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Characterization of Colletotrichum Nymphaeae Isolates, Causal Pathogen of Apple Bitter Rot, With Reduced Sensitivity to Fluazinam and Tebuconazole

Rulyu Meng
rulyum@g.clemson.edu

CHARACTERIZATION OF COLLETOTRICHUM NYMPHAEAE ISOLATES, CAUSAL PATHOGEN OF APPLE BITTER ROT, WITH REDUCED SENSITIVITY TO FLUAZINAM AND TEBUCONAZOLE

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Rulyu Meng
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Dr. Guido Schanabel, Committee Chair
Dr. Julia Kerrigan
Dr. James Faust

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CHAPTER ONE

1.LITERATURE REVIEW

1.1 The *Colletotrichum spp.*

Colletotrichum species cause diseases in a wide range of hosts (Freeman, 2008a; Afanador-Kafuri et al. 2003; Eastburn et al. 1990; Freeman et al. 2002b). They affect a wide range of economically important crops, including apple, peach, grape, blueberry, cranberry, and strawberry (Bernstein et al. 1995; Daykin 1984b; Howard 1972). There are different names for diseases caused by *Colletotrichum* spp. depending on the host and plant part. Anthracnose is the most commonly used disease name, while apple bitter rot or grape ripe rot are also used to describe diseases caused by *Colletotrichum* spp. Despite *Colletotrichum* being the eighth most important genus of phytopathogenic fungi in the world (Crouch et al. 2014; Dean et al. 2012), the systematics of *Colletotrichum* species has been difficult to establish (Hyde et al. 2009) because of changing taxonomy (Bernstein et al. 1995). Phylogenetic analysis of the genus *Colletotrichum* reveals that it is comprised of nine major clades, as well as several smaller clusters and isolated species (Cannon et al. 2012). Among the nine major clades of *Colletotrichum*, the *C. acutatum* species complex and the *C. gloeosporioides* species complex are the two most common clades containing species that cause bitter rot disease of apples (Jayawardena et al. 2016; Peres et al. 2005; Munir et al. 2016).

1.2 Importance of apples to the Brazilian economy

Apple (*Malus domestica* Borkh.), native to central Asia and then introduced to the west and other parts of the world (Dzhangaliev, 2003), is a major fruit crop cultivated in temperate regions today (Khodadadi et al. 2020). Four regions account for the bulk of Brazil's apple production: Santa Catarina state, Rio Grande do Sul state, Parana state, and Sao Paulo state (USDA, 2019). The apples produced in Brazil amounted to 983,247 tons in 2020 season (FAO, 2022). Apple crops are vulnerable to a wide range of diseases affecting yield and fruit quality (Khodadadi et al. 2020). The three most important fungal diseases occurring frequently in Brazilian apple orchards are apple bitter rot and Glomerella leaf spot, both caused by *Colletotrichum* species (Velho et al. 2015), and apple scab caused by *Venturia inaequalis* (Bogo et al. 2012).

Apple bitter rot was first described in England in 1856 and one decade later in the US (Taylor, 1971). Disease development depends on wet and warm weather conditions (Carraro et al. 2022). *C. nymphaeae*, *C. paranaense*, and *C. melonis* species causing apple bitter rot had optimal conidia germination at temperatures 20-25°C (Moreira et al. 2021). Optimal mycelium growth temperatures of individual *Colletotrichum* spp. range from 22 to 30°C (Dowling et al. 2020; Wang et al. 2015). The ideal weather for apple bitter rot infections is furnished by high moisture (80-10%) with frequent raining (John Roberts, 1918; Melanie L. Lewis Ivey, 2016). All currently available commercial apple cultivars are susceptible to the pathogen (Denardi et al. 2003). In Brazil, the disease was first described in the 1970s when commercial apple production was initiated in the country (Denardi et al. 2003). *C. nymphaeae* and *C. fioriniae* from the *Colletotrichum acutatum* species complex and *C. fructicola*, *C. aenigma*, and *C.*

siamense from the *Colletotrichum gloeosporioides* species complex are known to cause apple bitter rot worldwide (Weir et al. 2012; Jayawardena et al. 2016).

Apple bitter rot is a fungal apple fruit disease caused by *Colletotrichum* genus (Melanie L. Lewis Ivey, 2016). Glomerella leaf spot disease caused by *Glomerella cingulata* has been reported in Brazil and the United States (Gonzalez et al. 2004; Katsurayama et al. 2000), but *C. acutatum* is not known to attack apple leaves ((Peres et al. 2005). Apple bitter rot produces symptoms initially consisting of circular sunken lesions(Alan R. Biggs, 1995) and potentially appears with a halo in mature stage (Beever et al. 1995) on apples. Under optimum conditions, these lesions on apple surface subsequently enlarge rapidly and cover the entire fruit surface (Beever et al. 1995). Copious amounts of conidia are produced in fruiting bodies called acervuli. Under moist, humid conditions, the signs are the spore masses appear creamy and are orange to pink-colored arranged concentrically (Beever et al. 1995; Oo et al. 2018).

For the disease cycle, the branches and bud scales inoculation and sporulation (Jorunn Børve and Arne Stensvand, 2018) may contribute to infected buds opening in spring. Conidia from acervuli are rain-splash dispersed, germinate to form appressoria, and quiescent infections are established on fruit surface (Zaitalin et al. 2000), most of the quiescent infections develop during production season. Acervuli may also overwinter in mummified fruit in the canopy (Peres et al. 2005). In summer, conidia are most commonly rain-splashed from infected decaying petals, bud scales, twigs, and fruitlets; the conidia are also released from twig cankers and mummified fruit in the canopy (Everett et al. 2018). Results of survival of *C. acutatum* on infected plant litter

(Norman and Strandberg 1997; Stensvand et al. 2017; Wilson et al. 1992) indicate that infected fruit dropping from the tree in autumn could also contribute to infection in the following winter season. In Michigan, study shows that *C. acutatum* can be isolated from both healthy and damaged apple fruit trees tissue (Jones et al. 1996).

