubated at 25 °C in darkness, and radial growth of colonies was measured after 5 days of incubation. The diameter of each colony was measured in two perpendicular directions and the mean diameter was adjusted by subtracting the diameter of the original plugs used for inoculation. Fungicide sensitivity, as measured by the 50% effective concentration (EC_{50}) value, was estimated by fitting 3 parameter Log-Logistic curves to the colony diameter data and finding the inflexion point of the curve as it follows:

$$f(x) = \frac{\theta a - \theta}{1 + \exp\{\theta r \times [\log(x) - \log(\theta i)]\}}$$

Where: $f(x)$ = 3-parameter log-logistic function; θa = upper asymptote; θr = proportional value of maximum rate; θi = inflexion point of the curve (estimated EC_{50} value).

Differences between mean EC_{50} of *C. melonis* and *C. nymphaeae* were tested by ANOVA. The analyses were performed using R Version 4.0.4 (R Core Team 2020).

Results

Sampling and fungal isolation

A total of 143 single-spore isolates were obtained from three orchards (Figure 1). From 320 flowers incubated from each location, 29 isolates were recovered from Porto Amazonas (9.1% recovery), 14 from Palmeira (4.4% recovery), and 2 from Lapa (0.6% recovery). From 100 fruitlets incubated from each location, 39 isolates were recovered from Porto Amazonas (39% recovery), 28 from Palmeira (28% recovery), and 31 from Lapa (31% recovery).

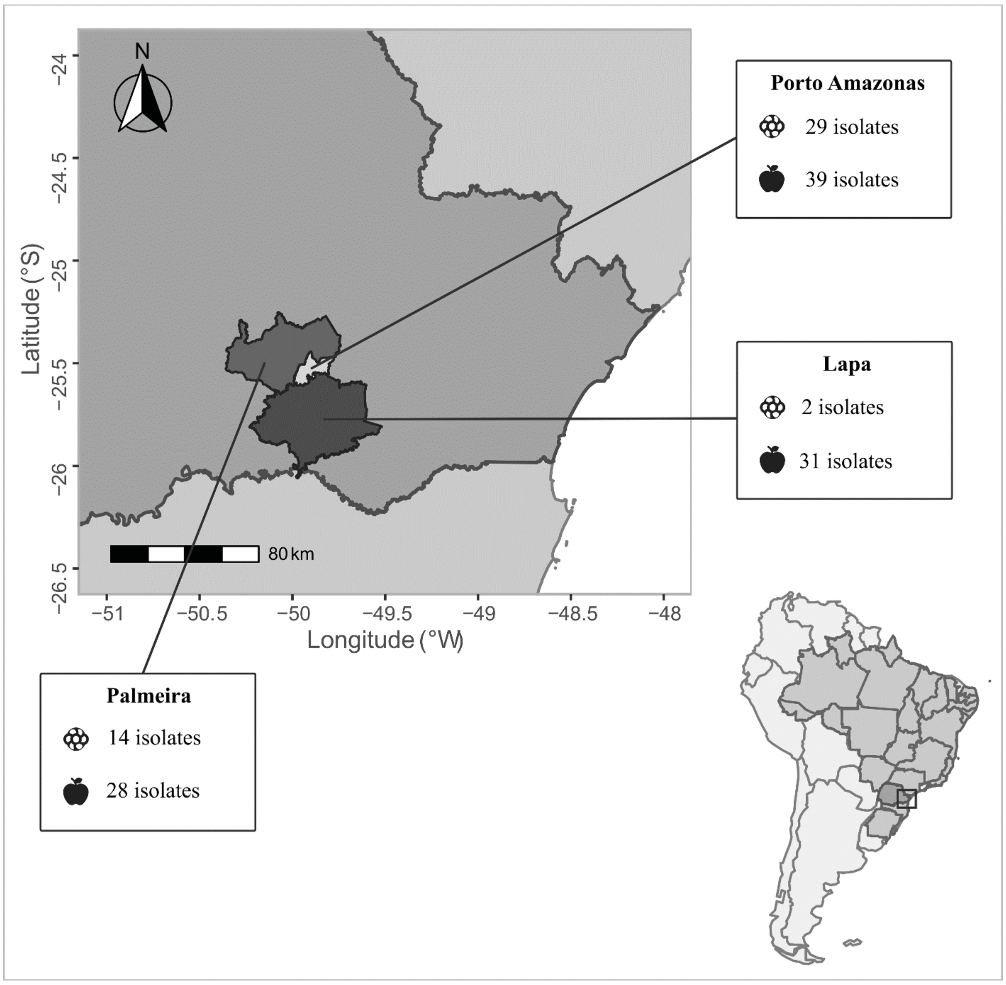


Figure 1. Isolates (143) obtained in 2017 from 'Eva' flowers (✿) and fruitlets (🍎) of apple orchards located in Lapa, Porto Amazonas and Palmeira, Paraná state, Brazil.

Molecular identification of Colletotrichum *species*

Based on the Bayesian multigenic phylogenetic analyses using *GAPDH* and *TUB2* nucleotide sequences, 3 *Colletotrichum* species were identified among the 88 isolates sequenced: *C. fructicola* (3 isolates) from the *C. gloeosporioides* species complex; *C. melonis* (30 isolates) and *C. nymphaeae* (57 isolates) from the *C. acutatum* species complex (Table 2). The *C. gloeosporioides* and *C. acutatum* species complex phylogenetic trees are shown in Supplementary 02 and 03, respectively. The isolate code names and tissue origins are listed in Supplementary 04. The concatenated *GAPDH* + *TUB2* sequences of one isolate did not clade with concatenated sequences from any *Colletotrichum* described species and was not included in further analysis in the present study.

Table 2. Characterization of the 88 sequenced *Colletotrichum* spp. isolates from 'Eva' apple trees collected in 2017.

Municipality	Plant tissue	Number of isolates		
		C. fructicola	*C. nymphaeae*	*C. melonis*
Lapa	Flower	0	1	0
	Fruitlet	0	24	4
Porto Amazonas	Flower	0	1	11
	Fruitlet	2	7	7
Palmeira	Flower	0	10	2
	Fruitlet	1	12	6

There was no significant difference between species occurrences in flower tissue. On the other way, *C. nymphaeae* was isolated significantly more (72,58 %) in thinned fruit than *C. melonis* (27,42 %) (Figure 2). Also, *C. nymphaeae* incidence was significantly higher (p-value ≤ 0.05) in apple fruitlets than in flower tissue ($p = 0.0346$) (Figure 2).

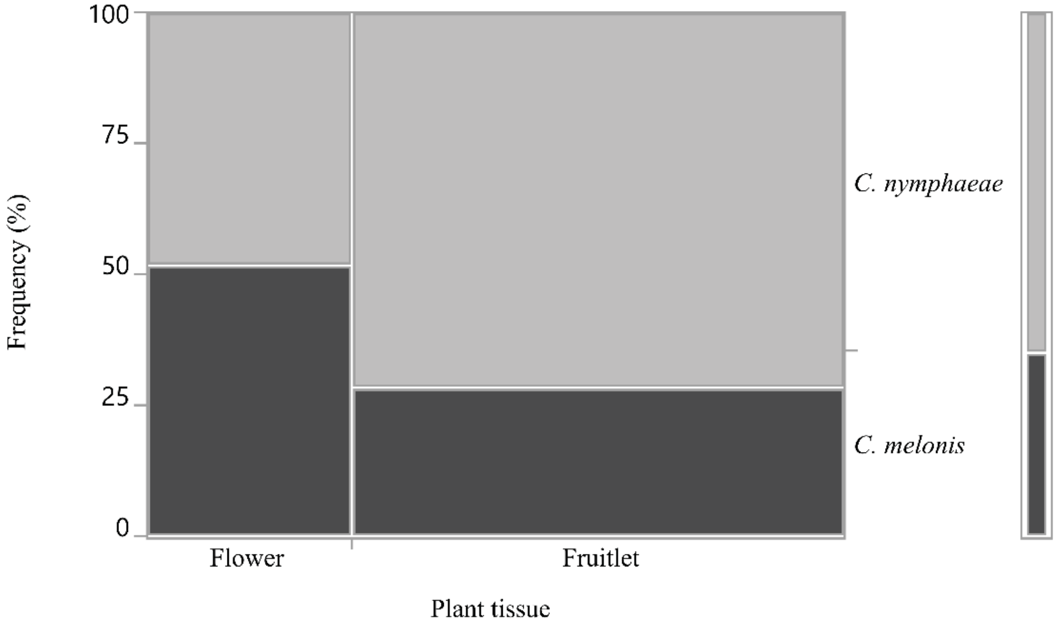


Figure 2. Mosaic Plot of Contingency Analysis of *Colletotrichum nymphaeae* and *C. melonis* by tissue sampled (flowers or fruit) of 'Eva' apple trees in Lapa, Porto Amazonas and Palmeira, Paraná state, Brazil.

Effect of fungicides on mycelial growth

The EC_{50} values and R^2 for each isolate of *C. melonis* and *C. nymphaeae* are shown in Supplementary 05. Among the fungicides tested, cyprodinil was the most effective against the two species complexes exhibiting EC_{50} values from 0.003 to 0.017 µg/ml for *C. melonis* isolates and from 0.001 to 0.052 µg/ml for *C. nymphaeae* isolates (Table 3). The EC_{50} values for benzovindiflupyr, fluazinam, tebuconazole, natamycin, and iprodione ranged from 0.011 to 0.096, 0.06 to 0.26, 0.013 – 0.168, 2.92 to 7.66, and 5.38 to 44.46 µg/ml for *C. melonis* and 0.013 to 0.46, 0.12 to 0.91, 0.038 to 0.894, 3.66 to 9.33, and 6.74 to 51.88 µg/ml for *C. nymphaeae*, respectively (Supplementary 05, Table 3). Seven isolates of *C. nymphaeae* (col012 - Lapa, col020 - Palmeira, col101 - Lapa, col117 - Lapa, col136 - Lapa, col142 – Lapa and col144 - Palmeira) had EC_{50} values for iprodione > 100 µg/ml. The mean EC_{50} values of *C. melonis* and *C. nymphaeae* isolates did not differ statistically for benzovindiflupyr (0.040 and 0.054 µg/ml), cyprodinil (0.010 and 0.014 µg/ml), iprodione (12.216 and 13.952 µg/ml), and natamycin (5.174 and 5.563 µg/ml; Table 3).

