

**UNIVERSIDADE FEDERAL DO PARANÁ**

**PAULO DOS SANTOS FARIA LICHTENBERG**

**DYNAMICS AND STABILITY OF RESISTANCE TO TEBUCONAZOLE IN  
*Monilinia fructicola* POPULATIONS FROM BRAZILIAN PEACH ORCHARDS**

**CURITIBA**

**2015**

**PAULO DOS SANTOS FARIA LICHTENBERG**

**DYNAMICS AND STABILITY OF RESISTANCE TO TEBUCONAZOLE IN  
*Monilinia fructicola* POPULATIONS FROM BRAZILIAN PEACH ORCHARDS**

Thesis presented to the Agronomy Post-Graduate Program, Crop Science Field Study, Department of Crop Protection, Universidade Federal do Paraná, as requirement to obtain the title of Doctor in Science.

**Advisor: Prof. PhD. Louise Larissa May De Mio**

**Co-advisor: Prof. PhD Walmes M. Zeviani**

**Co-advisor: Prof. PhD Themis J. Michailides**

**CURITIBA**

**2015**

L699 Lichtemberg, Paulo dos Santos Faria  
Dynamics and stability of resistance to tebuconazole in *Monilinia fructicola* populations from brazilian peach orchards. / Paulo dos Santos Faria Lichtemberg. – Curitiba : 2015.  
106 f. Il.

Orientadora: Louise Larissa May de Mlo.  
Tese (Doutorado) – Universidade Federal do Paraná.  
Setor de Ciências Agrárias. Programa de Pós-Graduação em Agronomia – Produção Vegetal.

1. Pêssego – Doenças e pragas. 2. Fungicidas. I. May de Mlo, Louise Larissa. II. Universidade Federal do Paraná. Setor de Ciências Agrárias. Programa de Pós-Graduação em Agronomia – Produção Vegetal. III. Título.

CDU 632:634.25



UNIVERSIDADE FEDERAL DO PARANÁ  
SETOR DE CIÊNCIAS AGRÁRIAS  
PROGRAMA DE PÓS-GRADUAÇÃO EM  
AGRONOMIA - PRODUÇÃO VEGETAL

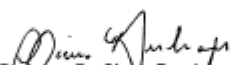


## PARECER

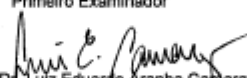
Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Agronomia - Produção Vegetal, reuniram-se para realizar a arguição da Tese de DOUTORADO, apresentada pelo candidato **PAULO DOS SANTOS FARIA LICHTENBERG**, sob o título "**DYNAMICS AND STABILITY OF RESISTANCE TO TEBUCONAZOLE IN *Monilinia fructicola* POPULATIONS FROM BRAZILIAN PEACH ORCHARDS**", para obtenção do grau de Doutor em Ciências do Programa de Pós-Graduação em Agronomia - Produção Vegetal do Setor de Ciências Agrárias da Universidade Federal do Paraná.


Após haver analisado o referido trabalho e argüido o candidato são de parecer pela **"APROVAÇÃO"** da Tese.

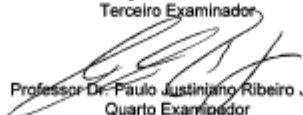
Curitiba, 23 de Março de 2015.

  
Professor Dr. Cícero Deschamps  
Coordenador do Programa

  
Professor Dr. Paulo Cezar Ceresini  
Primeiro Examinador

  
Professor Dr. Luiz Eduardo Aranha Cakobargo  
Segundo Examinador

  
Dr. Wagner Vicente Pereira  
Terceiro Examinador

  
Professor Dr. Paulo Justiniano Ribeiro Junior  
Quarto Examinador

  
Professora Dra. Louise Larissa May De Mio  
Presidente da Banca e Orientadora

Dedicated to Lindaura Melo Lichtenberg and Élia Francisca dos Santos Faria.

## ACKNOWLEDGEMENTS

To my parents and siblings for their unconditional support.

To Professors Larissa Louise May De Mio and Themis J. Michailides for all the great opportunities offered to me, their trust and guidance.

To Professor Walmes M. Zeviani, for sharing his knowledge with me and for all the online sessions that made this work possible.

To Lucimara Antunes, for all your collaboration during the doctorate.

To all Professors from the Agronomy Post-Graduate Program. To Professor Henrique Soares Koehler for offering an internship to teach in his subject.

Capes/CapesReuni for the funding that allowed this work to be done.

To Fernando Ramos and Karla Kudlawiec for the great assistance in all experiments performed.

To all Lemid personnel, I enjoyed these years shared with you, this is a memory that will stay with me. I wish success for all of us.

To the UC Kearney personnel, David, Dan, Mark, Loureen, Alejandro, Michael, Joel, Marcie, Diana, and specially Ryan Puckett for all reviews on my manuscripts and for his friendship.

Rodrigo Moriguti, Bruno Santana, and Patricia Barroso, for being great friends and for helping me on many of my decisions during this doctorate program.

To Lidia Lichtenberg and Hemerson Bassetto, for the amazing friendship, shelter and dinners.

To the visited peach farmers from southern Brazil, who were great collaborators on this work.

To my wife, Yunuen Belmonte my special thanks for the long hours of work in the laboratory, mounting evaluating and dismounting trials. For her support and encouragement particularly in the moments when it seemed that nothing was going right, and for loving everything we do together.

## ABSTRACT

The most widely used fungicides for the management of brown rot disease on peaches are those belonging to the demethylation inhibitor (DMI) chemical group. Their efficacy may be reduced in areas where rapid selection of resistant genotypes has occurred. The increased resistance of fungi populations to tebuconazole is of particular concern in areas with conditions conducive for disease development such as those found in Brazil. However, the dynamics of resistance development and how this process develops genetically in *Monilinia fructicola* isolates from Brazil remain unclear. *M. fructicola* is an important and high risk pathogen prompt to develop resistance to DMIs. Furthermore, little is known as to whether *M. fructicola* resistance to tebuconazole can be slowed, stopped, or reversed. Studies using isolates collected from commercial peach orchards from São Paulo, Paraná and Rio Grande do Sul revealed the resistance instability after a few disease cycles *in vivo*. In the absence of tebuconazole, sensitivity tends to be restored as sensitive isolates are more fit and competitive than resistant isolates. These disadvantages may be attributed to the lower fitness observed for resistant isolates. The resistance instability study is supportive of the *M. fructicola* shift toward sensitivity found in the current survey. An increase of fungicide alternation with quinone outside inhibitors, iminoctadine tris (albesilate), and iprodione in São Paulo and Rio Grande do Sul may have reduced the selection for DMI resistant isolates since the previous survey, and may be part of an important strategy for fungicide management. Concerning the molecular mechanism conferring DMI resistance for *M. fructicola* isolates from Brazil, it was observed that the relative expression of the *MfCYP51* gene was greater for the resistant isolate group than the sensitive group. It was also noticed that by inducing the gene expression with sub-lethal doses of tebuconazole, six out of seven resistant isolates increased their *MfCYP51* gene expression. The 65-bp insert located at the upstream regions of *MfCYP51* gene is absent from Brazilian isolates and it remains unknown as to what triggers the resistance mechanism for expression of the *MfCYP51* gene.

Key words: brown rot, demethylation inhibitor fungicides, disease management, DMI, population, sensitivity.

## RESUMO

O fungicida mais utilizado mundialmente no manejo da podridão parda em pêssago é pertencente ao grupo químico dos inibidores da demetilação (IDM). Sua eficácia é reduzida em áreas onde existe maior número de genótipos resistentes. O aumento de populações de isolados resistentes é um sério problema para as áreas produtivas que apresentam condições favoráveis ao desenvolvimento de doenças, como encontrado no Brasil. Entretanto, as dinâmicas do desenvolvimento da resistência e como estes processos se desenvolvem geneticamente, em isolados de *M. fructicola* no Brasil ainda não foram esclarecidas. *M. fructicola* é um importante patógeno com alto risco para o surgimento de isolados resistentes aos IDMs. Além disso, pouco se sabe sobre quando a resistência de *M. fructicola* ao tebuconazol pode ser diminuída, parada ou revertida. Estudos utilizando isolados coletados em pomares comerciais de São Paulo, Paraná e Rio Grande do Sul revelaram a instabilidade da resistência após poucos ciclos da doença *in vivo*. Na ausência de tebuconazol a sensibilidade tende a ser restaurada pois isolados sensíveis são mais adaptados e competitivos que isolados resistentes. Esta desvantagem devem ser atribuídas à menor adaptabilidade observada nos isolados resistentes. O estudo da instabilidade da resistência corrobora o movimento em direção à sensibilidade de isolados de *M. fructicola* obtido no levantamento atual. O aumento do uso intercalado dos inibidores da quinona, iminotadina tris (abesilate) e iprodiona em São Paulo e Rio Grande do Sul deve ter influenciado na redução de seleção para isolados resistentes aos inibidores da demetilação desde o levantamento anterior, tendo grande importância como estratégia no uso de fungicida. Em relação ao mecanismo molecular que confere resistência aos isolados de *M. fructicola* no Brasil, foi observado que a expressão relativa do gene *MfCYP51* entre grupos de sensibilidade de isolados resistentes e sensíveis foi superior no primeiro grupo. Também foi observado que a indução da expressão gênica com doses sub-letais de tebuconazole resultou no aumento na expressão do gene *MfCYP51* em seis de sete isolados resistentes. A inserção de 65-pb na região montante do gene *MfCYP51* é ausente em isolados do Brasil e a razão da regulação da expressão do gene *MfCYP51* é ainda desconhecida para isolados do Brasil.

Palavras-chave: fungicidas inibidores da demetilação, IDM, manejo de doenças, podridão parda, população, sensibilidade.

## LIST OF FIGURES

### 2. LITERATURE REVIEW

- FIGURE 1. Main areas of peach production in Brazil. Source: Region 1, 2 and 3 = Rio Grande do Sul (RS). Region 4 and 7 = Santa Catarina (SC). Region 5 and 6 = Paraná (PR). Region 8 and 9 = São Paulo (SP). Region 10 = Minas Gerais (MG). Source: Garrido et al. (2011). ..... 24
- FIGURE 2. Peach production, area and yield in Brazil. Source: SEAB (2013). Graphic representation: the author (2014). ..... 25
- FIGURE 3. Blossom blight (A) and brown rot/fruit decay (B) caused by *Monilinia* species in peaches. Photos: Themis J. Michailides (A) and Paulo S. F. Lichtemberg (B). ..... 26
- FIGURE 4. Disease cycle of brown rot of peach caused by *Monilinia fructicola*. Source: Adaskaveg et al. (2008). ..... 27
- FIGURE 5. Temperature, relative humidity, and rainfall of main peach production areas in subtropical (RS, PR, SC) and temperate climate (CA). RS = Rio Grande do Sul (Pelotas and Bento Golçaves). PR = Paraná (Curitiba and Lapa). SP = São Paulo (Jundiaí). CA = California (Parlier and Modesto). Dark grey = Summer, light grey = winter. Source: CIMIS (2011) and INMET (2014). Graphic representation: the author (2014). ..... 28
- FIGURE 6. Diagram showing the unimodal distributions of degree of sensitivity, characteristic of the multi-step patten of resistance development. Solid line = density distribution of baseline fungal population. Dashed line: initial population shift under the selective fungicide pressure toward lowered sensitivity. Source: Brent and Hollomon (2007a). ..... 29

### 3. CHAPTER I – SURVEY OF *Monilinia fructicola* SENSITIVITY TO TEBUCONAZOLE IN BRAZIL REVEALS A REVERSE SHIFT TOWARDS RESISTENCE

- FIGURE 1. Frequency of *Monilinia fructicola* sensitivity to tebuconazole for isolates collected between 2009 and 2011, using the relative growth under the discriminatory dose of 0.3 µg/ml method (A), the EC<sub>50</sub> value using 0.3 µg/ml as discriminatory dose (B), and the EC<sub>50</sub> value using the baseline value of 0.046 µg/ml as discriminatory dose. SP = São Paulo, PR = Paraná, and RS = Rio Grande do Sul, R = Resistant, S = Sensitive, entire values correspond to the sample size and values in between brackets correspond to percentage [n (%)]. ..... 42
- FIGURE 2. *Monilinia fructicola* density distribution (A) and cumulative relative frequency (B) for tebuconazole effective concentration for 50% population inhibition

(EC<sub>50</sub>) in Brazil, comparing baseline (BL, 2000-2004), historic (HP, 2005-2008) and current (CP, 2009-2011) populations. .... 43

FIGURE 3. *Monilinia fructicola* density distribution (A) and cumulative relative frequency (B) for tebuconazole effective concentration for 50% population inhibition (EC<sub>50</sub>) in Brazil, comparing origins within the actual population (2009-2011). SP = São Paulo, PR = Paraná, RS = Rio Grande do Sul. .... 44

**4. CHAPTER II – INSTABILITY OF RESISTANCE TO TEBUCONAZOLE OF *Monilinia fructicola* ISOLATES AND COMPETITIVE DISADVANTAGE OF RESISTANT GENOTYPES CAN FAVOR BROWN ROT MANAGEMENT IN BRAZIL**

FIGURE 1. Relation between the incubation period and the difference of latent and incubation period of *Monilinia fructicola* isolates..... 72

FIGURE 2. Relation between fitness variables and the EC<sub>50</sub> value of individual isolates of *Monilinia fructicola*. Experiment *in vitro*: Germination (A), sporulation (B), and Area under mycelial growth curve (C). Experiment *in vivo*: Germination (D), sporulation (E), and lesion size (F)..... 73

FIGURE 3. The frequency of resistant (R) and sensitive (S) spores of *Monilinia fructicola* after nine consecutive transfers *in vitro* (A, B, and C) and five consecutive transfers *in vivo* (C, D, and E). SP = São Paulo, PR = Paraná, RS = Rio Grande do Sul. Ratio R:S spores of 70:30 (A, D), 50:50 (B, E), and 30:70 (C, F). ... 74

**5. CHAPTER III – IN BRAZIL THE EXPRESSION OF *MfCYP51* GENE CONFERS RESISTANCE FOR TEBUCONAZOLE IN *Monilinia fructicola* ISOLATES OBTAINED FROM ORCHARDS WITH HIGH PRESSURE OF FUNGICIDE SELECTION**

FIGURE 1. Analysis of undigested (202, 204, 205, 208, 210, 212, 213, 316, 340, 346, 10, 21, 35, 39, 110, 112, 115, 122, 126, 152) and *Bsr*BI digested (Bmpc7). PCR fragments of 376-bp were amplified from *Monilinia fructicola* DMI-resistant and DMI-sensitive isolates with primers INS65-F and INS65-R. Ladder – 100-bp. Bmpc7 is DNA of resistant isolate used as reference. Lanes 202 to 346 and 10 to 152 are codes for each isolate as described in Table 1..... 94

FIGURE 2. The sequencing of 65-bp genetic ‘Mona’ element in the upstream region of *MfCYP51* in Brazilian isolates. Bmpc7 is DNA of resistant isolate used as reference. EU035301.1 is accession numbers available in GenBank to Bmpc7 ..... 94

## LIST OF TABLES

### 3. CHAPTER I – SURVEY OF *Monilinia fructicola* SENSITIVITY TO TEBUCONAZOLE IN BRAZIL REVEALS A REVERSE SHIFT TOWARDS RESISTENCE

TABLE 1.	List of <i>Monilinia fructicola</i> isolates collected in Brazil for tebuconazole resistance studies.....	45
TABLE 2.	Comparison of tebuconazole effective concentration value for 50% population inhibition (EC <sub>50</sub> value) of <i>Monilinia fructicola</i> isolates from three different populations in Brazil. ....	52
TABLE 3.	Comparison of tebuconazole effective concentration value for 50% population inhibition (EC <sub>50</sub> value) of <i>Monilinia fructicola</i> isolates collected between 2009-2011 in São Paulo (SP), Paraná (PR) and Rio Grande dos Sul (RS). ....	53
TABLE 4.	Mean of the tebuconazole sensitivity differences for <i>Monilinia fructicola</i> storage. ....	54

### 4. CHAPTER II – INSTABILITY OF RESISTANCE TO TEBUCONAZOLE OF *Monilinia fructicola* ISOLATES AND COMPETITIVE DISADVANTAGE OF RESISTANT GENOTYPES CAN FAVOR BROWN ROT MANAGEMENT IN BRAZIL

TABLE 1.	List of <i>Monilinia fructicola</i> isolates used to determine the sensitivity instability and competition capacity of resistant and sensitive phenotypes. ....	75
TABLE 2.	<i>Monilinia fructicola</i> EC <sub>50</sub> value after successive transfers in the absence of tebuconazole. ....	76
TABLE 3.	Transfer and sensitivity effect in <i>Monilinia fructicola</i> fitness variables <i>in vitro</i> . ....	77
TABLE 4.	Area under the mycelial growth curve of <i>Monilinia fructicola</i> isolates resistant and sensitive for tebuconazole.....	78
TABLE 5.	Transfer and sensitivity effect in <i>Monilinia fructicola</i> fitness variables <i>in vivo</i> . ....	79

### 5. CHAPTER III – IN BRAZIL THE EXPRESSION OF *MfCYP51* GENE CONFERS RESISTANCE FOR TEBUCONAZOLE IN *Monilinia fructicola* ISOLATES OBTAINED FROM ORCHARDS WITH HIGH PRESSURE OF FUNGICIDE SELECTION

TABLE 1.	<i>Monilinia fructicola</i> isolates used in the molecular experiments. ....	95
TABLE 2.	Nucleotide sequence and characteristics of primers used in this study. ....	96
TABLE 3.	Relative expression of the <i>MfABC1</i> gene in <i>Monilinia fructicola</i> groups of sensitivity.....	97
TABLE 4.	Relative expression of the gene <i>MfCYP51</i> in <i>Monilinia fructicola</i> groups of sensitivity.....	98

TABLE 5.	Relative expression of the <i>MfABC1</i> and <i>MfCYP51</i> genes in <i>Monilinia fructicola</i> mycelia untreated and treated with different concentration of tebuconazole.....	99
----------	--	----

## SUMMARY

1. GENERAL INTRODUCTION.....	15
2. LITERATURE REVIEW .....	18
2.1. PEACH ECONOMIC ASPECTS IN BRAZIL.....	18
2.2. BROWN ROT DISEASE .....	18
2.3. SYMPTOMS .....	19
2.4. DISEASE CYCLE AND EPIDEMIOLOGY .....	20
2.5. DISEASE CONTROL .....	20
2.6. DMI RESISTANCE .....	21
2.7. PERSPECTIVES ON <i>M. fructicola</i> STUDIES.....	22
3. CHAPTER I – SURVEY OF <i>Monilinia fructicola</i> SENSITIVITY TO TEBUCONAZOLE IN BRAZIL REVEALS A REVERSE SHIFT TOWARDS RESISTENCE .....	30
3.1. ABSTRACT .....	30
3.2. RESUMO .....	31
3.3. INTRODUCTION.....	32
3.4. MATERIAL AND METHODS .....	32
3.4.1. Isolate set and fungicide stock solution.....	33
3.4.2. Frequency of resistance .....	33
3.4.3. Density distribution of sensitivity .....	34
3.4.4. Storage sensitivity instability.....	34
3.4.5. Data analysis .....	35
3.5. RESULT.....	35
3.5.1. Frequency of resistance .....	35
3.5.2. Density distribution of sensitivity .....	36
3.5.3. Storage sensitivity instability.....	36
3.6. DISCUSSION .....	37
3.7. CONCLUSIONS .....	39
3.8. ACKNOWLEDGEMENTS .....	39
3.9. REFERENCES .....	39

4. CHAPTER II – INSTABILITY OF RESISTANCE TO TEBUCONAZOLE OF <i>Monilinia fructicola</i> ISOLATES AND COMPETITIVE DISADVANTAGE OF RESISTANT GENOTYPES CAN FAVOR BROWN ROT MANAGEMENT IN BRAZIL .....	55
4.1. ABSTRACT .....	55
4.2. RESUMO .....	56
4.3. INTRODUCTION .....	57
4.4. MATERIALS AND METHODS .....	58
4.4.1. Isolate set .....	58
4.4.2. Successive transfer effect on isolate sensitivity and fitness .....	58
4.4.3. Competitive ability of resistant and sensitive isolates .....	60
4.4.4. Data analysis .....	61
4.5. RESULTS .....	62
4.5.1. Successive transfer effect on isolate sensitivity .....	62
4.5.2. Effect of successive medium transfers on fitness .....	62
4.5.3. Effect of successive fruit transfers on fitness .....	63
4.5.4. Competitive ability of resistant and sensitive isolates .....	64
4.6. DISCUSSION .....	64
4.7. CONCLUSIONS .....	68
4.8. ACKNOWLEDGEMENTS .....	68
4.9. REFERENCES .....	68
5. CHAPTER III – IN BRAZIL THE EXPRESSION OF <i>MfCYP51</i> GENE CONFERS RESISTANCE FOR TEBUCONAZOLE IN <i>Monilinia fructicola</i> ISOLATES OBTAINED FROM ORCHARDS WITH HIGH PRESSURE OF FUNGICIDE SELECTION.....	80
5.1. ABSTRACT .....	80
5.2. RESUMO .....	81
5.3. INTRODUCTION .....	82
5.4. MATERIAL AND METHODS .....	82
5.4.1. Isolates .....	83
5.4.2. ‘Mona’ element identification .....	83
5.4.3. Mycelium preparation for relative expression experiments .....	84
5.4.4. RNA extraction, cDNA synthesis and PCR amplification .....	85
5.4.5. Expression quantification of <i>MfCYP51</i> and <i>MfABC1</i> genes .....	86
5.5. RESULTS .....	87
5.5.1. ‘Mona’ element identification .....	87

5.5.2. Amplification and sequencing of <i>MfCYP51</i> and <i>MfABC1</i> genes. ....	87
5.5.3. Expression of the <i>MfCYP51</i> and <i>MfABC1</i> genes. ....	87
5.6. DISCUSSION .....	88
5.7. CONCLUSION .....	90
5.8. ACKNOWLEDGEMENT .....	90
5.9. REFERENCES .....	91
6. GENERAL CONCLUSIONS.....	100
7. GENERAL REFERENCES .....	101

## 1. GENERAL INTRODUCTION

In Brazil, the peach tree *Prunus persica* (L.) Batsch is one of the most studied temperate crops in terms of its adaptability to subtropical conditions (ZANETTE; BIASI, 2004). This deciduous tree native to northwest China represents an important option to growers because of dual suitability for table and canned fruit as well as strong productivity (MAIDAL; RASEIRA, 2008; GARRIDO et al., 2011; MAY DE MIO et al., 2011a). Additionally, peach gross production value (GPV) ranks this crop among the most valuable crops in the country (SEAB, 2013). As a temperate crop, most of the production in Brazil is located in the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, and Minas Gerais, where temperatures favor cultivation (BIASI et al., 2004).

Among the most important limiting factors for the production of peaches in Brazil, the diseases are responsible for considerable economic losses (MAY DE MIO et al., 2008). According to Facchinello and Marodin (2004) the combination of rain, temperature, and winds in the spring and early summer is correlated to increased disease levels in southern Brazil. This was also confirmed by May De Mio et al. (2014) and Keske et al. (2011) who linked the occurrence of brown rot disease to climatic conditions. The temperature in peach production areas is usually constant, with little variation and ultimately results in more intense and uniform disease incidence (CALPOUZOS, 1962; SEQUEIRA, 1962; WELLMAN, 1962).

The brown rot disease includes blossom and twig blights in addition to pre and post-harvest fruit decay. It is mainly caused by *Monilinia fructicola* (Wint.) Honey, the most important fungal pathogen in Brazil (FORTES; MARTINS, 1998; MAY DE MIO et al., 2004) and North America (HARVEY et al., 1972; BATRA, 1991; OGAWA et al., 1995; ADASKAVEG et al., 2008). Two other species are known to cause brown rot in peaches as well: *Monilinia laxa* (Aderrh. & Ruhl.) and *Monilinia fructigena* Honey in Whetzel (BYRDE; WILLETTS, 1977; BATRA, 1991). So far, *M. fructicola* is the main brown rot pathogen in Brazil (BLEICHER, 1997; FORTES; MARTINS, 1998; MAY DE MIO et al., 2004), although *M. laxa* has been reported once in São Paulo (SOUZA et al., 2008).

Sanitation practices are important for brown rot control, aiming to reduce the primary inoculum and ultimately disease incidence. However, the best control of brown rot is achieved by spraying with protective fungicides (ADASKAVEG et al., 2008). The proper use of fungicides with some systemic activity protects flowers and fruits, reduces the amount of

sporulation formed on the infected tissue, and reduces sources of overwintering inoculum (OGAWA et al., 1995).

Demethylation inhibiting (DMI) fungicides are one of four classes that comprise the sterol biosynthesis inhibitors (SBI's) group. They inhibit the 14 $\alpha$ -demethylase enzyme in the fungal cell, interrupting the ergosterol biosynthesis, an important component of membrane fluidity and stability (JOSEPH-HORNE; HOLLOMON, 1997; SANGLARD et al., 1998). DMIs include a few different chemical groups with a broad spectrum and systemic activity against a range of economically important pathogens (BRENT, K. J.; HOLLOMON, 2007a). When introduced in early 80s, DMI fungicides replaced the benzimidazoles (ZEHR et al., 1999; CHEN et al., 2013a). Later, more effective products such as propiconazole, fenbuconazole, and tebuconazole were introduced and became the industry standard for brown rot control. In Brazil, tebuconazole is the most commonly sprayed DMI fungicide among peach growers for brown rot control (SILVA et al., 2011).

The almost exclusive use of DMI for brown rot control combined with multiple sprays applied in areas with high rainfall has resulted in a *M. fructicola* population shift towards resistance. The emergence of resistance was reported in New Zealand with triforine (ELMER et al., 1992), in the United States and Australia with propiconazole (ZEHR et al., 1999; WHERRETT et al., 2001; SCHNABEL et al., 2004; BURNETT et al., 2010; VILLANI; COX, 2011; HALBRENDT et al., 2013), and in Brazil with tebuconazole (MAY DE MIO et al., 2011b). Although, *M. fructicola* control failure associated with DMI are still rare, and have only been reported in Georgia and New York (BRANNEN et al., 2006; PARKER et al., 2006).

The emergence of resistance to DMI was considered less likely to occur due to fitness disadvantages of resistant genotypes. The gradual shift toward resistance is referred as quantitative or multistep resistance (GEORGOPOULOS, 1985; SCHEINPFLUG; KUCK, 1987; BRENT; HOLLOMON, 2007a). While new reports about *Monilinia* species resistant to DMIs are appearing, studies are focusing on practical solutions to problems of resistance such as fitness dynamics (GILPATRICK, 1981; ELMER et al., 1992; COX et al., 2007; CHEN et al., 2012) as well as documenting molecular changes that benefit resistance (SCHNABEL et al., 2003; LUO et al., 2008; LUO; SCHNABEL, 2008).

In southern Brazil, productivity remains low due to the lack of information regarding disease control and fungicide resistance management. With this in mind, research must be focused on the development of local information and techniques to support timely field decision making and to avoid or delay problems related to fungicide resistance. The objectives of this study were (i) to monitor the frequency of tebuconazole resistant isolates of *M. fructicola* in

peach producing areas of São Paulo, Paraná and Rio Grande do Sul, (ii) to determine changes in sensitivity of the current *M. fructicola* population compared to previous surveys, (iii) to determine the instability of resistance in the absence of tebuconazole fungicide, (iv) to evaluate the competitive ability of resistant versus sensitive *M. fructicola* isolates, (v) and to investigate the potential role of the 'Mona' element, and the expression of *MfCYP51* and *MfABC1* genes as molecular mechanisms conferring tebuconazole resistance in *M. fructicola* isolates in Brazil.

## 2. LITERATURE REVIEW

### 2.1. PEACH ECONOMIC ASPECTS IN BRAZIL

Most of the peach orchards in Brazil are located in the States of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, and Minas Gerais (Figure 1). In 2012, peach production was 232,987 t/year and Rio Grande do Sul accounted for 57% of the production destined for processing and fresh fruit consumption (SEAB, 2013). São Paulo, Santa Catarina, Minas Gerais and Paraná accounted for 16.2%, 10.8%, 8.6%, and 7.4% respectively (SEAB, 2013). In spite of a reduction in the production area of 21.8% over the last 10 years, productivity has increased from 8.992 t/ha to 12.163 t/ha over the same period (Figure 2). The States of São Paulo, Minas Gerais, and Santa Catarina exceed the national mean productivity with 22.401 t/ha, 21.539 t/ha, and 16.721 t/ha respectively (SEAB, 2013). The cultivation of varieties destined for fresh consumption, in conjunction with improved technical efficiency, are the main factors for the success of these regions (FACHINELLO et al., 2011).