1.3 Chemical control of apple bitter rot

Fungicides with multisite modes of action (MOA) such as captan and mancozeb as well as single-site MOA are used to control fungal diseases in apple production. Compared to single-site MOA fungicides, the multisites are typically less effective but broader in their activity spectrum (i.e. they work against pathogens of different genera). The most commonly used chemical classes with single site MOA for bitter rot control are the quinone outside inhibitors (QoIs) and benzimidazoles (MBC). *Colletotrichum* spp. have been reported to develop resistance to many fungicides, including QoIs and MBCs (Avila-Adame et al. 2003; Hu et al. 2015; Poti et al. 2020). QoI fungicides inhibit the cytochrome bc1 respiratory complex in the fungal mitochondria (Kim et al. 2007). QoI resistance has already been reported for *Colletotrichum graminicola* on turf (Avila-Adame et al. 2003) and was also found in *Venturia inaequalis* on apple (Lesniak et al. 2011). Control failure due to QoI resistance in *C. acutatum* was first documented in strawberries in the 2013-2014 season (Forcelini and Peres, 2018). QoI resistance in *C. nymphaeae* has been reported in isolates from Ohio, USA (Chechi, et al. 2020). Due to the increasing occurrence of QoI resistance in *Colletotrichum* species as well as reports of resistance to MBC

fungicides in the *C. gloeosporioides* complex (Hu et al., 2015), captan has become an important fungicide in resistance management and the management of common apple diseases such as apple scab (*Venturia inaequalis*), apple bitter rot (Abbott et al. 2017), and anthracnose of strawberry (MacKenzie and Peres 2012).

Previous studies suggest that *C. nymphaeae* isolates, belonging to *C. acutatum* species complex, were highly resistant to MBCs but sensitive to DMIs (Gelain et al. in press). To the best of our knowledge, no field resistance to tebuconazole and fluazinam has been found in *C. nymphaeae* (Gelain et al. in press). Because of the limited number of effective fungicides for bitter rot control in Brazil, chemicals with other MOA, such as fluazinam and tebuconazole, become more promising for the potential suppression of bitter rot.

Fluazinam is a fungicide used in agriculture against different diseases. The mode of action involves the uncoupling of oxidative phosphorylation in mitochondria (Guo et al. 1991) and high reactivity with thiols. Fluazinam is an aminopyridine inhibiting glucose oxidation in a dose-dependent manner and suppressing the energy production process through direct inhibition of the adenosine triphosphate (ATP) synthetase in fungal cells (Vitoratos et al. 2014). Based on recent studies, fluazinam successfully controlled strawberry crown rot caused by *C. gloeosporioides* (Oliveira et al. 2020) and seed decay by *C. trunctum* (Oh et al. 2022). Tebuconazole is a demethylation inhibitor (DMI) fungicide that targets the sterol 14α-demethylase and has become an integral part of apple scab management (Chen et al. 2018). Resistance

to DMI fungicides in *Colletotrichum truncatum* is possibly linked to *CYP51A* and *CYP51B* target gene expression (Chen et al. 2018). According to Oliveira et al. (2020) and Silvar-Junior et al. (2021), tebuconazole can inhibit strawberry crown rot caused by *C. gloeosporioides* and citrus anthracnose caused by *C. acutatum*.

Conventional fungicide applications in apple production are critical for disease management. However, the registration of new fungicides is hindered due to cost of product development and due to difficulty in new product registration. The use of existing products with suppressive action may therefore be beneficial at least in the short term for IPM (Integrated Pest Management) practices.

CHAPTER TWO

2.1 ABSTRACT

Apple bitter rot is caused by *Colletotrichum nymphaeae* and other *Colletotrichum* species and management relies primarily on synthetic pesticides. Very few fungicides are effective against the disease and resistance has further limited their usefulness. A recent study indicated a relatively low range of EC_{50} values (the concentration required to inhibit 50% of mycelial growth in vitro) of *C. nymphaeae* isolates from Brazilian apples to fluazinam and tebuconazole, two fungicides that are not routinely used for bitter rot control. Isolates on opposite sides of the range were designated either sensitive (lowest EC_{50} values) and reduced sensitive (highest EC_{50} values). The objective of this research was to confirm stability of EC_{50} values after storage, assess fluazinam and tebuconazole for apple bitter rot management in detached fruit studies, and to sequence the fungicide's target genes. Our research confirmed stability of EC_{50} values after 8 months of cold storage. Detached fruit studies revealed that protective treatment of cv. Gala apple fruit with fluazinam and tebuconazole controlled both sensitive and reduced sensitive phenotypes but if used curatively (after infection) reduced sensitive isolates were controlled less effectively. The complete *OS-1*, *CYP51A*, and *CYP51B* genes from *C. nymphaeae* isolates sensitive or reduced sensitive to fluazinam (potential target *OS-1*) and tebuconazole (CYP51A and CYP51B) were sequenced and two nucleotide changes in *OS-1* leading to amino acid changes were identified in reduced sensitive isolates. This study underscores the

potential usefulness for fluazinam and tebuconazole for bitter rot control of apple and provides the genetic basis for studies on potential resistance mechanisms.

2.2 INTRODUCTION

Apples (*Malus domestica* Borkh.) are an economically important fruit in countries with temperate climates, including large portions of southern Brazil. Four regions account for the bulk of Brazilian apple production: Santa Catarina state, Rio Grande do Sul state, Parana state, and Sao Paulo state (USDA, 2019). In 2020, Brazil produced 983,247 tons of fresh market and processing apples (FAO, 2022). According to the Brazilian Association of Apple Producers (ABPM), about 95% of fresh apples are sold to the Brazilian domestic market. A large portion of the processing apples, however, are exported in form of apple juice. In 2020, for example, Brazil exported 34,000 tons of apple juice (Companhia Nacional de Abastecimento – Conab – Relatório, 2020).