There were significant differences in sensitivity to fluazinam and tebuconazole between the two species (Table 4). In both cases, *C. nymphaeae* isolates had significantly higher mean

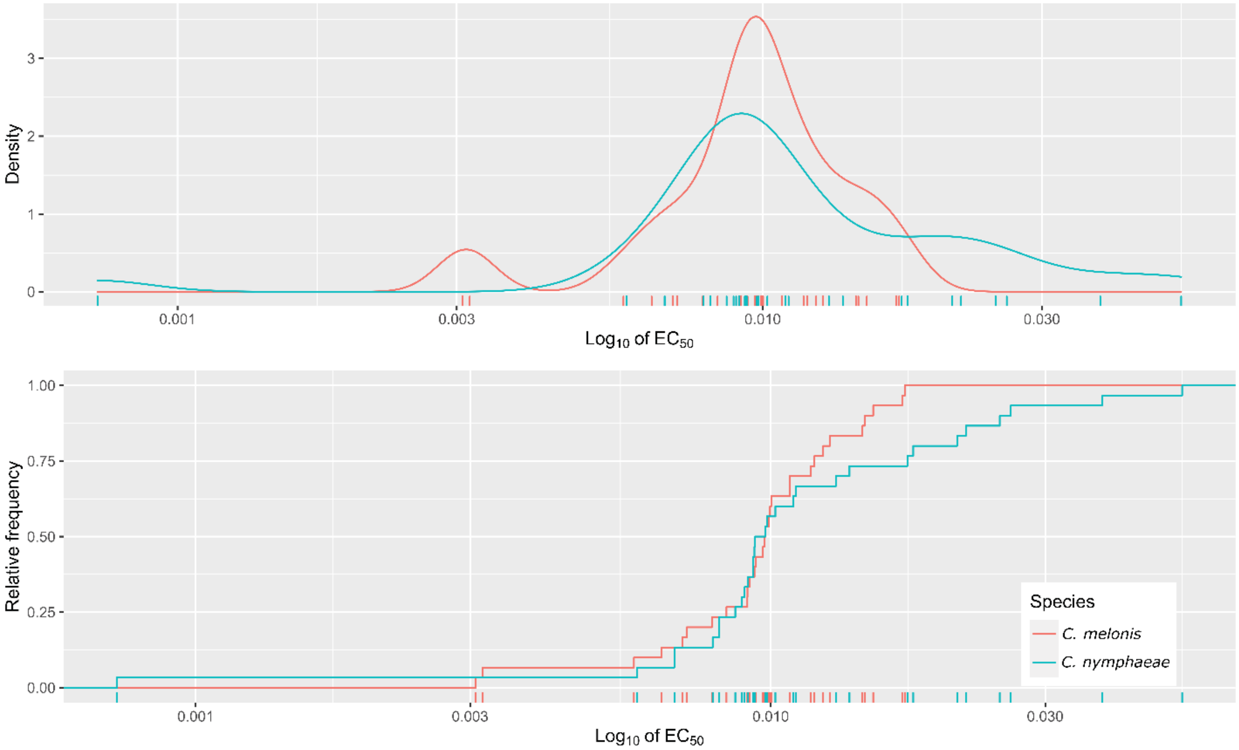
EC_{50} values (0.139 µg/ml and 0.330 µg/ml for tebuconazole and fluazinam, respectively) compared to *C. melonis* isolates (0.077 µg/ml and 0.116 µg/ml for tebuconazole and fluazinam, respectively) (Table 3).

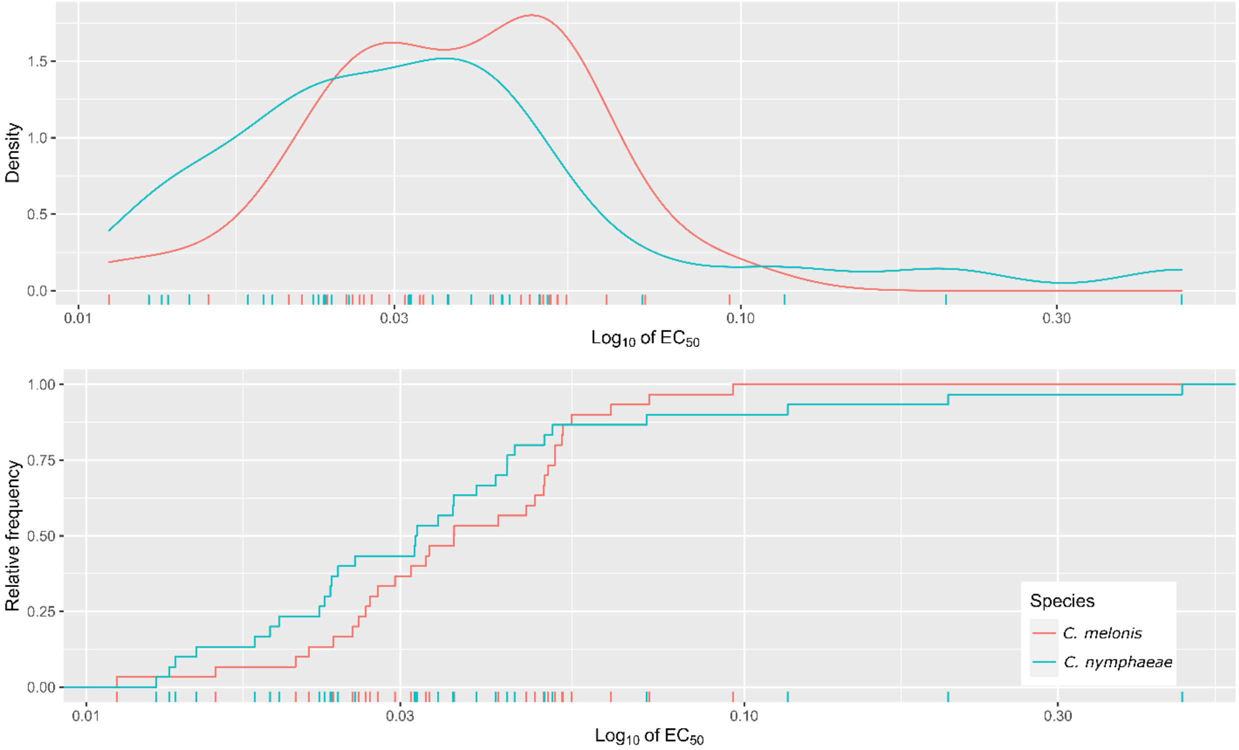
Table 3. Lowest, highest, and mean EC_{50} and Analysis of variance of mean EC_{50} values of *C. melonis* and *C. nymphaeae* for 6 fungicides from 'Eva' apple trees

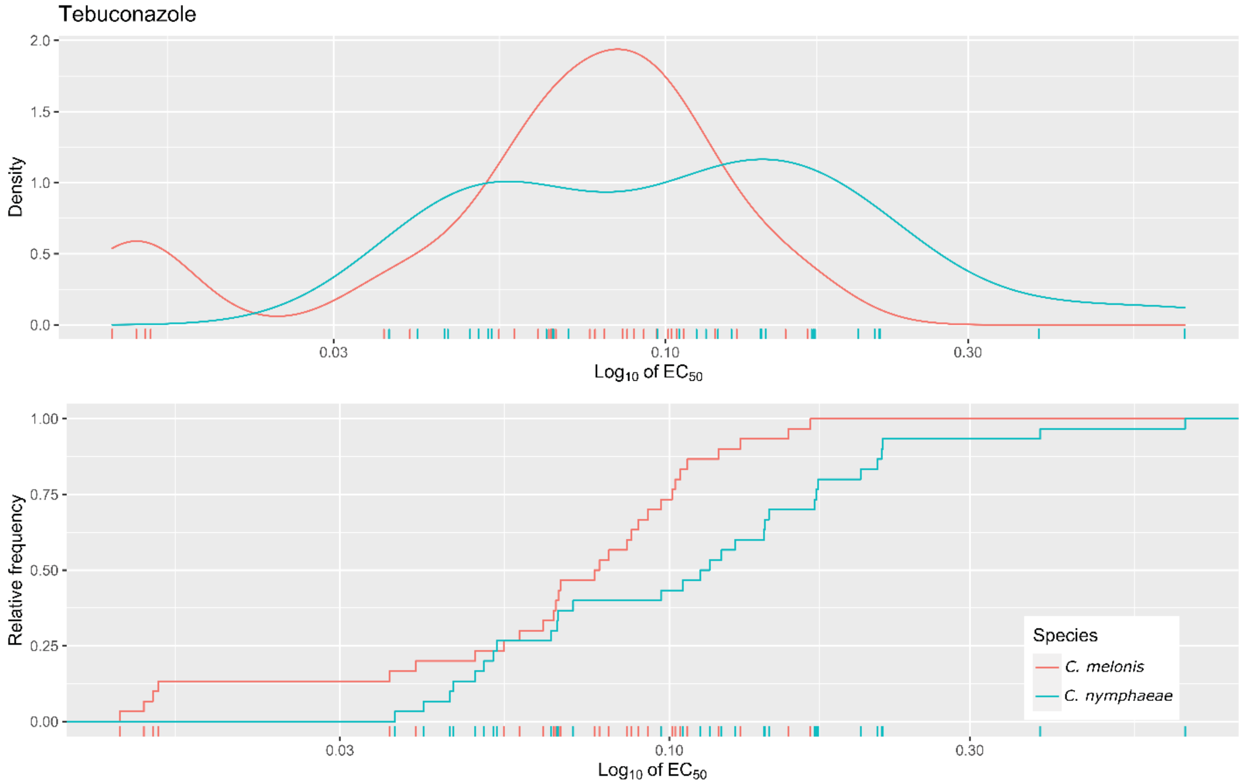
fungicide	**species**	**lowest EC_{50} (µg/ml)**	**highest EC_{50} (µg/ml)**	**mean EC_{50} (µg/ml)**	**Std error of the meanEC_{50}**	**F Ratio**	**prob > F**
cyprodinil	*C. melonis*	0.003	0.017	0.010	0.0014	3.52	0.07
	C. nymphaeae	0.001	0.052	0.014			
benzovindiflupyr	*C. melonis*	0.011	0.096	0.040	0.0113	0.76	0.39
	C. nymphaeae	0.013	0.463	0.054			
tebuconazole	*C. melonis*	0.013	0.167	0.077	0.0169	6.78	**0.0117***
	C. nymphaeae	0,037	0,659	0.139			
fluazinam	*C. melonis*	0,058	0,262	0.116	0.0240	39.83	**<.0001***
	C. nymphaeae	0,118	0,908	0.330			
natamycin	*C. melonis*	2,917	7,664	5.174	0.2014	1.87	0.18
	C. nymphaeae	3,663	9,326	5.563			
iprodione	*C. melonis*	5,375	44,463	12.216	15.481	0.55	0.46
	C. nymphaeae	6,736	2226.0	13.952			