From September through January, when Brazilian peaches are marketed, it is also possible to obtain imported fruits from Argentina, Chile, Spain, Greece and China (MAIDAL; RASEIRA, 2008). In 2013, Brazil imported 12,281 tons of unprocessed peaches (ALICEWEB2, 2014). This substantial volume of imported peaches is seen as potential for growth of national production (MARODIN et al., 2014). However, it is expected that the production area will remain stable with a gradual replacement of peaches designated for processing by those with dual purpose (FACHINELLO et al., 2011; MARODIN et al., 2014).

### 2.2. BROWN ROT DISEASE

Brown rot disease causes blossom and twig blights in addition to pre and post-harvest fruit decay (Figure 3). It is a major fungal disease of all commercially grown *Prunus* spp. in most regions of the world, and can result in extensive crop losses (BATRA, 1991). The disease is known to be caused by three species of the genus *Monilinia* which according Byrde and Willetts (1977) and Lane (2002) can be distinguished by cultural characteristics. However,

Lichtemberg et al. (2014) was not able to differentiate morphologically *Monilinia* species from Brazil recommending the molecular methods for specie identification. *Monilinia fructicola* causes serious crop losses in United States, Canada, Australia, New Zealand, Brazil and Japan; and is the main pathogen of peaches and other stone fruits in all production regions except Europe (OGAWA et al., 1995; ADASKAVEG et al., 2008). *M. fructigena* is not present in North and South America, but is present in Europe, where crop losses are lower than those caused by *M. laxa* (OGAWA et al., 1995; MAY DE MIO et al., 2004; ADASKAVEG et al., 2008). *M. laxa* causes severe blossom blight and fruit decay, and is found in Europe, South Africa, Chile, Canada and Iraq. In California, *M. laxa* is more widespread than it is in the midwestern and northeastern states, whereas it is absent in the southeastern states (ADASKAVEG et al., 2008). In China, where the three main *Monilinia* species are found (FAN et al., 2010), modern diagnostic tools have permitted the identification of two new species causing brown rot in Southeast Asia stone fruits: *Monilinia mumecola* and *Monilinia yunnanensis* (HARADA et al., 2004; HU et al., 2011).

### 2.3. SYMPTOMS

The first symptoms of blossom blight are the necrosis of anthers and browning of filaments that precede the floral tube, ovary, peduncle, and usually the twigs to which the peduncles are attached. Infected blossoms wilt, turn brown, and usually cling to the twig. Extension of the infection into the peduncle and twig results in a necrotic area in the woody tissue causing a canker. Sometimes succulent twigs and shoots become infected directly under extended periods of high humidity and warm temperatures (20 to 28°C). Under moist conditions, grayish colored sporodochia bearing conidia form on the surface of diseased blossom and twigs, serving as a new inoculum source. A gummy substance usually exudes from the cankers, causing the blighted flowers to adhere to the twig (RITCHIE, 2000).

Fruit rot is the most destructive phase in the life cycle of the disease. The first indication of fruit infection is the development of a small, superficial, and circular brown spot which gradually extends outwards. Under humid conditions, grayish masses of conidia develop in these lesions. Decay is visible within 48 hours of infection under optimum conditions (OGAWA et al., 1995; ADASKAVEG et al., 2008). Often, conidiophores are arranged in concentric rings around the source of infection and have a distinct appearance. There can be

thousands of conidia from each lesion, each capable of initiating a new infection. Diseased fruit that do not fall to the ground dehydrate and become shriveled mummies that cling to the branch (BYRDE; WILLETTS, 1977; RITCHIE, 2000).

#### 2.4. DISEASE CYCLE AND EPIDEMIOLOGY

The Brown rot fungi can survive the winter by several means (Figure 4). The mummified fruits clinging on trees, where conidia are produced on the fruit surface in the spring, represent the primary inoculum source in Brazil (MAY DE MIO et al., 2004). It can also survive on mummified fruits on the ground, producing stroma from which apothecia arise (BIGGS; NORTHOVER, 1985) and may discharge 1 million ascospores/day on average, for up to 20 days (HONG et al., 1997). Brown rot may also survive as mycelium in blossom parts, peduncles, and twigs killed by the pathogen the previous year (HOLB, 2008). In locations where the perfect stage occurs, ascospores are discharged at the time of the emergence of young shoots and blossoms of host plants (BYRDE; WILLETTS, 1977). Primary infection can also function as a source of latent infection in fruits, developing prior to harvest and during storage as confirmed by Luo and Michailides (2001), Moreira and May De Mio (2007) and Keske et al. (2011).

#### 2.5. DISEASE CONTROL

Orchard sanitation practices are important for brown rot control. During the winter, pruning is an important practice for removing mummified fruits, infected twigs, and limbs with cankers (ZEHR, 1982; BLEICHER, 1997; FORTES; MARTINS, 1998; ADASKAVEG et al., 2008). Harvey et al. (1972) mentioned that destroying or burying mummified fruits, cleaning grading and packing equipment, and destroying cull piles of fruits around orchards and packinghouses helps to decrease inoculum for future infections. Insect control is another means of avoiding dissemination (HARVEY et al., 1972). However, the most effective means for control are fungicide spray treatments (CHEN et al., 2012).

The class of fungicides known as demethylation inhibitors (DMI) have been the mainstay of producers for both pre- and post-harvest management of the brown rot pathogen since the 1980s (ZEHR et al., 1999) in the United States and later in Brazil (SILVA et al., 2011). The class of DMI fungicides offers effective results through systemic activity (BRENT; HOLLOMON, 2007b) and have brought forth a new era in disease control (NUNINGER-NEY et al., 1989). In the beginning, no more than 3 DMI fungicide applications per season were recommended in order to avoid problems relating to resistance (PRINCE et al., 1989; DE VICENZO et al., 1997). However, owing to the efficacy of DMI fungicides such as propiconazole and tebuconazole, many producers increased the number of applications to 4-6 sprays in the eastern United States and from 3-15 in Brazil, depending the production region (TIBOLA et al., 2005; MAY DE MIO et al., 2008; MAY DE MIO et al., 2011b; ZHU et al., 2012).

## 2.6. DMI RESISTANCE

In Brazil, climate conditions in the areas of peach production are conducive to high inoculum levels throughout the year; this is in contrast with locations where cold winters restrict the pathogen's population growth, as in California (Figure 5). According to Fachinello and Marodin (2004), in southern Brazil, precipitation can exceed 1,500 mm/year. Such conditions are known to increase inoculum and can lead to severe epidemics (BATRA, 1991). Consequently, fungicide pressure with a high amount of inoculum may lead to resistance build-up (BRENT; HOLLOMON, 2007a; BRENT; HOLLOMON, 2007b)

The DMIs inhibit the 14 $\alpha$ -demethylase (P450) enzyme, which is important in the fungal ergosterol biosynthesis pathway (ZHU et al., 2012). In ascomycete fungi, ergosterols and proteins are the structural components of cell membranes, and their deficiency leads to membrane disintegration, rupture, and ionic solute losses (LUPETTI et al., 2002). In Brazil, DMIs such as tebuconazole continue to be widely used to control *M. fructicola* in spite of resistance reports (MAY DE MIO et al., 2011b). The first reason is that although tebuconazole resistance was already occurring (MAY DE MIO et al., 2011b) no fungicide field failure was confirmed, and in the absence of field resistance, control is still possible by increasing the fungicide dose as suggested by GEORGOPOULOS (1985) and demonstrated by Brannen et al. (2008). Secondly, although other available alternatives such as methyl-benzimidazoles (MBC),

quinine outside inhibitors (QoIs), and dicarboximides are commonly used, these fungicides are also single-site inhibitors that have resistance issues already reported (ZEHR et al., 1991; ELMER; GAUNT, 1994; WHERRETT et al., 2001; MAY DE MIO et al., 2011b). Finally, while systemic fungicides start to show sensitivity shifting towards resistance in Brazil (MAY DE MIO et al., 2011b), the multi-site, contact fungicides are a means to alternate fungicides and avoid practical resistance, as demonstrated with guanidine and a few other fungicides by Moreira and May De Mio (2009).

DMI resistance was considered unlikely to occur due to a quantitative, or multistep resistance (Figure 6), implying that only a gradual, partial, and temporary loss of effectiveness may be observed (GEORGOPOULOS, 1985; BRENT; HOLLOMON, 2007b). However, DMI field failure for controlling brown rot was already reported in Georgia for propiconazole (BRANNEN et al., 2006) and in New York for myclobutanil and fenbuconazole (PARKER et al., 2006), highlighting the necessity to study topics related to the molecular mechanisms of resistance and resistance stability.

## 2.7. PERSPECTIVES ON *M. fructicola* STUDIES

Over the last few years, several studies were performed to elucidate the molecular mechanisms of azole resistance in fungi (JOSEPH-HORNE; HOLLOMON, 1997; GISI et al., 2000; MA; MICHAILIDES, 2005; COOLS et al., 2013). For *M. fructicola*, Luo and Schnabel (2008) found that propiconazole resistant isolates express the *MfCYP51* gene 5 to 11 times more than sensitive isolates, and interestingly, an insert called ‘Mona’ was found in the upstream region of the *MfCYP51* gene in resistant isolates. The same ‘Mona’ element, was later linked to propiconazole resistant phenotypes, revealing that overexpression of the *MfCYP51* gene is a common DMI fungicide resistance mechanism in *M. fructicola* throughout the eastern United States (LUO et al., 2008). However, Villani and Cox (2011) and Chen et al. (2013b) have demonstrated that resistance to DMIs are not always based on increased expression or mutation of the *MfCYP51* gene in the United States and China respectively, suggesting that overexpression can be triggered by means other than ‘Mona’. Other mechanisms such as the expression of the ATP-binding cassette transporter gene *MfABC1* were also examined, revealing a minor genetic determinant for DMI resistance in the United States (SCHNABEL et al., 2003; LUO; SCHNABEL, 2008).

The emergence of DMI resistant strains of *M. fructicola*, does not necessarily mean that failure to control disease will follow (GEORGOPOULOS, 1985; SCHEINPFLUG; KUCK, 1987; LINHARES; GHINI, 2001), for this would only occur if the frequency of resistant strains dominate the pathogen population (ELMER et al., 1992). For this reason, it is important to evaluate the mutant and wild type populations fitness and competitive ability to better establish fungicide management practices. So far, sparse and conflicting fitness information associated with DMIs is available for *M. fructicola* (GILPATRICK, 1981; NUNINGER-NEY et al., 1989; ELMER et al., 1992; COX et al., 2007).

Elmer et al. (1992) showed that triforine resistant isolates of *M. fructicola* in New Zealand were less virulent than sensitive isolates on untreated nectarines, but were pathogenic on triforine treated fruits. Nuninger-Ney et al. (1989) demonstrated decreased DMI resistant isolate fitness in comparison to sensitive isolates. Additionally, in the same study, the authors observed a reduced ability of resistant isolates to compete against sensitive isolates when inoculating peaches after three consecutive cycles in the absence of fungicide. On the other hand, results from Chen et al. (2013a) and Cox et al. (2007) revealed no fitness penalties for *M. fructicola* isolates resistant to propiconazole. In spite of the reported cases of DMI positive cross resistance (GILPATRICK, 1981; NUNINGER-NEY et al., 1989; PRINCE et al., 1989; BRAITHWAITE et al., 1995; HOLB; SCHNABEL, 2007), none of those fitness studies included *M. fructicola* populations from Brazil in conjunction with tebuconazole which is known as the most commonly used fungicide for brown rot control in the country (SILVA et al., 2011).

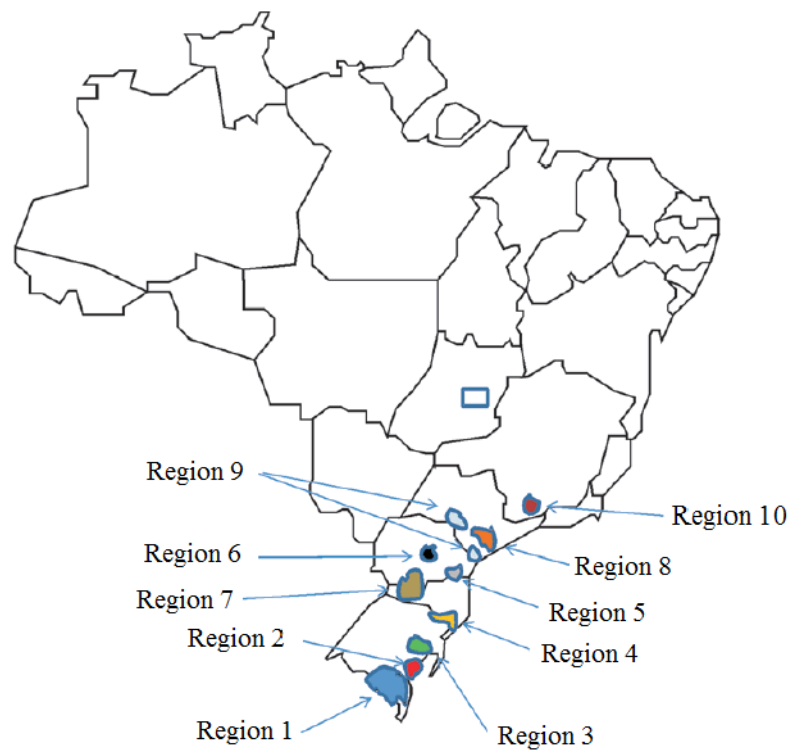


FIGURE 1. Main areas of peach production in Brazil. Source: Region 1, 2 and 3 = Rio Grande do Sul (RS). Region 4 and 7 = Santa Catarina (SC). Region 5 and 6 = Paraná (PR). Region 8 and 9 = São Paulo (SP). Region 10 = Minas Gerais (MG). Source: Garrido et al. (2011).

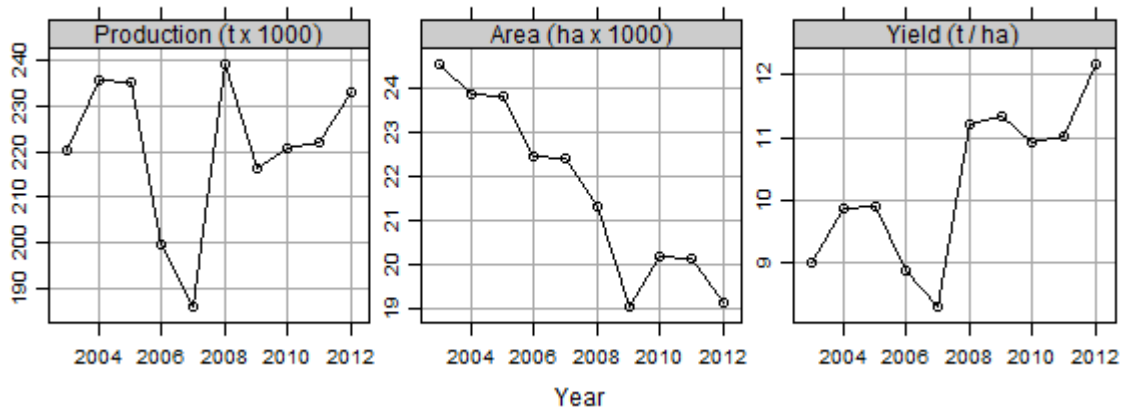


FIGURE 2. Peach production, area and yield in Brazil. Source: SEAB (2013). Graphic representation: the author (2014).

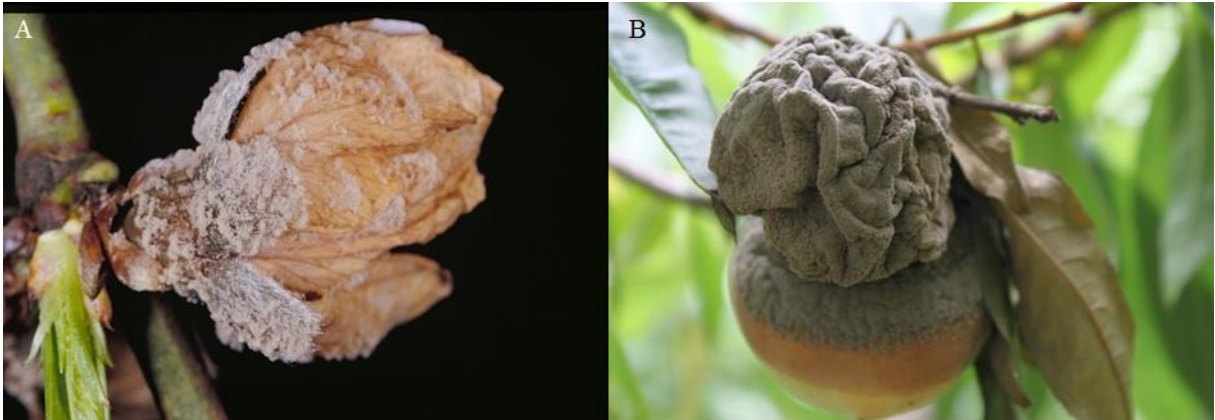


FIGURE 3. Blossom blight (A) and brown rot/fruit decay (B) caused by *Monilinia* species in peaches. Photos: Themis J. Michailides (A) and Paulo S. F. Lichtemberg (B).

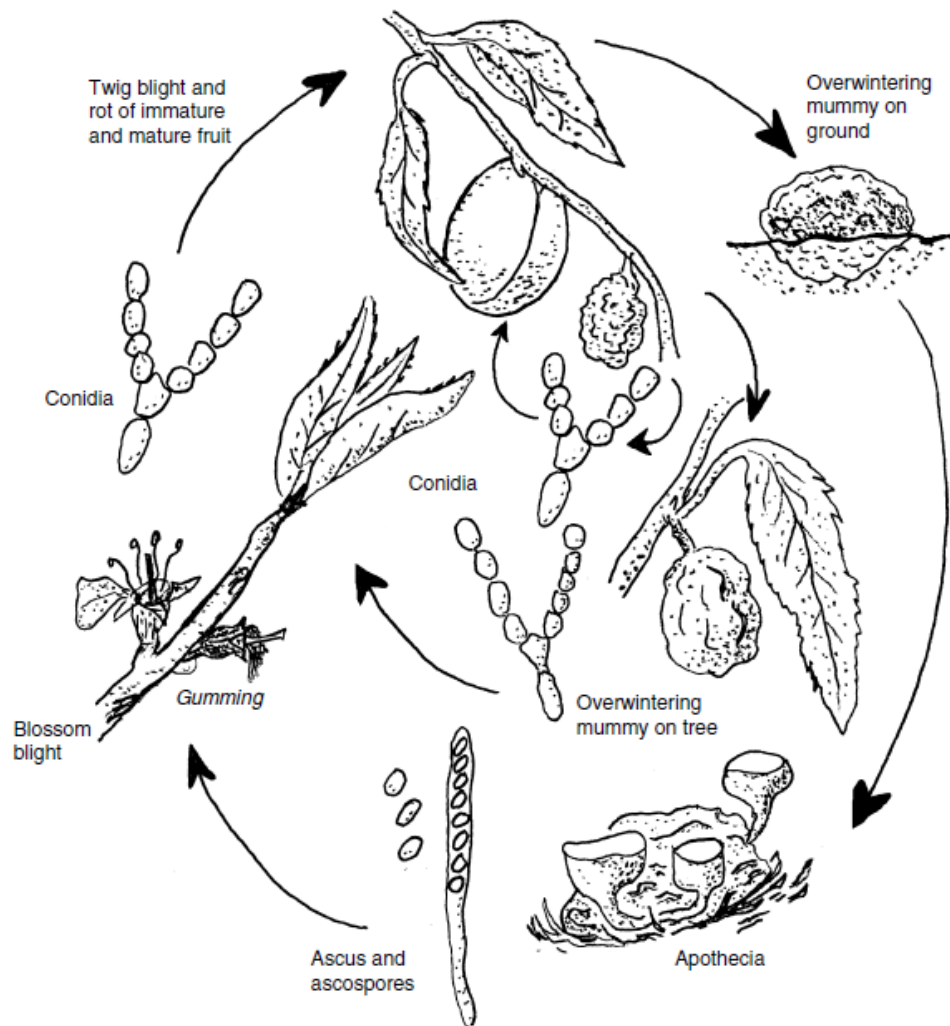


FIGURE 4. Disease cycle of brown rot of peach caused by *Monilinia fructicola*. Source: Adaskaveg et al. (2008).

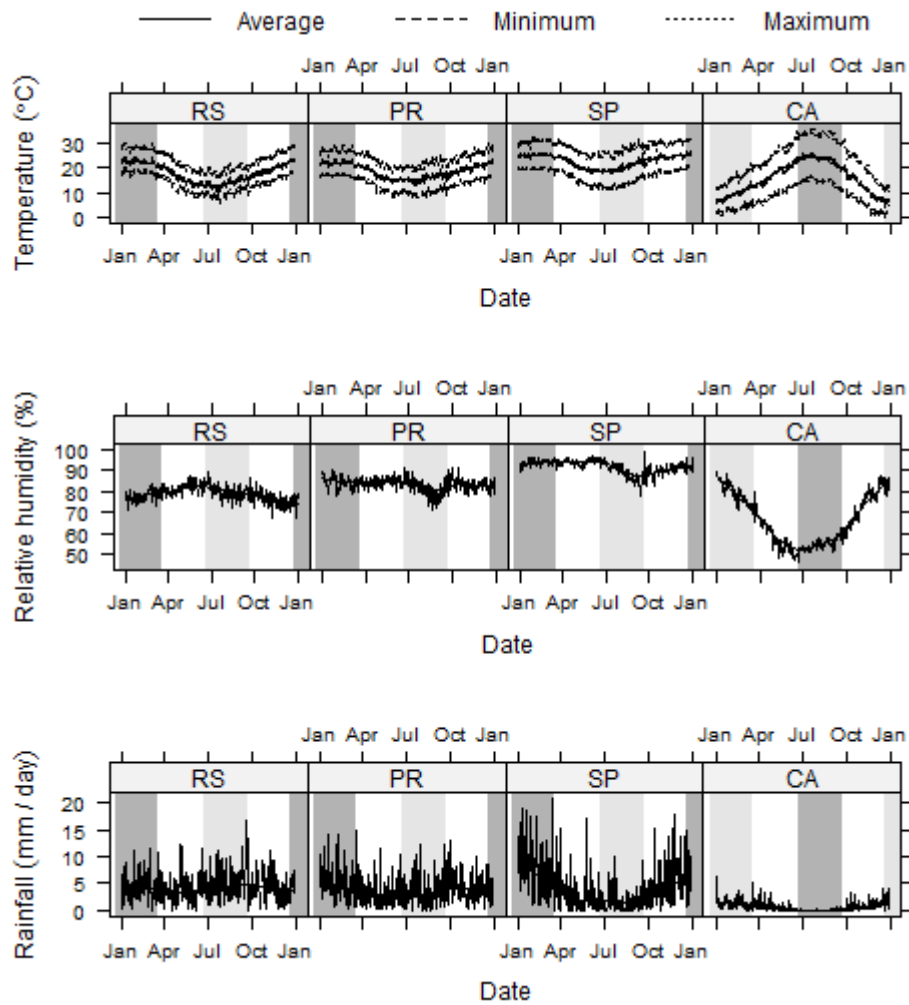


FIGURE 5. Temperature, relative humidity, and rainfall of main peach production areas in subtropical (RS, PR, SC) and temperate climate (CA). RS = Rio Grande do Sul (Pelotas and Bento Golçaves). PR = Paraná (Curitiba and Lapa). SP = São Paulo (Jundiaí). CA = California (Parlier and Modesto). Dark grey = Summer, light grey = winter. Source: CIMIS (2011) and INMET (2014). Graphic representation: the author (2014).

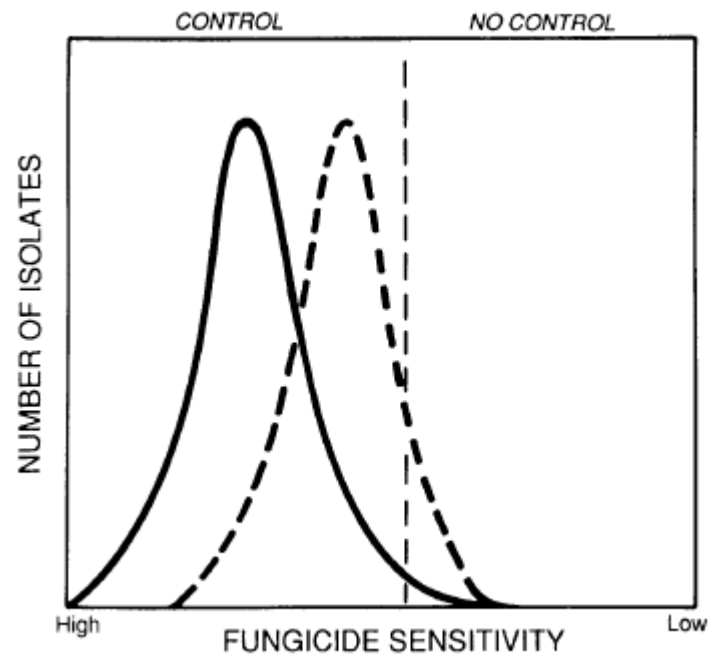


FIGURE 6. Diagram showing the unimodal distributions of degree of sensitivity, characteristic of the multi-step pattern of resistance development. Solid line = density distribution of baseline fungal population. Dashed line: initial population shift under the selective fungicide pressure toward lowered sensitivity. Source: Brent and Hollomon (2007a).

### 3. CHAPTER I – SURVEY OF *Monilinia fructicola* SENSITIVITY TO TEBUCONAZOLE IN BRAZIL REVEALS A REVERSE SHIFT TOWARDS RESISTENCE

#### 3.1. ABSTRACT

The frequency of resistant strains of *Monilinia fructicola* to tebuconazole was determined by three methods. The first involved testing 295 isolates for relative growth under the discriminatory dose of 0.3 µg/ml. The second and the third methods tested 120 isolates for EC<sub>50</sub> with one method using the discriminatory dose of 0.3 µg/ml and the other using the baseline value of 0.046 µg/ml to segregate sensitivity. Frequency of resistance among the isolates for the three methods was determined by measuring mycelial growth in fungicide-amended media. The shift for *M. fructicola* sensitivity was determined by analyzing the EC<sub>50</sub> density distribution between the baseline (2000-2004, n=31), historic (2005-2008, n=88), and current (2009-2011, n=120) isolate populations. The frequency of resistance to tebuconazole varied according to method, revealing a resistance frequency of 18.3% for the relative growth method, 40% for the baseline EC<sub>50</sub> method, and 8.33% for the EC<sub>50</sub> method segregated by the dose of 0.3 µg/ml. Application of EC<sub>50</sub> methods with discriminatory dose of 0.3 µg/ml revealed a decrease in the frequency of resistant genotypes. However, for future resistance surveys it is suggested the usage of baseline EC<sub>50</sub> method, considering its represent the mean sensitivity value before the tebuconazole use in Brazil. Additionally, the reverse shift of the current population towards resistance was observed when compared to the historic population. Finally, it was found that São Paulo is the state with the highest EC<sub>50</sub> values (0.186 µg/ml) compared to Paraná (0.053 µg/ml) and Rio Grande do Sul (0.026 µg/ml). These results suggest that *M. fructicola* resistance to tebuconazole can be decreased with anti-resistance strategies, but the study on resistance instability and isolate fitness are still necessary.

**Key words:** baseline sensitivity, brown rot, discriminatory dose, demethylation inhibitors, sensitivity segregation.

## MONITORAMENTO DA SENSIBILIDADE DE *Monilinia fructicola* AO TEBUCONAZOL NO BRASIL REVELA UMA MUDANÇA CONTRÁRIA AO SENTIDO DA RESISTÊNCIA.