Pests and diseases must be managed to produce high quality fresh fruit and processing apples. One of the most important diseases in all Brazilian apple production areas is bitter rot caused by several *Colletotrichum* species. The symptoms of apple bitter rot on fruit start with light brown, circular lesions. As the lesions expand, conidiophores rupture the fruit epidermis, forming small tufts (Henz et al. 1992). Acervuli are produced in abundance on the mature apple fruit and generate conidia that are rain splash-dispersed from one fruit to another. The conidia germinate with sufficient moisture and optimal temperature to form appressoria. The pathogen then enters the epidermis layers to either form lesions or remains quiescent on green

apples. Bitter rot occurs in all apple-producing regions of the Brazil and fruit losses often reached 9 to 15% (ABPM, 2020).

Numerous *Colletotrichum* species cause apple bitter rot in Brazil; they are also found in apple production areas in the United States, including South Carolina, North Carolina, Wisconsin, and Ohio (Khodadadi et al. 2020; Schnabel et al. 2018). *Colletotrichum* is a cosmopolitan fungal genus comprised of more than 189 species distributed throughout the world. The *Colletotrichum* genus comprises nine major clades, small clusters, and isolated species, and each clade represents a species complex (Cannon et al. 2012). Among species complexes, *Colletotrichum acutatum* and *Colletotrichum gloesporioides* are most commonly associated with bitter rot in Brazilian apple orchards (Moreira et al. 2019; Velho et al. 2015, 2018).

Colletotrichum acutatum species complex consists of about 30 species (Damm et al. 2012), including *Colletotrichum nymphaeae* and *Colletotrichum melonis*. They are the most common species found in Parana state (Moreira et al. 2019; Gelain et al. in press), one of the most important apple producing regions in Brazil. *C. nymphaea*, *C. fioriniae*, and *C. godetiae* from *Colletotrichum acutatum* species complex and *C. fructicola*, *C. aenigma*, *C. siamense* and *C. theobromicola* from *Colletotrichum gloesporioides* species complex, are known so far to cause bitter rot on apple worldwide.

Chemical control of bitter rot in Brazil is limited to the use of methyl benzimidazole carbamate (MBC; carbendazim, thiophanate-methyl) fungicides,

captan, quinone outside inhibitor (QoI; trifloxystrobin, kresoxim-methyl, and pyraclostrobin) fungicides, and demethylation inhibitor (DMI; tebuconazole) fungicides (Agrofit – MAPA, 2019). Despite these registered products, the chemical management of apple bitter rot is challenging because only a few are effective against this disease. For instance, *C. acutatum* species complex has inherent resistance to MBCs (Chung et al., 2006). The most active fungicides are still the QoIs, but *Colletotrichum* spp. have a high risk of developing resistance to QoIs with many reports on resistance in *C. acutatum* species already published (Dowling et al., 2020).

Because of the limited number of effective fungicides for bitter rot control and documented QoI resistance, chemicals with other MOA, such as fluazinam and tebuconazole, may become more important regarding suppression of bitter rot. Tebuconazole is a demethylation inhibitor (DMI) fungicide that targets the sterol 14α-demethylase and has become an integral part of apple disease like apple scab management (Chen et al. 2018) and not registered for apple bitter rot control in the U.S. In plant and human fungal pathogens, several major mechanisms of resistance to DMIs have been described. These include: (i) target-site modification in the *CYP51* gene that result in reduced affinity of DMIs for their target (Délye et al. 1997a, b); (ii) overexpression of the *CYP51* gene (Hamamoto et al. 2000; Ma et al. 2006; Schnabel and Jones 2001); (iii) reduced accumulation of DMIs in fungal cells through up-regulation of ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters in the membrane (Hayashi et al. 2003; Nakaune et al. 1998; Palani and Lalithakumari 1999; Reimann and Deising 2005); and (iv) multiple *CYP51* genes

generating varius versions of *CYP51* proteins or reduce the fitness cost associated with the *CYP51* structure change (Ziogas and Malandrakis 2015).

Fluazinam is an aminopyridine that inhibits glucose oxidation in a dose-dependent manner, and suppresses the energy production process through direct inhibition of the adenosine triphosphate (ATP) synthetase in fungal cells (Vitoratos et al. 2014). In *Sclerotinia sclerotiorum*, Mao et al. (2018) studied *Shk-1* gene expression and speculated other MAP/Histidine kinases in osmotic signal transduction may be involved in fluazinam resistance. Moreover, amino acid alterations in *OS-1* (named *Bos-1* for *B. cinerea*) previously resulted in fludioxonil resistance in *Botrytis cinerea* (Dowling et al. 2021). Therefore, the histidine kinase gene *OS-1* may affect fluazinam sensitivity. To the best of our knowledge, there is no report of resistance to fluazinam in *Colletotrichum* spp.

In a previous study, *C. nymphaeae* isolates from Brazilian apple orchards were characterized for their in vitro efficacy to tebuconazole and fluazinam(Gelain et al. in press). Results indicated the presence of a range of sensitivity including isolates that were reduced sensitive. The objective of this study was to further characterize these isolates and to investigate molecular mechanisms of reduced sensitivity.

2.3 MATERIALS AND METHODS

2.3.1 *Colletotrichum nymphaeae* isolates.

In 2017, isolates were obtained from 'Eva' apple fruit trees in the municipalities of Lapa, Porto Amazonas, and Palmeira, in the state of Paraná, southern Brazil (Table 1). In a previous study, the isolates had been collected from apple flowers or fruitlets with a tissue-isolation method and identified as *Colletotrichum* species (Gelain et al. in press). A total of 12 isolates with specific phenotypes were selected for this study, including fluazinam sensitive isolates (n=3), fluazinam reduced sensitive isolates (n=3), tebuconazole sensitive isolates (n=3), and tebuconazole reduced-sensitive isolates (n=3). The EC_{50} values determined in (Gelain et al. in press) are also listed in table 1.