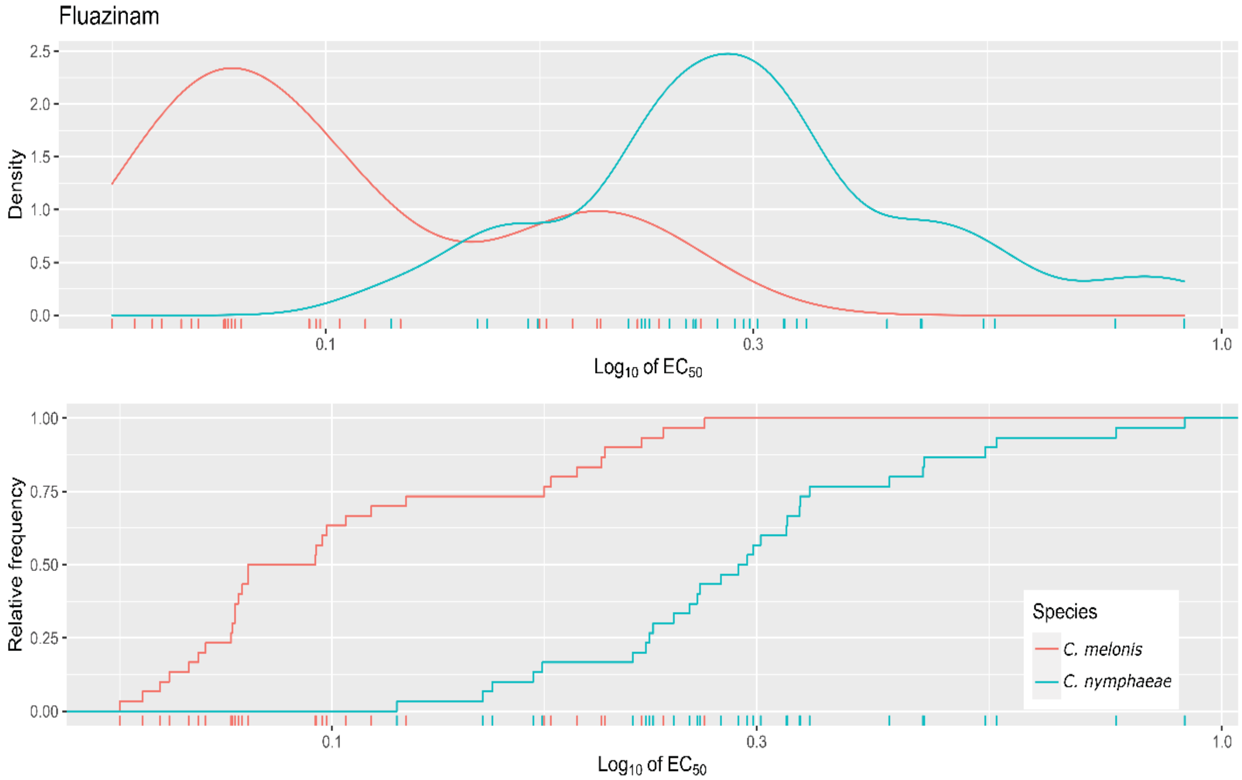
* values shown in bold indicate statistical differences between mean EC_{50} values of the two species (p-value ≤ 0.05).

For cyprodinil, benzovindiflupyr, natamycin and iprodione the two species exhibited similar sensitivity distributions patterns. However, a higher frequency of *C. nymphaeae* isolates clustered in the highest EC_{50} value categories, except for natamycin (Figure 3). In contrast to *C. melonis*, some *C. nymphaeae* isolates had EC_{50} values for cyprodinil > 0.3 µg/ml. Similarly, no *C. melonis* isolate presented EC_{50} values for benzovindiflupyr >0.1 µg/ml, while some *C. nymphaeae* isolates presented EC_{50} values >0.1 µg/ml. For tebuconazole, we can observe a distinct pattern of higher number of *C. melonis* isolates clustering in EC_{50} values from 0.03-0.1 µg/ml and drastic reduction of isolates with EC_{50} values >0.1 µg/ml, while for *C. nymphaeae* the distribution of isolates is more even from 0.03-0.3 µg/ml. For fluazinam, the difference of EC_{50} distribution between species is even more noticeable, with the density peaking at EC_{50} values <0.1 µg/ml for *C. melonis* and near 0.3 µg/ml for *C. nymphaeae*. Most isolates from both species had EC_{50} values below 7.9 µg/ml for natamycin and differently from the other fungicides more *C. melonis* isolates presented higher EC_{50} values (>7.0 µg/ml). For iprodione, only *C. nymphaeae* isolates presented EC_{50} values >100 µg/ml and most of the isolates from both species presented EC_{50} values near 10 µg/ml (Figure 3).


Cyprodinil


Benzovindiflupyr


Tebuconazole
Density
2.0
1.5
1.0
0.5
0.0
0.03
0.10
0.30
Log_{10} of EC_{50}
Relative frequency
1.00
0.75
0.50
0.25
0.00
Species
C. melonis
C. nymphaeae
0.03
0.10
0.30
Log_{10} of EC_{50}


Fluazinam
Density
2.5
2.0
1.5
1.0
0.5
0.0
0.1
0.3
1.0
Log_{10} of EC_{50}
Relative frequency
1.00
0.75
0.50
0.25
0.00
Species
C. melonis
C. nymphaeae
0.1
0.3
1.0
Log_{10} of EC_{50}

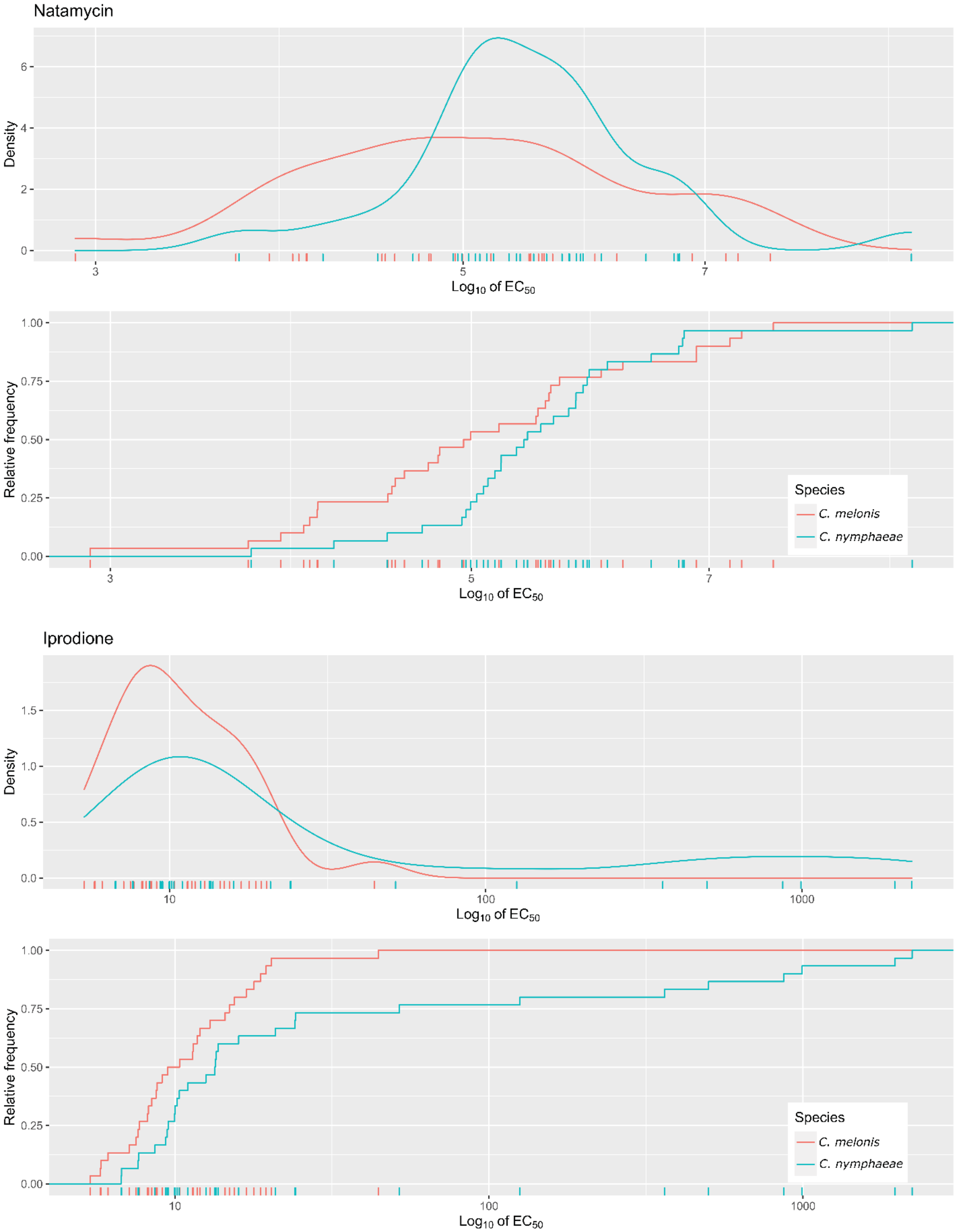


Figure 3. Density and Relative frequency distribution of 50% effective concentration (EC$_{50}$) values for cyprodinil, benzovindiflupyr, tebuconazole, fluazinam, natamycin, and iprodione, from the top to the bottom, for 30 *Colletotrichum melonis* and 30 *C. nymphaeae* isolates collected from 'Eva' apple orchards in Paraná state, Brazil.