### 3.2. RESUMO

A frequência da resistência de isolados de *Monilinia fructicola* para o fungicida tebuconazol foi estabelecida utilizando-se três diferentes métodos. O método do crescimento relativo foi utilizado para determinar a frequência da sensibilidade em 295 isolados, sendo estes segregados a partir da dose discriminatória de 0,3 µg/ml. Foram, também, testados dois métodos de determinação da DL<sub>50</sub> para segregar a sensibilidade em 120 isolados. O primeiro método da DL<sub>50</sub> utilizou a dose discriminatória de 0,3 µg/ml, enquanto o segundo método da DL<sub>50</sub> utilizou o valor de referência baseline para tebuconazol de 0,046 µg/ml. A frequência na resistência de isolados de *M. fructicola* foram obtidos com a medida miceliais das colônias de cada isolado, cultivados em meio de cultura solidificada com a presença de fungicida. As mudanças na sensibilidade de *M. fructicola* foram determinadas analisando-se as distribuições de densidade da DL<sub>50</sub> entre as populações baseline (2000-2004, n=31), histórica (2005-2008, n=88) e atual (2009-2011, n=120). A frequência de genótipos resistentes ao tebuconazol variou conforme o método utilizado, revelando valores de 18,3% para o método do crescimento relativo, 40% para o método da DL<sub>50</sub> baseline, e 8,3% para o método da DL<sub>50</sub>, segregado com a dose discriminatória de 0,3 µg/ml. A utilização do segundo método da DL<sub>50</sub> revelou uma diminuição na frequência de genótipos resistentes de *M. fructicola*. Entretanto, para futuros monitoramentos é sugerida a utilização do método da DL<sub>50</sub> baseline, pois este considera o valor médio da sensibilidade ao tebuconazol em uma população de *M. fructicola* que nunca teve contato com o fungicida. Adicionalmente, foi observada uma mudança contrária ao sentido da resistência na população atual em relação ao monitoramento anterior. Para a população atual, os isolados coletados em São Paulo apresentaram os maiores valores médios de DL<sub>50</sub> (0,186 µg/ml) comparados aos isolados coletados no estado do Paraná (0,053 µg/ml) e o Rio Grande do Sul (0,026 µg/ml). Estes resultados sugerem que a resistência de *M. fructicola* para o tebuconazol no Brasil pode ser reduzida através de corretas estratégias anti-resistência, porém estudos da instabilidade da resistência e adaptabilidade deverão ser realizados.

**Palavras-chave:** dose discriminatória, inibidores da demetilação, podridão parda, sensibilidade baseline, segregação da sensibilidade

### 3.3. INTRODUCTION

In Brazil, brown rot disease is caused by *Monilinia fructicola* (BLEICHER, 1997). The control of this disease depends on fungicide applications (MAY DE MIO et al., 2008) that are tied to cultural practices that start in the winter to target primary infections (FORTES; MARTINS, 1998). According to May De Mio et al. (2011) the number of applications per season may vary, depending on location and environmental conditions. The number of sprayings can reach 8 applications in Rio Grande do Sul (TIBOLA et al., 2005), 10 in Paraná (MAY DE MIO et al., 2008) and up to 15 in São Paulo (MAY DE MIO et al., 2011). With so many sprays, it is difficult to determine how many times tebuconazole is applied. In the meantime, the selection of resistant isolates of *M. fructicola* is occurring, due to the overuse of tebuconazole in commercial peach orchards in Brazil (MAY DE MIO et al., 2011). During the time when resistance to tebuconazole was observed, Silva et al. (2011) reported that 60% of peach growers from Rio Grande do Sul confirmed to spray tebuconazole to control *M. fructicola*. Considering these reports, it is believed that tebuconazole resistance frequency has increased in Brazil and that field monitoring is critical to establish a fungicide management program that will help to maintain low frequencies of resistant genotypes. Furthermore, the preservation of isolates are important for continued scientific studies to permit a follow up for status on fungi resistance development. The effect of laboratorial storage of *M. fructicola* was shown by Zhu et al. (2012) to cause a reversion on DMI sensitivity and that could be not avoided by using different methods and temperatures. These effects are extremilly important because could interfere on the interpretation of data collected from isolates kept under storage conditions. The objectives of this study were to (i) determine the frequency of *M. fructicola* resistant isolates according to three different methods, (ii) verify differences of *M. fructicola* tebuconazole sensitivity distribution in populations collected in the last 11 years, (iii) verify differences for *M. fructicola* tebuconazole sensitivity distribution among the origins where the current populations were collected, and (iv) investigate the sensitivity instability effects of *M. fructicola* isolates storage.

### 3.4. MATERIAL AND METHODS

#### 3.4.1. Isolate set and fungicide stock solution

A total of 295 isolates of *M. fructicola* were obtained from symptomatic and mummified peach fruit; collected in the states of São Paulo (SP), Paraná (PR), and Rio Grande do Sul (RS) from 2009 to 2011 (Table 1). Single-spore isolates were cultured in full strength acidified potato dextrose agar (APDA 2.5 ml of lactic acid at 25% [w/v] from Hi-Media Laboratories, Mumbai) and incubated for 5 days at 22°C with 12 h light regime. Inoculum was started from this medium. Tebuconazole stock solution was prepared by dissolving the commercial product with 100% ethanol then diluting to 10 and 100 mg a.i./ml concentrations. The *M. fructicola* baseline (2000 – 2003) and historic (2004 – 2008) populations were used for sensitivity shift comparison were fully described by May De Mio et al. (2011). All three isolate collections mentioned, current (2009-2011), historic (2005-2008) and baseline (2000-2004) were obtained from the same location to allow the population follow up.

#### 3.4.2. Frequency of resistance

The frequency of *M. fructicola* resistance was determined by three different methods for isolates collected from 2009 to 2011. The first method, tested was the relative growth under the discriminatory dose method (RG), suggested by Russell (2002) and applied to 295 isolates. The RG method was previously used for propiconazole resistance studies on *M. fructicola* (COX et al., 2007; AMIRI et al., 2009). This method consists of the addition of tebuconazole stock solution to APDA cooled to 45°C after sterilization, to produce a final discriminatory concentration of 0.3 µg/ml. A 4 mm mycelial plug was transferred to the amended and unamended media, with three repetitions per sample. Colony growth in amended media was recorded and expressed relative to its growth in unamended media as a percentage value; measurements were taken after 4 days of incubation at 24°C with 12 h light regime. Resistant isolates were defined as those with at least 50% the colony growth size obtained in unamended media (RUSSELL, 2002).

The second and third methods utilized the EC<sub>50</sub> values for sensitivity determination of 120 randomly selected isolates (Table 1). Stock solution was added to APDA and cooled to 45°C after sterilization to produce final concentrations of 0, 0.01, 0.04, 0.16, 0.64, 2.56 and

10.24 µg/ml. For each isolate, a 4 mm mycelial plug was transferred from a 5-day-old colony and placed onto amended APDA media with one of the above fungicide concentrations. Two repetitions were prepared for each concentration and plugs were placed upside down, allowing contact between mycelium and media. The Petri plates were incubated at 24°C with 12 h light regime for 4 days. The colony diameter was measured in two perpendicular directions, and the plug size was subtracted. For each concentration, the inhibition of colony growth ( $L_i$ ) of isolate  $i$  was calculated by  $L_i = (C_{ck} - C_i) / C_{ck} \cdot 100$ , where  $C_{ck}$  is the mean colony diameter for the control with no fungicide, and  $C_i$  is the mean colony diameter for isolate  $i$  on amended medium (MAY DE MIO et al., 2011).

Differences between the second and third method relate to the way by which sensitivity frequencies were inferred. For the second method, *M. fructicola* isolates were considered resistant when their  $EC_{50}$  value was greater than 0.3 µg/ml as used by May De Mio et al. (2011). The third method used the baseline value for tebuconazole (0.046 µg/ml) to separate resistant from sensitive isolates. The *M. fructicola* baseline (BL) value was suggested by May De Mio et al. (2011) for populations collected between 2000-2004, before tebuconazole usage in Brazil.

### 3.4.3. Density distribution of sensitivity

The *M. fructicola* distribution of sensitivity to tebuconazole was plotted by the density and the cumulative relative frequency, using log transformed  $EC_{50}$  values. First, three different populations were compared: 2000-2004 (baseline - BL), 2005-2008 (historical population - HP) and 2009-2011 (current population - CP); then a comparison of origin (SP, PR and RS) was made within the actual population (Table 1). The BL and HP  $EC_{50}$  values were retrieved from May De Mio et al. (2011).

### 3.4.4. Storage sensitivity instability

To evaluate the effect of isolate storage on *M. fructicola* resistance to tebuconazole, 32 isolates were randomly selected from the HP (17 isolates) and CP (15 isolates). Isolates belonging to the HP had their  $EC_{50}$  values firstly evaluated in 2010 by May De Mio et al. (2011).

The second EC<sub>50</sub> evaluation was performed after four years of long term storage in 25% glycerol at -80°C. The 15 isolates belonging to the CP had their EC<sub>50</sub> value calculated for the first time in 2012. Later in 2013, a second EC<sub>50</sub> evaluation was performed after one year storage on sterile filter paper at 4°C. The *M. fructicola* EC<sub>50</sub> value was calculated for tebuconazole according to May De Mio et al. (2011).

#### 3.4.5. Data analysis

The EC<sub>50</sub> value was analyzed by logarithm (log<sub>10</sub>) transforming the fungicide concentrations, and then generating a linear regression of the colony inhibition values (*L*) by the log<sub>10</sub> concentrations. The tebuconazole EC<sub>50</sub> value was calculated from the corresponding linear regression equations for each isolate where the regression coefficient was significant at  $P < 0.05$ . Changes in sensitivity density curves were analyzed by using the two sample Kolmogorov-Smirnov test. The nonparametric Kolmogorov-Smirnov test works by comparing the cumulative frequency distributions of two data sets at a time, and reporting the maximum difference between distributions (KIRKMAN, 1996). Arithmetic EC<sub>50</sub> means for population, origin, storage method, and date were calculated separately. Statistical differences in population sensitivity were verified by the Welch two sample *t*-test, while differences for storage methods were verified by the Paired *t*-test, both at  $P < 0.05$ . The statistical software R (version 3.1.1) was used for data analysis and graphical representation.

### 3.5. RESULT

#### 3.5.1. Frequency of resistance

The frequency of *M. fructicola* resistance to tebuconazole varied according to the method of evaluation used. The baseline EC<sub>50</sub> method indicated an overall resistance frequency of 40%. In SP, PR and RS, the baseline EC<sub>50</sub> method revealed resistance frequencies of 71.1%, 25%, and 18.6% respectively (Fig.1C). The relative growth method gave 18.3% for the

frequency of resistant isolates within the surveyed population from peach orchards in Brazil. In SP, PR and RS, resistant isolates had frequencies of 37.3%, 5.3%, and 2.7%, respectively (Fig. 1A). The EC<sub>50</sub> method used by May De Mio et al. (2011) has yielded the lowest resistance frequency among the three evaluation methods (not statistically compared), with a total of 8.3% of resistant isolates in the surveyed population. This latter method revealed 20% resistance in SP, 3.1% in PR and no resistance observed in RS (Fig. 1B).

### 3.5.2. Density distribution of sensitivity

The tebuconazole sensitivity distribution revealed that all tested populations differed from each other according to the statistic-D and corresponding P-value (Fig. 2). The BL differed from the HP, ( $p = 0.002$ ,  $D = 0.38$ ), and from the CP ( $p = 0.009$ ,  $D = 0.32$ ; Fig. 2). The HP and CP showed significant differences regarding sensitivity distribution ( $p = 8.8 \times 10^{-7}$ ,  $D = 0.38$ ; Fig. 2). The mean EC<sub>50</sub> value obtained for BL, HP and CP were 0.046, 5.593, and 0.093  $\mu\text{g/ml}$  respectively (Table 2). Significant differences for mean EC<sub>50</sub> were observed between the BL and HP ( $p = 0.052$ ), BL and CP ( $p = 0.006$ ), and HP and CP ( $p = 0.054$ ) (Table 2).

Within the current population, the sensitivity distribution from SP isolates differed significantly from PR isolates ( $p = 5.8 \times 10^{-5}$ ,  $D = 0.51$ ) and RS isolates ( $p = 1.9 \times 10^{-7}$ ,  $D = 0.57$ ). Also, a similar distribution was observed between PR and RS ( $p = 0.198$ ,  $D = 0.23$ ; Fig. 3). The mean EC<sub>50</sub> values obtained for SP, PR and RS were 0.186, 0.053 and 0.026  $\mu\text{g/ml}$  respectively (Table 3). Significant differences for mean EC<sub>50</sub> were observed between SP and PR ( $p = 0.001$ ), SP and RS ( $p = 1.24 \times 10^{-5}$ ), but not between PR and RS ( $p = 0.143$ ) (Table 3).

### 3.5.3. Storage sensitivity instability

A storage effect on *M. fructicola* sensitivity to tebuconazole was observed for the HP ( $p = 1.8 \times 10^{-6}$ ) and CP ( $p = 1.4 \times 10^{-5}$ ) after four years of storage at  $-80^\circ\text{C}$  in 25% glycerol and one year of storage at  $4^\circ\text{C}$  on filter paper, respectively (Table 4). In both storage methods, the mean EC<sub>50</sub> value has decreased from the first to the second evaluation.

### 3.6. DISCUSSION

In Brazil, *Monilinia fructicola* isolates resistant to tebuconazole account for 40% of the surveyed population. The baseline EC<sub>50</sub> method was suitable for the survey of resistance to tebuconazole in Brazil because it was based on specific data retrieved from a local *M. fructicola* population prior to tebuconazole usage. The relative growth method, using the discriminatory dose of 0.3 µg/ml, is a useful method for initial screening of resistant isolates because it demands less time and material to be performed. However, the relative growth method should be avoided for determining tebuconazole resistance frequencies in isolates from Brazil, because some resistant isolates may be misclassified as sensitive. Different methods for calculating the relative growth of *M. fructicola* isolates have been reported for propiconazole in order to segregate them by sensitivity based on the discriminatory dose (RUSSELL, 2002; AMIRI et al., 2008; VILLANI; COX, 2011). The discriminatory dose for tebuconazole in *M. fructicola* isolates from Brazil is still unknown and difficult to determine because of the continuous resistance pattern of this fungicide and due to the absence of an isolate collection from fields where tebuconazole failed to control brown rot. In order to refine the relative growth method for resistance surveys, it is necessary to determine the tebuconazole discriminatory dose in Brazil or change the percentual relative growth used as sensitivity segregation parameter until the match to frequencies obtained with the baseline EC<sub>50</sub> method.

According to May De Mio et al. (2011), a total resistance frequency of 15.8% was reported for the isolate population surveyed between 2005 to 2008 (HP). By reproducing the sensitivity segregation method used by May De Mio et al. (2011), an overall resistance frequency of 8.33% was determined for the CP. Therefore, it was possible to observe a reverse shift towards resistance in the CP as demonstrated by the EC<sub>50</sub> density distribution analysis. If the previous tebuconazole survey performed by May De Mio et al. (2011) had used the mean EC<sub>50</sub> baseline value to segregate resistant and sensitive isolate frequencies, the reduction would also be observed for the population within this study (data not shown).

The decrease of resistance frequency noticed between the current and previous surveys can be explained by changes to brown rot fungicide management programs that peach growers have followed in these surveyed areas. In SP, sprays of azoxystrobin fungicide have increased in order to prevent peach rust caused by *Tranzschelia discolor*, while brown rot control has been given secondary importance. In PR and RS, the usage of iminoctadine tris (albesilate) and iprodione has demonstrated satisfactory control in controlling brown rot disease (MOREIRA;

MAY DE MIO, 2009) and therefore have been included in the spray program. Those changes to fungicide management may reduce the selection for resistant isolates and affect disease incidence, consequently reducing the frequency of DMI resistant isolates, as demonstrated by Staub (1991) and Brannen et al. (2006), and suggested by Brent and Hollomon (2007).

The sensitivity instability observed by the reduction of EC<sub>50</sub> values for isolates recovered from both storage methods, may affect the degree of resistance reported in this paper. On the other hand, this survey and the one from May De Mio et al. (2011) would have been similarly affected by the storage instability. For both populations (HP and CP), isolates were stored over a similar period and by the same method, thereby reducing sensitivity heterogeneity caused by storage. A similar storage effect was previously reported in other DMI studies for different pathogens (KÖLLER et al., 1991; KARAOGLANIDIS; THANASSOULOPOULOS, 2002; ZHU et al., 2012). Zhu et al. (2012), also in studies with *M. fructicola*, has suggested that changes during cold storage at -80°C were more likely a result of cellular damage, rather than deleterious effects on DNA, since mutation related to resistance was identified before and after storage experiments. Regarding changes observed from filter paper storage, differences may be attributed to continuous growth that has been observed at 4°C (OGAWA; ENGLISH, 1991; MOREIRA; MAY DE MIO, 2007), causing a similar effect to those observed after successive colony transfers in the absence of fungicide (COX et al., 2007).

Similar to the study from May De Mio et al. (2011), SP showed the highest mean EC<sub>50</sub> value and different sensitivity distribution when compared with isolates from PR and RS. Those differences were attributed to higher mean temperature, rainfall and air humidity found in SP, in comparison to the other locations where peaches are produced. High fungicide pressure caused by the intense spray for controlling peach rust in SP (ALVES; MAY DE MIO, 2008) in an environment with higher inoculum pressure caused by warmer average temperatures (BERGAMIN FILHO; AMORIM, 2001) were the most probable causes for the observed differences.

In short, the baseline EC<sub>50</sub> value of 0.046 µg/ml is recommended to determine the frequency of *M. fructicola* isolates resistant to tebuconazole in Brazil. The relative growth method with discriminatory dose of 0.3 µg/ml can be used for a fast screening of resistant isolates, but additional studies are necessary for its application on resistance frequency studies. Additionally, the sensitivity distribution of resistant *M. fructicola* isolates indicates a reverse shift toward resistance. This finding gave us an important insight about anti-resistance strategies that can avoid or delay practical resistance to tebuconazole in Brazilian peach orchards. Studies

including those that investigate sensitivity instability after successive isolate transfers in the absence of fungicide and those investigating competitive ability and fitness between resistant and sensitive isolates are essential for supporting new anti-resistance strategies.

### 3.7. CONCLUSIONS

Current population of *M. fructicola* isolate has shown a reverse shift toward resistance in comparison to the previous survey.

The tebuconazole baseline EC<sub>50</sub> method revealed that 40% of the current *M. fructicola* population is composed by resistant isolate genotypes.

In São Paulo, 71.1% of *M. fructicola* population are resistant to tebuconazole, and higher mean EC<sub>50</sub> value was observed in comparison to isolates recovered from Paraná and Rio Grande do Sul peach orchards.

Laboratorial storage methods cause decreased sensitivity in *M. fructicola* isolates.

### 3.8. ACKNOWLEDGEMENTS

We thank Karla Kudlawiek, Fernando Ramos and Ryan Puckett for technical assistance, and the Capes/Reuni doctoral fellowship. This material is based upon work supported by grants nº 479041/2010-5 Universal/CNPq.

### 3.9. REFERENCES

ALVES, G.; MAY DE MIO, L. L. Efeito da desfolha causada pela ferrugem na floração e produtividade do pessegueiro. **Revista Brasileira de Fruticultura**, v. 30, n. 4, p. 907-912, 2008.

AMIRI, A.; BRANNEN, P. M.; SCHNABEL, G. Monitoring resistance in *Monilinia fructicola* populations of the southeastern United States for enhanced brown rot control in peach. **Phytopathology**, p.S12, 2008.

AMIRI, A.; BRANNEN, P. M.; SCHNABEL, G. Validation of the lipbalm tube assay for evaluation of fungicide sensitivity in field isolates of *Monilinia fructicola*. **Plant Health Progress**, v. 10, 2009.

BERGAMIN FILHO, A.; AMORIM, L. Epidemiologia comparativa entre os patossistemas temperado e tropical: conseqüências para a resistência a fungicidas. **Fitopatologia Brasileira**, v. 26, n. 2, p. 119-127, 2001.

BLEICHER, J. Doenças de rosáceas de caroço. In: KIMATI, H.; AMORIM, L. REZENDE, J. A. M.; BERGAMIN FILHO, A. (Ed.). **Manual de fitopatologia: Doenças das plantas cultivadas**, v.2, p.621-627, 1997.

BRANNEN, P.; HOTCHKISS, M.; REILLY, C. C.; SCHNABEL, G. Evaluation of fungicide programs to manage a DMI-resistant *Monilinia fructicola* population in a Georgia peach research block, 2005. **F&N Tests**, v. 61, 2006.

BRENT, K. J.; HOLLOMON, D. W. **Fungicide resistance in crop pathogens: how can it be managed?** 2nd. Fungicide Resistance Action Committee, 2007.

COX, K.; BRYSON, P.; SCHNABEL, G. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. **Phytopathology**, v. 97, n. 4, p. 448-453, 2007.

FORTES, J.; MARTINS, O. Sintomatologia e controle das principais doenças. In: MEDEROS, C. e RASEIRA, M. D. C. (Ed.). **A cultura do pessegueiro**: Embrapa-CPACT/Embrapa-SPI, p.243-264, 1998.

KARAOGLANIDIS, G.; THANASSOULOPOULOS, C. Phenotypic Instability of *Cercospora beticola* Sacc. Strains Expressing Resistance to the Sterol Demethylation-Inhibiting (DMI) Fungicide Flutriafol after Cold Exposure. **Journal of Phytopathology**, v. 150, n. 11-12, p.692-696, 2002.

KIRKMAN, T. Statistics to use. <http://www.physics.csbsju.edu/stats/>, 1996. Acesso em: Jan/03/2006.

KÖLLER, W.; SMITH, F.; REYNOLDS, K. Phenotypic instability of flusilazole sensitivity in *Venturia inaequalis*. **Plant pathology**, v. 40, n. 4, p. 608-611, 1991.

MAY DE MIO, L.; LUO, Y.; MICHAELIDES, T. J. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management. **Plant Disease**, v. 95, n. 7, p. 821-827, 2011.

MAY DE MIO, L.; MOREIRA, L. M.; MONTEIRO, L. B.; JUSTINIANO, P. Infecção de *Monilinia fructicola* no período da floração e incidência de podridão parda em frutos de pessegueiro em dois sistemas de produção. **Tropical Plant Pathology, Brasília**, v. 33, n. 03, p. 227-234, 2008.

MOREIRA, L.; MAY DE MIO, L. L. Crescimento Micelial de *Monilinia fructicola* e *Trichothecium roseum* em diferentes temperaturas e sensibilidade do antagonista a fungicidas e fosfitos. **Scientia Agraria**, v. 8, n. 3, p. 337-341, 2007.

MOREIRA, L.; MAY DE MIO, L. L. Controle da podridão parda do pessegueiro com fungicidas e fosfitos avaliados em pré e pós-colheita. **Ciênc. agrotec.**, v. 33, n. 2, p. 405-411, 2009.

OGAWA, J. M.; ENGLISH, H. **Diseases of temperate zone tree fruit and nut crops**. UCANR Publications, 1991.

RUSSELL, P. **Sensitivity baselines in fungicide resistance research and management**. Cambridge UK: FRAC, 2002.

SILVA, S. J. P.; KOHLS, V. K.; MANICA-BERTO, R.; RIGATTO, P.; ROMBALDI, C. V. Apropriação tecnológica da produção integrada de pêssegos na região de Pelotas no Estado do Rio Grande do Sul. **Ciência Rural**, v. 41, n. 9, p. 1667-1673, 2011.

STAUB, T. Fungicide resistance: Practical experience with antiresistance strategies and the role of integrated use. **Annual review of Phytopathology**, v. 29, n. 1, p. 421-442, 1991.

TIBOLA, C. S. FACHINELLO, J. C.; GRÜTZMACHER, A. D.; PICOLOTTO, L.; KRÜGER, L. Manejo de pragas e doenças na produção integrada e convencional de pêssegos. **Revista Brasileira de Fruticultura**, v. 27, n. 2, p. 215-218, 2005.

VILLANI, S. M.; COX, K. D. Characterizing fenbuconazole and propiconazole sensitivity and prevalence of 'Mona' in isolates of *Monilinia fructicola* from New York. **Plant Disease**, v. 95, n. 7, p. 828-834, 2011.

ZHU, F.; BRYSON, P. K.; SCHNABEL, G. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. **Pest management science**, v. 68, n. 7, p. 1003-1009, 2012.

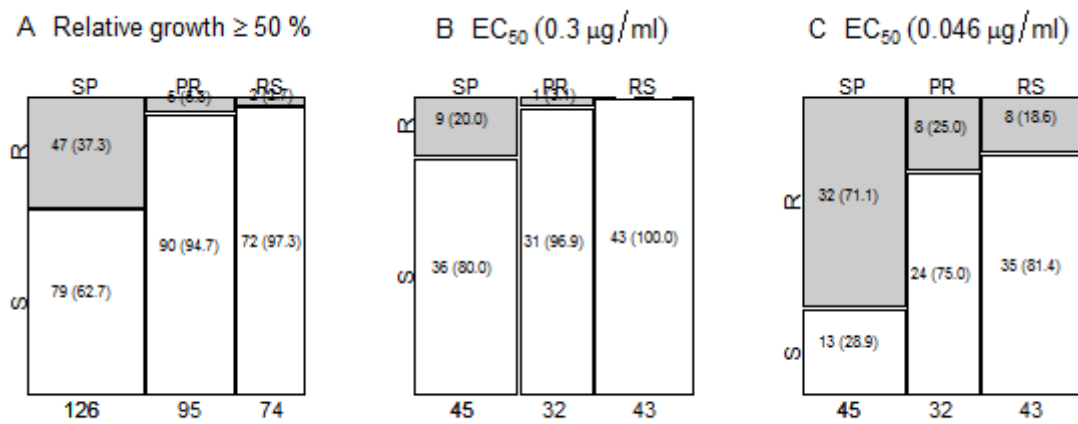


FIGURE 1. Frequency of *Monilinia fructicola* sensitivity to tebuconazole for isolates collected between 2009 and 2011, using the relative growth under the discriminatory dose of 0.3  $\mu\text{g/ml}$  method (A), the  $EC_{50}$  value using 0.3  $\mu\text{g/ml}$  as discriminatory dose (B), and the  $EC_{50}$  value using the baseline value of 0.046  $\mu\text{g/ml}$  as discriminatory dose. SP = São Paulo, PR = Paraná, and RS = Rio Grande do Sul, R = Resistant, S = Sensitive, entire values correspond to the sample size and values in between brackets correspond to percentage [n (%)].

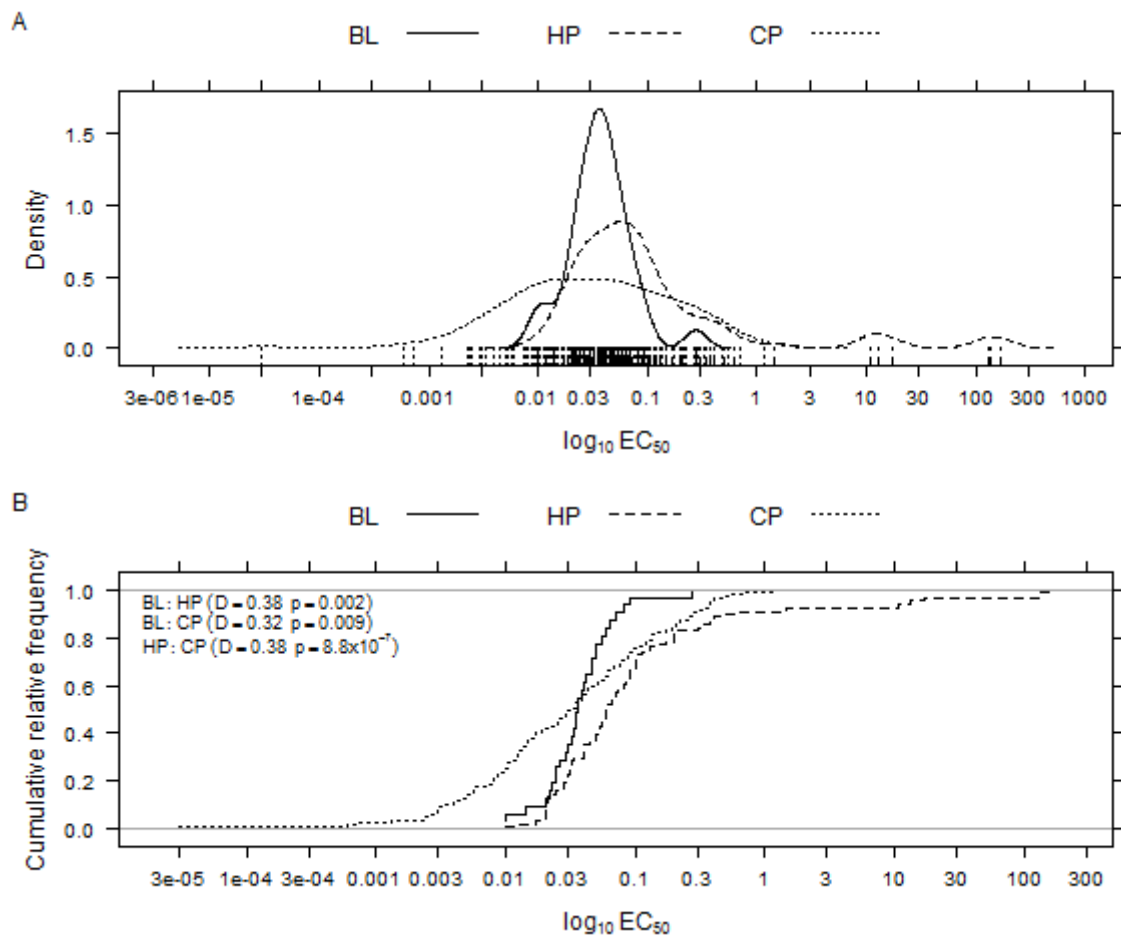


FIGURE 2. *Monilinia fructicola* density distribution (A) and cumulative relative frequency (B) for tebuconazole effective concentration for 50% population inhibition ( $EC_{50}$ ) in Brazil, comparing baseline (BL, 2000-2004), historic (HP, 2005-2008) and current (CP, 2009-2011) populations.

