Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Identification of pathogens causing brown rot of stone fruit in Cuneo province (Italy) and assessment of sensitivity to azoxystrobin, cyprodinil, fenhexamid, fludioxonil, and tebuconazole

Greta DARDANI[1,2], Vladimiro GUARNACCIA[1,2,*], Luca NARI[3], Stefanos I. TESTEMPASIS[4], George S. KARAOGLANIDIS[4], M. Lodovica GULLINO[2]

[1] *Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy*
[2] *Interdipartimental Centre for Innovation in the Agro-Environmental Sector, AGROINNOVA, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy*
[3] *AGRION, The Foundation for Research, Innovation and Technological Development of Piedmont Agriculture, 12030 Manta, Italy*
[4] *Laboratory of Plant Pathology, Aristotle University of Thessaloniki, P.O. Box 269, 54124, Thessaloniki, Greece*
*Corresponding author. E-mail: vladimiro.guarnaccia@unito.it

Summary. *Monilinia* spp. cause brown rot and blossom blight of stone fruit. This study characterized the diversity of *Monilinia* spp. associated with stone fruit rots in the Cuneo province, the major fruit production area in Piedmont, and assessed their sensitivity to azoxystrobin, cyprodinil, fenhexamid, fludioxonil and tebuconazole. Species diversity was determined by PCR amplification and sequencing of isolate internal transcribed spacer (ITS) regions. Sensitivity to fungicides was determined by measuring *in vitro* mycelium growth on fungicide-amended media. Fifty isolates were obtained from apricot, cherry, or peach fruits with typical brown rot symptoms. Thirteen isolates were identified as *M. fructicola*, and 37 as *M. laxa*. Nine isolates of *Monilinia laxa* and two of *M. fructicola* had resistance factor (RF) values greater than 10 for different fungicides. The greatest (RF) value (48.96) was measured for azoxystrobin against the *M. fructicola* isolate CVG 1514. Among the *M. laxa* isolates, isolate CVG 1547 had the greatest RF value to cyprodinil, while isolate CVG 1709 had RF values greater than 10 for cyprodinil and tebuconazole. A systematic and wider sampling should be carried out in the Piedmont region to determine the distribution of fungicide resistant *Monilinia* spp. in stone fruit crops. The use of site-specific fungicides remains the most effective strategy for control brown rot, and continued monitoring for fungicide resistance within *Monilinia* spp. populations is recommended.

Keywords. *Monilinia*, fungus characterization, chemical control.

OPEN ACCESS

Citation: G. Dardani, V. Guarnaccia, L. Nari, S.I. Testempasis, G.S. Karaoglanidis, M.L. Gullino (2023) Identification of pathogens causing brown rot of stone fruit in Cuneo province (Italy) and assessment of sensitivity to azoxystrobin, cyprodinil, fenhexamid, fludioxonil, and tebuconazole. *Phytopathologia Mediterranea* 62(3): 455-465. doi: 10.36253/phyto-14399

Accepted: December 5, 2023

Published: December 30, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Anne-Sophie Walker, Bioger, Inrae, Thiverval-Grignon, France.

ORCID:

GD: 0000-0002-9146-2168
VG: 0000-0003-3188-7743
SIT: 0000-0002-1892-8523
GSK: 0000-0002-7413-2052
MLG: 0000-0002-7706-1915

INTRODUCTION

Monilinia fructicola, M. laxa, M. fructigena and *M. polystroma* are the causal agents of blossom blight and brown rot of stone fruit (peach, nectarine, plum, apricot and cherry) (Holb, 2008; Chen *et al.*, 2013; Abate *et al.*, 2018). Chemical control of these diseases is the most effective strategy to reduce pathogen inoculum and disease incidence (Mustafa *et al.*, 2021). Site-specific fungicides are currently available against brown rot in Europe (Commission Implementing Regulation (EU), No. 540/2011), where one to three spray applications are applied from flowering to ripening stages. Frequent use of site-specific fungicides increases the risk of selection of fungicide resistant pathogen populations, reducing fungicide effectiveness and disease control.