2.3.2 In vitro sensitivity to fluazinam and tebuconazole.

An in vitro assay was performed three times to confirm the sensitivity of *Colletotrichum nymphaeae* isolates to fluazinam and tebuconazole. The single spore stocks used in this study were stored under -20 °C by silica gel method for 8 months. The fungicide resistance may vary with long term silica gel cold storage method (Delcán et al. 2002). Therefore, it is necessary to determine if the resistance of *Colletotrichum nymphaeae* isolates has been changed.

The isolates were cultured on potato dextrose agar (PDA) for 8 days in the dark at 25°C (Gelain et al. in press). Mycelial disks (5 mm in diameter) from the margins of actively growing cultures were transferred onto fungicide-amended PDA in petri

dishes (90 mm) with three replications for each concentration and two agar plugs per plate located 4 cm apart and equidistantly from petri dish margins. The fungicide-amended PDA had been autoclaved and cooled to 60 °C before being amended either with fluazinam at 0.03, 0.1, 0.3, 1.0, and 3.0 μg /mL (Chen et al. 2016) or with tebuconazole at 0, 0.02, 0.1, 0.5, 2.5, and 12.5 μg /mL (Gang et al. 2015). The cultures were incubated at 25 °C for five days in darkness before the diameter of each colony was measured in two perpendicular directions. EC_{50} values were calculated by linear regression using R software (4.0.4, ec50estimator package). The means of EC_{50} values of different phenotypes were compared by one-way ANOVA (P<0.05).

2.3.3 Fungicide sensitivity assays using detached fruits.

Asymptomatic and organic 'Gala' apples of commercial maturity were purchased from a local grocery store and stored in a walk-in cooler at 4°C before experiments were conducted. Each fruit was rinsed with tap water for 15 s and then dipped in water for 30 mins to remove any organic pesticide residues from the fruit surface. All fruit were dried in the running laminar flow hood and exposed to 5 mins of ultraviolet treatment (Janisiewicz et al. 2016). The apples were randomly placed in lidded plastic boxes (40 x 20 x 20 cm), kept in place by plastic cups, and kept at 100 % relative humidity. Apples were stab-wounded with a 1 cc syringe needle to a 5 mm depth and then inoculated at the wound site with 50 μL of a 10^5/ mL spore suspension. Spore suspensions were prepared as described previously (Suzaki et al. 2011). To verify the viability of spores, a portion of the same spore suspension (50

μL) of each isolate was pipetted onto water agar medium (20 g/L) and spread out with a glass rod (Tao et al. 2019). Germination was verified 12 hrs later. Fungicide treatments were applied 12 hrs before inoculation (protective treatment) and 12 and 36 hrs after inoculation (curative treatments). Apples were sprayed with at 200 μg/ml fluazinam, 0.5 mL / L (Secure, Syngenta Crop Protection LLC, Greensboro, NC) and tebuconazole at 67.5 μg /mL, 0.15 g / L (Elite 45DF, Bayer CropScience, Leverkusen, Germany) following label recommended rate. Sterile water was applied to untreated inoculated control group apples. Experimental apples were kept at 25°C, 8 hrs light 16 hrs dark incubation procedure (Rodríguez-Pires et al. 2020). Bitter rot lesion diameters were measured in two perpendicular directions after 7 days of incubation, and the entire experiment was performed two times.

2.3.4 DNA extraction.

Genomic DNA from *C. nymphaeae* isolates were extracted following the protocol described by Chi et al. (2009) with minor modifications. The mycelium for extraction was produced on cellophane placed on PDA plates (Cassago et al. 2002). After 7 days of 25°C in dark incubation, mycelium originating from mycelial plugs was harvested from the cellophane with autoclaved toothpicks and suspended in 500 μL of DNA extraction buffer. Fungal tissue was pulverized in centrifuge tubes using the Dremel tool with plastic tips. Samples were centrifuged for 10 minutes at 5,000 rpm and the supernatant was mixed with 300 μL of isopropanol by inverting the tubes 25 times followed by centrifugation for 10 mins at 1,2000 rpm. The supernatant was

removed, and the pellet was washed with 800 µL of 70% ethanol and centrifuged for 2 mins at 1,4000 rpm. The supernatant was removed, and open-lid tubes were left in the laminar flow hood for 12 hrs (overnight) before 50 µL DNAse-free water was added to suspend the DNA.

2.3.5 Primer design and PCR amplification.

CYP51A (MK990546.1), *CYP51B* (MK990546.2), and *OS*-1 (XM_035483032.1) complete genome sequences and the NCBI primer blast online designing tool (National Center for Biotechnology Information, Bethesda, MD) was used for the design of primers specific to *Colletotrichum nymphaeae* gene sequences. All primer options were left on standard settings (product size range: 1000 to 1200 bp; primer size range: 0-30 nucleotides; GC content: 40-60%; and melting temperature range, 58-61°C). For the design of *CYP51A&B* and *OS-1* genes expression analysis, settings were switched to product size: 100 to 200 bp; 0-30 nucleotides; GC content: 40-60%; and melting temperature range 60-63°C. 19 primer pair sets were designed for *CYP51A&B* and *OS-1* genes amplification, sequencing, and overexpression study (Table 2).

PCR amplifications were done in Bio-Rad Laboratories PCR Thermal Cycler T100™ (Bio-Rad Laboratories, Hercules, CA). The *CYP51A&B* genes amplification was performed with the following procedure: initial denaturation at 94 °C for 10 mins, followed by 35 cycles at 94 °C for 40 s, 56 °C for 160s, and at 72 °C for 2 mins, with final elongation at 72 °C for 10 mins. The parameters were used to amplify *OS-1* gene fragment: 94 °C for 40 s, 55 °C for 165 s, and at 72 °C for 2 mins, with final

elongation at 72 °C for 10 mins; 94 °C for 40 s, 55 °C for 2 mins, and at 72 °C for 2 mins, with final elongation at 72 °C for 10 mins. The PCRs were performed in a final volume of 40 µL using the AccuPower® PyroHotStart Taq PCR PreMix (Bioneer, Oakland, CA) with the following reagent: 2 µL genomic DNA (50-80 ng), 2 µL forward primer (1 µM), 2 µL reverse primer (1 µM), 36 µL nuclease-free water. After the PCR program finished, all PCR product samples were purified with Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA).