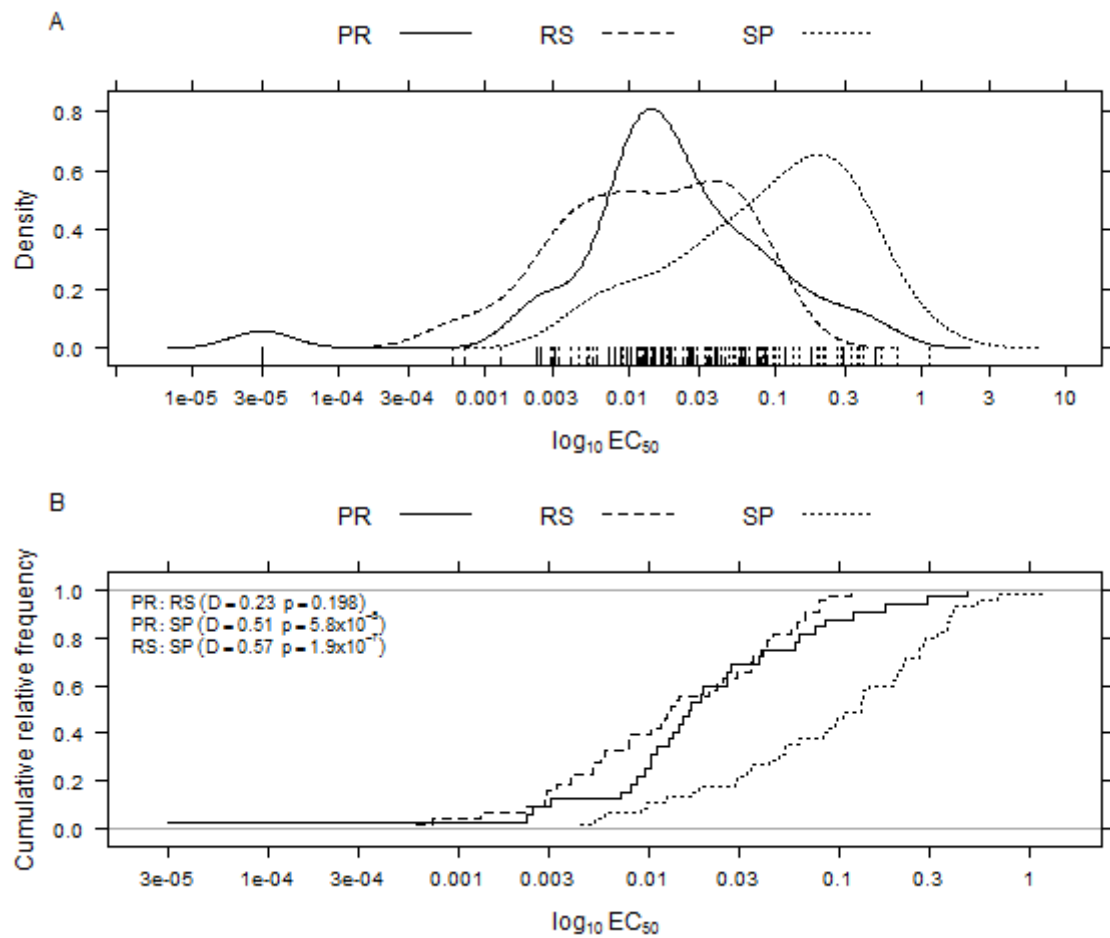


FIGURE 3. *Monilinia fructicola* density distribution (A) and cumulative relative frequency (B) for tebuconazole effective concentration for 50% population inhibition ( $EC_{50}$ ) in Brazil, comparing origins within the actual population (2009-2011). SP = São Paulo, PR = Paraná, RS = Rio Grande do Sul.

TABLE 1. List of *Monilinia fructicola* isolates collected in Brazil for tebuconazole resistance studies.

Isolate	Year	Origin <sup>a</sup>	EC <sub>50</sub> <sup>b</sup>	RG <sup>c</sup>	EC <sub>50</sub> (0.3µg/ml) <sup>d</sup>	EC <sub>50</sub> (0.046 µg/ml) <sup>e</sup>
638	2009	PR (Lapa)	0.0000	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
639	2009	PR (Lapa)	NA <sup>f</sup>	S <sub>teb</sub>	NA	NA
643	2009	PR (Lapa)	0.0858	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
646	2009	PR (Lapa)	0.1746	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
722	2009	SP (Holambra)	0.0097	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
750	2009	SP (Holambra)	0.0183	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
752	2009	SP (Holambra)	0.1346	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
753	2009	SP (Holambra)	0.0091	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
754	2009	SP (Holambra)	0.3740	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
755	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
756	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
757	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
758	2009	SP (Holambra)	0.0294	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
761	2009	SP (Holambra)	0.5320	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
775	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
777	2009	SP (Holambra)	0.1327	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
781	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
786	2009	SP (Holambra)	0.2011	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
787	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
792	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
793	2009	SP (Holambra)	0.3796	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
794	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
795	2009	SP (Holambra)	0.2164	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
796	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
797	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
802	2009	SP (Holambra)	0.3341	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
806	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
807	2009	SP (Holambra)	1.1644	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
809	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
811	2009	SP (Holambra)	0.1945	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
826	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
827	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
828	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
829	2009	SP (Holambra)	0.1331	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
831	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
832	2009	SP (Holambra)	0.0617	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
834	2009	SP (Holambra)	0.1453	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
839	2009	SP (Holambra)	0.2813	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
850	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
851	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
854	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA

855	2009	SP (Holambra)	0.2669	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
856	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
857	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
859	2009	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
860	2009	SP (Holambra)	0.0426	R <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
862	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
864	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
868	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
869	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
870	2009	SP (Holambra)	0.2032	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
888	2009	SP (Holambra)	0.2640	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
911	2009	SP (Holambra)	0.0328	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
912	2009	SP (Holambra)	0.0986	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
914	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
915	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
927	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
929	2009	SP (Holambra)	0.3685	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
940	2009	SP (Holambra)	0.4026	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
941	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
945	2009	SP (Holambra)	0.4086	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
950	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
951	2009	SP (Holambra)	0.2833	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
952	2009	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
956	2009	SP (Holambra)	0.2239	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1023	2010	PR (Araucária)	0.0031	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1024	2010	PR (Araucária)	0.0381	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1025	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1026	2010	PR (Araucária)	0.0023	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1027	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1028	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1030	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1033	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1035	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1036	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1038	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1039	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1040	2010	PR (Araucária)	0.0104	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1041	2010	PR (Araucária)	0.0271	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1042	2010	PR (Araucária)	0.0111	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1043	2010	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
1044	2010	PR (Araucária)	0.0195	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1049	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
1051	2010	PR (Campo Largo)	0.0127	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1052	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
1053	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
1054	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA

1056	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
1057	2010	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
1058	2010	PR (Campo Largo)	NA	R <sub>teb</sub>	NA	NA
1060	2010	PR (Faxinal dos Castilhos)	NA	S <sub>teb</sub>	NA	NA
1062	2010	PR (Faxinal dos Castilhos)	0.0133	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1063	2010	PR (Faxinal dos Castilhos)	NA	S <sub>teb</sub>	NA	NA
1067	2010	PR (Lapa)	0.0103	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1068	2010	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
1069	2010	PR (Lapa)	0.0614	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1070	2010	PR (Lapa)	NA	R <sub>teb</sub>	NA	NA
1071	2010	PR (Lapa)	NA	R <sub>teb</sub>	NA	NA
1073	2010	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
1075	2010	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
1077	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1079	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1080	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1081	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1083	2010	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
1085	2010	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
1086	2010	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
1088	2010	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
1089	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1090	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1092	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
1093	2010	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
959	2010	RS (Dourado do Sul)	NA	S <sub>teb</sub>	NA	NA
961	2010	RS (Dourado do Sul)	NA	S <sub>teb</sub>	NA	NA
962	2010	RS (Dourado do Sul)	NA	R <sub>teb</sub>	NA	NA
964	2010	RS (Dourado do Sul)	0.0029	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
965	2010	RS (Dourado do Sul)	NA	S <sub>teb</sub>	NA	NA
968	2010	RS (Dourado do Sul)	0.0786	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
974	2010	RS (Farroupilha)	0.0121	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
979	2010	RS (Farroupilha)	0.0424	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
982	2010	RS (Farroupilha)	0.0039	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
983	2010	RS (Farroupilha)	NA	S <sub>teb</sub>	NA	NA
989	2010	RS (Pinto Bandeira)	NA	S <sub>teb</sub>	NA	NA
993	2010	RS (Bento Gonçalves)	NA	S <sub>teb</sub>	NA	NA
996	2010	RS (Bento Gonçalves)	0.0103	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
997	2010	RS (Bento Gonçalves)	0.0006	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
998	2010	RS (Bento Gonçalves)	NA	S <sub>teb</sub>	NA	NA
999	2010	RS (Bento Gonçalves)	0.0421	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1000	2010	RS (Picada Café)	0.0346	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1002	2010	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
1004	2010	RS (Pelotas)	0.0051	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>

1006	2010	RS (Pelotas)	0.0144	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1007	2010	RS (Pelotas)	0.0051	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1009	2010	RS (Pelotas)	0.1161	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1010	2010	RS (Pelotas)	0.0079	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1013	2010	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
1016	2010	RS (Capão do Leão)	0.0603	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1019	2010	RS (Capão do Leão)	0.0232	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1020	2010	RS (Capão do Leão)	0.0794	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1022	2010	RS (Capão do Leão)	NA	S <sub>teb</sub>	NA	NA
1094	2010	SP (Holambra)	0.0517	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1095	2010	SP (Holambra)	0.0052	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1096	2010	SP (Holambra)	0.0838	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1097	2010	SP (Holambra)	0.0490	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1098	2010	SP (Holambra)	0.0059	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1099	2010	SP (Holambra)	0.0044	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1100	2010	SP (Holambra)	0.0883	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
1101	2010	SP (Holambra)	0.0167	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
1	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
2	2011	PR (Araucária)	0.0072	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
3	2011	PR (Araucária)	0.0087	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
4	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
6	2011	PR (Araucária)	0.2908	R <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
7	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
8	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
9	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
10	2011	PR (Araucária)	0.0405	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
11	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
12	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
13	2011	PR (Araucária)	0.0584	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
14	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
15	2011	PR (Araucária)	0.4807	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
16	2011	PR (Araucária)	NA	S <sub>teb</sub>	NA	NA
17	2011	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
18	2011	PR (Campo Largo)	NA	S <sub>teb</sub>	NA	NA
19	2011	PR (Campo Largo)	0.0151	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
21	2011	PR (Lapa)	0.0080	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
22	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
23	2011	PR (Lapa)	0.0259	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
24	2011	PR (Lapa)	0.0145	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
25	2011	PR (Lapa)	0.0164	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
26	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
27	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
28	2011	PR (Lapa)	0.0761	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
29	2011	PR (Lapa)	0.1189	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
30	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
31	2011	PR (Lapa)	0.0262	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>

32	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
34	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
35	2011	PR (Lapa)	0.0024	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
36	2011	PR (Lapa)	NA	S <sub>teb</sub>	NA	NA
38	2011	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
39	2011	PR (Campo do Tenente)	0.0096	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
40	2011	PR (Campo do Tenente)	NA	S <sub>teb</sub>	NA	NA
41	2011	PR (Rio Negro)	NA	S <sub>teb</sub>	NA	NA
42	2011	PR (Rio Negro)	0.0188	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
43	2011	PR (Rio Negro)	NA	S <sub>teb</sub>	NA	NA
44	2011	PR (Rio Negro)	NA	S <sub>teb</sub>	NA	NA
45	2011	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
46	2011	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
47	2011	PR (Mandirituba)	0.0168	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
48	2011	PR (Mandirituba)	NA	S <sub>teb</sub>	NA	NA
97	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
98	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
99	2011	RS (Pelotas)	0.0344	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
100	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
101	2011	RS (Pelotas)	0.0667	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
102	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
103	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
104	2011	RS (Pelotas)	0.0247	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
105	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
106	2011	RS (Pelotas)	0.0033	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
107	2011	RS (Pelotas)	0.0079	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
108	2011	RS (Pelotas)	0.0566	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
109	2011	RS (Pelotas)	0.0057	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
110	2011	RS (Pelotas)	0.0029	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
111	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
112	2011	RS (Pelotas)	0.0142	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
113	2011	RS (Pelotas)	0.0290	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
114	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
115	2011	RS (Pelotas)	0.0358	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
116	2011	RS (Pelotas)	0.0030	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
117	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
118	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
119	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
120	2011	RS (Pelotas)	0.0392	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
121	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
122	2011	RS (Pelotas)	0.0039	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
123	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
124	2011	RS (Pelotas)	0.0125	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
125	2011	RS (Capão do Leão)	0.0117	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
126	2011	RS (Capão do Leão)	0.0078	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
127	2011	RS (Capão do Leão)	NA	S <sub>teb</sub>	NA	NA

128	2011	RS (Bento Gonçalves)	NA	S <sub>teb</sub>	NA	NA
129	2011	RS (Bento Gonçalves)	0.0007	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
130	2011	RS (Bento Gonçalves)	0.0013	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
132	2011	RS (Bento Gonçalves)	0.0130	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
133	2011	RS (Bento Gonçalves)	NA	S <sub>teb</sub>	NA	NA
134	2011	RS (Bento Gonçalves)	0.0872	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
135	2011	RS (Bento Gonçalves)	0.0207	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
136	2011	RS (Bento Gonçalves)	NA	S <sub>teb</sub>	NA	NA
138	2011	RS (Pinto Bandeira)	0.0059	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
139	2011	RS (Pinto Bandeira)	0.0672	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
140	2011	RS (Pelotas)	0.0450	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
141	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
142	2011	RS (Pelotas)	0.0023	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
143	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
144	2011	RS (Pelotas)	NA	S <sub>teb</sub>	NA	NA
145	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
146	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
147	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
148	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
149	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
150	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
151	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
152	2011	SP (Holambra)	0.6888	R <sub>teb</sub>	R <sub>teb</sub>	R <sub>teb</sub>
153	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
154	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
155	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
156	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
157	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
158	2011	SP (Holambra)	0.0959	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
159	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
160	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
161	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
162	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
163	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
164	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
165	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
166	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
167	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
168	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
169	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
170	2011	SP (Holambra)	0.1069	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
171	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
172	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
173	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
174	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
175	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA

176	2011	SP (Holambra)	0.0125	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
177	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
178	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
179	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
180	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
181	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
182	2011	SP (Holambra)	0.0526	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>
183	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
184	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
186	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
187	2011	SP (Jarinú)	NA	S <sub>teb</sub>	NA	NA
188	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
189	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
190	2011	SP (Holambra)	0.0287	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
191	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
192	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
193	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
194	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
195	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
196	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
197	2011	SP (Holambra)	NA	R <sub>teb</sub>	NA	NA
198	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
199	2011	SP (Holambra)	0.0347	S <sub>teb</sub>	S <sub>teb</sub>	S <sub>teb</sub>
200	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
201	2011	SP (Holambra)	NA	S <sub>teb</sub>	NA	NA
203	2011	SP (Holambra)	0.1314	S <sub>teb</sub>	S <sub>teb</sub>	R <sub>teb</sub>

<sup>a</sup> Isolate origins, State(City). SP = São Paulo, PR = Paraná, RS = Rio Grande do Sul.

<sup>b</sup> EC<sub>50</sub> value determined according May De Mío et al. (2011).

<sup>c</sup> Sensitivity determined with Relative Growth method (n=295), according Russel (2002). S<sub>teb</sub> and R<sub>teb</sub> stands for tebuconazole sensitive and resistant isolates respectively.

<sup>d</sup> Sensitivity determined with EC<sub>50</sub> value (n=120), where 0.3 µg/ml was used as the discriminatory dosage according May De Mío et al. (2011).

<sup>e</sup> Sensitivity determined with EC<sub>50</sub> value (n=120), where 0.046 µg/ml (Baseline) was used as the discriminatory dosage.

<sup>f</sup> Not available data (NA).

TABLE 2. Comparison of tebuconazole effective concentration value for 50% population inhibition (EC<sub>50</sub> value) of *Monilinia fruticola* isolates from three different populations in Brazil.

Populations <sup>a</sup>	n	Mean EC <sub>50</sub> <sup>b</sup> values (µg/ml) and p-value <sup>c</sup>			Median	Percentiles (10 % - 90 %)
		BL	HP	CP		
BL	31	<b>0.046<sup>b</sup></b>	0.052 <sup>c</sup>	0.006 <sup>c</sup>	0.036	0.02 and 0.07
HP	88	-	<b>5.593<sup>b</sup></b>	0.054 <sup>c</sup>	0.06	0.02 and 0.53
CP	120	-	-	<b>0.093<sup>b</sup></b>	0.031	0.003 and 0.28

<sup>a</sup> BL = baseline population (2000-2004). HP = historic population (2005-2008). CP = current population (2009-2011).

<sup>b</sup> Mean EC<sub>50</sub> value in bold.

<sup>c</sup> p-value obtained with the Welch *t*-test.

TABLE 3. Comparison of tebuconazole effective concentration value for 50% population inhibition (EC<sub>50</sub> value) of *Monilinia fructicola* isolates collected between 2009-2011 in São Paulo (SP), Paraná (PR) and Rio Grande dos Sul (RS).

Origin	n	Mean EC <sub>50</sub> values <sup>a</sup> (µg/ml) and p-value <sup>b</sup>			Median	Percentiles (10 % and 90 %)
		SP	PR	RS		
SP	45	<b>0.186<sup>a</sup></b>	0.001 <sup>b</sup>	1.24 × 10 <sup>-5</sup> <sup>b</sup>	0.131	0.010 and 0.393
PR	32	-	<b>0.053<sup>a</sup></b>	0.143 <sup>b</sup>	0.016	0.003 and 0.115
RS	43	-	-	<b>0.026<sup>a</sup></b>	0.013	0.002 and 0.067

<sup>a</sup> Mean EC<sub>50</sub> value in bold.

<sup>b</sup> p-value obtained with the Welch *t*-test.

TABLE 4. Mean of the tebuconazole sensitivity differences for *Monilinia fructicola* storage.

Evaluation years	Storage method	n	Mean of the differences (µg/ml) <sup>a</sup>	p-value <sup>b</sup>	CI <sub>95%</sub> <sup>c</sup>
2010-2014	25% glycerol	17	3.76 (2010>2014)	$1.8 \times 10^{-6}$	2.66 - 4.85
2012-2013	filter paper	15	2.2 (2012>2013)	$1.4 \times 10^{-5}$	1.47 - 2.93

<sup>a</sup> Mean of the differences between the earlier and later EC<sub>50</sub> value evaluations.

<sup>b</sup> p-value obtained with the Paired *t*-test.

<sup>c</sup> 95% confidence interval of the mean.

#### 4. CHAPTER II – INSTABILITY OF RESISTANCE TO TEBUCONAZOLE OF *Monilinia fructicola* ISOLATES AND COMPETITIVE DISADVANTAGE OF RESISTANT GENOTYPES CAN FAVOR BROWN ROT MANAGEMENT IN BRAZIL

##### 4.1. ABSTRACT

In this study, we investigated the components of *Monilinia fructicola* resistance to tebuconazole. These components included persistence of resistance over time, isolate fitness, and competitive disadvantage among sensitive and resistant isolates. Studies were conducted both *in vitro* (in culture) and *in vivo* (on peach fruits) in order to reproduce natural conditions. For testing the resistance stability and fitness parameters, isolates were successive transferred 9 times *in vitro* and 5 times *in vivo* in the absence of tebuconazole. After the 2<sup>nd</sup>, 5<sup>th</sup> and 9<sup>th</sup> transfer, evaluations were performed. Competitive studies were performed by mixing three pairs of resistant (R) and sensitive (S) isolates with same spore concentration. Frequencies of R and S isolates were tested after successive transfers without tebuconazole in three different mixtures rates, 70%:30%, 50%:50%, and 30%:70% of R and S isolates. Successive transfers in the absence of tebuconazole showed that after nine cycles *in vitro*, despite the numerical reduction, the differences of EC<sub>50</sub> values within the resistant population were not significant. However, resistance instability was confirmed *in vivo*, as a significant reduction was observed after 5 transfer cycles. Experiments on competitive capacity revealed the disadvantage of resistant isolates when competing against sensitive isolates. This disadvantage may be attributed to the lower fitness observed for resistant isolates, which included lower germination, sporulation and area under mycelial growth curve. For all those variables, the sensitive isolates presented higher values in experiments *in vitro* and *in vivo* (except germination *in vivo*). Regarding the pathogenic capacity of tebuconazole-sensitive and -resistant isolates, the first group revealed shorter incubation period and greater lesion size, which are considered important advantages for their establishment in the environment. However, similar latent periods were observed for both groups of sensitivity. Additionally, fitness costs attributed to resistance were significant for germination *in vitro* and lesion size *in vivo*. The results presented here suggest that the frequency of *M. fructicola* isolates resistant to tebuconazole tends to decrease in the absence of fungicide pressure, representing valuable information for the establishment of anti-resistance strategies.

**Key words:** brown rot disease, peach, demethylation-inhibiting fungicides.

## **INSTABILIDADE DA RESISTÊNCIA PARA TEBUCONAZOL EM ISOLADOS DE *Monilinia fructicola* E DESVANTAGEM COMPETITIVA PODEM FAVORECER O MANEJO DA PODRIDÃO PARDA NO BRASIL.**

### **4.2. RESUMO**

Neste estudo, foram investigados componentes da resistência de *Monilinia fructicola* ao tebuconazol. Dos componentes estudados, incluíram-se a persistência da resistência no tempo, a adaptabilidade de isolados, e a desvantagem competitiva entre isolados sensíveis e resistentes. Estudos foram conduzidos em ambos *in vitro* (meio de cultura) e *in vivo* (em pêssego) para poder reproduzir as condições naturais. Para testar a estabilidade da resistência e parâmetros de adaptabilidade, isolados foram transferidos sucessivamente 9 vezes *in vitro* e 5 vezes *in vivo* na ausência de tebuconazol. Avaliações foram realizadas após a 2<sup>o</sup>, 5<sup>o</sup> e 9<sup>o</sup> transferência. Estudos de competição foram realizados com a mistura de três pares de isolados resistentes (R) e sensíveis (S) com mesma concentração de esporos. Frequências de isolados R e S foram testados após transferências sucessivas sem tebuconazol em três diferentes proporções de mistura de isolados R e S, 70%:30%, 50%:50%, and 30%:70%. Transferências sucessivas na ausência de tebuconazol mostraram que após nove ciclos *in vitro*, apesar da redução numérica, a diferença dos valores de DL<sub>50</sub> dentro da população resistente não foram significante. Entretanto, a instabilidade da resistência foi confirmada *in vivo*, ao se observar uma redução após cinco ciclos de transferências. Experimentos da capacidade competitiva revelaram desvantagens de isolados resistentes contra isolados sensíveis, que foram atribuídas à menor adaptabilidade observada demonstrada pelos resultados de germinação, esporulação e área abaixo da curva de crescimento micelial. Para todas estas variáveis mencionadas, isolados sensíveis obtiveram maiores valores em experimentos realizados *in vitro* e *in vivo* (exceto para a germinação *in vivo*). Referente à capacidade patogênica de isolados sensíveis e resistentes ao tebuconazol, o primeiro grupo revelou um período de incubação mais curto e maior tamanho de lesão, considerada importante vantagem para o seu estabelecimento no ambiente. Adicionalmente, o custo da adaptabilidade atribuído à resistência foi significativo para a germinação *in vitro* e para o tamanho de lesão *in vivo*. Os resultados aqui apresentados sugerem que a frequência de genótipos resistentes ao tebuconazol tende a diminuir na ausência da pressão do fungicida, representando uma valiosa informação para o estabelecimento de estratégias anti-resistência.

**Palavras-chave:** fungicidas inibidores da demetilação, podridão parda, pêssego.

### 4.3. INTRODUCTION

Blossom blight and brown rot disease are caused in Brazil by the ascomycete *Monilinia fructicola* (G Wint) Honey (MAY DE MIO et al., 2004; ADASKAVEG et al., 2008). This disease can become a limiting factor for peach and other stone fruit production when not controlled (BYRDE; WILLETTS, 1977; ADASKAVEG et al., 2008). Most current commercial peach varieties are very susceptible, and disease control is mainly accomplished with fungicide applications (MOREIRA; MAY DE MIO, 2007; MAY DE MIO et al., 2011). Fungicides registered for use against brown rot disease of peaches in Brazil include methyl benzimidazole carbamates, dicarboximides, quinone outside inhibitors, and demethylation inhibitor (DMI) (MAPA/AGROFIT, 2014). Tebuconazole is among the most widely used DMI in Brazil for controlling brown rot disease (SILVA et al., 2011). Therefore, under intense fungicide pressure, resistant strains can be selected among the pathogen populations in only three years as was observed for propiconazole in South Carolina (ZEHR et al., 1999). In Brazil, tebuconazole resistance was observed after 5 years of use in São Paulo State, and after 10 years in Paraná and Rio Grande do Sul States (MAY DE MIO et al., 2011; LICHTEMBERG et al., 2014). Since then, no other studies have been conducted to elucidate the resistance components of *M. fructicola* to support the establishment of tebuconazole anti-resistance management strategies.

Within a resistant DMI population which tend to be the most unfit, resistance mechanisms are generally controlled by several genes (GEORGOPOULOS, 1985). Fitness was well defined by Pringle and Taylor (2002) as the survival and reproductive success conferred by an allele, individual, or group of isolates. By that, the fitness concept means the extent to which an individual contributes genes to a future generation (ANDERSON, 2005). This approach became a common comparative tool in epidemiological studies (HOLMES; ECKERT, 1995; KARAOGLANIDIS et al., 2001; COX et al., 2007) for testing the practical relevance of resistance effects of certain populations under extensive fungicide exposure. In *M. fructicola*, fitness studies have differed in methodology, source of isolates, and fungicides tested (PENROSE et al., 1979; SONODA; OGAWA, 1982; SANOAMUANG; GAUNT, 1995; ZHU et al., 2012). The determination of a population fitness is an important tool for development of anti-resistance strategies, which consist of understanding how changes in the population sensitivity occur in the absence of fungicides. In Brazil, tebuconazole resistant isolates of *M. fructicola* were found in the main peach production regions (MAY DE MIO et al., 2011; LICHTEMBERG et al., 2014). In our conditions, the climate favors the continuous presence of

inoculum in the field (SEQUEIRA, 1962; VANDERPLANK, 1984), and resistant isolates are selected because of multiple DMI sprays which are necessary for decreasing the conducive effects of excessive rainfall (OGAWA et al., 1995; KESKE et al., 2011), and to control stone fruit diseases other than caused by *M. fructicola* (ALVES et al., 2008). Considering these factors, selection pressure for resistance may also occur over the winter for inoculum that develops on mummified fruit. Therefore, knowledge concerning fitness and resistance instability is necessary for the understanding if in the absence of tebuconazole the resistant population will be less fit than the sensitive one. This would support the current usage of more effective use of selective fungicides such as tebuconazole in Brazil.