Discussion

The present study identified sources of inoculum for secondary infections of *C. nymphaeae*, the predominant species causing Glomerella leaf spot and bitter rot in Paraná, Brazil (Moreira et al. 2019c). The isolates collected for this study were not obtained from leaves symptomatic for Glomerella leaf spot, but from early season flower and fruitlets. Molecular analysis revealed that the isolates belonged to *Colletotrichum* species previously identified to be among the causal agents of Glomerella leaf spot (*C. nymphaeae*, *C. melonis* and *C. fructicola*) and bitter rot (*C. nymphaeae* and *C. fructicola*) (Khodadadi et al. 2020). Inoculum sources for *C. acutatum*, including *C. nymphaeae*, are reported to be leaves on the trees (Børve and Stensvand 2017) or leaf litter on the soil (Hamada and May De Mio 2017), twigs (Hamada and May De Mio 2017); Gelain et al., in press) and buds (Børve and Stensvand 2017; Hamada and May De Mio 2017), and can rapidly disseminate under favorable conditions, requiring several fungicide sprays throughout a season. A previous study identified 3 isolates (2010-2011 season) from 'Eva' flowers with quiescent infections as belonging to *C. acutatum* species complex (Moreira et al. 2019c), and they were pathogenic to wounded and unwounded apple detached fruit (Hamada et al. 2020). The confirmation of flowers and fruitlets as inoculum sources in the present study has significant implications on disease management. Spores from these sources may cause bitter rot in mature fruit in the field, when substances that inhibit the activity of pathogenicity factors are degraded, and when changes in pH and nutrients (mainly nitrogen and sugars) occur (Prusky 1996; Prusky and Lichter 2007), or during storage, appearing as postharvest rot (Wenneker and Thomma 2020). Sporulation on diseased apple fruit is a source of secondary inoculum for new infections on fruit and leaves. Management of bitter rot starts with the reduction of the inoculum early in the season; considering that the disease can cause crop losses up to 50% (Sutton 1990), reviewing the spraying program in apple orchards with focus on avoiding pathogen infection in flowers is necessary.

The higher recovery of isolates from fruitlets than from flowers is most likely related to the faster degradation of flower tissues and growth of many other fungi that prevented the observation and collection of *Colletotrichum* spp.

About 1/3 of all flower and fruitlet-derived isolates were *C. melonis*, which is in sharp contrast to the rare occurrence reported for this species in Glomerella leaf spot symptomatic leaf samples from 'Gala' and 'Eva', in which *C. melonis* occurrence among other species was < 2% (Moreira et al. 2019c). Perhaps *C. melonis* has a competitive advantage on 'Eva' flowers and fruitlets or a competitive disadvantage on 'Eva' leaves. The species has not been isolated from bitter rot field lesions but can cause bitter rot lesions on wounded and unwounded 'Gala' and 'Eva' detached fruit (Moreira et al. 2021). More studies are needed to examine tissue preference within a cultivar and preference between cultivars of *Colletotrichum* species. Also, more epidemiological studies are needed to document adaptability of species to regional climates. Another explanation for the discrepancy of species occurrence in Parana apple orchards between studies includes the impact of fungicide applications by the grower leading up to sampling dates. Given the differential sensitivity to site-specific fungicides between *Colletotrichum* species (Dowling et al. 2020); this study), some of the products applied may have imposed selection pressure onto population structure.

The two species *C. melonis* and *C. nymphaeae* differed in sensitivity to some but not all fungicides tested. *C. melonis* was more sensitive to tebuconazole and fluazinam than *C. nymphaeae* in this study. Differences in sensitivities among species complex and even between species within the same complex have been reported. *C. acutatum* species complex isolates were less sensitive to thiophanate-methyl, myclobutanil, trifloxystrobin, and captan compared with the isolates from the *C. gloeosporioides* species complex (Munir et al. 2016). Moreover, in the same study, *C. siamense* was more sensitive to the tested fungicides compared with *C. fructicola* and *C. theobromicola*, all three species from the *C. gloeosporioides* complex (Munir et al. 2016). On peach, *C. nymphaeae* isolates were resistant to the DMI fungicides flutriafol and fenbuconazole while *C. fioriniae* isolates were sensitive (Chen et al. 2016). These findings highlight the importance of the accurate identification of local populations of *Colletotrichum* to the species level and an understanding of fungicide sensitivity differences among species. Such information will aid in disease and in resistance management. The lower sensitivity of *C. nymphaeae* may have been inherent, similar to previously reported inherent fungicide tolerance of *C. gloeosporioides* species compared to *C. acutatum* species (Schnabel et al. 2021) or it may have evolved due to a quantitative shift following selection pressure. *C. melonis* was reported more recently than *C. nymphaeae* on apple and may have entered commercial apple orchards later. It may therefore have been exposed to fewer DMI and fluazinam fungicide applications.

Despite some species-specific fungicide sensitivity, the two species tested showed greatest sensitivity to cyprodinil and benzovindiflupyr, medium sensitivity to fluazinam and tebuconazole, and least sensitivity to natamycin and iprodione. Despite being registered for apple scab (*Venturia inaequalis)* control, cyprodinil has not been used in any production areas evaluated in this study. In many crops, including peach, anilinopyrimidines are recommended for use early in season to increase their curative effect and limit their volatility (Creemers 1994;

Creemers and Vanmechelen 1999). Therefore, it stands to reason that cyprodinil may aid in an effort to reduce inoculum of *Colletotrichum* species, including the inoculum discovered in this study. The SDHI fungicide benzovindiflupyr is one of the few SDHIs with documented activity against *Colletotrichum* species (Ishii et al. 2016). The relatively low EC_{50} values for benzovindiflupyr found in this study agree with previous study, that reported EC_{50} values for multiple *Colletotrichum* species below 0.1 µg/ml in mycelial growth assays (Ishii et al. 2016). However, the same molecule exhibited higher average EC_{50} values of 0.3, 0.17, and 0.26 µg/ml for *C. fioriniae, C. noveboracense* and *C. chrysophilum*, respectively (Khodadadi et al. 2020). Our results suggest that together with cyprodinil, benzovindiflupyr may be an effective tool for Glomerella leaf spot and bitter rot management in Brazil (Acheampong and Babadoost 2020; Kreis and Villani 2020; Parker and Ritchie 2016; Villani et al. 2018). Regarding fluazinam, the EC_{50} values were consistently lower than 1 µg/ml. This fungicide has shown efficacy against *C. gloeosporioides* and *C. acutatum* species complexes *in vitro* (Gang et al. 2015; Leroux 1996). It also performed well in field trials, providing similar levels of control to the commercial mixture of pyraclostrobin and fluxapyroxad (Ritchie et al. 2016). It also has particularly low risk of resistance development. Despite these good results, the high sensitivity found in the present study, and it being registered to control other apple diseases, no study has considered fluazinam for Glomerella leaf spot and bitter rot control in Brazil and few studies are available worldwide. On the other hand, many studies have been published to examine *Colletotrichum* species for their sensitivity to DMI fungicides, including tebuconazole. However, only one study examined *C. nymphaeae* isolates and none examined *C. melonis* isolates. Mean EC_{50} values were 0.8 µg/ml for 5 *C. nymphaeae* isolates (Chen et al. 2020, 2016), which is higher than the 0.139 µg/ml reported here. The data suggest that fluazinam and DMIs such as tebuconazole may have a place in Glomerella leaf spot and bitter rot management if integrated into a disease management program.

The EC_{50} values of *C. nymphaeae* and *C. melonis* isolates for natamycin were similar to those reported previously (Khodadadi et al. 2020). In both studies mean EC_{50} values ranged from 4 to 5 µg/ml. Both studies were performed with mycelial growth inhibition assays, but there is evidence of an even higher effect on spore germination (He et al. 2019; Saito et al. 2020). Despite the comparably poor *in vitro* performance, the field tests conducted with formulated product with comparably high rates document control success. For example, incidence of anthracnose crown rot caused by *C. acutatum* after natamycin dip-treatments of strawberry transplants (using 500 µg/ml solutions) was significantly reduced and similar in efficacy to the commercial standard fludioxonil (Haack et al. 2018). Natamycin binds specifically to ergosterol without permeabilizing the membrane (te Welscher et al. 2010), leading to the release of intracellular contents and cell death (He et al. 2019). Its broad-spectrum activity, the lack of development of resistance (te Welscher et al. 2008), lack of cross-resistance with other postharvest fungicides (Saito et al. 2020), and low oral and topical toxicity to mammals (Arima et al. 2014) make it a promising molecule for *Colletotrichum* disease management. Sensitivity to iprodione was generally low and multiple isolates from both species revealed EC_{50} values for iprodione > 100 µg/ml. Similarly high EC_{50} values were previously reported for *C. gloeosporioides* isolates (LaMondia 2001) substantiate the low efficacy of this molecule for *Colletotrichum* species.

The detection of *C. acutatum* complex in apple flowers and fruitlets in this study corroborates the findings of the same species complex in apple leaves and buds from previous studies and underscores the importance of detecting sources of inoculum in disease management. The *in vitro* efficacy of the fungicides cyprodinil and fluazinam against *C. melonis* and *C. nymphaeae* are of special interest because both fungicides are already registered for controlling other apple pathogens. Amending an existing label with additional diseases is typically less problematic from a regulation standpoint than requesting a new label for a yet unregistered product. Benzovindiflupyr and the biological fungicide natamycin were also relatively effective in mycelial growth inhibition of *C. melonis* and *C. nymphaeae.* The results of this study may be useful efficacy data for potential future registration of these molecules against Glomerella leaf spot and bitter rot.