Table 1. Primers used for amplification of *CYP51A, CYP51B,* and *OS-1* gene

Primers	Sequence (5'-3')	Description
CYP51A-1	TCACAGTGATTTCCTAGTGCTTAT	Amplify and sequence *CYP51A* gene fragment
CYP51A-8	GCCTTTCGCTTAGACCAAACTT	
CYP51A-2	GCACGGTACTTGACGAAGAA	Sequence *CYP51A* gene fragment
CYP51A-3	AGGTTGCTAGACTTGGCTCC	Sequence *CYP51A* gene fragment
CYP51A-4	TAGGCGGTCAAGCTGTCAAT	Sequence *CYP51A* gene fragment
CYP51A-5	GTTTGGACTTACGCAAAAGGCT	Sequence *CYP51A* gene fragment
CYP51A-6	TTGGCATCAACCATGCCCT	Sequence *CYP51A* gene fragment
CYP51A-7	GCAGAAGAGCTTTATCAAGAGCAG	Sequence *CYP51A* gene fragment
CYP51B-1	CAAAAGGAAGAAGAGCTTGCG	Amplify and sequence *CYP51B* gene fragment
CYP51B-8	TCTGCACTGGTTGGTGGAT	
CYP51B-2	ACTTCTTGGAGGAGACCCAT	Sequence *CYP51B* gene fragment
CYP51B-3	CATTAGCTTAGCTGCTGGCTG	Sequence *CYP51B* gene fragment
CYP51B-4	CAGATTGACGACGCCAGAC	Sequence *CYP51B* gene fragment
CYP51B-5	TGCGGAGGATATCTACACTGTT	Sequence *CYP51B* gene fragment
CYP51B-6	GGGCTCGGGGAAGTAGAC	Sequence *CYP51B* gene fragment
CYP51B-7	GCCAACATGATGATTGCCCT	Sequence *CYP51B* gene fragment
OS-1-1	AGCGCTGTACTTCTTACGC	Amplify *OS-1* gene fragment with primer OS-1-6 and OS-1-10, and sequence
OS-1-2	TTTTCGATTCTTTGGGCAAGC	Sequence *OS-1* gene fragment
OS-1-3	CTGCTGGTTGACCACAACTC	Sequence *OS-1* gene fragment
OS-1-4	CTTTCCAAGTACCGTCGACAC	Sequence *OS-1* gene fragment
OS-1-5	CGAGACAGGAAATCAGCAGC	Sequence *OS-1* gene fragment
OS-1-6	ACCTCTCGGGCAATCTTGG	Amplify *OS-1* gene fragment with primer OS-1-10 and sequence

OS-1-7	CTTACGATACAGGTCCGCAGT	Amplify *OS-1* gene fragment with primer OS-1-12 and sequence
OS-1-8	AGACCCAATGTCATAGACTGTTT	Sequence *OS-1* gene fragment
OS-1-9	TGTGGAATGAACTAACGGTCA	Amplify *OS-1* gene fragment with primer OS-1-16 and sequence
OS-1-10	CAGGATCGGCCTTCTTAATAGTCA	Amplify *OS-1* gene fragment with primer OS-1-1 and sequence
OS-1-11	CTGGCGAACAGTCTACTCAC	Amplify *OS-1* gene fragment with primer OS-1-16 and sequence
OS-1-12	GGTTCTTCGAGAGCAGGAATC	Amplify *OS-1* gene fragment with primer OS-1-7 and sequence
OS-1-13	CGAGACAATGCTGTCTTTGATTG	Sequence *OS-1* gene fragment
OS-1-14	GAACTATGTGACCTCAGTAGCA	Sequence *OS-1* gene fragment
OS-1-15	ATCCAGACCATTCTCAAGTGTG	Sequence *OS-1* gene fragment
OS-1-16	GAAGCTCTTAGCCACTGAAGG	Amplify *OS-1* gene fragment with primer OS-1-9 OS-1-11, and sequence
OS-1-F	AACGTCAACGCAATGGCAAA	Amplify 605 bp *OS-1* gene fragment
OS-1-R	GTCGAGATACTGCGGACCTG	