The objectives of this study were (i) to determine the sensitivity instability of *M. fructicola* isolates to tebuconazole after consecutive transfers in the fungicide absence, (ii) to evaluate the competitive disadvantages of resistant and sensitive strains, and (iii) to measure the fitness cost associated with resistance *in vitro* and *in vivo*.

#### 4.4. MATERIALS AND METHODS

##### 4.4.1. Isolate set

*M. fructicola* isolates were randomly selected according to their EC<sub>50</sub> value, previously determined by Lichtemberg et al. (2014). In total, 15 isolates (12 resistant – DMI-R and 3 sensitive – DMI-S) were used in the instability experiment, and six of these were used in the competition experiment (Table 1). Single-spore isolates stored in sterile filter paper were cultured on acidified potato dextrose agar (APDA, 2.5 ml of 25 % lactic acid [w/v]) at 25°C and 12 h light regime for 7 days.

##### 4.4.2. Successive transfer effect on isolate sensitivity and fitness

In order to measure the isolate fitness and tebuconazole sensitivity instability *in vitro*, nine successive transfers were made on APDA with evaluations performed after the 2<sup>nd</sup>, 5<sup>th</sup>,

and 9<sup>th</sup> transfer cycles. The isolate cultures were renewed every 7 days, by transferring a 4-mm mycelial plug onto the center of new APDA plate, and incubated at 25°C with 12 h light regime. Mycelial plugs were placed upside down. Each evaluation included the area under mycelial growth curve (AUMGC), sporulation, germination, and the effective concentration for 50% population inhibition by tebuconazole (EC<sub>50</sub> value). Two repetitions were made per isolate and the experiment was repeated twice.

For fitness and tebuconazole sensitivity instability determined *in vivo*, detached peach fruit of cv. ‘Sun Ryan’ were surface disinfested with 70% ethanol (1 min), 0.5 % sodium hypochlorite (1 min) and rinsed three times in sterile distillate water. Inoculum was taken from a conidial suspension ( $5 \times 10^4$  conidia/ml) prepared from 7-day old cultures. Fruit inoculations were made with 20 µL inoculum aliquots placed into a wound punctured at 3 mm depth. After inoculation, fruits were placed into humidified transparent plastic containers (32 × 24 × 10 cm), and incubated for 3-days at room temperature prior to a subsequent transfer. In total, five transfers were performed, and evaluations were made after the 2<sup>nd</sup> and 5<sup>th</sup> transfer cycles. The variables that were evaluated were incubation and latent periods, lesion size, sporulation, germination, and the EC<sub>50</sub> value. Four repetitions were made per isolate and the experiment was repeated twice.

Conidial production was measured from 2 cm<sup>2</sup> fruit slices (*in vivo*) and six 5 mm diameter plugs (*in vitro*) taken 1 cm from the center of the lesion and colony culture respectively. Sampled material was transferred into a water vial with 1 ml sterile distilled water, homogenized and counted with a hemacytometer. Results were expressed as the number of conidia per ml. Two droplets of 10 µL were counted for each conidial suspension.

Germination frequency was evaluated by transferring 40 µL onto a depression slide (2.5 × 7.5 cm × 1.2 mm thick, with a depression of 16 mm in diameter and 0.50 mm max depth). Depression slides were placed inside a humidified plastic container (30 × 13 × 7 cm) and incubated for 5 h at 25°C in the dark. The frequency of germination for each isolate was calculated by evaluating 200 randomly selected conidia from each of two conidia suspensions. The AUMGC was determined by measuring the colony dimensions in two perpendicular directions over the course of seven days, followed by the calculation of the area under the growth curve. Lesion size was determined at the end of the third day with two perpendicular measurements.

Incubation and latent period were assessed by monitoring the inoculated fruits every 3 h for the appearance of brown rot symptoms and every 6 h for structures of *M. fructicola* over the course of 51 hours. The incubation period was defined as the time from the inoculation to

the initial appearance of symptoms and the latent period as the time from inoculation to the first signs of reproductive activity. Four repetitions were made per isolate and the experiment was repeated twice.

A commercial product of tebuconazole was dissolved in sterile water, and adjusted to concentrations of 100 µg a.i./ml and 500 µg a.i./ml. Stock solutions were added to APDA cooled to 45°C after sterilization, to produce final concentrations of 0, 0.005, 0.015, 0.045, 0.135, 0.405, 1.215, 3.645, and 10.935 µg/ml. For each isolate, a 4-mm plug was transferred from the edge of a 5-day-old colony and placed onto the center of APDA media amended with one of the above fungicide concentrations. Two repetitions were prepared for each concentration. The Petri plates were incubated at 25°C with 12 h light regime for 4 days. The colony diameter was measured in two perpendicular directions, and 5-mm was subtracted for the plug. For each concentration, the inhibition of colony growth ( $L_i$ ) of isolate  $i$  was calculated by  $L_i = (C_{ck} - C_i) / C_{ck} \cdot 100$ , where  $C_{ck}$  is the mean colony diameter for the control with no fungicide, and  $C_i$  is the mean colony diameter for isolate  $i$  on amended media.

#### 4.4.3. Competitive ability of resistant and sensitive isolates

Three resistant (SP-R1, PR-R2, and RS-R3) and three sensitive (SP-S1, PR-S2, and RS-S3) *M. fructicola* isolates were selected according to their  $EC_{50}$  values and place of origin. Isolates were cultured in APDA at 25°C with 12 h light regime over 7 days. Conidia were harvested by adding 2 ml of sterile distilled water in each plate. Isolate inoculum was adjusted to  $5 \times 10^4$  conidia/ml and prepared by mixing volumes of conidial suspensions of SP-R1 with SP-S1, PR-R2 with PR-S2, and RS-R3 with RS-S3 to produce final conidial suspensions containing R:S ratios of 70:30, 50:50, and 30:70.

The *in vitro* experiment was started by transferring a 100 µL aliquot of mixed conidial suspension into a 3 mm well made in the center of a fresh APDA plate. Plates were incubated at 25°C with 12 h light regime for 7 days. Nine successive transfers were prepared in the absence of fungicide before experiment evaluation. A 4-mm diameter mycelial plug was used as subsequent source of inoculum, by transferring it from the edge of the previous colony, into the center of a fresh APDA plate. Two repetitions were made per isolate pair and ratio and the experiment was repeated twice.

For the *in vivo* experiment, the same isolate pairs and ratios mentioned above were used to inoculate the surface of disinfested peach fruit cv. 'Sun Ryan'. Fruits were placed on the top of a plastic support inside transparent plastic containers (32 × 24 × 10 cm), water was added in the bottom of each container without contact with the fruits, which were punctured at 3 mm depth prior to conidia inoculation (20 µL at 5 × 10<sup>4</sup> conidia/ml) in the absence of fungicide. Peaches were incubated for 3 days at room temperature and conidia were subsequently harvested from two equidistant lesion slices (1 cm<sup>2</sup> size each) from the center of the lesion. Lesion slices were suspended in 2 ml distilled water serving as the new source of inoculum. Five successive inoculation cycles were made before experiment evaluation. Four repetitions were made for each isolate and ratio and the experiment was repeated twice.

The frequency of tebuconazole sensitivity was evaluated for 30 and 12 recovered single-spore isolates from each repetition after the 9<sup>th</sup> transfer *in vitro* and 5<sup>th</sup> transfer *in vivo* respectively. Single-spores were cultured on fresh APDA plates at 25°C and 12 h light regime for 4 days, and subsequently tested for their relative growth at the discriminatory dose of 0.3 µg/ml according to Lichtemberg et al. (2014). Resistant isolates were determined by those with growth equal to or above 50% of growth in the control without fungicide (RUSSELL, 2002). For each of the single-spore colonies, three replicate plates were prepared for each isolate pair and ratio. Pure isolates that were not mixed were used as absolute controls.

#### 4.4.4. Data analysis

Fitness variables were analyzed by the correspondent linear model imposed by the experimental design with effects tested by ANOVA, evaluating the significance of sensitivity groups and successive transfer effects. Visual inspections of the model assumptions were conducted to verify the need for data transformation. When departures were observed, a data transformation was made as suggested by graphical interpretation of the  $\lambda$  log-likelihood profile (BOX; COX, 1964). The EC<sub>50</sub> value was analyzed by transforming fungicide concentrations at log<sub>10</sub>, and a linear regression of the colony inhibition values on the logarithm concentrations was made. The tebuconazole EC<sub>50</sub> value was calculated from the corresponding linear regression equation for each isolate where the regression coefficient was significant. Arithmetic means of EC<sub>50</sub> values were calculated separately for transfers and isolate sensitivity and statistical differences were verified by ANOVA. The incubation and latent periods were

analyzed by adjusting the parametric sequential Weibull survival models for the censored time to physiological event variable and the importance of the extra term, as it relates to experimental factors, was evaluated by the likelihood ratio test. For any class of parametric models used (Gaussian ANOVA or Weibull survival), mean comparisons were made using proper linear functions and P-value adjustments for multiple hypotheses were done by the single-step method (HOTHORN et al., 2008). For all inferences, P-value < 0.05 was considered a nominal significance level and the statistical software R (version 3.1.1.) was used in the entire work for all graphical representations.

## 4.5. RESULTS

### 4.5.1. Successive transfer effect on isolate sensitivity

The instability of *M. fructicola* sensitivity to tebuconazole after successive transfers in the absence of fungicide was observed *in vitro* ( $p = 0.016$ ), with significant interaction ( $p = 0.0001$ ) between successive transfers and sensitivity groups (groups of R- and S-isolates) (Table 2). From the interaction observed *in vitro*, differences in sensitivity were observed only in sensitive isolates, as noticed from the 5<sup>th</sup> to the 9<sup>th</sup> transfer cycle. However, no sensitivity differences were noticed from the 2<sup>nd</sup> to the 9<sup>th</sup> transfer cycle in the sensitive isolates (Table 2). In the *in vitro* same experiment, the resistant isolates revealed no significant reduction from the 2<sup>nd</sup> to the 9<sup>th</sup> transfer cycle, even with mean  $EC_{50}$  values changing from 0.03 to 0.0153  $\mu\text{g/ml}$  (Table 2). In the absence of interaction ( $p = 0.861$ ) between successive transfer and sensitivity groups, the *in vivo* experiment only demonstrated a significant transfer main effect ( $p = 0.0001$ ; Table 2). Values of  $EC_{50}$  decreased from 0.0112 to 0.0007  $\mu\text{g/ml}$  within the interval of three successive transfers (Table 2). In both experiments, significant  $EC_{50}$  differences ( $p = 2.2 \times 10^{-16}$ ) between resistant and sensitive isolates were observed (data not showed).

### 4.5.2. Effect of successive medium transfers on fitness

Prior to statistical analysis, datum for germination, sporulation and AUMGC were raised to the power of 1.5, square root transformed, and logarithm transformed respectively. No significant interactions between transfer and sensitivity group were observed for germination ( $p = 0.829$ ) and sporulation ( $p = 0.618$ ) (data not shown). Instead, germination results have shown transfer ( $p = 8.72 \times 10^{-5}$ ) and sensitivity ( $p = 0.003$ ) main effects, while for sporulation, significance was observed for transfer main effect ( $p = 5.11 \times 10^{-6}$ ), and at 10% probability for sensitivity main effect ( $p = 0.068$ ; Table 3).

No germination differences were observed from the 2<sup>nd</sup> (67.4%) to the 5<sup>th</sup> transfer cycles (61.1%), but from the 5<sup>th</sup> to the 9<sup>th</sup> transfer cycle, germination frequencies increased to 73.3%. (Table 3). Sporulation differences were observed between the 2<sup>nd</sup> (98,663.19 conidia/cm<sup>2</sup>) and 5<sup>th</sup> (52,353.89 conidia/cm<sup>2</sup>) transfer cycle, but not for the 9<sup>th</sup> transfer cycle (80,779.59 conidia/cm<sup>2</sup>; Table 3). Sensitive groups of isolates germinated at a rate of 71.2% and produced 86,156.5 conidia/cm<sup>2</sup>, while the resistant group of isolates had germination levels of 63.4% and sporulation of 66,511.7 conidia/cm<sup>2</sup> (Table 3).

The AUMGC yielded a significant interaction ( $p = 0.003$ ) between transfer and sensitivity groups. It was observed that the resistant group of isolates increased the mean value from 100.6 (5<sup>th</sup> transfer cycle) to 109.8 (9<sup>th</sup> transfer cycle). Within the sensitive group of isolates, the AUMGC remained similar throughout successive transfers (Table 4).

#### 4.5.3. Effect of successive fruit transfers on fitness

Prior to statistical analysis, germination datum were cubic root transformed; while sporulation, incubation and latent periods were logarithm transformed. The transfer effect was significant for germination ( $p = 2.2 \times 10^{-16}$ ), sporulation ( $p = 0.032$ ), and lesion size ( $p = 3.68 \times 10^{-7}$ ; Table 5). Germination and conidial production increased from 78.3 to 93.4% and from 99,013.4 to 120,247.8 conidia/cm<sup>2</sup>, respectively, while lesion size reduced from 48.7 to 44.9 mm diameter after three successive transfers (Table 5). The sensitive group main effect was significant for sporulation ( $p = 1.33 \times 10^{-7}$ ) and lesion size ( $p = 5 \times 10^{-4}$ ), but not for germination ( $p = 0.8895$ ; Table 5). The sensitive group of isolates exceeded the resistant group for sporulation (201,122.6 against 59,197.8 conidia/cm<sup>2</sup>), and lesion size (48.1 against 45.5 mm; Table 5). No interactions between successive transfers and group of sensitivity were observed (data not shown).

No successive transfer effects were observed for incubation ( $p = 0.154$ ) or latent periods ( $p = 0.117$ ). Shorter ( $p = 0.016$ ) incubation period was found in the sensitive group (14.5 h) compared to the resistant group (17.2 h), but no significant differences were observed for the latent period ( $p = 0.884$ ; Table 5). The interaction between successive transfer and sensitivity group was not significant for incubation ( $p = 0.312$ ) and latent period ( $p = 0.373$ ). Significant ( $p = 1.28 \times 10^{-6}$ ) linear regression (R-squared of 84%) between the mean incubation period and the difference between latent and incubation periods was observed among the isolate samples. This relation between the two variables revealed that isolates with longer incubation period had shorter latent period (Fig. 1).

The linear regression analysis between the  $EC_{50}$  value from 15 isolates (Table 1) and the fitness variables, revealed that fitness cost was associated with germination ( $p = 0.014$ ; Fig. 2A) *in vitro* and lesion size ( $p = 0.014$ ; Fig. 2F) *in vivo*. All other variables *in vitro* and *in vivo* showed no statistical significance for the linear regression.

#### 4.5.4. Competitive ability of resistant and sensitive isolates

Changes in the frequency of resistance were largely dependent on which isolates were in competition. In two out of three isolate pairing (from São Paulo and Rio Grande do Sul states), the sensitive isolates surpassed the resistant isolates by 100% after nine successive transfers *in vitro* (Fig. 3A-C) and five successive transfers *in vivo* (Fig. 3D-F). The case of the isolate pair from Paraná, the competition of the resistant and sensitive isolate occurred at different frequencies *in vitro*, since sensitive isolates were always recovered at higher frequency (Fig. 3A-C). Recovered resistant isolates was only a majority *in vivo*, when sensitive isolates were outnumbered in  $R_{70}:S_{30}$  (Fig. 3D). For the other ratios tested *in vivo*, isolate pairs from Paraná resulted in sensitive frequencies of 64.5 % at  $R_{50}:S_{50}$  (Fig. 3E), and 65.5 % at  $R_{30}:S_{70}$  (Fig. 3F). The controls prepared without any conidial mixture have confirmed their sensitivities after 9 cycles *in vitro* and 5 cycles *in vivo* (data not shown).

#### 4.6. DISCUSSION

In this study, we found that in the absence of tebuconazole, resistant populations of *M. fructicola* are more likely to decline when competing against sensitive isolates. It was observed that after successive transfers (*in vivo*) both sensitivity groups (resistant and sensitive isolates) had their EC<sub>50</sub> value reduced towards sensitivity. Furthermore, the *in vitro* experiments revealed an numeric reduction on its EC<sub>50</sub> value indicating that resistance may be reduced with few more cycles.

Tebuconazole resistance in *M. fructicola* was unstable through the first five cycles when inoculated in peaches. However, the non-significant EC<sub>50</sub> reduction, observed for the resistant group *in vitro*, was different from observations recorded by Cox et al. (2007), where sensitivity of resistant *M. fructicola* isolates for propiconazole increased after a few transfers on artificial media. In agreement with our results, Zhu et al. (2012) have observed that after 36 weeks of successive transfers in PDA, no significant reduction was observed for three propiconazole resistant isolates. In the same study, Zhu et al. (2012) noted that successive transfers on canned peach halves showed no sensitivity reduction as well. In our study, the absence of significant *in vitro* differences for isolates resistant to tebuconazole, can be explained by a phenomenon reported for the apple scab pathogen *Venturia inaequalis* (KÖLLER et al., 1991). Köller et al. (1991), suggest that the late loss of resistance would be related to a spontaneous alteration of one of the genes, which accounts for mitotic instability of the isolates. This alteration was described by Keller et al. (1991), explaining that mitotic instability frequency depends on the DNA configuration, and may take several transfers *in vitro* to occur. The processes that modulate the frequency and quality of DNA replication and mitosis are known as regulation, and their mechanisms were reviewed by Kelly and Brown (2000).

During the *in vitro* experiment, the sensitive isolate group showed an unexpected EC<sub>50</sub> mean value increase verified after the 5<sup>th</sup> transfer cycle. Köller et al. (1991) has also observed the same event in 12% of tested samples including flusilazole sensitive isolates and Zhu et al. (2012) has reported a significant EC<sub>50</sub> decline for one of two sensitive isolates during 36 successive transfers in the absence of propiconazole. The method of comparing relative growth between amended and unamended media applied by Cox et al. (2007), revealed small variations in the propiconazole inhibition levels due to the individual isolates plasticity. The sensitivity fluctuation of sensitive populations was explained by Köller et al. (1991) as a competitive advantage or disadvantage for individual phenotypes governed by environmental conditions.

Germination and sporulation data *in vitro* demonstrated an initial reduction observed (not significant for germination) between the 2<sup>nd</sup> and 5<sup>th</sup> transfers, followed by a final recovery. Despite the similarity between the 2<sup>nd</sup> and 9<sup>th</sup> transfers for both germination and sporulation,

the *in vivo* experiment supports the conclusion that in the absence of tebuconazole, the isolates of *M. fructicola* regain their reproductive vigor. The AUMGC and lesion size data, demonstrate the advantage of sensitive over resistant isolates. The fitness evaluation performed by Cox et al. (2007) revealed a significant variability in the germination, sporulation, and growth rates during successive transfers of *M. fructicola*. However, when sensitive and resistant isolates were compared, the former group had higher germination and sporulation values than the latter group (COX et al., 2007). In our study, for both experiments (*in vitro* and *in vivo*), sensitive isolates were fitter than resistant isolates (with the exception of germination *in vivo* which indicated similar rates among groups). Similar to our results, Ritchie (1983), Penrose et al. (1985), and Sanoamuang and Gaunt (1995) found that dicarboximide resistant isolates of *M. fructicola* produced fewer spores than sensitive isolates when inoculated in fruits. Smaller fruit lesions were also observed for *M. fructicola* isolates resistant to benomyl (SONODA; OGAWA, 1982) and dicarboximide (RITCHIE, 1983; PENROSE et al., 1985). On the other hand, no differences between benomyl sensitive and resistant isolates of *Sclerotinia fructicola* were found for germination, sporulation, and growth rate (PENROSE et al., 1979). In different pathosystems, it was also found that DMI-resistant isolates were less fit when compared to sensitive isolates (SCHEPERS, 1985; KARAOGLANIDIS et al., 2001). Despite the fitness cost attributed to resistance only being verified for germination *in vitro* and lesion size *in vivo*, these results suggest a possible relation with regard to fungicide resistance and fitness. The lack of fitness cost associated with other variables could be caused by the specificity of the study, as suggested by Cox et al. (2007) in his work on *M. fructicola* resistance to propiconazole.

Under optimum temperature, *M. fructicola* produces symptoms and signs on infected fruit within two days (OGAWA; ENGLISH, 1991; OGAWA et al., 1995) as confirmed in our study. The faster incubation period observed in the sensitive population of *M. fructicola*, highlights other important competitive advantages over the resistant population. However, successive transfers in the absence of tebuconazole did not impose any effect on the incubation and latent time. Similarly, Sanoamuang and Gaunt (1995) have found that the length of the incubation and latent periods were not affected by *M. fructicola* being exposed to an over-summering period on nectarine twigs in the absence of fungicide. Besides, Sanoamuang and Gaunt (1995) have only found shorter latent periods for sensitive isolates in comparison to carbendazim- and iprodione-resistant isolates. When studying other sterol biosynthesis inhibitors, Peever and Milgroom (1994) could not find any difference between sensitive and resistant isolates of *Pyrenophora teres* regarding their latent periods. The relationship between the time of incubation and the difference between of latent and incubation period, demonstrates

that isolates with slow incubation periods can reproduce faster. Lichtemberg et al. (unpublished data) have previously observed this event in *M. fructicola* when comparing epidemiological variables between Californian and south Brazilian populations. In that study, the phenomenon was inferred as an inherent compensation mechanism to facilitate species survival. Considering results for isolates with incubation of 14.5 hours, sensitive isolates were generally observed with greater intervals between both events since they have a shorter incubation period than resistant isolates.

Few studies on competitive ability between fungicide resistant and sensitive isolates have been published for *M. fructicola* or other pathogens. Benomyl-resistant isolates of *S. fructicola*, were shown to persist after consecutive transfers in fruit and PDA, even when the conidia from susceptible isolates outnumbered the resistant conidia by 9:1 (PENROSE et al., 1979). Carbendazim and dicarboximides resistant isolates of *M. fructicola* were found to compete well against sensitive isolates (SANOAMUANG; GAUNT, 1995). But when studying DMIs competitive ability, Nuninger-Ney et al. (1989) reported higher frequencies of sensitive *M. fructicola* isolates recovered after successive transfers in peaches. Also corroborating our results, resistant isolates were less frequently re-isolated in DMI studies with *Penicillium digitatum* (HOLMES; ECKERT, 1995), *Cercospora beticola* (KARAOGLANIDIS et al., 2001), and *Sphaerotheca fuliginea* (SCHEPERS, 1985). The prevalence of sensitive spore frequency could be attributed to the slower incubation period and lower fitness of the resistant isolates which results in the inferior ability to adapt in an environment without tebuconazole.

In conclusion, tebuconazole resistant isolates of *M. fructicola* in Brazil showed lowered fitness and competitive disadvantages in comparison to sensitive isolates. The selection of sensitive isolates is likely to proceed more rapidly due to greater fitness and pathogenicity. Additionally, the sensitivity instability which moves towards reduced resistance in the absence of fungicide, represents valuable information for anti-resistance strategy implementation for peach orchards in Brazil. Our results have been in accordance with the statement of Georgopoulos (1985) and Georgopoulos and Skylakakis (1986) which have reported that in cases of multistep resistance, as for DMIs, resistant members of a population tend to be less fit, and that alternating fungicides can help to achieve good control and delay resistance, as shown by Staub (1991). Field experiments are highly recommended in order to confirm these observations and to further our understanding of resistance dynamics in a non-controlled environment.

#### 4.7. CONCLUSIONS

In the absence of tebuconazole, sensitivity of *M. fructicola* isolate tends to decline.

Sensitive isolates have shown better fitness than resistant isolates in the absence of tebuconazole.

Sensitive isolates demonstrated higher competitive capacity than resistant isolates when competing in the absence of tebuconazole.

The relationship between incubation time and the interval between latent and incubation periods demonstrate that isolates with slow incubation capacity can reproduce faster.

#### 4.8. ACKNOWLEDGEMENTS

We thank Ryan Puckett, David Morgan and Michael Luna from the University of California Davis/Kearney Agricultural Research and Extension Center for their technical assistance and Bayer SA for supplying the fungicide used in this study. A special appreciation to Capes doctoral fellowship for making this study possible both in Brazil and in California. This study is based upon work supported by grants n° 479041/2010-5 Universal/CNPq.

#### 4.9. REFERENCES

ADASKAVEG, J.; SCHNABEL, G.; FÖRSTER, H. Disease of peach caused by fungi and fungal-like organisms: biology, epidemiology and management. In: LAYNE, D. R. e BASSI, D. (Ed.). **The peach: botany, production and uses**. Wallingford: CABI, p.352-406, 2008.

ALVES, G.; MAY DE MIO, L. L.; ZANETTE, F.; MARISA, C. O. Ferrugem do pessegueiro e seu efeito na desfolha e na concentração de carboidratos em ramos e gemas. **Tropical Plant Pathology**, v. 33, n. 5, p. 347-353, 2008.

ANDERSON, J. B. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. **Nature Reviews Microbiology**, v. 3, n. 7, p. 547-556, 2005.

BOX, G. E.; COX, D. R. An analysis of transformations. **Journal of the Royal Statistical Society, Series B**, v. 26, n. 2, p. 211-252, 1964.

- BYRDE, R. J. W.; WILLETTS, H. J. The brown rot fungi of fruit. Their biology and control. **The brown rot fungi of fruit. Their biology and control.** 1977.
- COX, K.; BRYSON, P.; SCHNABEL, G. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. **Phytopathology**, v. 97, n. 4, p. 448-453, 2007.
- GEORGOPOULOS, S. The genetic basis of classification of fungicides according to resistance risk. **EPPO Bulletin**, v. 15, n. 4, p. 513-517, 1985.
- GEORGOPOULOS, S.; SKYLAKAKIS, G. Genetic variability in the fungi and the problem of fungicide resistance. **Crop Protection**, v. 5, n. 5, p. 299-305, 1986.
- HOLMES, G.; ECKERT, J. Relative fitness of imazalil-resistant and-sensitive biotypes of *Penicillium digitatum*. **Plant Disease**, v. 79, n. 10, p. 1068-1073, 1995.
- HOTHORN, T.; BRETZ, F.; WESTFALL, P. Simultaneous inference in general parametric models. **Biometrical Journal**, v. 50, n. 3, p. 346-363, 2008.
- KARAOGLANIDIS, G.; THANASSOULOPOULOS, C.; IOANNIDIS, P. Fitness of *Cercospora beticola* field isolates—resistant and—sensitive to demethylation inhibitor fungicides. **European journal of plant pathology**, v. 107, n. 3, p. 337-347, 2001.
- KELLER, N. P.; BERGSTROM, G. C.; YODER, O. Mitotic stability of transforming DNA is determined by its chromosomal configuration in the fungus *Cochliobolus heterostrophus*. **Current genetics**, v. 19, n. 3, p. 227-233, 1991.
- KELLY, T. J.; BROWN, G. W. Regulation of chromosome replication. **Annual review of biochemistry**, v. 69, n. 1, p. 829-880, 2000.
- KESKE, C.; AMORIM, L.; MAY DE MIO, L. L. Peach brown rot incidence related to pathogen infection at different stages of fruit development in an organic peach production system. **Crop Protection**, v. 30, n. 7, p. 802-806, 2011.
- KÖLLER, W.; SMITH, F.; REYNOLDS, K. Phenotypic instability of flusilazole sensitivity in *Venturia inaequalis*. **Plant pathology**, v. 40, n. 4, p. 608-611, 1991.
- LICHTENBERG, P. S.; MICHAILIDES, T. J.; MORALES, R. G. F.; ZEVIANI, W. M.; MAY DE MIO, L. L. Shift in *Monilinia fructicola* sensitivity to tebuconazole fungicide, and resistant isolate survey in southern Brazil. In: DEHNE HW, D. H., FRAAIJE B, GISI U, HERMANN D, MEHL A, OERKE EC, RUSSELL PE, STAMMLER G, KUCK KH, LYR H (Ed.). **Modern fungicides and antifungal compounds**. Braunschweig, Germany: Deutsch Phytomedizinische Gesellschaft, v. Vol II, p.173-178, 2014.
- MAPA/AGROFIT. Sistema de agrotóxicos e fitossanitários. **Ministério da Agricultura**, <http://www.agricultura.gov.br/servicos-e-sistemas/sistemas/agrofit>, 2014. Acesso em: October 21st.
- MAY DE MIO, L.; GARRIDO, L.; UENO, B. Doenças de Fruteiras de Carço. In: MONTEIRO, L. B.; MAY DE MIO, L. L.; SERRAT, B. M.; MOTTA, A. C.; CUQUEL, F. L. (Ed.). **Fruteiras de carço: uma visão ecológica**. Curitiba, PR: UFPR, p.169–185, 2004.