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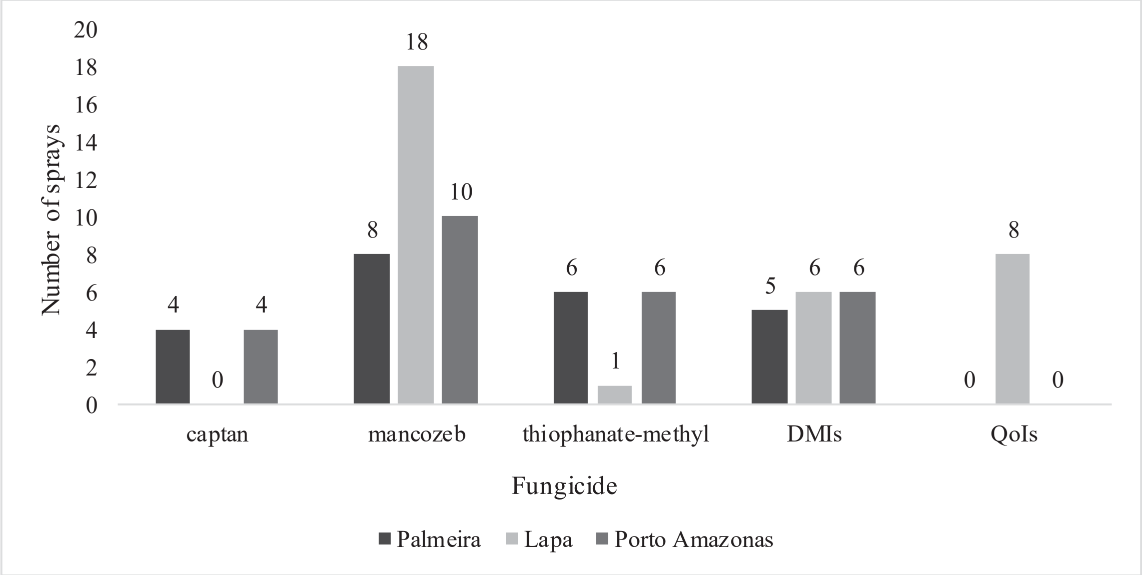
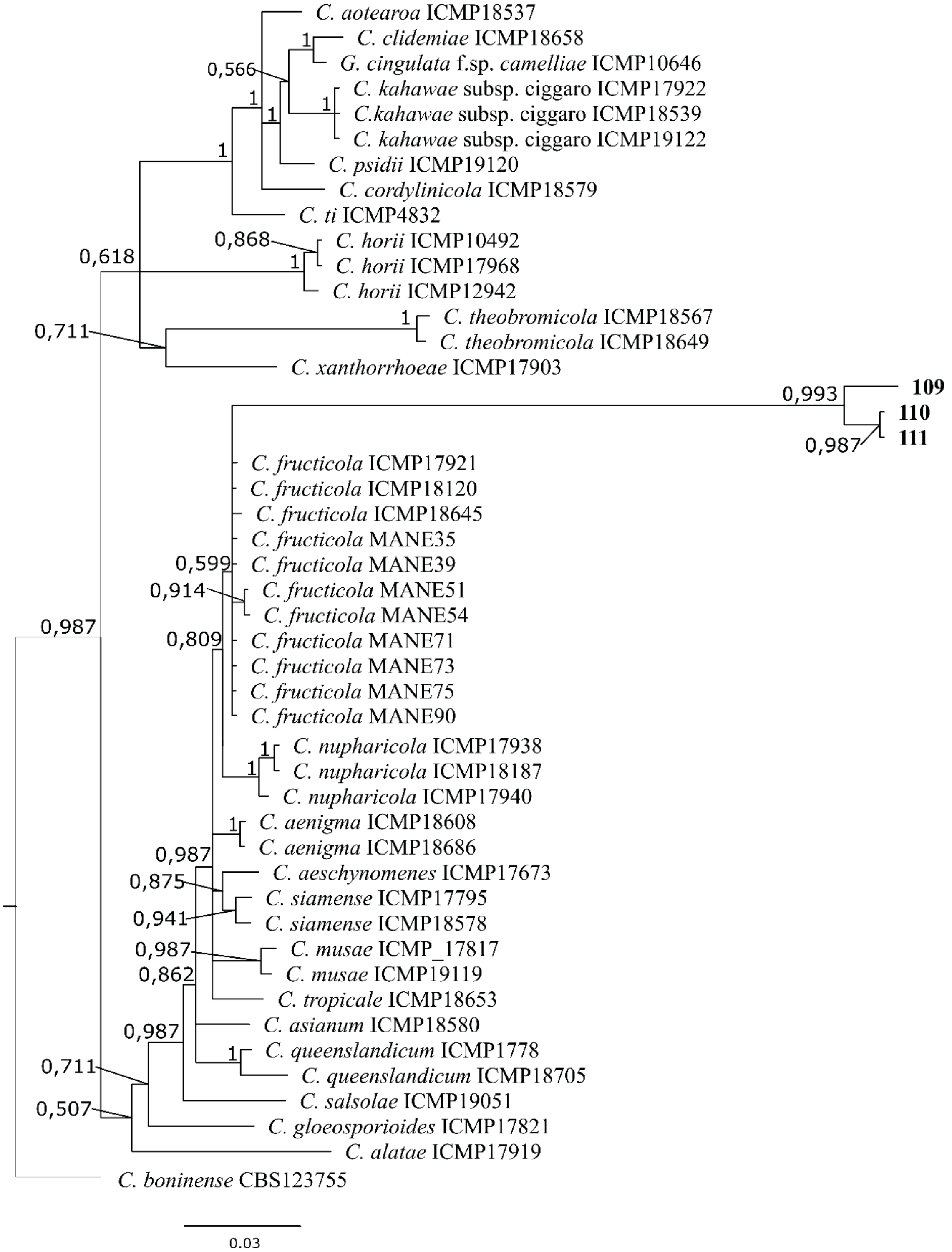
Supplementary 01 – number of applications of fungicides used in 'Eva' apple orchards located in Palmeira (total applications 23), Lapa (total applications 33) and Porto Amazonas (total applications 26).



DMIs: demethylation inhibitor fungicides (difenoconazole, tebuconazole)
QoIs: quinone-outside inhibitor fungicides (pyraclostrobin, trifloxystrobin)

Supplementary 02 - Phylogenetic analysis by Bayesian inference of combined *GAPDH* and *TUB2* sequence data of *Colletotrichum gloeosporioides* species complex. Bayesian posterior probabilities scores (> 0,50) from 1000 replicates are shown at the nodes. The scale bar represents the number of expected changes per site. The tree is rooted in *Colletotrichum boninense* (CBS123755). The scale bar indicates the number of expected changes per site. The isolates in the present study are shown in bold, and the numbers are part of the complete code shown in Supplementary 04 (LEMID-MdCm17-nnn for *C. melonis* and MdCn17-nnn for *C. nymphaeae*).



Supplementary 03 - Phylogenetic analysis by Bayesian inference of combined *GAPDH* and *TUB2* sequence data of *Colletotrichum acutatum* species complex. Bayesian posterior probabilities scores (>0,50) from 1000 replicates are shown at the nodes. The scale bar represents the number of expected changes per site. The tree is rooted in *Colletotrichum orchidophilum* (CBS632.80). The scale bar indicates the number of expected changes per site. The isolates in the present study are shown in bold, and the numbers are part of the complete code shown in Supplementary 04 (LEMID-MdCm17-nnn for *C. melonis* and MdCn17-nnn for *C. nymphaeae*).

1
C. acerbum CBS1285.30
0,783
C. rhombiforme CBS129953
0,908
C. kinghornii CBS198.35
0,993
C. phormii CBS118194
C. australe CBS116478
1
C. salicis CBS607.94
1
C. godetiae CBS13344
C. godetiae CBS155.25
1
0,921
C. johnstonii CBS1285.32
C. pyricola CBS128531
101
112
113
114
115
117
118
122
124
125
135
27
29
66
0,513
73
74
76
78
80
82
83
86
91
93
94
12
127
130
132
134
136
138
139
14
140
141
0,586
142
143
144
16
0,796
20
87
49
65
69
77
85
88
89
90
92
C. nymphaeae CBS126513
C. nymphaeae CBS127608
C. nymphaeae CBS127609
C. nymphaeae CBS127610
C. nymphaeae CBS129918
C. nymphaeae CBS129928
0,98
C. nymphaeae CBS129929
C. nymphaeae CBS129933
C. nymphaeae CBS129936
C. nymphaeae CBS129937
C. nymphaeae CBS129945
C. nymphaeae IMI301119
0,816
C. nymphaeae CBS48282
C. nymphaeae CBS51578
C. nymphaeae CBS51678
C. nymphaeae CBS52677
C. nymphaeae CSL455
C. nymphaeae IMI299103
1
116
55
C. nymphaeae CBS126528
0,895
C. nymphaeae CBS127612
C. nymphaeae CBS129935
C. nymphaeae CBS361.79
C. nymphaeae CBS129926
106
107
108
119
120
121
123
126
0,947
129
34
38
47
54
64
128
131
133
0,809
137
21
28
32
39
41
42
52
0,954
56
59
6
1
61
9
C. melonis LEMIDPR18-11
C. melonis COL20
C. melonis CBS159.84
C. abscissum CDA918
1
C. abscissum CPC208.94
0,855
C. abscissum STF-FTP10
C. costaricense CBS330.75
1
C. limetticola CBS114.14
1
C. paranaense CPC209.01
C. paranaense CPC209.28
0,928
C. sp CBS101611
1
C. tamarilloi CBS129814
0,993
C. tamarilloi CBS129954
0,803
C. cuscutae IMI304802
1
C. lupini CBS109225
C. lupini CBS109226
2
0,928
C. brisbanense CBS292.67
C. sloanei IMI364297
1
C. indonesiense CBS127551
1
1
C. paxtonii IMI1657.53
1
C. simmondsii CBS126524
0,908
C. simmondsii CBS294.67
C. laticiphilium CBS112989
1
C. cosmi CBS853.73
C. walleri CBS125472
0,592
C. guajavae IMI350839
1
C. scovillei CBS120708
0,993
C. scovillei CBS126529
1
C. sp CBS126518
C. sp CBS126519
C. acutatum CBS112994
1
C. acutatum CBS112996
C. acutatum CBS129924
1
C. fioriniae CBS112995
0,98
C. fioriniae CBS119186
C. fioriniae IMI345575
C. orchidophilum CBS632.80

Supplementary 04 – *Colletotrichum* spp. isolates sequenced (87 total) codes and origins.