In Italy, brown rot is the most important fungal disease of stone fruit, both in orchards and post-harvest storage. Before 2008, *M. laxa* and *M. fructigena* were the only recorded brown rot pathogens (Pratella, 1996). In 2008, Pellegrino *et al.* (2009) reported *M. fructicola* in Cuneo province (Piedmont) for the first time, which was included in the EPPO A2 list (no. 153, OEPP/EPPO, 1997) as a quarantine pest. Later, *M. fructicola* was reported in additional Italian regions (Martinelli *et al.*, 2013; Landi *et al.*, 2016; Martini *et al.*, 2016; Montuschi *et al.*, 2016; Abate *et al.*, 2018), while in 2014, *M. polystroma* was also first reported in Italy, causing brown rot of peach (Martini *et al.*, 2014).

Demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), amino acids and protein synthesis inhibitors and signal transduction inhibitors fungicides are classified according to the Fungicide Resistance Action Committee (FRAC, 2022). In Italy, site-specific fungicides used in stone fruit orchards against brown rot include anilinopyrimidines (e.g. cyprodinil, pyrimethanil), phenylpyrroles (e.g. fludioxonil), triazoles within the DMIs (e.g. tebuconazole, penconazole), SDHIs (e.g. boscalid, penthiopyrad), hydroxyanilides (e.g. fenhexamid), and QoIs (e.g. azoxystrobin, pyraclostrobin, trifloxystrobin). Postharvest fungicide applications are not approved for stone fruit in Italy.

The FRAC considers the three main species of *Monilinia* as pathogens of moderate risk for development of fungicide resistance, as resistant isolates have been reported both under field and laboratory conditions (FRAC, 2020). Reductions in sensitivity of *Monilinia* spp. to QoIs has been reported in Brazil (May-De Mio *et al.*, 2011; Pereira *et al.*, 2017) and in the United States of America (Holb and Schnabel, 2007; Amiri *et al.*, 2010). Isolates resistant to DMIs were reported in North America (Schnabel *et al.*, 2004; Chen *et al.*, 2013) and South America (Lichtemberg *et al.*, 2016; Pereira *et al.*, 2020). In Europe, reductions in sensitivity of *Monilinia* spp. to several fungicide classes, including dicarboximides, DMIs and hydroxyanilides, have been reported in Spain (Egüen *et al.*, 2015), Italy (Bustos Lòpez *et al.*, 2012), Greece (Malandrakis *et al.*, 2013) and Serbia (Hrustić *et al.*, 2018).

Some information is available on *Monilinia* spp. distribution in Italy, but little is known about the fungicide sensitivity of these pathogens. (Abate *et al.*, 2018; Mancini *et al.*, 2021). For this reason, a survey was carried out in Piedmont to obtain isolates associated with affected fruit, to characterize pathogen species diversity and determine their sensitivity to fungicides. This study aimed to: a) monitor presence and species of *Monilinia* spp. associated with brown rot in stone fruit orchards in the Cuneo province, the major Piedmont stone fruit production area; and b) determine sensitivity of obtained *Monilinia* isolates to azoxystrobin, tebuconazole, fenhexamid, cyprodinil and fludioxonil.

MATERIALS AND METHODS

Field survey, sampling and fungus isolations

In June and July 2021, samples were collected from commercial stone fruit orchards (cherry, peach and apricot; Table 1). Single sampled orchards, representative of a small subset of this stone fruit production area, were situated in four towns in the Cuneo province. Isolations were carried out from brown rot affected fruit of different cultivars (Table 1). Portions (5–8 mm) of each symptomatic fruit were surface sterilized with 1% sodium hypochlorite for 30 sec, then rinsed in sterile distilled water for 1 min, and dried on sterile absorbent paper. Small fragments (2–3 mm) were cut from lesion margins and plated on potato dextrose agar (PDA, Merck) amended with 25 ppm of streptomycin sulphate (PDA-S). The plates were incubated at 25±1°C, and after 48 to 72 h incubation, single hyphal tips from margin of resulting colonies were cut and placed individually on PDA plates to establish pure cultures. The obtained isolates were used for determinations of *in vitro* sensitivity to fungicides and molecular identification (Table 1). Stock cultures of isolates are kept at -80°C in the University of Torino (Italy) culture collection.

DNA extraction, PCR amplification and sequencing

Mycelium was scraped from surfaces of 10-d-old cultures grown on PDA, and placed into 2 mL capacity

Table 1. Isolate details and GenBank accession numbers for isolates included in this study.