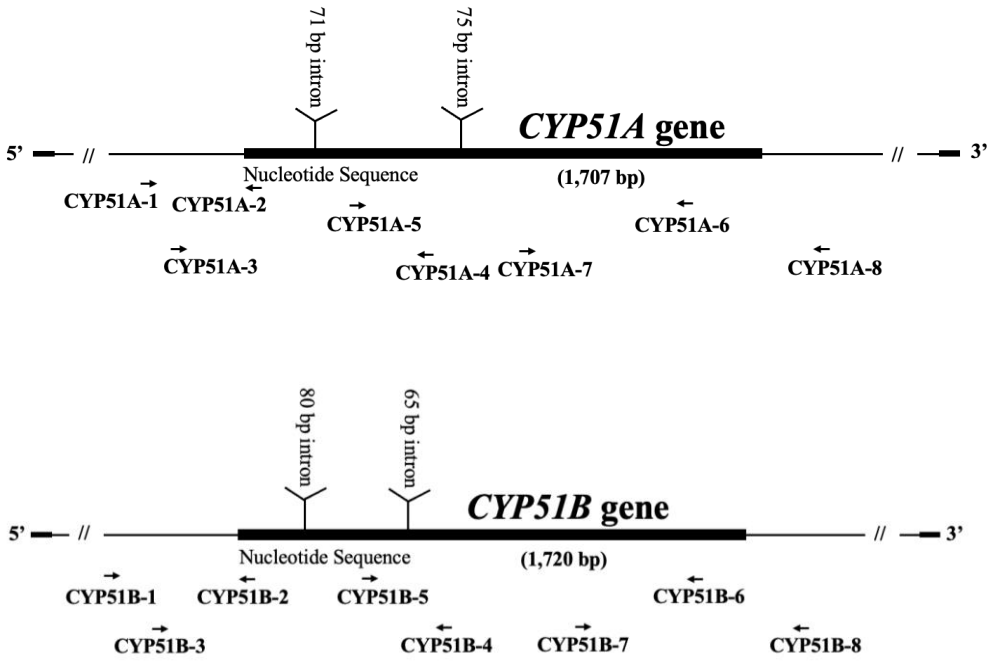

Fig 1. *CYP51A* (upper schematic) and *CYP51B* (lower schematic) gene length, intron locations, and locations of primers used in this study.

2.3.6 Sequencing analysis.

The sequences of *CYP51A&B* and *OS-1* genes were commercially sequenced using Eurofins USA service (Louisville, KY) . Gene sequences were compared and assembled using Seqman Pro (DNASTAR Inc). Coding region nucleotide sequence alignments were done with MegAlign (DNASTAR Inc). Sequence reversions were achieved with SeqBuilder Pro (DNASTAR Inc). The positions of primers and Sanger sequence reaction directions are shown in Fig 1 and Fig 2.

2.3.7 Statistical analysis.

Statistical significance was determined in JMP Pro 15 (V15; 2019 SAS Institute Inc.) by one way ANOVA, Tukey, and least significant difference tests at $P = 0.05$. R software version 4.0.4 (R Core Team, 2020) with the ec50estimator R package (v0.1.0; Alves, 2020) was used to estimate the EC_{50} values.

2.4 RESULTS

2.4.1 *Colletotrichum nymphaeae* sensitivity to fluazinam and tebuconazole.

Eleven *Colletotrichum nymphaeae* isolates collected in 2017 from Lapa, Palmeira, and Porto Amazonas, Brazil (Gelain et al. in press) were used in this study. The previously reported EC_{50} values were similar to the EC_{50} values obtained in this study for fluazinam and tebuconazole sensitive and reduced-sensitive isolates (Table 2; P=0.3). Based on the EC_{50} values, isolates of *C. nymphaeae* were categorized as Flu^{RS} (EC_{50} ranging from 0.5-0.76 mg/L), Flu^{S} (EC_{50} values ranging from 0.1-0.15

mg/L), Teb^{RS} (EC_{50} values ranging from 0.22-0.69 mg/L), and Teb^{S} (EC_{50} values ranging from 0.04-0.05 mg/L) (Table 1). The overall range and mean for fluazinam sensitive and reduced sensitive isolates were 0.10 to 0.64 mg/L and 0.32 mg/L, respectively (Table 2). For tebuconazole, the overall range and mean for sensitive and reduced-sensitive isolates were 0.04 to 0.69 mg/L and 0.24 mg/L, respectively (Table 2).

Table 2. Isolate names, fungicide sensitivity phenotype, geographical origin, year of isolation and EC_{50} values obtained before and after long-term storage of *Colletotrichum nymphaeae* isolates from Brazilian apple orchards

Isolate	**Phenotype[x]**	**Origin (Municipality)**	**Year of isolation**	**Before storage[y]**	**After storage[z]**
				EC_{50} value (mg/L)	
col136	Flu^{S}	Lapa	2017	0.12	0.10
col142	Flu^{S}	Lapa	2017	0.15	0.13
col112	Flu^{S}	Lapa	2017	0.15	0.14
col049	Flu^{RS}	Lapa	2017	0.54	0.50
col139	Flu^{RS}	Lapa	2017	0.56	0.41
col140	Flu^{RS}	Porto Amazonas	2017	0.76	0.64
col134	Teb^{S}	Palmeira	2017	0.04	0.04
col113	Teb^{S}	Lapa	2017	0.04	0.05
col077	Teb^{S}	Lapa	2017	0.04	0.05
col083	Teb^{RS}	Palmeira	2017	0.22	0.26
col139	Teb^{RS}	Lapa	2017	0.39	0.37
col012	Teb^{RS}	Lapa	2017	0.66	0.69

[x]Flu = fluazinam; Teb = tebuconazole; RS = Reduced Sensitive S = Sensitive.
[y]Determined by Gelain et al. in press.
[z]EC_{50} values after 8 months of storage on filter paper at -20°C.

2.4.2 Detached apple fruit assay.

The first lesions of apple bitter rot were observed within one week after inoculation and measured when approximately 24 mm wide in diameter. The Flu^S and Flu^R isolates were controlled effectively with the protective 12h treatment (Fig. 2). However, in the curative 12h treatment the Flu^{RS} isolates were significantly harder to control compared to the Flu^S isolates. Although not statistically significant at the 0.05 level, the control efficacy was numerically lower for the Flu^{RS} isolates in the curative 36h treatment compared to the Flu^S isolate group.

Teb^S and Teb^{RS} isolates were equally well controlled when tebuconazole was applied protectively (protective 12h treatment) and curatively when tebuconazole was applied 12 h after inoculation (curative 12h treatment). However, when tebuconazole was applied 36 h after inoculation, the Teb^{RS} isolates were significantly harder to control compared to the Teb^S isolates according to the ANOVA analysis (Fig. 2; $P \leq 0.001$).