Municipality	Plant tissue	*C. fructicola*		*C. nymphaeae*		*C. melonis*	
		Isolate codes	n	Isolate codes	n	Isolate codes	n
Lapa	Flower	-	0	MdCnPR17-113	1	-	0
	Fruitlet	-	0	MdCnPR17-012	24	MdCmPR17-021	4
				MdCnPR17-016		MdCmPR17-039	
				MdCnPR17-049		MdCmPR17-042	
				MdCnPR17-050		MdCmPR17-137	
				MdCnPR17-055			
				MdCnPR17-065			
				MdCnPR17-069			
				MdCnPR17-077			
				MdCnPR17-087			
				MdCnPR17-088			
				MdCnPR17-089			
				MdCnPR17-090			
				MdCnPR17-092			
				MdCnPR17-101			
				MdCnPR17-102			
				MdCnPR17-112			
				MdCnPR17-116			
				MdCnPR17-117			
				MdCnPR17-136			
				MdCnPR17-138			
				MdCnPR17-139			
				MdCnPR17-141			
				MdCnPR17-142			
				MdCnPR17-143			
Porto Amazonas	Flower	-	0	MdCnPR17-057	1	MdCmPR17-006	11
						MdCmPR17-009	
						MdCmPR17-032	
						MdCmPR17-038	
						MdCmPR17-041	
						MdCmPR17-052	
						MdCmPR17-054	
						MdCmPR17-056	
						MdCmPR17-059	
						MdCmPR17-061	
						MdCmPR17-064	
	Fruitlet	MdCfPR17-109	2	MdCnPR17-014	7	MdCmPR17-107	7
		MdCfPR17-111		MdCnPR17-027		MdCmPR17-108	
				MdCnPR17-091		MdCmPR17-120	

				MdCnPR17-115		MdCmPR17-121	
				MdCnPR17-124		MdCmPR17-126	
				MdCnPR17-135		MdCmPR17-131	
				MdCnPR17-140		MdCmPR17-133	
Palmeira	Flower	-	0	MdCnPR17-029	9	MdCmPR17-028	2
				MdCnPR17-066		MdCmPR17-034	
				MdCnPR17-073			
				MdCnPR17-078			
				MdCnPR17-080			
				MdCnPR17-083			
				MdCnPR17-093			
				MdCnPR17-114			
				MdCnPR17-122			
	Fruitlet	MdCfPR17-110	1	MdCnPR17-020	12	MdCmPR17-047	6
				MdCnPR17-074		MdCmPR17-106	
				MdCnPR17-076		MdCmPR17-119	
				MdCnPR17-082		MdCmPR17-123	
				MdCnPR17-085		MdCmPR17-128	
				MdCnPR17-086		MdCmPR17-129	
				MdCnPR17-094			
				MdCnPR17-118			
				MdCnPR17-125			
				MdCnPR17-130			
				MdCnPR17-134			
				MdCnPR17-144			

Md = Malus domestica.
Cf = *Colletotrichum fructicola*.
Cm = *Colletotrichum melonis*.
Cn = *Colletotrichum nymphaeae*.
PR = Paraná state.
17 = year of collection (2017).

Supplementary 05. EC$_{50}$ and R^2 values for cyprodinil, benzovindiflupyr, fluazinam, iprodione, natamycin, and tebuconazole of *Colletotrichum melonis* (n = 30) and *C. nymphaeae* (n = 30) isolates. The 3-parameter log logistic model was fitted to the data.

Isolate[1]	cyprodinil		benzovindiflupyr		fluazinam		iprodione		natamycin		tebuconazole	
	EC$_{50}$[2]	R^2[3]	EC$_{50}$	R^2	EC$_{50}$	R^2	EC$_{50}$	R^2	EC$_{50}$	R^2	EC$_{50}$	R^2
MdCmPR17-006	0.008	0.97	0.033	0.97	0.078	0.99	12.922	0.81	4.704	0.99	0.089	0.96
MdCmPR17-009	0.010	0.97	0.050	0.86	0.064	0.99	14.457	0.75	4.489	0.97	0.058	0.97
MdCmPR17-021	0.011	0.96	0.050	0.96	0.097	0.97	16.868	0.63	3.819	0.99	0.092	0.99
MdCmPR17-028	0.009	0.98	0.011	0.94	0.058	0.99	11.438	0.69	4.548	0.98	0.049	0.99
MdCmPR17-032	0.006	0.94	0.096	0.77	0.069	0.98	9.102	0.79	5.596	0.98	0.066	0.98
MdCmPR17-034	0.010	0.98	0.047	0.97	0.066	0.99	19.491	0.67	4.770	0.98	0.063	0.97
MdCmPR17-038	0.009	0.97	0.029	0.98	0.078	0.98	18.744	0.77	5.581	0.99	0.076	0.99
MdCmPR17-039	0.009	0.99	0.028	0.97	0.061	0.99	8.171	0.82	4.996	0.99	0.120	0.98
MdCmPR17-041	0.010	0.93	0.031	0.99	0.071	0.98	11.394	0.76	5.199	0.97	0.040	1.00
MdCmPR17-042	0.017	0.97	0.072	0.94	0.077	0.97	44.463	0.65	7.207	0.97	0.087	0.99
MdCmPR17-047	0.012	0.92	0.033	0.89	0.203	0.98	7.644	0.95	5.664	0.93	0.015	0.98
MdCmPR17-052	0.011	0.96	0.027	0.97	0.096	0.98	15.462	0.65	3.946	0.91	0.036	0.99
MdCmPR17-054	0.010	0.98	0.024	0.93	0.099	0.98	12.040	0.84	4.020	0.91	0.066	0.99
MdCmPR17-056	0.010	0.99	0.026	0.85	0.078	0.99	14.903	0.69	2.917	0.87	0.067	0.99
MdCmPR17-059	0.008	0.95	0.048	0.82	0.080	0.98	17.868	0.69	3.647	0.87	0.077	0.96
MdCmPR17-061	0.009	1.00	0.021	0.96	0.077	0.99	8.236	0.78	3.979	0.94	0.055	1.00
MdCmPR17-064	0.003	0.98	0.022	0.98	0.072	0.99	20.269	0.61	4.468	0.88	0.013	0.99
MdCmPR17-106	0.007	0.96	0.025	0.97	0.262	0.98	7.704	0.95	7.329	0.97	0.155	0.98
MdCmPR17-107	0.015	0.98	0.036	0.99	0.111	0.97	5.820	0.95	5.554	0.98	0.130	0.89
MdCmPR17-108	0.010	0.97	0.016	0.98	0.236	0.98	11.763	0.89	5.492	0.99	0.067	0.87
MdCmPR17-119	0.014	0.93	0.053	0.98	0.176	0.96	7.520	0.98	6.876	0.98	0.167	0.97
MdCmPR17-120	0.017	0.97	0.052	0.98	0.096	0.97	5.791	0.92	4.946	0.97	0.015	0.99
MdCmPR17-121	0.012	0.96	0.042	0.97	0.079	0.96	5.375	0.94	4.443	0.97	0.104	0.99
MdCmPR17-123	0.012	0.92	0.052	0.97	0.201	0.95	8.442	0.94	5.480	0.96	0.102	0.99
MdCmPR17-126	0.015	0.96	0.036	0.97	0.104	0.97	8.708	0.94	6.193	0.98	0.107	0.99
MdCmPR17-128	0.009	0.93	0.027	0.94	0.189	0.97	8.781	0.92	7.664	0.95	0.101	0.99
MdCmPR17-129	0.003	0.95	0.055	0.96	0.173	0.98	10.355	0.94	6.007	0.97	0.015	0.97
MdCmPR17-131	0.013	0.94	0.063	0.94	0.121	0.96	7.163	0.92	4.781	0.98	0.086	0.98
MdCmPR17-133	0.006	0.91	0.050	0.97	0.080	0.98	6.124	0.97	4.026	0.99	0.097	0.99
MdCmPR17-137	0.007	0.95	0.053	0.93	0.223	0.97	9.472	0.95	6.876	0.80	0.080	0.98
MdCnPR17-012	0.022	0.91	0.463	0.69	0.227	0.96	500.62	0.56	6.755	0.95	0.659	0.89
MdCnPR17-014	0.013	0.92	0.023	0.95	0.344	0.95	13.373	0.90	5.854	0.99	0.045	0.97
MdCnPR17-020	0.052	0.76	0.036	0.96	0.461	0.95	1966.07	0.30	9.326	0.97	0.127	0.97
MdCnPR17-027	0.008	0.99	0.013	0.97	0.286	0.98	8.647	0.98	5.168	0.99	0.051	0.99
MdCnPR17-029	0.011	0.99	0.014	0.97	0.325	0.97	13.730	0.74	6.449	0.99	0.170	0.95
MdCnPR17-049	0.007	0.97	0.051	0.88	0.542	0.96	24.105	0.72	5.216	0.99	0.070	0.94
MdCnPR17-066	0.008	0.98	0.044	0.92	0.303	0.96	7.641	0.97	5.738	0.99	0.053	0.99
MdCnPR17-073	0.010	0.99	0.013	0.97	0.252	0.98	7.667	0.95	4.964	0.99	0.171	0.98
MdCnPR17-077	0.009	0.97	0.015	0.90	0.335	0.97	12.54	0.75	4.994	0.99	0.045	0.98
MdCnPR17-083	0.010	0.99	0.020	0.96	0.257	0.98	6.761	0.94	5.414	0.99	0.218	0.92
MdCnPR17-089	0.007	0.98	0.204	0.77	0.336	0.97	20.862	0.73	5.216	0.99	0.097	0.97