Species	Isolate	Host	Cultivar	Origin	GenBank No. ITS
Monilinia laxa	CVG 1506	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317580
	CVG 1507	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317581
	CVG 1508	Cherry	M2029	Manta, Cuneo, Italy	OP317582
	CVG 1509	Cherry	Kordia	Manta, Cuneo, Italy	OP317583
	CVG 1513	Cherry	Selah®	Manta, Cuneo, Italy	OP317586
	CVG 1535	Cherry	Giant Red	Manta, Cuneo, Italy	OP317588
	CVG 1536	Apricot	Tom Cot	Manta, Cuneo, Italy	OP317589
	CVG 1540	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317593
	CVG 1541	Cherry	Coralise	Manta, Cuneo, Italy	OP317594
	CVG 1542	Cherry	Coralise	Manta, Cuneo, Italy	OP317595
	CVG 1543	Cherry	M2003	Manta, Cuneo, Italy	OP317596
	CVG 1544	Cherry	M2003	Manta, Cuneo, Italy	OP317597
	CVG 1566	Cherry	Giant Red	Manta, Cuneo, Italy	OP317602
	CVG 1567	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317603
	CVG 1568	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317604
	CVG 1569	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317605
	CVG 1633	Peach		Falicetto, Cuneo, Italy	OP317606
	CVG 1642	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317608
	CVG 1643	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317609
	CVG 1644	Peach		Falicetto, Cuneo, Italy	OP317610
	CVG 1645	Peach		Falicetto, Cuneo, Italy	OP317611
	CVG 1648	Peach		Falicetto, Cuneo, Italy	OP317613
	CVG 1650	Peach		Falicetto, Cuneo, Italy	OP317615
	CVG 1692	Peach		Falicetto, Cuneo, Italy	OP317616
	CVG 1693	Peach		Falicetto, Cuneo, Italy	OP317617
	CVG 1699	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317618
	CVG 1702	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317620
	CVG 1703	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317621
	CVG 1705	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317622
	CVG 1707	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317623
	CVG 1709	Peach		Manta, Cuneo, Italy	OP317624
	CVG 1712	Peach		Manta, Cuneo, Italy	OP317625
	CVG 1713	Peach		Manta, Cuneo, Italy	OP317626
	CVG 1714	Peach		Manta, Cuneo, Italy	OP317627
	CVG 1715	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317619
	CVG 1716	Peach		Manta, Cuneo, Italy	OP317628
	CVG 1717	Peach	N/A	Manta, Cuneo, Italy	OP317629
M. laxa	CBS 298.31	N/A	N/A	Ireland	HQ856917
	BPZK	Peach	N/A	Serbia	KC544793
	MDA12	N/A	N/A	United States	HQ846948
Monilinia fructicola	CVG 1510	Cherry	Kordia	Manta, Cuneo, Italy	OP317584
	CVG 1511	Cherry	Kordia	Manta, Cuneo, Italy	OP317585
	CVG 1514	Cherry	Selah®	Manta, Cuneo, Italy	OP317587
	CVG 1537	Cherry	M2043	Manta, Cuneo, Italy	OP317590
	CVG 1538	Cherry	M2043	Manta, Cuneo, Italy	OP317591
	CVG 1539	Cherry	M2043	Manta, Cuneo, Italy	OP317592
	CVG 1545	Cherry	M2003	Manta, Cuneo, Italy	OP317598
	CVG 1546	Cherry	M2003	Manta, Cuneo, Italy	OP317599

(Continued)

Table 1. (Continued).

Species	Isolate	Host	Cultivar	Origin	GenBank No. ITS
	CVG 1547	Cherry	Selah®	Manta, Cuneo, Italy	OP317600
	CVG 1563	Cherry	M2043	Manta, Cuneo, Italy	OP317601
	CVG 1635	Peach		Falicetto, Cuneo, Italy	OP317607
	CVG 1647	Peach		Falicetto, Cuneo, Italy	OP317612
	CVG 1649	Peach		Falicetto, Cuneo, Italy	OP317614
M. fructicola	Ft	N/A	N/A	France	HQ846967
	XP1	Peach	N/A	Chaoyang, Beijing	KR778937
	P169	Nectarine	N/A	Italy	FJ411109
Monilinia fructigena	CBS 101500	N/A	N/A	Poland	KR778933
	CBS 101499	N/A	N/A	Spain	KR778932
	SPBA	Plum	N/A	Serbia	KC544805
Monilinia polystroma	CBS 102686	N/A	N/A	Japan	HQ846944
	HML-3	Plum	N/A	China	GU067539
	09-G4	Apricot	N/A	Switzerland	JN128835
Monilia yunnanensis	GP18	Peach	N/A	Yanqing, Beijing	HQ856917
Botrytis cinerea	BCE4	Tomato	N/A	Beijing	HQ856917