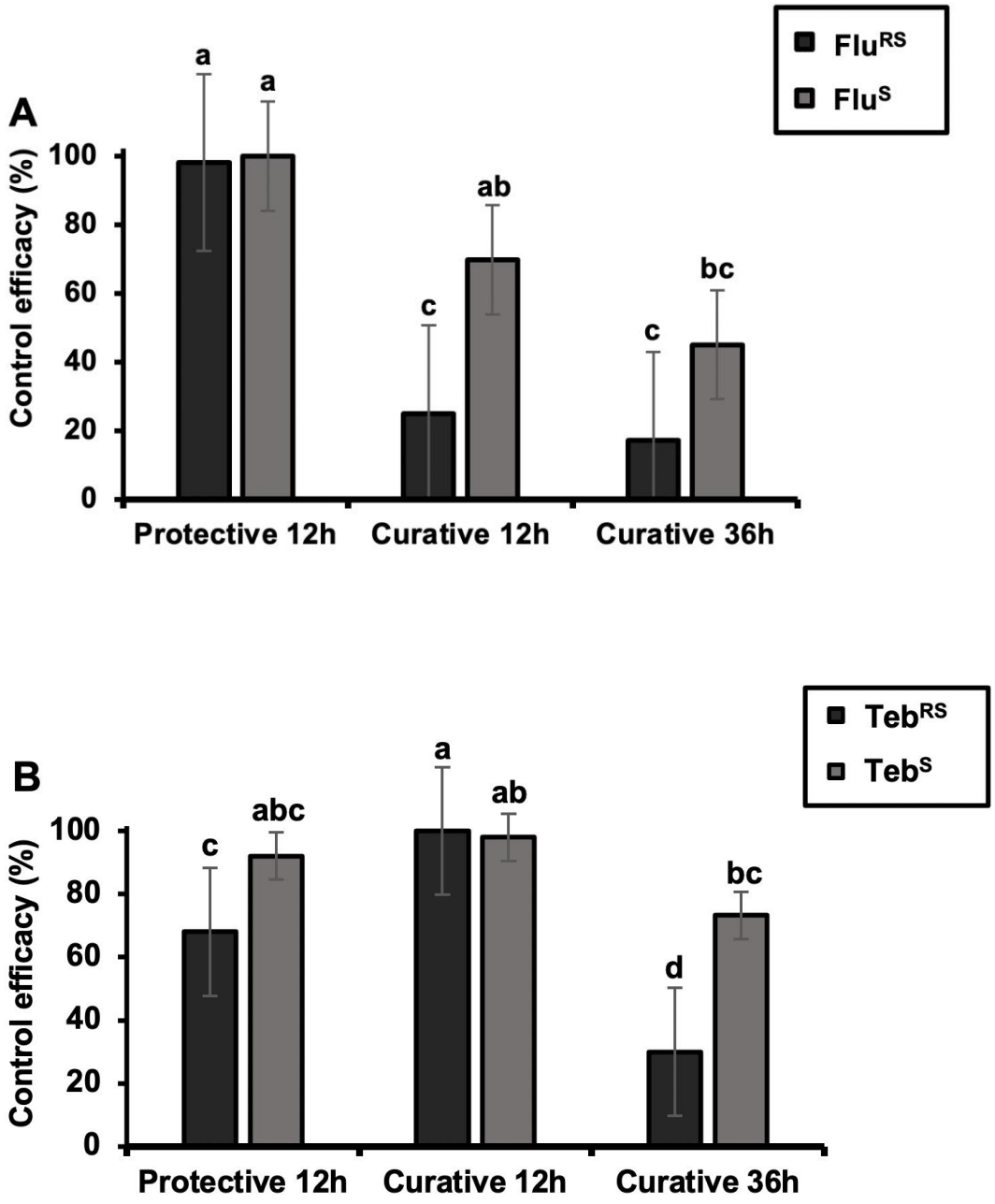

Fig 2. Protective and curative activity of (A) fluazinam (Flu) and (B) tebuconazole (Teb) against *Colletotrichum nymphaeae* isolates with different fungicide sensitivity phenotypes on detached apple fruit. Each bar represents the mean of two independent experiments with three replicates per experiment and three isolates per phenotype. ^{RS} = Reduced sensitive isolates ^{S} = Sensitive isolates. Different letters indicate

significant differences between treatments using Tukey's honest significant difference ($\alpha = 0.05$).

2.4.3 Sequencing of the *OS-1, CYP51A, and CYP51B* genes.

The complete *OS-1* gene contained 4 introns of 56 bp, 52 bp, 56 bp, and 65 bp length and 5 exons of 844bp, 33bp, 1094bp, 1503bp, and 441bp length (Fig. 3). The entire *OS-1* gene was 4,144 bp long and was sequenced for one sensitive (col136) and one reduced-sensitive (col139) isolate. Between the two genes there were 16 nucleotide variations in exons (15) and introns (1). Most of these variations were silent mutations, however, there were four that led to amino acid substitutions. They were located at positions 1336, 1348, 1379, and 1516. This variable region of the gene was further amplified with primers OS-1-F and OS-1-R (Table 1) and sequenced for two more fluazinam sensitive isolates (col142 and col112) and two more fluazinam reduced-sensitive isolates (col49 and col140). Results indicated that reduced-sensitive isolates had two amino acid substitutions (L446V, A450P/S) compared to the sensitive isolates (Fig. 3).

The length of the *CYP51A* gene was 1,707 bp and contained 3 exons and 2 introns 71 bp and 75 bp in length (Fig. 1). The *CYP51B* gene length was 1,720 bp with 2 introns of 80 bp and 65 bp in length and 3 exons (Fig. 1). No nucleotide or amino acid variations were detected in the *CYP51A* or in the *CYP51B* genes between tebuconazole sensitive isolate col134 and reduced-sensitive isolate col139.