MdCnPR17-091	0.011	1.00	0.024	0.96	0.230	0.98	9.345	0.91	4.932	1.00	0.116	0.66
MdCnPR17-093	0.009	0.98	0.019	0.95	0.218	0.98	10.160	0.96	5.039	0.99	0.121	0.99
MdCnPR17-094	0.014	0.96	0.026	0.93	0.225	0.96	10.974	0.82	5.330	0.98	0.217	0.97
MdCnPR17-101	0.001	0.74	0.045	0.70	0.908	0.77	125.39	0.30	4.440	0.94	0.172	0.87
MdCnPR17-112	0.025	0.94	0.116	0.90	0.151	0.93	24.239	0.50	6.740	0.98	0.141	0.93
MdCnPR17-113	0.009	0.53	0.032	0.90	0.273	0.98	15.959	0.77	5.118	0.99	0.041	0.97
MdCnPR17-114	0.009	0.96	0.071	0.95	0.324	0.90	6.737	0.70	5.908	0.98	0.066	0.98
MdCnPR17-115	0.009	1.00	0.050	0.89	0.297	0.96	10.009	0.97	5.798	0.98	0.065	0.98
MdCnPR17-117	0.006	0.90	0.023	0.95	0.168	0.98	363.00	0.47	4.664	0.99	0.049	0.98
MdCnPR17-122	0.009	0.97	0.032	0.95	0.259	0.98	9.510	0.93	5.088	0.98	0.053	0.99
MdCnPR17-124	0.009	0.96	0.044	0.84	0.463	0.95	9.982	0.96	3.663	0.97	0.112	0.99
MdCnPR17-127	0.018	0.90	0.024	0.95	0.293	0.98	10.333	0.97	5.516	0.98	0.144	0.98
MdCnPR17-134	0.008	0.91	0.034	0.96	0.423	0.98	13.532	0.92	5.793	0.98	0.037	0.99
MdCnPR17-135	0.009	0.97	0.018	0.95	0.242	0.99	9.455	0.99	5.387	0.97	0.214	0.95
MdCnPR17-136	0.026	0.90	0.042	0.93	0.118	0.97	869.71	0.45	6.062	0.96	0.142	0.93
MdCnPR17-139	0.021	0.98	0.039	0.86	0.558	0.90	51.879	0.38	4.116	0.99	0.388	0.89
MdCnPR17-140	0.010	0.84	0.032	0.96	0.760	0.98	13.446	0.78	5.889	0.99	0.201	0.96
MdCnPR17-142	0.038	0.93	0.023	0.93	0.148	0.95	993.10	0.51	6.706	0.99	0.105	0.98
MdCnPR17-144	0.017	0.76	0.036	0.91	0.172	0.95	2225.97	0.25	5.616	0.98	0.067	0.95

[1] Cm = *Colletotrichum melonis*; Cn = *Colletotrichum nymphaeae.*

[2] EC_{50} values estimated with 3-parameter log-logistic model.

[3] R^2 = coefficient of determination.

6 CHAPTER 4: *IN VITRO* SENSITIVITY OF *Colletotrichum melonis* AND *C. nymphaeae* FROM APPLE TREES TO PYRACLOSTROBIN IN PARANÁ STATE[4]

Abstract

In Paraná state, southern Brazil, the environmental conditions are especially favorable for Glomerella leaf spot and bitter rot in apple trees, caused mainly by species within the *Colletotrichum acutatum* species complexes. Fungicides management is based on mancozeb but the use of quinone-outside inhibitor (QoI) fungicides has been increasing in the last years. Pyraclostrobin is a QoI single-site fungicide already registered for controlling *Colletotrichum* spp. in Brazil; however, no information is available on the sensitivity profile of this fungicide for Brazilian isolates. The objectives of this study were to evaluate and compare the *in vitro* sensitivity with EC_{50} estimation of *Colletotrichum melonis* and *C. nymphaeae* isolates from apple trees in Paraná state for the QoI fungicide pyraclostrobin with spore germination inhibition assays, as well as to characterize the isolates phenotypically. The species *C. melonis* presented EC_{50} values from 0.016 – 0.722 µg/ml (mean = 0.207 µg/ml) and *C. nymphaeae* from 0.034 – 0.700 µg/ml (mean = 0.241 µg/ml). *C. melonis* and *C. nymphaeae* did not differ in sensitivity to pyraclostrobin. Most of the tested isolates presented low sensitive phenotype to this fungicide. For *C. melonis*, 53.3% of the isolates presented low sensitive and 46.7% sensitive phenotypes. For *C. nymphaeae*, 83.3% presented low sensitive and 26.7% sensitive phenotypes. This study indicates that pyraclostrobin would not be a suitable option to be used in Glomerella leaf spot and bitter rot management in Paraná apple orchards, and studies to assess practical resistance and also to characterize the genotype of the isolates with low sensitive phenotypes are recommended.

Keywords: bitter rot; Glomerella leaf spot; chemical control; quinone-outside inhibitor; single-site fungicide.

Colletotrichum species are associated with the two most important diseases of apples in subtropical climate, Glomerella leaf spot and bitter rot. Symptoms of Glomerella leaf spot include severe defoliation that could reduce productivity in subsequent seasons, which occurs with greater intensity in apple producing regions with humid subtropical climate, as southern Brazil (Becker et al. 2000). Glomerella leaf spot is associated with severe necrotic, irregular sized, brown-reddish lesions on leaves, which can exceed 70% of leaf area (Moreira et al.

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2019c). In fruit, bitter rot is the most common symptom leading to crop losses up to 50% (Becker et al. 2000; Moreira et al. 2021). The disease initially appear as small circular lesions that are light to dark brown in color, evolving into circular, sunken, and light brown to brown lesions formed on the fruit surface; in humid conditions, erumpent, mucilaginous, pink conidia masses are formed (González et al. 2006).

In Paraná state, the predominant species causing both bitter rot and Glomerella leaf spot are from the *C. acutatum* complex, as shown in 2010-2011 season (Moreira et al. 2019c) and confirmed in the 2017-2018 season (data from chapter 3 of this thesis). Chemical control is the main method for management of the diseases in Brazilian apple orchards. Differences in sensitivities to fungicides among *Colletotrichum* species can occur (Yokosawa et al. 2017; Munir et al. 2016), but susceptibility profiles of *Colletotrichum* species across Brazilian apple producing regions are very limited. The lack of information lead growers to repetitive use of multisite protective fungicides (Hamada and May De Mio 2017) and calendar spraying. Regarding QoIs, the most effective mode of action against *Colletotrichum* spp. in fruit crops (Dowling et al. 2020), only one study tested the sensitivity of *Colletotrichum acutatum* species complex isolates from the 2010-2011 season to azoxystrobin and found some highly resistant isolates (Moreira et al. 2019a). The highly specific mode of action of the QoIs led to resistance in a significant number of target pathogens, usually due to modification of the target site (Fernández-Ortuño et al. 2016), so monitoring for shifts in sensitivity is crucial. However, *Colletotrichum* spp. sensitivity was not assessed after 2011 season, and the sensitivity of the pathogen to pyraclostrobin, one of the most used QoI in apple orchards in Brazil, has never been evaluated.

Therefore, the objectives of this study were to evaluate and compare the *in vitro* sensitivity of *Colletotrichum melonis* and *C. nymphaeae* isolates from apple trees collected on 2017-2018 season in Paraná state to the QoI fungicide pyraclostrobin with EC_{50} estimation, as well as to characterize the isolates phenotypically.

The monosporic isolates used in the present study (30 *C. melonis* and 30 *C. nymphaeae*) were obtained from 'Eva' apple flowers and fruitlets in Lapa, Porto Amazonas and Palmeira municipalities, in Paraná state, southern Brazil, in the 2017-2018 season. The isolates were previously identified by phylogenetic trees from concatenated Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-tubulin (*TUB2*) sequences to identify isolates to the species level. The municipality and tissue of origin of the isolates are described in Supplementary 5 from Chapter 3 of this thesis. From budbreak until thinning in the season of collection, Porto

Amazonas and Palmeira orchards were sprayed with fungicides but not with QoIs, while Lapa orchard was sprayed 3 times with pyraclostrobin from budbreak until the fruitlet collection in the 2017-2018 season.

Cabrio (BASF®), with pyraclostrobin as active ingredient, was added to sterile distilled water and adjusted to the concentration of 1,000 μg/ml for the stock solution. Different amounts of the stock solution were added to autoclaved medium cooled to 60°C to obtain the final concentrations of 0, 0.01, 0.05, 0.25, 1.25 and 6.25 μg/ml. Fresh conidial suspensions from 8-day-old colonies were pipetted onto WA plates, 100 μl per plate, with a final concentration of conidia of 1×10^4 conidia/ml and spread with a Drigalski spatula. The plates were incubated at 25 °C for 24 h. The germination was halted, and conidia and germ tubes stained with Lactophenol Cotton Blue solution (Sigma-Aldrich®). Conidia were then observed at 100 x magnification and scored as either non-germinated or germinated (germ tube at least the length of the conidium). Petri dishes were divided into quadrants and 100 conidia per quadrant were counted from microscope fields arbitrarily located within each quadrant (n = 400). The experiment was performed twice. Fungicide sensitivity, as measured by the 50% effective concentration (EC_{50}) value, was estimated by fitting 3 parameter Log-Logistic curves to the germination data (average germination of each plate) and finding the inflexion point of the curve as it follows:

$$f(x) = \frac{\theta a - \theta}{1 + \exp\{\theta r \times [\log(x) - \log(\theta i)]\}}$$

Where: $f(x)$ = 3-parameter log-logistic function; θa = upper asymptote; θr = proportional value of maximum rate; θi = inflexion point of the curve (estimated EC_{50} value).

Differences between mean EC_{50} of *C. melonis* and *C. nymphaeae* were tested by ANOVA. The analyses were performed using R Version 4.0.4 (R Core Team 2020).