MAY DE MIO, L.; LUO, Y.; MICHAILIDES, T. J. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management. **Plant Disease**, v. 95, n. 7, p. 821-827, 2011.

MOREIRA, L.; MAY DE MIO, L. L. Metodologia para detecção de infecções latentes de *Monilinia fructicola* em frutas de caroço. **Ciência Rural**, v. 37, n. 3, p. 628-633, 2007.

NUNINGER-NEY, C.; SCHWINN, F.-J.; STAUB, T. In vitro selection of sterol-biosynthesis-inhibitor (SBI)-resistant mutants in *Monilinia fructicola* (Wint.) Honey. **Netherlands Journal of Plant Pathology**, v. 95, n. 1, p. 137-150, 1989.

OGAWA, J. M.; ENGLISH, H. **Diseases of temperate zone tree fruit and nut crops**. UCANR Publications, 1991.

OGAWA, J. M. ZEHR, E. I.; BIRD, G. W.; RITCHIE, D. F.; UIRO, K.; UYEMOTO, J. K. **Compendium of stone fruit diseases**. American Phytopathological Society, 1995.

PEEVER, T. L.; MILGROOM, M. G. Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. **Phytopathology**, v. 84, n. 5, p. 515-519, 1994.

PENROSE, L.; DAVIS, K.; KOFFMANN, W. The distribution of benomyl-tolerant *Sclerotinia fructicola* (Wint.) Rehm. in stone fruit orchards in New South Wales and comparative studies with susceptible isolates. **Crop and Pasture Science**, v. 30, n. 2, p. 307-319, 1979.

PENROSE, L.; KOFFMANN, W.; NICHOLLS, M. Field occurrence of vinclozolin resistance in *Monilinia fructicola*. **Plant pathology**, v. 34, n. 2, p. 228-234, 1985.

PRINGLE, A.; TAYLOR, J. W. The fitness of filamentous fungi. **Trends in microbiology**, v. 10, n. 10, p. 474-481, 2002.

RITCHIE, D. Mycelial growth, peach fruit-rotting capability, and sporulation of strains of *Monilinia fructicola* resistant to dichloran, iprodione, procymidone, and vinclozolin. **Phytopathology**, v. 73, n. 1, p.44-47, 1983.

RUSSELL, P. **Sensitivity baselines in fungicide resistance research and management**. Cambridge UK: FRAC, 2002.

SANOAMUANG, N.; GAUNT, R. Persistence and fitness of carbendazim- and dicarboximide-resistant isolates of *Monilinia fructicola* (Wint.) Honey in flowers, shoots and fruit of stone fruit. **Plant pathology**, v. 44, n. 3, p. 448-457, 1995.

SCHEPERS, H. Fitness of isolates of *Sphaerotheca fuliginea* resistant or sensitive to fungicides which inhibit ergosterol biosynthesis. **Netherlands journal of plant pathology**, v. 91, n. 2, p. 65-76, 1985.

SEQUEIRA, L. Special contributions from the tropics to phytopathological mycology. **Phytopathology**, n. 52, p. 936-942, 1962.

SILVA, S. J. P.; KOHLS, V. K.; MANICA-BERTO, R.; RIGATTO, P.; ROMBALDI, C. V. Apropriação tecnológica da produção integrada de pêssegos na região de Pelotas no Estado do Rio Grande do Sul. **Ciência Rural**, v. 41, n. 9, p. 1667-1673, 2011.

SONODA, R.; OGAWA, J. Growth rate of *Monilinia fructicola* resistant and sensitive to benomyl on potato-dextrose agar and on peach fruit. **Plant disease**, v. 66, n 12, 1982.

STAUB, T. Fungicide resistance: Practical experience with antiresistance strategies and the role of integrated use. **Annual review of Phytopathology**, v. 29, n. 1, p. 421-442, 1991.

VANDERPLANK, J. **Disease resistance in plants**. 2nd. Orlando: Academic Press, 1984.

ZEHR, E. I.; LUSZCZ, L. A.; OLIEN, W. C.; NEWALL, W. C.; TOLER, J. E. Reduced sensitivity in *Monilinia fructicola* to propiconazole following prolonged exposure in peach orchards. **Plant disease**, v. 83, n. 10, p. 913-916, 1999.

ZHU, F.; BRYSON, P. K.; SCHNABEL, G. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. **Pest management science**, v. 68, n. 7, p. 1003-1009, 2012.

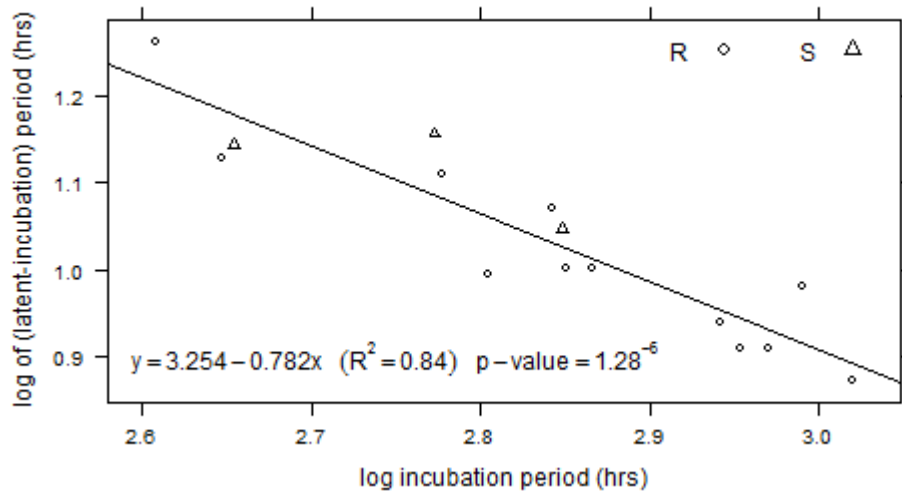


FIGURE 1. Relation between the incubation period and the difference of latent and incubation period of *Monilinia fructicola* isolates.

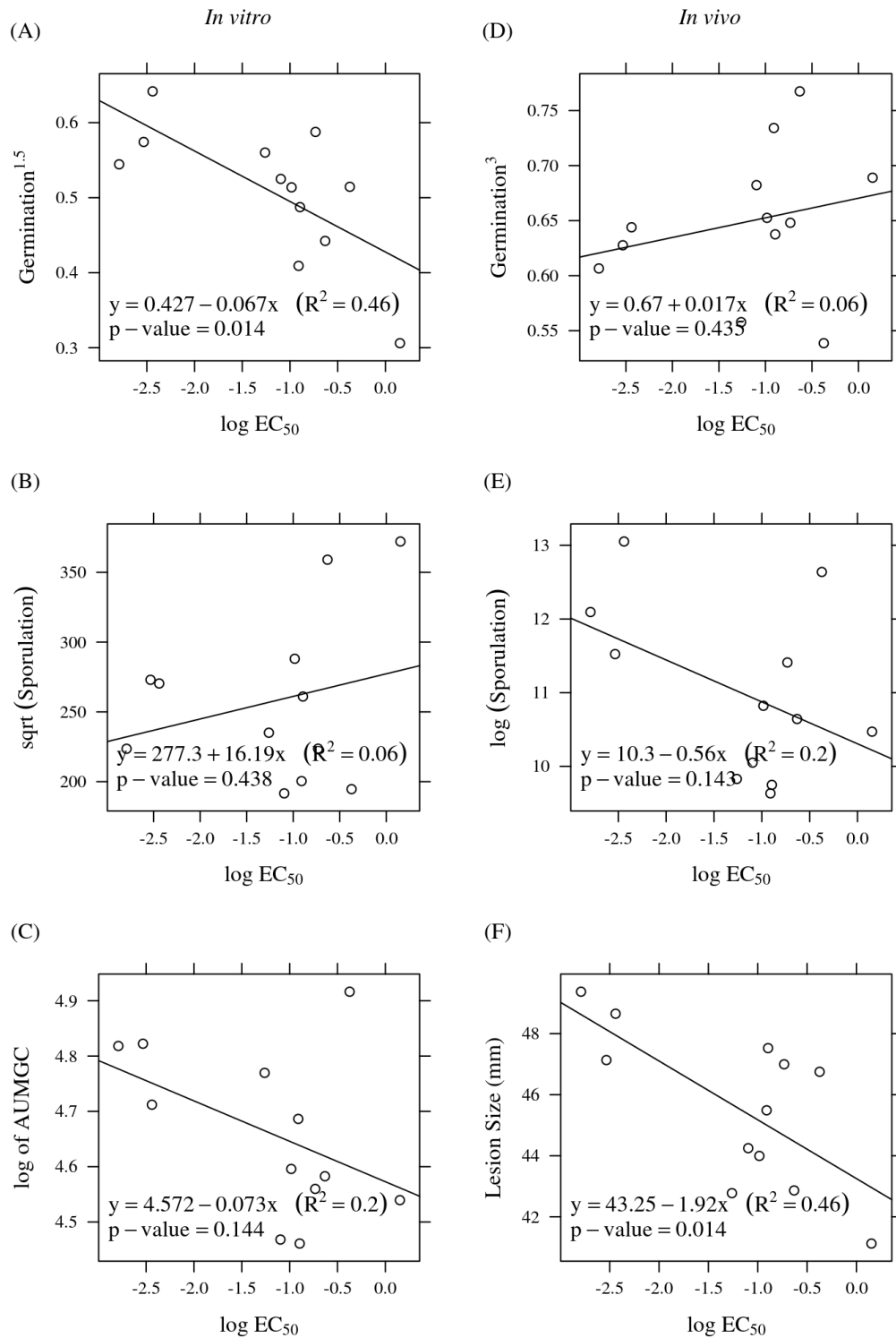


FIGURE 2. Relation between fitness variables and the EC<sub>50</sub> value of individual isolates of *Monilinia fructicola*. Experiment *in vitro*: Germination (A), sporulation (B), and Area under mycelial growth curve (C). Experiment *in vivo*: Germination (D), sporulation (E), and lesion size (F)

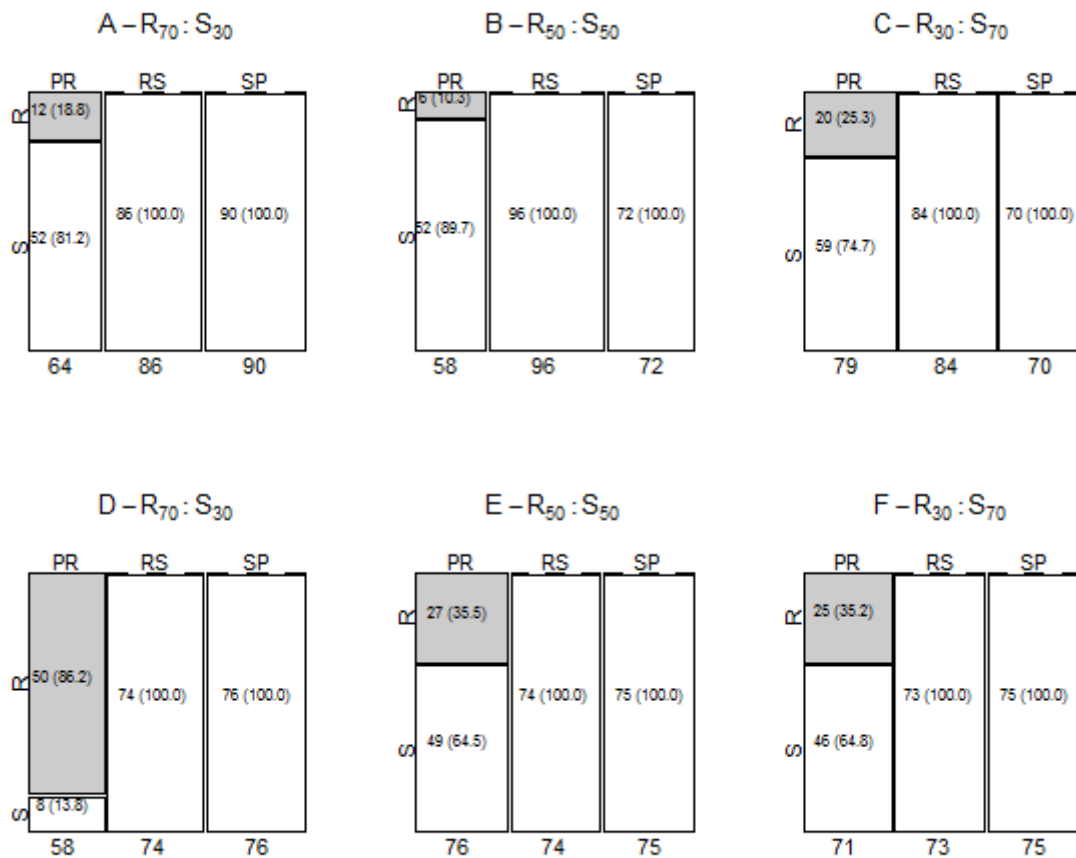


FIGURE 3. The frequency of resistant (R) and sensitive (S) spores of *Monilinia fructicola* after nine consecutive transfers *in vitro* (A, B, and C) and five consecutive transfers *in vivo* (C, D, and E). SP = São Paulo, PR = Paraná, RS = Rio Grande do Sul. Ratio R:S spores of 70:30 (A, D), 50:50 (B, E), and 30:70 (C, F).

TABLE 1. List of *Monilinia fructicola* isolates used to determine the sensitivity instability and competition capacity of resistant and sensitive phenotypes.

Isolate <sup>a</sup>	Origin <sup>b</sup>	Year	Sensitivity (EC <sub>50</sub> ) <sup>c</sup>	Competition <sup>d</sup>
1069	PR	2010	R (0.0614)	-
15	PR	2011	R (0.4807)	PR-R2
1023	PR	2010	S (0.0031)	PR-S2
1020	RS	2010	R (0.0794)	RS-R3
134	RS	2011	R (0.0872)	-
106	RS	2011	S (0.0033)	RS-S3
754	SP	2009	R (0.3740)	-
761	SP	2009	R (0.5320)	-
802	SP	2009	R (0.3341)	-
807	SP	2009	R (1.1644)	SP-R1
940	SP	2009	R (0.4026)	-
945	SP	2009	R (0.4086)	-
951	SP	2009	R (0.2833)	-
152	SP	2011	R (0.6888)	-
1099	SP	2010	S (0.0044)	SP-S1

<sup>a</sup> Fifteen isolates used for the sensitivity instability experiment.

<sup>b</sup> Origin of isolate samples (PR = Paraná, RS = Rio Grande do Sul and SP = São Paulo).

<sup>c</sup> Sensitivity of *M. fructicola* isolates (R=resistant, S=sensitive), and EC<sub>50</sub> values (µg/ml) determined previously by Lichtemberg et al. (2014).

<sup>d</sup> Six isolates used for the competition capacity experiment. Paired according its origin.

TABLE 2. *Monilinia fructicola* EC<sub>50</sub> value after successive transfers in the absence of tebuconazole.

<b>Sensitivity <i>in vitro</i><sup>a</sup></b>					
<b>Sensitivity</b>	<b>Transfer</b>	<b>n</b>	<b>EC<sub>50</sub><sup>c</sup></b>		<b>IC<sub>95</sub> %<sup>d</sup></b>
R	2 <sup>nd</sup>	45	0.0300	a	0.0165 - 0.0546
	5 <sup>th</sup>		0.0223	a	0.0123 - 0.0407
	9 <sup>th</sup>		0.0153	a	0.0084 - 0.0278
S	2 <sup>nd</sup>	15	0.0019	<b>ab</b>	0.0004 - 0.0087
	5 <sup>th</sup>		0.0043	<b>a</b>	0.0009 - 0.0195
	9 <sup>th</sup>		0.0003	<b>b</b>	0.0001 - 0.0016
p-value (transfer)		<b>0.016</b>			
p-value (transfer : sensitivity)		<b>0.0001</b>			
<b>Sensitivity <i>in vivo</i><sup>b</sup></b>					
<b>Transfer</b>		<b>n</b>	<b>EC<sub>50</sub></b>		<b>IC<sub>95</sub> %</b>
2 <sup>nd</sup>		60	0.0112	a	0.008 - 0.016
5 <sup>th</sup>		60	0.0077	b	0.005 - 0.011
p-value (transfer)		<b>0.0001</b>			
p-value (transfer : sensitivity)		<b>0.861</b>			

<sup>a</sup> Three evaluations were carried out for *in vitro* experiment, at the end of 2<sup>nd</sup>, 5<sup>th</sup>, and 9<sup>th</sup> transfer cycles.

<sup>b</sup> Two evaluations were carried out for *in vivo* experiment, at the end of 2<sup>nd</sup>, and 5<sup>th</sup> transfer cycles.

<sup>c</sup> EC<sub>50</sub> value (µg/ml) determined according May De Mio et al. (2011). Means were compared with single-step method.

<sup>d</sup> 95% confidence interval for the mean.

TABLE 3. Transfer and sensitivity effect in *Monilinia fructicola* fitness variables *in vitro*.

Transfer <sup>a</sup>	Germination (%)			Sporulation (conidia/cm <sup>2</sup> )		
	n	mean <sup>b</sup>	IC <sub>95%</sub> <sup>c</sup>	n	mean <sup>b</sup>	IC <sub>95%</sub> <sup>c</sup>
2 <sup>nd</sup>	59	67.4 ab	62.5-72.1	59	98,663.2 a	77,299.2 - 122,630.6
5 <sup>th</sup>	57	61.1 b	55.9-66.1	60	52,353.9 b	37,174.6 - 70,125.6
9 <sup>th</sup>	59	73.3 a	68.6-77.8	60	80,779.6 ab	61,610.6 - 102,540.9
p-value	<b>8.72 × 10<sup>-5</sup></b>			<b>5.11 × 10<sup>-6</sup></b>		
Sensibility	n	mean	IC <sub>95%</sub>	n	mean	IC <sub>95%</sub>
R	139	63.4 b	60.8-66.0	143	66,511.7 a	57,236.1 - 76,483.5
S	36	71.2 a	66.3-75.9	36	86,156.5 a	65,710.6 - 109,367.5
p-value	<b>0.003</b>			0.068		

<sup>a</sup> Three evaluations were carried out for *in vitro* experiment, at the end of 2<sup>nd</sup>, 5<sup>th</sup>, and 9<sup>th</sup> transfer cycles.

<sup>b</sup> Means were compared with the single-step method.

<sup>c</sup> 95% confidence interval for the mean.

TABLE 4. Area under the mycelial growth curve of *Monilinia fructicola* isolates resistant and sensitive for tebuconazole.

Sensitivity <sup>a</sup>	Transfer <sup>b</sup>	n	AUMGC <sup>c</sup>		IC <sub>95%</sub> <sup>d</sup>
R	2 <sup>nd</sup>	48	100.7	b	93.63 - 108.3
	5 <sup>th</sup>		100.6	b	93.58 - 108.2
	9 <sup>th</sup>		109.8	a	102.14 - 118.1
S	2 <sup>nd</sup>	12	199.8	<b>a</b>	166.48 - 239.9
	5 <sup>th</sup>		171.5	<b>a</b>	142.86 - 205.9
	9 <sup>th</sup>		166.6	<b>a</b>	138.78 - 200.0
p-value (transfer : sensitivity)				<b>0.003</b>	

<sup>a</sup> Sensitivity of *M. fructicola* isolates (R=resistant, S=sensitive).

<sup>b</sup> Three evaluations were carried out for *in vitro* experiment, at the end of 2<sup>nd</sup>, 5<sup>th</sup>, and 9<sup>th</sup> transfer cycles.

<sup>c</sup> Means were compared with the single-step method.

<sup>d</sup> 95% confidence interval for the mean.

TABLE 5. Transfer and sensitivity effect in *Monilinia fructicola* fitness variables *in vivo*.

Fitness Parameters ( <i>in vivo</i> )		Transfer <sup>a</sup>		Sensitivity	
		2 <sup>nd</sup>	5 <sup>th</sup>	R	S
<b>Germination (%)</b>	n	110	112	176	46
	mean <sup>b</sup>	78.3 b	93.4 a	86.4 a	86.6 a
	IC <sub>95%</sub> <sup>c</sup>	75.4 - 81.0	91.3 - 95.3	84.8 - 87.8	83.6 - 89.4
	p-value	<b>2.2 × 10<sup>-16</sup></b>		0.8890	
<b>Sporulation (spores/cm<sup>2</sup>)</b>	n	112	118	183	47
	mean <sup>b</sup>	99,013.4 b	120,247.8 a	59,197.8 b	201,122.6 a
	IC <sub>95%</sub>	65,819.4 - 148,947.6	80,648.4 - 179,211.2	45,727.3 - 76,637.4	120,880.8 - 334,629.8
	p-value	<b>0.032</b>		<b>1.33 × 10<sup>-7</sup></b>	
<b>Lesion size (mm)</b>	n	116	118	187	47
	mean <sup>b</sup>	48.7 a	44.9 b	45.5 b	48.1 a
	IC <sub>95%</sub>	47.6 - 49.8	43.8 - 46.0	44.8 - 46.2	46.7 - 49.4
	p-value	<b>3.68 × 10<sup>-7</sup></b>		<b>5 × 10<sup>-4</sup></b>	
<b>Incubation Period (h)</b>	n	120	120	192	48
	mean <sup>b</sup>	16.2 a	15.4 a	17.2 a	14.5 b
	IC <sub>95%</sub>	14.87-17.71	14.09-16.83	16.44-18.03	12.94-16.29
	p-value	0.1540		<b>0.016</b>	
<b>Latent Period (h)</b>	n	120	120	192	48
	mean <sup>b</sup>	46.8 a	45.8 a	47.3 a	45.3 a
	IC <sub>95%</sub>	45.35-48.24	44.30-47.34	46.51-48.11	43.42-47.24
	p-value	0.117		0.884	

<sup>a</sup> Two evaluations were carried out for *in vivo* experiment, at the end of 2nd, and 5th transfer cycles.

<sup>b</sup> Means were compared with the single-step method.

<sup>c</sup> 95% confidence interval for the mean.

## 5. CHAPTER III – IN BRAZIL THE EXPRESSION OF *MfCYP51* GENE CONFERS RESISTANCE FOR TEBUCONAZOLE IN *Monilinia fructicola* ISOLATES OBTAINED FROM ORCHARDS WITH HIGH PRESSURE OF FUNGICIDE SELECTION

### 5.1. ABSTRACT

The aim of this study was to investigate the mechanism associated with *Monilinia fructicola* resistance to demethylation inhibitor fungicide (DMI) tebuconazole. Isolates used in this study differs on sampling locations, tebuconazole sensitivity (0.002 - >10 µg/ml), and sampling dates (2002 – 2011). In this study, a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method was used to identify the 65-bp insertion called ‘Mona’ element, and the real-time PCR technique was used to quantify expression levels of the ABC transporter gene *MfABC1* and the gene encoding the target enzyme 14  $\alpha$ -demethylase *MfCYP51*. The induced expression of the *MfABC1* gene with tebuconazole on isolate mycelium not always increased the levels of mRNA. No differences of *MfABC1* gene expression were observed between the groups of sensitivity (DMI-R and -S isolates), even when isolates were exposed to tebuconazole. The expression of the *MfCYP51* gene in DMI-R group of isolates was higher than DMI-S group, with significant difference observed in samples exposed to tebuconazole *in vitro*. Exposure to tebuconazole increased the expression of the *MfCYP51* gene in 6 out of 7 *M. fructicola* isolates resistant to tebuconazole. The ‘Mona’ element, was tested and not identified for 58 isolates from Brazil based on the digestion of the gene with endonuclease *BsrBI*. This is the first report on molecular mechanisms for DMI resistance identification for *M. fructicola* isolates from Brazil; providing an important advancement for risk assessment of DMI fungicides used on brown rot management.

**Key words:** Brown rot, demethylation inhibitor fungicide, gene expression, *MfABC1* and *MfCYP51* genes, ‘Mona’ element.

## NO BRASIL A EXPRESSÃO DO GENE *MfCYP51* CONFERE RESISTÊNCIA PARA TEBUCONAZOL EM ISOLADOS DE *Monilinia fructicola* OBTIDOS DE POMARES COM ALTA PRESSÃO DE SELEÇÃO POR FUNGICIDAS.

### 5.2. RESUMO

O objetivo deste estudo foi investigar o mecanismo associado com a resistência de isolados de *Monilinia fructicola* ao fungicidas inibidores da demetilação (IDM) tebuconazol. Isolados utilizados neste estudo deferem-se nas localidades de amostragem, sensibilidade ao tebuconazol (0.002 - >10 µg/ml), e datas de amostragem (2002 – 2011). Neste estudo, o método da reação em cadeia da polimerase – polimorfismo de fragmentos de restrição (PCR-RFLP) foi usado para identificar a inserção de 65-pb chamado elemento ‘Mona’, e a técnica de PCR de tempo-real foi usada para quantificar níveis de expressão do gene transportador ABC *MfABC1* e do gene que codifica a enzima alvo 14  $\alpha$ -demethylase *MfCYP51*. A expressão induzida do gene *MfABC1* com tebuconazol em micelio de isolados nem sempre aumentou os níveis de mRNA. Nenhuma diferença na expressão do gene *MfABC1* foi observado entre os grupos de sensibilidade (isolados IDM-R e -S), mesmo quando isolados foram expostos ao tebuconazol. A expressão do gene *MfCYP51* no grupo de isolados IDM-R foi maior que a dos grupo de IDM-S, com diferença significativa observado em amostras expostas ao tebuconazol *in vitro*. A exposição ao tebuconazol aumentou a expressão do gene *MfCYP51* em 6 de 7 isolados de *M. fructicola* resistentes ao tebuconazol. O elemento ‘Mona’, foi testado e não encontrado para 58 isolados do Brasil, baseado na digestão do gene com a enzima de restrição *BsrBI*. Este é o primeiro estudo dos mecanismos moleculares para identificar resistência de IDM em isolados de *M. fructicola* no Brasil, gerando um importante avanço para determinação dos riscos do uso de IDM no manejo da podridão parda.

**Palavras-chave:** fungicidas inibidores da demetilação, podridão parda, expressão gênica, genes *MfABC1* e *MfCYP51*, elemento ‘Mona’.

### 5.3. INTRODUCTION

The sterol demethylation inhibitor (DMI) fungicides are the largest and most important group of antifungal agents used in agriculture (MA et al., 2006). The azoles function by targeting the ergosterol biosynthetic enzyme lanesterol demethylase, blocking the production of ergosterol which leads to accumulation of toxic sterols that exert a severe stress on the cell membrane (SHAPIRO et al., 2011). These fungicides have been widely used for more than 30 years to control brown rot in stone fruits around the world (CHEN et al., 2013) and reduced sensitivity to DMIs has been reported since then (SCHNABEL et al., 2004; AMIRI et al., 2010; MAY DE MIO et al., 2011; CHEN et al., 2012).

Molecular mechanisms leading to DMI resistance in fungal pathogens involve several mechanisms, and have been intensively studied (DE WAARD, 1996; LUPETTI et al., 2002). The most common mechanisms conferring resistance include a mutation in the *CYP51* gene encoding the target enzyme sterol 14  $\alpha$ -demethylase (DELYE et al., 1997; ASAI et al., 1999), overexpression or increased copy number of the *CYP51* gene (SCHNABEL; JONES, 2001; LUO; SCHNABEL, 2008), and overexpression of ATP-binding cassette (ABC) transporters encoding efflux pumps (HAYASHI et al., 2002; ZWIERS et al., 2002; SCHNABEL et al., 2003).