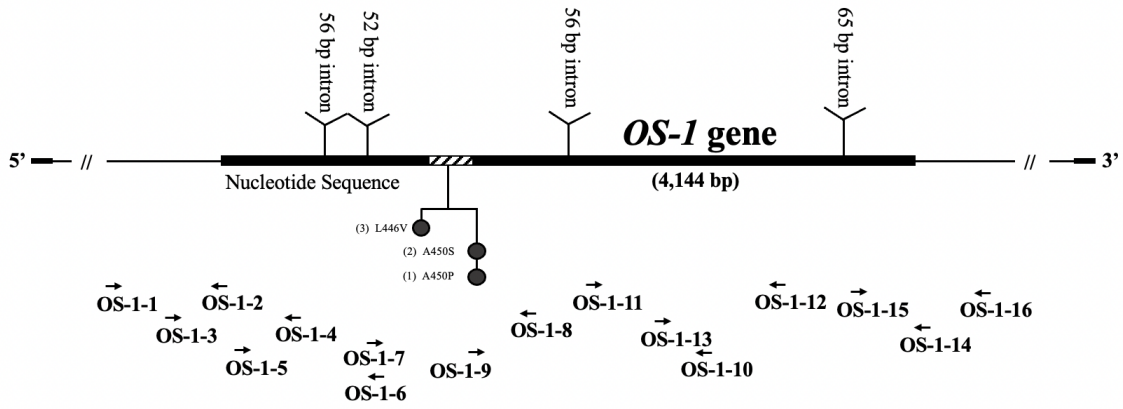

Fig 3. *OS-1* gene length, intron locations, variable nucleotide sequence locations leading to amino acid changes, and locations of primers used in this study.

2.5 DISCUSSION

Detached fruit assays show differential efficacy of fluazinam against sensitive and reduced sensitive *C. nymphaeae* isolates. Like many other fungicides, fluazinam is used as a protectant and applied in the field prior to infection events. Our study shows that in detached fruit assays protective treatments achieved high efficacy against both fluazinam phenotypes suggesting that field applications may be effective against fluazinam sensitive and reduced-sensitive phenotypes. However, our study also showed weaknesses of curative applications, which did not control reduced-sensitive isolates as well as sensitive isolates. Curative applications of fungicides are sometimes used to prevent disease progression in the field after extensive rainfalls may have washed off previous fungicide residues on the fruit or leaf tissues. In the field, such performance weaknesses would lead to faster relative growth of reduced-

sensitive strains compared to sensitive strains and thus lead to greater speed of selection for resistant isolates (Neve P. and Powles S. 2005).

Detached fruit assays also show differential efficacy of tebuconazole against sensitive and reduced-sensitive *C. nymphaeae* isolates. Tebuconazole is a fungicide that can be used for both preventative and curative control of fungal diseases because it kills fungi on the surface of the plant tissue and because it is systemic, killing fungi that have already penetrated the plant epidermis. Results of this study showed that protective and 12h curative treatments had high efficacy against both sensitive and reduced-sensitive phenotypes suggesting that typical preventative applications in orchards would be effective against strains with multiple tebuconazole sensitivity phenotypes. However, our research showed weaknesses of 36h curative applications, which indicated that Teb^{RS} isolates may survive better such treatments in the field. This may lead to greater speed selection of reduced sensitive strains compared to sensitive strains.

The reduced sensitivity to fluazinam and tebuconazole was stable after 8 months of long-term storage, indicating there must be a molecular basis for the phenotypes. Reduced sensitivity and resistance to fluazinam has been described in field isolates of *Botrytis cinerea* from Japanese bean fields (Tamura et al. 2000) and in *Phytophthora infestans* from Dutch potato fields (Schepers et al. 2018), respectively. However, no information on the stability of reduced sensitivity or resistance was reported. Various studies have been performed to study DMI fungicide resistance. Reduced sensitivity

to flutriafol in *Cercospora beticola* remained stable after 5 months in storage (Karaoglanidis and Thanassoulopoulos 2002) and flusilazole resistance in *Venturia inaequalis* was found to be stable over 6 months of consecutive transfers but became unstable after 10 months (Koeller et al. 1991). Decreased resistance to flusilazole was reported for *V. inaequalis* isolates after 7 months of cold storage and in *Monilinia fructicola* isolates with reduced sensitivity to propiconazole after 34 months of cold storage (Cox et al. 2007).

Tebuconazole is registered for apple bitter rot control in Brazil, but not commonly used for this purpose; fluazinam is registered only for other diseases of apple (USDA, 2019). None of them is recommended for apple bitte rot control in the USA. The lack of effective fungicides and current resistance problems with existing fungicides (i.e. quionone outside inhibitors) may make it more difficult in the future to control bitter rot in commercial apple fields production. The availability of additional options with the ability to at least suppress the disease may increase bitter rot control success and may also extend the productive life of existing products if they are not used as often.

The molecular mechanisms of resistance or reduced sensitivity to fluazinam are not yet known, but some studies suggest involvement of MAP/Histidine kinase genes. Previous research showed the possible MAP/Histidine kinase *Shk-1* gene, involved in osmotic signal transduction, involvement in fluazinam resistance (Mao et al. 2018). Specifically, the authors found the *Shk-1* gene overexpressed in some but not all

Sclerotinia sclerotiorum isolates from oilseed rape resistant to fluazinam (Mao et al. 2018). The sequencing of the *Shk-1* homologue of *C. nymphaeae* reported in this study provides the molecular basis for further examination of expression patterns in isolates with reduced sensitivity to fluazinam. Although point mutations in MAP/Histidine Kinase genes have not been found to confer resistance to fluazinam, our study provides preliminary evidence in form of three amino acid variations between sensitive and reduced sensitive isolates. More studies are warranted to confirm the relationship between these amino acid alterations and the reduced-sensitivity phenotype.

In summary, *C. nymphaeae* from apples appear to have natural resistance (reduced sensitivity) to fluazinam and tebuconazole. Fluazinam and tebuconazole inhibited mycelial growth of *C. nymphaeae* in vitro and was as effective in reducing apple bitter rot development on detached fruits. The apple growers will benefit from fluazinam and tebuconazole application and registration. With limited number of fungicides available for apple bitter rot management and the risk of fungicide resistance selection, further studies with other alternative fungicides are still necessary.

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