The isolates were classified phenotypically as sensitive (EC_{50} <0.110 μg/ml) or low sensitive (EC_{50} > 0.110 μg/ml), adapted from a previous methodology described for *Colletotrichum acutatum* in strawberry (Forcelini et al. 2016).

The species *C. melonis* presented EC_{50} values from 0.016 – 0.722 μg/ml and *C. nymphaeae* from 0.034 – 0.700 μg/ml for the QoI fungicide pyraclostrobin. The mean EC_{50} of the isolates of *C. melonis* and *C. nymphaeae* for pyraclostrobin were 0.207 and 0.241 μg/ml, respectively, and did not differ statistically (p=0.05) (Table 1).

Table 1. Lowest, highest, and mean EC$_{50}$ and Analysis of variance of EC$_{50}$ values of 30 *C. melonis* and 30 *C. nymphaeae* isolates for pyraclostrobin

species	lowest EC$_{50}$ (µg/ml)	highest EC$_{50}$ (µg/ml)	mean EC$_{50}$ (µg/ml)	std error of the mean EC$_{50}$	F Ratio	prob > F
C. melonis	0,016	0,722	0.207	0.0318	0.56	0.46
C. nymphaeae	0,034	0,700	0.241			

From the total *C. melonis* isolates (n = 30), 16 presented low sensitive and 14 sensitive phenotypes. For *C. nymphaeae* (n = 30), the number of isolates for each phenotype were 25 low sensitive and 5 sensitive. This represents more than 68% of the isolates from both species with low sensitive phenotype. From Lapa, 50% of the *C. melonis* isolates were sensitive and 50% low sensitive, while *C. nymphaeae* were 25% sensitive and 75% low sensitive. From Porto Amazonas, 66.7% of the *C. melonis* isolates were highly sensitive and 33.3% low sensitivity, while *C. nymphaeae* were 28.6% sensitive and 71.4% low sensitive. All isolates from Palmeira, regardless of the species, were classified as low sensitive (Table 2).

QoI fungicides have been crucial to disease control since azoxystrobin was released in 1992, followed by kresoxim-methyl in 1998, and trifloxystrobin and pyraclostrobin in 2000 (Balba 2007; Bartlett et al. 2002). However, only one study regarding sensitivity to QoIs with Brazilian *Colletotrichum* isolates from apples from the 2010-2011 season is available, that found 3 isolates with resistant or highly resistant phenotypes to azoxystrobin among the 16 *C. nymphaeae* isolates tested (Moreira et al. 2019a). On the other hand, the present study revealed that the majority of *C. nymphaeae* isolates from Paraná apple orchards present low sensitive phenotype to pyraclostrobin. The EC$_{50}$ values from the isolates collected in the 2017/2018 season and tested in the present study may indicate a shift in sensitivity in the *C. nymphaeae* population from Paraná orchards to QoI fungicides when compared to the previous study. EC$_{50}$ values of *C. acutatum* in strawberry for pyraclostrobin varied from 0.011 to > 0.11 µg/ml, 8 isolates with sensitive and 4 with resistant phenotypes (Forcelini et al. 2016), also showing predominance of isolates with sensitive phenotype, differently from the present study.

Despite reported differences in sensitivities among *Colletotrichum* species (Yokosawa et al. 2017; Munir et al. 2016), *Colletotrichum melonis* and *C. nymphaeae* did not differ in sensitivity to this fungicide.

Table 2. EC_{50} values and phenotypes of 30 *Colletotrichum melonis* and 30 *C. nymphaeae* isolates for pyraclostrobin fungicide *in vitro*

Species							
Colletotrichum melonis				*Colletotrichum nymphaeae*			
isolate	origin	EC_{50}[1]	phenotype[2]	isolate	origin	EC_{50}[1]	phenotype[2]
MdCmPR17-021	Lapa	0.027	S	MdCnPR17-142	Lapa	0.073	S
MdCmPR17-039		0.094	S	MdCnPR17-113		0.079	S
MdCmPR17-042		0.165	LS	MdCnPR17-136		0.097	S
MdCmPR17-137		0.574	LS	MdCnPR17-089		0.119	LS
MdCmPR17-034	Palmeira	0.128	LS	MdCnPR17-077		0.186	LS
MdCmPR17-028		0.230	LS	MdCnPR17-112		0.202	LS
MdCmPR17-106		0.260	LS	MdCnPR17-012		0.300	LS
MdCmPR17-047		0.266	LS	MdCnPR17-127		0.330	LS
MdCmPR17-128		0.369	LS	MdCnPR17-101		0.369	LS
MdCmPR17-123		0.492	LS	MdCnPR17-049		0.376	LS
MdCmPR17-129		0.510	LS	MdCnPR17-117		0.409	LS
MdCmPR17-119		0.570	LS	MdCnPR17-139		0.434	LS
MdCmPR17-061	Porto Amazonas	0.016	S	MdCnPR17-114	Palmeira	0.121	LS
MdCmPR17-009		0.021	S	MdCnPR17-029		0.134	LS
MdCmPR17-052		0.022	S	MdCnPR17-083		0.135	LS
MdCmPR17-032		0.041	S	MdCnPR17-122		0.135	LS
MdCmPR17-006		0.051	S	MdCnPR17-093		0.181	LS
MdCmPR17-038		0.055	S	MdCnPR17-144		0.217	LS
MdCmPR17-059		0.056	S	MdCnPR17-094		0.261	LS
MdCmPR17-108		0.065	S	MdCnPR17-134		0.353	LS
MdCmPR17-064		0.082	S	MdCnPR17-073		0.395	LS
MdCmPR17-107		0.090	S	MdCnPR17-066		0.494	LS
MdCmPR17-054		0.099	S	MdCnPR17-020		0.700	LS
MdCmPR17-120		0.108	S	MdCnPR17-115	Porto Amazonas	0.034	S
MdCmPR17-041		0.119	LS	MdCnPR17-140		0.053	S
MdCmPR17-121		0.150	LS	MdCnPR17-027		0.129	LS
MdCmPR17-056		0.227	LS	MdCnPR17-091		0.164	LS
MdCmPR17-131		0.284	LS	MdCnPR17-124		0.213	LS
MdCmPR17-126		0.312	LS	MdCnPR17-135		0.241	LS
MdCmPR17-133		0.722	LS	MdCnPR17-014		0.280	LS

[1] EC_{50} = effective concentration where growth was reduced by 50%.

[2] S = sensitive *Colletotrichum melonis* and *C. nymphaeae* isolates for pyraclostrobin ($EC_{50} < 0.110$ mg/ml). LS = low sensitive *Colletotrichum melonis* and *C. nymphaeae* isolates for pyraclostrobin ($EC_{50} > 0.110$) mg/ml (Forcelini et al. 2016).

Pyraclostrobin was the most effective fungicide tested among azoxystrobin, pyraclostrobin and picoxystrobin in inhibiting spore germination of *C. acutatum* (Gao et al. 2017). Besides pyraclostrobin, trifloxystrobin and kresoxim-methyl are registered for apple crop in Brazil. Cross-resistance between QoI fungicides is common and expected, according to FRAC, so not only pyraclostrobin efficacy in the Paraná orchards may be compromised but also the other two registered QoI fungicides.

QoI fungicides specifically bind to the quinol oxidation (Qo) site of cytochrome b to inhibit mitochondrial respiration, blocking electron transfer between cytochrome b and cytochrome c1 and inhibiting the synthesis of nicotinamide adenine dinucleotide (NADH) and the mitochondrial membrane protein adenosine triphosphate (ATP) (Balba 2007; Feng et al. 2020; Fernández-Ortuño et al. 2016; Bartlett et al. 2002), leading to a block in the energy production and eventual death of the fungus (Balba 2007). This highly specific mode of action of the QoIs led to resistance in *Colletotrichum* species in several fruit crops, such as mango (Kongtragoul 2020), strawberry (Forcelini and Peres 2018; Ali et al. 2020; Chechi et al. 2020), peach and blueberry (Hu et al. 2015). In apples, resistance has been reported in isolates with point mutations on cytb gene (Yokosawa et al. 2017) and in isolates that did not present mutations on cytb gene (Chechi et al. 2020; Moreira et al. 2019a), indicating that other resistance mechanisms may be involved. The presence of point mutations on the cytb gene in the isolates with low sensitive phenotype from the present study should be assessed in future studies.

Salicylhydroxamic acid (SHAM) is usually added to the medium in *in vitro* QoI fungicide sensitivity assays because it blocks the alternative respiration pathway (Wood and Hollomon 2003). However, no effect of the addition of SHAM was observed on *Colletotrichum acutatum* germination (Forcelini et al. 2016). In the present study, no SHAM was added to the medium, and the presence of sensitive and resistant phenotypes within our population to pyraclostrobin suggests that SHAM does not affect *C. melonis* and *C. nymphaeae* germination under different doses of pyraclostrobin. Therefore, SHAM does not need to be added in *in vitro* experiments with these species and fungicide.

In favorable climates, such as in Parana State, spraying for Glomerella leaf spot is extended until the postharvest period in mid-autumn to avoid early defoliation of the plant and to ensure growth and production during the following year. This regimen results in >20 multisite fungicide sprays per season of protective fungicides such as mancozeb (Hamada and May De Mio 2017). However, mancozeb is being banned from the EU, so growers will have to use every fungicide available and already registered in the control of Glomerella leaf spot and bitter rot to avoid residues that could impact fruit commercialization. Species from the *Colletotrichum acutatum* complex, the predominant in Paraná state, are naturally resistant to methyl-benzimidazole carbamates (MBCs) as thiophanate methyl (Chung et al. 2006; Peres et al. 2004) and this study showed also low sensitivity to pyraclostrobin, which reduces considerably the management options regarding single site fungicides in the state.

The present study showed that the sensitivity of *Colletotrichum melonis* and *C. nymphaeae* to pyraclostrobin is reduced, and even though this fungicide is already registered for *Colletotrichum* control in Brazilian apple orchards, it is no longer a good option to be used in bitter rot and Glomerella leaf spot disease management in Eva orchards. Therefore, other single-site fungicides should be tested to reduce dependance on mancozeb sprays to control the diseases.

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