In Brazil, the *M. fructicola* mechanisms of resistance to DMI fungicides are unknown, thus the use of sensitive methods for large scale detection and quantification of resistance are impaired. The objective of this study was (i) to characterize the molecular mechanism of DMI resistance in *M. fructicola*. Our hypothesis was that the resistance mechanism of DMI resistance in this pathogen would be similar to mechanisms previously reported in United States. Additional knowledge of DMI resistance mechanisms will increase our understanding of the evolution of fungicide resistance, and could result in development of an effective detection method for resistant isolates in Brazil while providing new strategies for fungicide management programs for brown rot control.

### 5.4. MATERIAL AND METHODS

#### 5.4.1. Isolates

From the 58 isolates tested for the presence of the ‘Mona’ element, 48 were sampled from 2002-2008, and 10 were sampled in 2011. Table 1 describes 20 of the 58 samples tested for the ‘Mona’ element. For the expression experiment of the *MfCYP51* and *MfABC1* genes, a total of 10 isolates were selected (half sampled from 2006-2008 and other half from 2009-2011, Table 1). The isolate EC<sub>50</sub> values were previously determined by May De Mio et al. (2011) and Lichtemberg et al. (2014). All isolates were obtained from mummified fruit in commercial stone fruit orchards of São Paulo (SP), Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS), Brazil. Isolates were single spore cultured and plugs were stored in sterile water vials at 4°C and 25% glycerol at -80°C for later use. Species identification was done according to methods described previously by Côté et al. (2004).

#### 5.4.2. ‘Mona’ element identification

All 58 isolates were grown on acidified dextrose agar (APDA) plates covered with a cellophane disc for 5 days at 22°C with a 12 h light regime. Approximately 2 cm<sup>2</sup> mycelium was removed using a sterile toothpick and placed into a 1.5 ml sterile tube. DNA was extracted using the UltraClean Microbial DNA extraction kit (MoBio Laboratories, Solana, CA) following the manufacturer's protocols. The DNA of the resistant isolate Bmpc7 from Georgia (United States) was used as reference due to the availability of the sequencing of its *MfCYP51* gene by Luo and Schnabel (2008).

PCR primers INS65-F and INS65-R (Table 2) were designed based on a previously cloned *MfCYP51* upstream sequence (GenBank accession number EU035301) (LUO et al., 2008). PCR reaction volumes were 15 µl containing 1 × PCR buffer provided by the manufacturer, 20 ng genomic DNA, 0.5 µM of each primer, 200 µM of each dNTP, and 0.625 unit of Taq DNA polymerase (Invitrogen®). Amplifications were performed in an “iCycler” thermal cycler (Mastercycler Gradient) programmed for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and followed by a final extension step of 5 min at 72°C. The PCR products were stained with GelRed (Invitrogen®) and separated on a 1.5% agarose gel in 1 × TBE buffer (44.5 mM Trisborate, 1 mM EDTA, pH 8.0) at 100 V for 1 h and with a 100-

bp ladder size standard (Invitrogen®). Gels were visualized with ultraviolet light and photographed with an Electrophoresis Documentation and Analysis System.

Presence or absence of the 'Mona' element was confirmed by digesting the PCR product with the restriction endonuclease *BsrBI*. Only fragments containing the 'Mona' element are digestible with this enzyme resulting in two restriction fragments of 236-bp and 140-bp in size (LUO et al., 2008). PCR fragments were submitted to restriction digestions with *BsrBI* (FastDigest - MbiI) at 37°C for 10 min according to the manufacturer's recommendations. Digested PCR products were separated on a 1.5% agarose gel, and the gel was photographed as described above. The PCR reaction and PCR product digestion with restriction endonuclease *BsrBI* were performed twice, for the 20 isolates selected by EC<sub>50</sub> (Table 1).

Five isolates (21, 152, 316, 202 and 340) were selected for sequencing the *MfCYP51* gene region where element 'Mona' is inserted. Sequencing of the 'Mona' element was performed with the same primers used in the amplification. The PCR product was purified with ammonium acetate and ethanol (CROUSE; AMORESE, 1987). The sequencing was performed by the chain termination method with the ET sequencing mix (kit: "DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE"; Amersham Biosciences®) and purified with Sephadex™ G-50 medium (GE Healthcare®). The sequencing was performed in a MegaBACE™ 1000 DNA Analysis Systems (Amersham Biosciences®). Sequence editing and inspection were performed using the BioEdit program, version 7.0 (HALL, 1999), and then aligned and compared with sequences from the GenBank by using the BLAST program (ALTSCHUL et al., 1997).

#### 5.4.3. Mycelium preparation for relative expression experiments

In total for the *in vitro* experiment, 10 isolates of *M. fructicola* were cultured on APDA plates covered with a cellophane disc and incubated for 5 days at 24°C and 12 h light regimen. A 5-days-old active growing culture (1 cm<sup>2</sup>) were transferred into eppendorf tubes containing sterile water (1 ml). Subsequently, the tube contents were vigorously agitated and transferred into 250 ml flasks with acidified potato dextrose broth (APDB; 50 ml). The liquid cultures were incubated for 4 days at room temperature on a rotary shaker at 100 rev/min. Prior to RNA extraction, samples were exposed for one hour to 0.5 µg/ml of commercial tebuconazole product suspended with sterile water. Controls were not exposed to fungicide.

For the *in vitro* experiment, 4 isolates of *M. fructicola* were tested on detached peaches (Table 1). Peaches of the variety ‘Maycrest’ were harvested from an untreated orchard, and surface sterilized by 1 minute immersion in 70% ethanol and 0.5% sodium hypochlorite and then rinsed three times in sterile deionized water. The fruits were allowed to dry before spraying with 0.5 and 1 µg/ml of tebuconazole until run-off, and kept inside the hood for 12 h. Control peaches were sprayed with sterile water only. Three small and equally distributed punctures were made on each fruit before inoculation with 30 µl of spore suspension at  $5 \times 10^4$  conidia/ml. Fruits were incubated inside humidified plastic containers at room temperature for 3 days before spore harvest.

#### 5.4.4. RNA extraction, cDNA synthesis and PCR amplification

After 1 hour of fungicide treatment *in vitro*, mycelial pellets were harvested on filter paper by suction (Whatman® n° 4), dried under hood conditions for 2 hours, and weighed. The total RNA was extracted from 100 mg of *M. fructicola* mycelia using the FastRNA™ Pro Red kit (MP biomedical, Solon, OH) and FastPrep® Instrument (MP biomedical, Solon, OH) for 40 s at a speed setting of 6.0. RNA (2 µl) and was readily checked for concentration using NanoDrop 1000 Spectrophotometer (Fischer Scientific, Pittsburgh, PA). Integrity was checked using a 1.5% formaldehyde agarose gel, with samples stained with GelRed (Invitrogen®) and RNA 2 x sample Loading Buffer (Invitrogen®). For experiments *in vivo*, spores were harvested directly into the tube provided by the kit.

The cDNA was synthesized with GoScript™ Reverse Transcription System (Promega, Madison, WI, US) following the manufacturer’s protocol with modifications. For a 20 µl reaction, 2 µl of experimental RNA at 500 ng/µl and 1 µl of primer Oligo (dT)<sub>15</sub> was used; and for the reverse transcription reaction mix, 3.2 µl of 0.4 mM MgCl<sub>2</sub> was used.

Primer pair MfABC6-F and MfABC6-R (Table 2) were designed from the previously described *MfABC1* gene sequence (GeneBank accession number AY077839) (SCHNABEL et al., 2003), while primer pair RealCYP-F2 – RealCYP-R2 were retrieved from Luo and Schnabel (2008). A reaction of 25 µl contained 12.5 µl PCR Master Mix (Promega, Madison, WI), 2 µl experimental cDNA, 0.5 µl for each primer at 10 mM (Invitrogen®), and nuclease free water was prepared for the following program: one cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 20 s, and a final extension of 72°C for 5 min. A thermocycler

Eppendorf Mastercycle (Hamburg, Germany) was used. PCR product was visualized on 1.5% formaldehyde agarose gel, with samples stained with GelRed (Invitrogen®) and DNA ladder TrackIt™ (Invitrogen®). ExoSAP-it® product (Affymetrix Inc., Cleveland, OH) was used for PCR product purification following manufacturer's protocol prior to the sequencing at UC Davis, CA. Controls were made in the absence of cDNA templates.

#### 5.4.5. Expression quantification of *MfCYP51* and *MfABC1* genes

Real-Time PCR amplifications were performed in the DNA Engine Opticon 2 system (MJ Research, Waltman, MA) using the GoTaq® qPCR Master Mix (Promega, Madison, WI) containing BRYT Green® as a fluorescent DNA-binding dye. For quantification of mRNA levels for *MfCYP51* and *MfABC1* genes, the respective primer pairs were used: RealCYP-F2 – RealCYP-R2, and MfABC6-F – MfABC6-R. The quantification of the normalization gene *MfActin* were done with the primer pair MfActin-F – MfActin-R (Table 2). Reactions were performed in 25 µl volumes, containing 2 µl of cDNA template 10 × diluted, 12.5 µl of GoTaq® qPCR Master Mix, 0.5 µl of each primer pair (10 µM), and nuclease free water. All PCR were amplified in quadruplicates *in vitro* and duplicates *in vivo* with the program: one cycle of 95°C for 3 min, 40 cycles of 95°C for 10 s, 55°C for 45 s, and one cycle of 95°C for 1 min and 55°C for 1 min.

To confirm the correct amplification in these reactions, a melting curve was constructed for each primer pair (100 cycles with increasing temperature by 0.4°C increments starting at 55°C) to verify the presence of one gene-specific peak and the absence of primer dimmer.

The comparative Ct method (separate tubes) proposed by Livak and Schmittgen (2001) was used to determine the *MfCYP51* and *MfABC1* expression relative to the expression of the untreated sample, using *MfActin* expression to normalize the data. The assumption for experiment validation indicated equal efficiency of the target amplification and the reference amplification for *MfABC1* (slope = 0.0208) and *MfCYP51* (slope = 0.057) (data not shown). The expression was calculated by the equation:  $2^{-\Delta\Delta Ct} = 2^{-(Ct, \text{gene} - Ct, \text{MfActin gene})_x - (Ct, \text{gene} - Ct, \text{MfActin gene})_y}$ , where x is the tebuconazole treated sample, and y is the untreated isolate sample, used as calibrator. This method was used to evaluate the tebuconazole effect for each individual sample. Differences between the expressions from tebuconazole treatment for samples tested

*in vivo* were analyzed with analysis of variance (ANOVA). A variation proposed by Schmittgen and Livak (2008) on the comparative Ct method was used to compare the expression between sensitivity groups, such those composed by DMI-R and DMI-S isolates. The expression of DMI-R and DMI-S was determined separately for each group by  $2^{-\Delta Ct}$  [ $2^{-(Ct, \text{gene} - Ct, \text{MfActin gene})}$ ], and ANOVA was performed to determine significant differences. For all inferences, 5% was considered a nominal significance level and the statistical software R (version 3.1.1.) was used in the entire analysis. All experiments were twice repeated.

## 5.5. RESULTS

### 5.5.1. 'Mona' element identification

No differences in fragment size between DMI-R and -S isolates were observed on gel electrophoresis of the 20 tested isolates (Fig.1). All 58 assessed isolates (20 from Table 1 and 38 not showed) revealed fragment size of 376-bp when amplified with primer pair INS65-F and INS65-R. The classification of isolates as DMI-R or DMI-S had no correlation with fragment size. The restriction of PCR product with endonuclease *Bsr*BI, occurred only in fragments of control *Bmpc7* containing the 'Mona' element as shows the two restriction fragments of 236-bp and 140-bp in size (Fig.1). Brazilian isolates showed no 'Mona' element as confirm the sequence of the upstream region of *MfCYP51* gene (Fig. 2).

### 5.5.2. Amplification and sequencing of *MfCYP51* and *MfABC1* genes.

The specificity of primer pairs RealCYP-F2 – RealCYP-R2 and MfABC6-F – MfABC6-R for *M. fructicola* was confirmed after sequencing, showing an expected 120-bp and 176-bp PCR product respectively (data not shown).

### 5.5.3. Expression of the *MfCYP51* and *MfABC1* genes.

The relative expression of the *MfABC1* gene between DMI-R and -S isolate groups were similar for experiments *in vitro* ( $p = 0.70$ ) and *in vivo* ( $p = 0.34$ ). The expression of the *MfABC1* gene revealed a DMI-R and -S fold change of 1.2 and 0.47, for experiments performed *in vitro* and *in vivo* respectively (Table 3). The mycelium exposure to non-lethal dose of tebuconazole led to a slight increase ( $p = 0.05$ ) on the gene expression of the *MfCYP51* gene in DMI-R in comparison to DMI-S group. This result revealed a fold change of 1.65 when tested *in vitro*. No differences ( $p = 0.324$ ) were observed *in vivo* (Table 4).

For the *in vitro* experiment on the *MfABC1* gene, the mean  $2^{-\Delta\Delta Ct}$  values for DMI-R isolates 204, 208, 213 and 945 treated with tebuconazole at 0.5  $\mu\text{g/ml}$  increased the expression value in relation to the untreated sample (used as calibrator), while the fungicide exposure to the other four DMI-R isolates (15, 210, 802 and 807) decreased did not resulted in increased expression in relation to the untreated sample (Table 5). For the DMI-S, isolate 106 showed increased expression, while isolate 349 demonstrated a reduction in relation to the untreated sample (Table 5). When the expression of the *MfABC1* gene was tested *in vivo*, the  $2^{-\Delta\Delta Ct}$  values of DMI-R isolates 204 and 208 increased to 29.30 and 2.04 respectively in samples exposed to tebuconazole at 0.5  $\mu\text{g/ml}$  (Table 5). When samples were exposed to 1  $\mu\text{g/ml}$  of tebuconazole, only isolate 208 revealed a significant difference relative to the previous treatment dose (Table 5). The DMI-S isolate 349 showed a significant ( $p = 0.009$ ) increase for  $2^{-\Delta\Delta Ct}$  values at 1  $\mu\text{g/ml}$  compared to 0.5  $\mu\text{g/ml}$  of tebuconazole, while isolate 106 showed no differences ( $p = 0.99$ ) between fungicide dosages (Table 5).

For the *in vitro* experiment on the *MfCYP51* gene expression, all DMI-R isolates except 807 showed an increased  $2^{-\Delta\Delta Ct}$  value in relation to the untreated sample used as calibrator. The DMI-S isolates 106 and 349 showed decreased  $2^{-\Delta\Delta Ct}$  values (Table 5). When tested *in vivo*, the expression of the *MfCYP51* gene in samples exposed to tebuconazole at 0.5  $\mu\text{g/ml}$  increased only for the DMI-R isolate 204 and DMI-S 349 in relation to untreated control, and no significant differences ( $p = 0.26$ ) were observed in the  $2^{-\Delta\Delta Ct}$  values of isolates exposed to increased dose of tebuconazole at 1  $\mu\text{g/ml}$  in relation to the previous tested dosage (Table 5).

## 5.6. DISCUSSION

Increased expression of *CYP51* is an important mechanism of DMI resistance, and it was extensively reported in several plant pathogens (VAN DEN BRINK et al., 1996; HAMAMOTO et al., 2000; SCHNABEL; JONES, 2001; MA et al., 2006). In our *in vitro* study, the isolates exposed to tebuconazole have resulted in increased *MfCYP51* gene expression in six out of nine tested samples, while DMI-S isolates showed no increased expression. When isolates were evaluated within groups of sensitivity, it was observed that by inducing expression with tebuconazole at 0.5 µg/ml resulted in higher mRNA levels on DMI-R group in relation to DMI-S group in order of 1.65 fold. There is no rule to determine the amount of fold change necessary to a mechanism be associated or not to the DMI fungicide resistance. However, changes of 2- up to 5-fold were enough to associate the expression of *CYP51* gene as the azole mechanism of resistance in *Aspergillus niger* (VAN DEN BRINK et al., 1996) and *Venturia inaequalis* (SCHNABEL; JONES, 2001). In *M. fructicola* the expression of *MfCYP51* gene was 5- to 11 fold higher in DMI-R than –S isolate (LUO; SCHNABEL, 2008).

The driving factors for increased *CYP51* mRNA levels are usually associated to insertions in the *CYP51* gene. Sequencing confirmed that there is no 65-bp region related to the ‘Mona’ element in Brazilian DMI-R isolates. The method developed by (LUO et al., 2008) using the restriction enzyme *BsrBI* is fundamental for identification of the ‘Mona’ element. Absence of the ‘Mona’ element in Brazilian isolates cannot be the result of possible losses due to storage or maintenance performed by subcultures of isolates *in vitro*, as already observed by Zhu et al. (2012). Therefore, these results indicated that the mechanism which triggers *MfCYP51* expression in Brazilian isolates is another from that reported for isolates from Georgia, South Carolina and New York in United States (LUO et al., 2008; VILLANI; COX, 2011). No insertions other than the ‘Mona’ element were studied in our current work. Insertions are known to regulate gene expression and have been reported in *Venturia inaequalis* (SCHNABEL; JONES, 2001), *Penicillium digitatum* (HAMAMOTO et al., 2000), *Blumeriella jaapii* (MA et al., 2006) and also *M. fructicola* from United States (LUO et al., 2008).

The ABC transporters involved in energy-dependent efflux of DMIs have been described for *Aspergillus nidulans* (DEL SORBO et al., 2000), *Candida albicans* (PRASAD et al., 1995), *Mycosphaerella graminicola* (ZWIERS et al., 2002), and *Penicillium digitatum* (NAKAUNE et al., 1998). In *M. fructicola*, the role of ABC transporters as a mechanism which grants DMI resistance was shown as a minor genetic determinant (SCHNABEL et al., 2003; LUO; SCHNABEL, 2008). In our study, the low expression of *MfABC1* for isolates treated with tebuconazole may be associated with the wide fungicide sensitivity of our tested isolates. According to Zwiers et al. (2002) changes in susceptibility to azoles could cause profound

changes in the levels of expression of the ABC transporter genes. This reason is more likely to explain the low expression than the rapid decline of the mycelium exposure to fungicide, since for the same samples results on *MfCYP51* gene were conclusive. Furthermore, when isolates were studied by group of sensitivity (DMI-R and DMI-S) no effect has been demonstrated after fungicide induction or between groups, as it was also observed by (LUO; SCHNABEL, 2008).

In conclusion, DMI tebuconazole resistance in *M. fructicola* isolates from Brazil is likely a result of increased expression of the *MfCYP51* gene but it is still unknown what could be triggering such a mechanism. Since the DMI-R Brazilian isolates are absent for the 65-bp insertion, the effect of a common ancestor could, in part, explain the ‘Mona’ absence in Brazil. Further investigations should consider the full sequencing of *MfCYP51* gene for Brazilian isolates, in order to evaluate other target site mutations in *CYP51*, such as the Y136F mutation occurring in *M. fructicola* resistant isolates of SYP-Z048 in China, which have demonstrated positive cross-resistance to propiconazole (CHEN et al., 2012). It would also be ideal to target other transporters that could be involved in azole transport in *M. fructicola* that are not yet identified.

## 5.7. CONCLUSION

DMI resistance in *M. fructicola* in Brazil is correlates with increased expression of *MfCYP51* gene.

Its still unknown what could be triggering the resistance mechanism of *MfCYP51* gene expression in isolates from Brazil.

Increased in gene expression is not due to the presence of the ‘Mona’ element.

## 5.8. ACKNOWLEDGEMENT

We thank Ryan Puckett, Joel Quattrini, and Herve Avenot, for their technical assistance. To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) doctoral fellowship. This study was supported by CNPq grants n° 479041/2010-5 Universal/CNPq.

## 5.9. REFERENCES

- ALTSCHUL, S. F.; MADDEN, T. L.; SCHAFFER, A. A.; ZHANG, J.; ZHANG, Z.; MILLER, W.; LIPMANN, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Research**, v. 25, n. 17, p. 3389-3402, September 1, 1997.
- AMIRI, A.; BRANNEN, P. M.; SCHNABEL, G. Reduced Sensitivity in *Monilinia fructicola* Field Isolates from South Carolina and Georgia to Respiration Inhibitor Fungicides. **Plant Disease**, v. 94, n. 6, p. 737-743, 2010.
- ASAI, K.; TSUCHIMORI, N.; OKONOJI, K.; PERFECT, J. R.; GOTOH, O.; YOSHIDA, Y. Formation of azole-resistant *Candida albicans* by mutation of sterol 14-demethylase P450. **Antimicrobial agents and chemotherapy**, v. 43, n. 5, p. 1163-1169, 1999.
- CHEN, F.; FAN, J. R.; ZHOU, T.; LIU, X. L.; LIU, J. L.; SCHNABEL, G. Baseline sensitivity of *Monilinia fructicola* from China to the DMI fungicide SYP-Z048 and analysis of DMI-resistant mutants. **Plant Disease**, v. 96, n. 3, p. 416-422, 2012.
- CHEN, F.; LIU, X. L.; CHEN, S.; SCHNABEL, E.; SCHNABEL, G. Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. **Plant Disease**, v. 97, n. 5, p. 645-651, 2013.
- CÔTÉ, M. J.; TARDIF, M. C.; MELDRUM, A. J. Identification of *Monilinia fructigena*, *M. fructicola*, *Monilinia laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. **Plant Disease**, v. 88, n. 11, p. 1219-1225, 2004.
- CROUSE, J.; AMORESE, D. Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. **Focus**, v. 9, n. 2, p. 3-5, 1987.
- DE WAARD, M. A. Molecular genetics of resistance in fungi to azole fungicides. **ACS Symp. Ser.**, n. 645, p. 62-71, 1996.
- DEL SORBO, G.; SCHOONBEEK, H.-J.; DE WAARD, M. A. Fungal Transporters Involved in Efflux of Natural Toxic Compounds and Fungicides. **Fungal Genetics and Biology**, v. 30, n. 1, p. 1-15, 2000.
- DELYE, C.; LAIGRET, F.; CORIO-COSTET, M.-F. A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. **Applied and Environmental Microbiology**, v. 63, n. 8, p. 2966-2970, 1997.
- HALL, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symposium Series**, v. 41, p. 95-98, 1999.

HAMAMOTO, H.; HASEGAWA, K.; NAKAUNE, R.; LEE, Y. J.; MAKIZUMI, Y.; AKUTZU, K.; HIBI, T. Tandem repeat of a transcriptional enhancer upstream of the sterol 14 $\alpha$ -demethylase gene (*CYP51*) in *Penicillium digitatum*. **Applied and environmental microbiology**, v. 66, n. 8, p. 3421-3426, 2000.

HAYASHI, K.; SCHOONBEEK, H.-J.; DE WAARD, M. A. Expression of the ABC transporter BcatrD from *Botrytis cinerea* reduces sensitivity to sterol demethylation inhibitor fungicides. **Pesticide Biochemistry and Physiology**, v. 73, n. 2, p. 110-121, 2002.

LICHTENBERG, P. S. F.; MICHAILIDES, T. J.; MORALES, R. G. F.; ZEVIANI, W. M.; MAY DE MIO, L. L. Shift in *Monilinia fructicola* sensitivity to tebuconazole fungicide, and resistant isolate survey in southern Brazil. In: DEHNE, H. W.; DEISING, D. H.; FRAAIJE, B.; GISI, U.; HERMANN, D.; MEHL, A.; OERKE, E. C.; RUSSELL, P. E.; STAMMLER, G.; KUCK, K. H.; LYR, H. (Ed.). **Modern fungicides and antifungal compounds**. Braunschweig, Germany: Deutsch Phytomedizinische Gesellschaft, v. Vol II, p.173-178, 2014

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. **methods**, v. 25, n. 4, p. 402-408, 2001.

LUO, C. X.; COX, K. D.; AMIRI, A.; SCHNABEL, G. Occurrence and detection of the DMI resistance-associated genetic element 'Mona' in *Monilinia fructicola*. **Plant Disease**, v. 92, n. 7, p. 1099-1103, 2008.

LUO, C. X.; SCHNABEL, G. The cytochrome P450 lanosterol 14 $\alpha$ -demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. **Applied and environmental microbiology**, v. 74, n. 2, p. 359-366, 2008.

LUPETTI, A.; DANESI, R.; CAMPA, M.; DEL TACCA, M.; STEVEN, K. Molecular basis of resistance to azole antifungals. **Trends in molecular medicine**, v. 8, n. 2, p. 76-81, 2002.

MA, Z.; PROFFER, T. J.; JACOBS, J. L.; SUNDIN, G. W. Overexpression of the 14 $\alpha$ -demethylase target gene (*CYP51*) mediates fungicide resistance in *Blumeriella jaapii*. **Applied and environmental microbiology**, v. 72, n. 4, p. 2581-2585, 2006.

MAY DE MIO, L. L.; LUO, Y.; MICHAILIDES, T. J. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management. **Plant Disease**, v. 95, n. 7, p. 821-827, 2011.

NAKAUNE, R.; ADACHI, K.; NAWATA, O.; TOMIYAMA, M.; AKUTSU, K.; HIBI, T. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. **Applied and environmental microbiology**, v. 64, n. 10, p. 3983-3988, 1998.

PRASAD, R.; DE WERGIFOSSE, P.; GOFFEAU, A.; BALZI, E. Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. **Current genetics**, v. 27, n. 4, p. 320-329, 1995.

SCHMITTGEN, T. D.; LIVAK, K. J. Analyzing real-time PCR data by the comparative CT method. **Nature protocols**, v. 3, n. 6, p. 1101-1108, 2008.

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

SCHNABEL, G.; DAI, Q.; PARADKAR, M. R. Cloning and expression analysis of the ATP-binding cassette transporter gene *MFABC1* and the alternative oxidase gene *MfAOX1* from *Monilinia fructicola*. **Pest management science**, v. 59, n. 10, p. 1143-1151, 2003.

SCHNABEL, G.; JONES, A. The 14 $\alpha$ -demethylase (CYP51A1) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. **Phytopathology**, v. 91, n. 1, p. 102-110, 2001.

SHAPIRO, R. S.; ROBBINS, N.; COWEN, L. E. Regulatory circuitry governing fungal development, drug resistance, and disease. **Microbiology and Molecular Biology Reviews**, v. 75, n. 2, p. 213-267, 2011.

VAN DEN BRINK, H. J. M.; VAN NISTELROOY, H. J. G. M.; DE WAARD, M. A.; VAN DEN HONDE, C. A. M. J. J.; VAN GORCOM, R. F. M. Increased resistance to 14 $\alpha$ -demethylase inhibitors (DMIs) in *Aspergillus niger* by coexpression of the *Penicillium italicum* eburicol 14 $\alpha$ -demethylase (*cyp51*) and the *A. niger* cytochrome P450 reductase (*cprA*) genes. **Journal of Biotechnology**, v. 49, n. 1-3, p. 13-18, 1996.

VILLANI, S. M.; COX, K. D. Characterizing Fenbuconazole and Propiconazole Sensitivity and Prevalence of 'Mona' in Isolates of *Monilinia fructicola* from New York. **Plant Disease**, v. 95, n. 7, p. 828-834, 2011.

ZHU, F.; BRYSON, P. K.; SCHNABEL, G. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. **Pest management science**, v. 68, n. 7, p. 1003-1009, 2012.

ZWIERS, L.H.; STERGIPOULOS, I.; VAN NISTELROOY, J. G. M.; DE WAARD, M. A. ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. **Antimicrobial agents and chemotherapy**, v. 46, n. 12, p. 3900-3906, 2002.



TABLE 1. *Monilinia fructicola* isolates used in the molecular experiments.

Collection <sup>a</sup>	Isolate	Crop - Origin <sup>b</sup>	EC <sub>50</sub> <sup>c</sup>	Sensitivity <sup>d</sup>	test <sup>e</sup>
HP	212	2002-SP	0.406	R	1
HP	202	2006-SP	0.611	R	1
HP	204	2006-SP	>10	R	1,2,3
HP	205	2006-SP	>10	R	1
HP	208	2006-SP	>10	R	1,2,3
HP	210	2006-SP	>10	R	1,2
HP	213	2006-SP	>10	R	1,2
HP	316	2007-SP	0.506	R	1
HP	340	2008-SP	>10	R	1
HP	346	2008-SP	0.633	R	1
HP	349	2008-RS	0.010	S	2,3
CP	802	2009-SP	0.334	R	2
CP	807	2009-SP	1.164	R	2
CP	945	2009-SP	0.409	R	2
CP	10	2011-PR	0.047	R	1
CP	15	2011-PR	0.480	R	2
CP	21	2011-PR	0.008	S	1
CP	35	2011-PR	0.002	S	1
CP	39	2011-PR	0.009	S	1
CP	106	2011-PR	0.003	S	2,3
CP	110	2011-RS	0.003	S	1
CP	112	2011-RS	0.014	S	1
CP	115	2011-RS	0.358	R	1
CP	122	2011-RS	0.039	S	1
CP	126	2011-RS	0.003	S	1
CP	152	2011-SP	0.689	R	1

<sup>a</sup> *M. fructicola* collection from LEMID-UFPR, Historical (2000-2008) and Actual (2009-2011).

<sup>b</sup> Place of origin where isolate was obtained. SP = São Paulo, PR = Paraná, SC = Santa Catarina, RS = Rio Grande do Sul.

<sup>c</sup> EC<sub>50</sub> values determined by May De Mio et al. (2011) for historic population (HP) and Lichtemberg et al. (2014) for current population (CP).

<sup>d</sup> Sensitivity based on EC<sub>50</sub> value according Lichtemberg et al. (2014). Sensitive (S) ≤ 0.04 µg/ml < Resistant (R).

<sup>e</sup> Isolates tested for 'Mona' element (1), *CYP51* and ABC transporter *in vitro* (2) and *in vivo* (3). Before RNA isolation, isolates were treated with tebuconazole at 0 and 0.5 µg/ml (2) and 0, 0.5 and 1 µg/ml (3).

TABLE 2. Nucleotide sequence and characteristics of primers used in this study.

Primer	5' -> 3' sequence	Template strand	Primer length (bp)	Product length (bp)	Citation
INS65-F	TCGATGTTGCGTGGTCTGTCA	plus	21	376	Luo et al. (2008)
INS65-R	CGGAGAGCGTCGATGTAGGTG	minus	21	376	Luo et al. (2008)
MfABC6-F	GCCAACAATCGAGCGGGATA	plus	20	176	This study
MfABC6-R	GGACCCTTCATTCTGGTCGC	minus	20	176	This study
RealCYP-F2	TCTTGGGCGCCGACCTTC	plus	19	120	Luo and Schanbel (2008)
RealCYP-R2	CTTTGCGCATGATAGAGTGGAT	minus	22	120	Luo and Schanbel (2008)
MfActin-F	GTGTTGATATGGCCGGTCGTGATT	plus	24	85	Schnabel and Dai (2004)
MfActin-R	TCGGCAGTGGTGGAGAAAGTGTA	minus	24	85	Schnabel and Dai (2004)

TABLE 3. Relative expression of the *MfABC1* gene in *Monilinia fructicola* groups of sensitivity.

Group	n	mean <sup>a</sup>	CI95% <sup>b</sup>
R <sup>c</sup>	8	$1.06 \times 10^{-2}$	$6.69 \times 10^{-3} - 1.69 \times 10^{-2}$
S <sup>c</sup>	2	$8.76 \times 10^{-3}$	$3.45 \times 10^{-3} - 2.21 \times 10^{-2}$
FC <sup>d</sup> <i>in vitro</i>		1.2	p-value = 0.70
R	8	$2.59 \times 10^{-2}$	$8.82 \times 10^{-3} - 7.64 \times 10^{-2}$
S	2	$5.48 \times 10^{-2}$	$1.93 \times 10^{-2} - 1.55 \times 10^{-1}$
FC <i>in vivo</i>		0.47	p-value = 0.34

<sup>a</sup> Values correspond to the mRNA levels on *MfABC1* gene. The amount of target, normalized to an endogenous reference, is given by  $2^{-\Delta C_t}$ . Means of four and two different reaction *in vitro* and *in vivo* respectively.

<sup>b</sup> 95% confidence interval of the mean.

<sup>c</sup> R=resistant and S=sensitive isolate.

<sup>d</sup> FC= fold change, according the comparison Ct method (Schmittgen and Livak 2008).

TABLE 4. Relative expression of the gene *MfCYP51* in *Monilinia fructicola* groups of sensitivity.

Group	n	mean <sup>a</sup>	CI95% <sup>b</sup>
0 µg/ml - R <sup>c</sup>	8	$4.48 \times 10^{-5}$	$2.36 \times 10^{-5} - 8.53 \times 10^{-5}$
0 µg/ml - S <sup>c</sup>	2	$3.59 \times 10^{-4}$	$8.30 \times 10^{-5} - 1.55 \times 10^{-3}$
FC <sup>d</sup> <i>in vitro</i>		0.12	p-value = 0.92
0.5 µg/ml - R	8	$1.06 \times 10^{-2}$	$6.69 \times 10^{-3} - 1.69 \times 10^{-2}$
0.5 µg/ml - S	2	$8.76 \times 10^{-3}$	$3.45 \times 10^{-3} - 2.21 \times 10^{-2}$
FC <i>in vitro</i>		1.65	p-value = 0.05
<i>in vivo</i> R	8	$9.05 \times 10^{-5}$	$3.10 \times 10^{-5} - 2.63 \times 10^{-4}$
<i>in vivo</i> S	2	$7.49 \times 10^{-5}$	$3.21 \times 10^{-5} - 1.74 \times 10^{-4}$
FC <i>in vivo</i>		1.20	p-value = 0.324

<sup>a</sup> Values correspond to the mRNA levels of *MfABC1* gene. The amount of target, normalized to an endogenous reference, is given by  $2^{-\Delta C_t}$ . Means of four and two different reaction *in vitro* and *in vivo* respectively.

<sup>b</sup> 95% confidence interval of the mean.

<sup>c</sup> R=resistant and S=sensitive isolate.

<sup>d</sup> FC= fold change, according the comparison Ct method (Schmittgen and Livak 2008).

TABLE 5. Relative expression of the *MfABC1* and *MfCYP51* genes in *Monilinia fructicola* mycelia untreated and treated with different concentration of tebuconazole.

Isolates <sup>a</sup>	Sensitivity <sup>b</sup>	<i>MfABC1</i> <sup>c</sup>			<i>MfCYP51</i> <sup>c</sup>			
		0 µg/ml	0.5 µg/ml	1 µg/ml	0 µg/ml	0.5 µg/ml	1 µg/ml	
15	R	1.0	0.06	NA <sup>d</sup>	1.0	1.06	NA <sup>d</sup>	
106	S	1.0	2.13	NA	1.0	0.14	NA	
204	R	1.0	1.40	NA	1.0	1.12	NA	
208	R	1.0	2.41	NA	1.0	4.94	NA	
210	R	1.0	0.03	NA	1.0	1.14	NA	
213	R	1.0	72.20	NA	1.0	9.08	NA	
349	S	1.0	0.06	NA	1.0	0.46	NA	
802	R	1.0	0.33	NA	1.0	1.30	NA	
807	R	1.0	0.13	NA	1.0	0.07	NA	
945	R	1.0	5.35	NA	1.0	NA	NA	
204	R	1.0	29.30	17.64	1.0	3.21	4.74	ns <sup>e</sup>
208	R	1.0	2.04	0.39	1.0	0.44	0.11	ns
349	S	1.0	0.15	3.68	1.0	0.15	2.15	ns
106	S	1.0	1.26	0.06	1.0	0.76	0.03	ns

<sup>a</sup> First 10 isolates from experiment *in vitro*, and 4 last isolates from experiment *in vivo*.

<sup>b</sup> Sensitivity based on EC<sub>50</sub> value according Lichtemberg et al. (2014). Sensitive (S) ≤ 0.04 µg/ml < Resistant (R).

<sup>c</sup> Values correspond to the mRNA levels of *MfABC1* and *MfCYP51* gene as compared with the mRNA level from each individual calibrator on dose of 0 µg/ml of tebuconazole. The amount of target, normalized to an endogenous reference, and relative to a calibrator, is given by  $2^{-\Delta\Delta C_t}$ . Means of four and two different reaction *in vitro* and *in vivo* respectively.

<sup>d</sup> Dosage not used for *in vitro* experiments.

<sup>e</sup> Means comparison between tebuconazole dosage are significantly different at 5%. Significance codes: '\*\*\*' 0.01, '\*' 0.05, 'ns' not significant.

## 6. GENERAL CONCLUSIONS

Current population of *M. fructicola* isolate has shown a reverse shift toward resistance in comparison to the previous survey.

The tebuconazole baseline EC<sub>50</sub> method revealed that 40% of the current *M. fructicola* population is composed by resistant isolate genotypes.

In São Paulo, 71.1% of *M. fructicola* population are resistant to tebuconazole, and higher mean EC<sub>50</sub> value was observed in comparison to isolates recovered from Paraná and Rio Grande do Sul peach orchards.

Laboratorial storage methods cause decreased sensitivity in *M. fructicola* isolates.

In the absence of tebuconazole, sensitivity of *M. fructicola* isolate tends to decline.

Sensitive isolates have shown better fitness than resistant isolates in the absence of tebuconazole.

Sensitive isolates demonstrated higher competitive capacity than resistant isolates when competing in the absence of tebuconazole.

The relationship between incubation time and the interval between latent and incubation periods demonstrate that isolates with slow incubation capacity can reproduce faster.

DMI resistance in *M. fructicola* in Brazil is correlates with increased expression of *MfCYP51* gene.

Its still unknown what could be triggering the resistance mechanism of *MfCYP51* gene expression in isolates from Brazil.

Increased in gene expression is not due to the presence of the 'Mona' element.

## 7. GENERAL REFERENCES

ADASKAVEG, J.; SCHNABEL, G.; FÖRSTER, H. Disease of peach caused by fungi and fungal-like organisms: biology, epidemiology and management. In: LAYNE, D. R. e BASSI, D. (Ed.). **The peach: botany, production and uses**. Wallingford: CABI, p.352-406, 2008.

ALICEWEB2. Sistema de análise das informações de comércio exterior. <http://alicesweb.mdic.gov.br/>, 2014. Acesso em: 09/16/2014.

BATRA, L. R. **World species of Monilinia (Fungi): their ecology, biosystematics and control**. 1991.

BIASI, L. A.; ZANETTE, F.; PETRI, J. L.; MARODIN, G. A. B. Cultivares de fruteiras de caroço. In: MONTEIRO, L. B.; MAY DE MIO, L. L.; SERRAT, B. M.; MOTTA, A. C.; CUQUEL, F. L. (Ed.). **Fruteiras de caroço uma visão ecológica**. Curitiba: UFPR, p.5-32, 2004.

BIGGS, A.; NORTHOVER, J. Inoculum sources for *Monilinia fructicola* in Ontario peach orchards. **Canadian Journal of plant pathology**, v. 7, n. 3, p. 302-307, 1985.

BLEICHER, J. Doenças de rosáceas de caroço. In: KIMATI, H.; AMORIM, L. REZENDE, J. A. M.; BERGAMIN FILHO, A. (Ed.). **Manual de fitopatologia: Doenças das plantas cultivadas**, v.2, p.621-627, 1997.

BRAITHWAITE, M.; ELMER, P. A. G.; SAVILLE, D. J.; BOYD-WILSON, K. S. H.; WHELAN, H. G. Reduced sensitivity to DMI fungicides in *Monilinia fructicola* and their efficacy of DMI fungicides for blossom blight control. **Proceedings 48th New Zealand Plant Protection Conference**, p.17-21, 1995.

BRANNEN, P.; HOTCHKISS, H.; REILLY, C. C.; SCHNABEL, G. Evaluation of fungicide programs to manage a DMI-resistant *Monilinia fructicola* population in a Georgia peach research block, 2005. **F&N Tests**, v. 61, 2006.

BRANNEN, P. M.; TAYLOR, K.; HOTCHKISS, H.; REILLY, C. C.; SCHNABEL, G.; AMIRI, A. Evolving management strategies for *Monilinia fructicola* populations with reduced sensitivity to DMIs in Georgia and implications for brown rot control recommendations. APS Centennial Meeting 2008. Minneapolis. **Phytopathology**. p.S25, 2008.

BRENT, K.; HOLLOMON, D. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograph No. 1. **Fungicide Resistance Action Committee**. 2007a.

BRENT, K. J.; HOLLOMON, D. Fungicide resistance: the assessment of risk. 2nd. **Fungicide Resistance Action Committee**, 2007b.

BURNETT, A.; LALANCETTE, N.; MCFARLAND, K. First report of the peach brown rot fungus *Monilinia fructicola* resistant to demethylation inhibitor fungicides in New Jersey. **Plant Disease**, v. 94, n. 1, p. 126-126, 2010.

BYRDE, R. J. W.; WILLETTS, H. J. The brown rot fungi of fruit. Their biology and control. **The brown rot fungi of fruit. Their biology and control.**, 1977.

CALPOUZOS, L. Some contributions from the tropics to plant disease control methods. **Phytopathology**, v. 52, p. 942-945, 1962.

CHEN, F.; FAN, J. R.; ZHOU, T.; LIU, X. L.; LIU, J. L.; SCHNABEL, G. Baseline sensitivity of *Monilinia fructicola* from China to the DMI fungicide SYP-Z048 and analysis of DMI-resistant mutants. **Plant Disease**, v. 96, n. 3, p. 416-422, 2012.

CHEN, F.; LIU, X. L.; CHEN, S.; SCHNABEL, E.; SCHNABEL, G. Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. **Plant Disease**, v. 97, n. 5, p.645-651, 2013a.

CHEN, F.; LIU, X.; SCHNABEL, G. Field Strains of *Monilinia fructicola* Resistant to Both MBC and DMI Fungicides Isolated from Stone Fruit Orchards in the Eastern United States. **Plant Disease**, v. 97, n. 8, p.1063-1068, 2013b.

CIMIS. Montly report. **California Irrigation Management Information System**, <http://www.cimis.water.ca.gov>, 2011. Acesso em: July 18.

COOLS, H.; HAWKINS, N.; FRAAIJE, B. Constraints on the evolution of azole resistance in plant pathogenic fungi. **Plant Pathology**, v. 62, n. S1, p.36-42, 2013.

COX, K.; BRYSON, P.; SCHNABEL, G. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. **Phytopathology**, v. 97, n. 4, p.448-453, 2007.

DE VICENZO, M.; VEIGA, J.; DARIO, G. Controle da podridão parda (*Monilinia fructicola*) na cultura do pêssego (*Prunus persica*) com o fungicida tebuconazole. **Horticultura Brasileira**, v. 15, n. supl., p.88, 1997.

ELMER, P.; BRAITHWAITE, M.; SAVILLE, D. Changes in triforine sensitivity in populations of *Monilinia fructicola* from Hawkes Bay orchards. **Proceedings of the New Zealand Plant Protection Conference**, p.138-140, 1992.

ELMER, P. A.; GAUNT, R. The biological characteristics of dicarboximide-resistant isolates of *Monilinia fructicola* from New Zealand stone-fruit orchards. **Plant Pathology**, v. 43, n. 1, p. 130-137, 1994.

FACHINELLO, J. C.; MARODIN, G. Implantação de pomares. In: MONTEIRO, L. B.;MAY DE MIO, L. L.; SERRAT, B. M.; MOTTA, A. C.; CUQUEL, F. L. (Ed.). **Fruteiras de caroço uma visão ecológica**. Curitiba: UFPR, p.33-48, 2004.

FACHINELLO, J. C.; PASA, M. S.; SCHMTZ, J. D.; BETEMPS, D. L.. Situação e perspectivas da fruticultura de clima temperado no Brasil. **Revista Brasileira de Fruticultura**, v. 33, n. S1, p. 109-120, 2011.

FAN, J. Y.; GUO, L. Y.; XU, J. P.; LUO, Y.; MICHAILIDES, T. J.. Genetic diversity of populations of *Monilinia fructicola* (Fungi, Ascomycota, Helotiales) from China. **Journal of eukaryotic microbiology**, v. 57, n. 2, p. 206-212, 2010.

FORTES, J.; MARTINS, O. Sintomatologia e controle das principais doenças. In: MEDEROS, C. e RASEIRA, M. D. C. (Ed.). **A cultura do pessegueiro**: Embrapa-CPACT/Embrapa-SPI, p.243-264, 1998.

GARRIDO, L.; MAY DE MIO, L. L.; UENO, B.; CAMPOS, A. D. Impacto das mudanças climáticas sobre as doenças de fruteiras de caroço no Brasil. In: GHINI, R.; HAMADA, E.; BETTIOL, W. (Ed.). **Impacto das mudanças climáticas sobre doenças de importantes culturas no Brasil**. Jaguariúna: Embrapa Meio Ambiente, p.229-247, 2011.

GEORGOPOULOS, S. The genetic basis of classification of fungicides according to resistance risk1. **EPPO Bulletin**, v. 15, n. 4, p. 513-517, 1985.

GILPATRICK, J. Resistance to ergosterol biosynthesis-inhibiting fungicides in laboratory strains of *Monilinia fructicola*. **Netherlands Journal of Plant Pathology**, p.240, 1981.

GISI, U. CHIN, K. M.; KNAPOVA, G.; KUNG, F. R.; MOHR, U.; PARISI, S.; SIEROTZKI, H.; STEINFELD, U. Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. **Crop Protection**, v. 19, n. 8, p. 863-872, 2000.

HALBRENDT, N.; NGUGI, H. K.; MAGDAMA, F.; JIMENEZ-GASCO, M.. Multi-drug resistance to site-specific fungicides in populations *Monilinia fructicola* in Pennsylvania orchards. APS-MSA Joint Meeting. Austin TX. **Phytopathology**. p.S2.55, 2013.

HARADA, Y.; NAKAO, S.; SASAKI, M.; SASAKI, Y.; ICHIHASHI, Y.; SANO, T. *Monilia mumecola*, a new brown rot fungus on *Prunus mume* in Japan. **Journal of General Plant Pathology**, v. 70, n. 6, p. 297-307, 2004.

HARVEY, J. M.; SMITH, W. L.; KAUFMAN, J. **Market diseases of stone fruits: cherries, peaches, nectarines, apricots, and plums**. Washington: United States Department of Agriculture, 1972.

HOLB, I. Brown rot blossom blight of pome and stone fruits: symptom, disease cycle, host resistance, and biological control. **International journal of horticultural science**, v. 14, n. 3, p. 15-21, 2008.

HOLB, I.; SCHNABEL, G. Can DMI fungicides differ in controlling *Monilinia fructicola* isolates with reduced sensitivity to propiconazole? APS Annual Meeting, 2007. San Diego CA. **Phytopathology**. p.S47, 2007.

HONG, C. MICHAILIDES, T. J.; KONG, P.; HOLTZ, B, A. Discharge and quantification of ascospores from apothecia of *Monilinia fructicola*., APS anual meeting, 1997. Rochester, NY. **Phytopathology**. p.S43, 1997.

HU, M.-J.; COX, K. D.; SCHNABEL, G.; LUO, C. X. *Monilinia* species causing brown rot of peach in China. **PloS one**, v. 6, n. 9, p. e24990, 2011.

INMET. Banco de dados meteorológicos para ensino e pesquisa **Instituto Nacional de Meteorologia**, <http://www.inmet.gov.br/>, 2014. Acesso em: April 18.

JOSEPH-HORNE, T.; HOLLOWAY, D. W. Molecular mechanisms of azole resistance in fungi. **FEMS microbiology letters**, v. 149, n. 2, p. 141-149, 1997.

KESKE, C.; AMORIM, L.; MAY-DE MIO, L. Peach brown rot incidence related to pathogen infection at different stages of fruit development in an organic peach production system. **Crop Protection**, v. 30, n. 7, p. 802-806, 2011.

LANE, C. A synoptic key for differentiation of *Monilinia fructicola*, *M. fructigena* and *M. laxa*, based on examination of cultural characters. **EPP0 Bulletin**, v. 32, n. 3, p. 489-493, 2002.

LICHTENBERG, P. S. F.; SILVA, F. A.; ZEVIANI, W. M.; MAY DE MIO, L. L. Comparison of macro-morphological and physiological methods for *Monilinia* species identification in Paraná State, Brazil. **Canadian Journal of Plant Pathology**, v. 36, n. 1, p. 38-47, 2014.

LINHARES, A. I.; GHINI, R. **Resistência de fungos fitopatogênicos a fungicidas inibidores da demetilação (DMI): uma revisão**. Jaguariúna, SP: 2001.

LUO, C. X.; COX, K. D.; AMIRI, A.; SCHNABEL, G. Occurrence and detection of the DMI resistance-associated genetic element 'Mona' in *Monilinia fructicola*. **Plant Disease**, v. 92, n. 7, p. 1099-1103, 2008.

LUO, C.-X.; SCHNABEL, G. The cytochrome P450 lanosterol 14 $\alpha$ -demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. **Applied and environmental microbiology**, v. 74, n. 2, p. 359-366, 2008.

LUO, Y.; MICHAILIDES, T. J. Risk analysis for latent infection of prune by *Monilinia fructicola* in California. **Phytopathology**, v. 91, n. 12, p. 1197-1208, 2001.

LUPETTI, A.; DANESI, R.; CAMPA, M.; DEL TACCA, M.; STEVEN, K. Molecular basis of resistance to azole antifungals. **Trends in molecular medicine**, v. 8, n. 2, p. 76-81, 2002.

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

MAIDAL, J.; RASEIRA, M. D. C. B. **Aspectos da produção e mercado de pêsego no Brasil**. EMBRAPA. Pelotas: Embrapa: 14 p. 2008.

MARODIN, G.; ALMEIDA, G.; MARODIN, F. Mercado mundial de frutas de caroço. VI **Simpósio Internacional de Fruticultura Temperada em Região Subtropical**. Avaré SP. 11-13/September/2014. p.10. 2014.

MAY DE MIO, L. L.; CUQUEL, F. L.; MONTEIRO, L. B.; MOTTA, A. C. V.; SERRAT, B. M. Peach productivity, brown rot incidence, and pest damage under integrated production system. **Revista Brasileira de Fruticultura**, v. 33, n. SPE1, p. 424-428, 2011a.

MAY DE MIO, L. L.; GARRIDO, L.; UENO, B. Doenças de Fruteiras de Caroço. In: MONTEIRO, L. B.; MAY DE MIO, L. L.; SERRAT, B. M.; MOTTA, A. C.; CUQUEL, F. L. (Ed.). **Fruteiras de caroço: uma visão ecológica**. Curitiba, PR: UFPR, p.169-185, 2004.

MAY DE MIO, L. L.; LUO, Y.; MICHAILIDES, T. J. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management. **Plant Disease**, v. 95, n. 7, p. 821-827, 2011b.

MAY DE MIO, L. L.; MOREIRA, L. M.; MONTEIRO, L. B.; JUSTINIANO, P. Infecção de *Monilinia fructicola* no período da floração e incidência de podridão parda em frutos de pessegueiro em dois sistemas de produção. **Tropical Plant Pathology, Brasília**, v. 33, n. 03, p. 227-234, 2008.

MAY DE MIO, L. L.; NEGRI, G.; MICHAILIDES, T. J. Effect of *Trichothecium roseum*, lime sulfur and phosphites to control blossom blight and brown rot on peach. **Canadian Journal of Plant Pathology**, n. just-accepted, 2014.

MOREIRA, L.; MAY DE MIO, L. L. Metodologia para detecção de infecções latentes de *Monilinia fructicola* em frutas de caroço. **Ciência Rural**, v. 37, n. 3, p. 628-633, 2007.

MOREIRA, L.; MAY DE MIO, L. L. Controle da podridão parda do pessegueiro com fungicidas e fosfitos avaliados em pré e pós-colheita; Control of peach tree brown rot by fungicides and phosphites evaluated during preharvest and postharvest. **Ciênc. agrotec.,(Impr.)**, v. 33, n. 2, p. 405-411, 2009.

NUNINGER-NEY, C.; SCHWINN, F.-J.; STAUB, T. In vitro selection of sterol-biosynthesis-inhibitor (SBI)-resistant mutants in *Monilinia fructicola* (Wint.) Honey. **Netherlands Journal of Plant Pathology**, v. 95, n. 1, p. 137-150, 1989.

OGAWA, J. M. ZEHR, E. I.; BIRD, G. W.; RITCHIE, D. F.; URIU, K.; UYEMOTO, J. K. Compendium of stone fruit diseases. **American Phytopathological Society**, 1995.

PARKER, D. ZHANG, N.; SMART, C. D.; KOLLER, W. D. Polymorphism of 14 alpha-demethylase gene (CYP51) in brown rot pathogen *Monilinia fructicola* from a resistant orchard in New York State. **Phytopathology**, p.S90-S90, 2006.

PRINCE, R.; MOORE, M.; WEARING, C. The New Zealand committee on pesticide resistance 1989 summary. **Proc. 42nd NZ Weed and Pest Control Conf.** p.278-292, 1989.

RITCHIE, D. **Brown rot of stone fruit**. American Phytopathological Society, p.8. 2000

SANGLARD, D.; ISHER, F.; KOYMANS, L.; BILLE, J. Amino acid substitutions in the cytochrome P-450 lanosterol 14 $\alpha$ -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. **Antimicrobial Agents and Chemotherapy**, v. 42, n. 2, p. 241-253, 1998.

SCHEINPFLUG, H.; KUCK, K. Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azoles fungicides. In: LYR, H. (Ed.). **Modern selective fungicides: properties, applications and mechanisms of action**: Gustav Fischer Verlag, p.173-204. 1987.

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

SCHNABEL, G.; DAI, Q.; PARADKAR, M. R. Cloning and expression analysis of the ATP-binding cassette transporter gene MFABC1 and the alternative oxidase gene MfAOX1 from *Monilinia fructicola*. **Pest management science**, v. 59, n. 10, p. 1143-1151, 2003.

SEAB. Produção Agropecuária. <http://www.agricultura.pr.gov.br/>, 2013. Acesso em: 10/10/2014.

SEQUEIRA, L. Special contributions from the tropics to phytopathological mycology. **Phytopathology**, n. 52, p. 936-942, 1962.

SILVA, S. J. P. KOHLS, V. K.; MANICA-BERTO, R.; RIGATTO, P.; ROMBALDI, C. V. Apropriação tecnológica da produção integrada de pêssegos na região de Pelotas no Estado do Rio Grande do Sul. **Ciência Rural**, v. 41, n. 9, p. 1667-1673, 2011.

SOUZA, D.; FAZZA, A. Z.; CAMARGO, L. A.; MAY DE MIO, L. L.; ANGELI, S. S.; AMORIM, L. First report of *Monilinia laxa* causing brown rot on peaches in Brazil. (Abstr.) **Phytopathology** (Supplement), p.S148-S149, 2008.

TIBOLA, C. S.; FACHINELLO, J. C.; GRUTZMACHER, A. D.; PICOLOTTO, L.; KRUGER, L. Manejo de pragas e doenças na produção integrada e convencional de pêssegos. **Revista Brasileira de Fruticultura**, v. 27, n. 2, p. 215-218, 2005.

VILLANI, S. M.; COX, K. D. Characterizing Fenbuconazole and Propiconazole Sensitivity and Prevalence of Mona'in Isolates of *Monilinia fructicola* from New York. **Plant Disease**, v. 95, n. 7, p. 828-834, 2011.

WELLMAN, F. L. A few introductory features of tropical plant pathology. **Phytopathology**, v. 52, p. 928-930, 1962.

WHERRETT, A.; SIVASITHAMPARAM, K.; KUMAR, S. detection of possible systemic fungicide resistance in Western Australian *Monilinia* population. APS/MSA/SON Join Meeting, 2001. Salt Lake City. **Phytopathology**. p.S95.

ZANETTE, F.; BIASI, L. A. Introdução à fruteiras de caroço. In: MONTEIRO, L. B.; MAY DE MIO, L. L.; SERRAT, B. M.; MOTTA, A. C.; CUQUEL, F. L. (Ed.). **Fruteiras de caroço uma visão ecológica**. Curitiba: UFPR, p.1-4, 2004.

ZEHR, E.; TOLER, J.; LUSZCZ, L. Spread and persistence of benomyl-resistant *Monilinia fructicola* in South Carolina peach orchards. **Plant disease**, v. 75, n. 6, p. 590-593, 1991.

ZEHR, E. I. Control of brown rot in peach orchards. **Plant Disease**, v. 66, n. 12, p. 1101-1105, 1982.

ZEHR, E. I.; LUSZCZ, L. A.; OLIEN, W. C.; NEWALL, W. C.; TOLER, J. E. Reduced sensitivity in *Monilinia fructicola* to propiconazole following prolonged exposure in peach orchards. **Plant disease**, v. 83, n. 10, p. 913-916, 1999.

ZHU, F.; BRYSON, P. K.; SCHNABEL, G. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. **Pest management science**, v. 68, n. 7, p. 1003-1009, 2012.