centrifuge tubes. Total DNA was extracted from all isolates using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. Species identification was achieved by DNA amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) regions of the isolates. For each isolate, ITS was amplified using universal primers ITS1 and ITS4 (White *et al*., 1990). Reactions were each carried out using a Taq DNA polymerase kit (Qiagen), in a final volume 25 µL, containing 2.5 µL of Qiagen PCR buffer 10×, 1.4 µL of 25mM $MgCl_2$, 0.5 µL of each dNTP (10µM), 0.5 µL of each primer (10µM), 0.2 µL of Taq DNA polymerase, and 25 ng of DNA. Amplification was carried out using the following conditions: initial preheating for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min and with a final extension at 72°C for 7 min. An aliquot (5 µL) of PCR product for each reaction was separated by electrophoresis at 100V in a 1% agarose gel (VWR Life Science AMRESCO® Biochemicals), and then stained with GelRed™ in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0). PCR products were sequenced in forward directions by Eurofins Genomics Service. Obtained sequences were analyzed using Geneious v. 11.1.5 software (Auckland, New Zealand), and were blasted against the NCBI's GenBank nucleotide database to determine the closest relatives of the studied isolates. Isolate sequences, including sequences downloaded from GenBank, were initially aligned with the software MAFFT v. 7 online server (Katoh and Standley, 2013), and were then manually adjusted in MEGA v.7 (Kumar *et al*., 2016). Phylogenic analysis, based on Maximum Parsimony (MP), was carried out using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Swofford, 2003).

Fungicides and in vitro *sensitivity of isolates*

Commercial formulations of azoxystrobin (Ortiva®, 250 g L^{-1} active ingredient (a.i.), Syngenta), cyprodinil (Chorus® 50%. a.i., Syngenta), fenhexamid (Teldor Plus®, 500 g L^{-1} a.i. Bayer CropScience), fludioxonil (Geoxe® 50% a.i., Syngenta) and tebuconazole (Folicur® WG 25% a.i., Bayer CropScience) were used in this study. These fungicides were each dissolved in sterilized water, and stock solutions were prepared and stored at 4°C. Sensitivity of fungal isolates was assessed at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 µg mL^1 for each fungicide. Autoclaved agar medium was cooled to 50°C, and 10 mL of fungicide-amended medium was dispensed in each Petri plates. Sensitivities to azoxystrobin, fenhexamid, fludioxonil and tebuconazole were assessed on PDA. Salicylhydroxamic acid (SHAM; Sigma-Aldrich) was added to azoxystrobin amended PDA at 100 µg mL^{-1} to prevent test fungi from commencing alternative respiration. To determine isolate sensitivity to an anilinopyrimidine fungicide, as reported by Myresiotis *et al.* (2007), cyprodinil was added to minimal medium containing (per liter) 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)2SO_4$, 0.5 g $MgSO_4.7H_2O$, and 12.5

Table 2. Concentrations and resistance factors (RF) of azoxystrobin, cyprodinil, fenhexamid, fludioxonil and tebuconazole at which mycelium growth of *Monilinia laxa* and *Monilinia fructicola* were inhibited by 50% (EC_{50} values).

Species	Parameter	*Monilinia laxa*	*Monilinia fructicola*
Azoxystronbin	EC_{50} (μg mL^{-1}) max/min	3.44/0.05	7.47/0.03
	$EC_{50\ mean}$	0.37	1.19
	RF max/min	22.54/0.35	48.96/0.19
Cyprodinil	EC_{50} (μg mL^{-1}) max/min	1.11/0.03	0.29/0.06
	$EC_{50\ mean}$	0.18	0.12
	RF max/min	25.33/0.62	6.59/1.32
Fenhexamid	EC_{50} (μg mL^{-1}) max/min	0.49/0.04	1.09/0.12
	$EC_{50\ mean}$	0.14	0.28
	RF max/min	4.53/0.41	9.97/1.13
Fludioxonil	EC_{50} (μg mL^{-1}) max/min	0.12/0.02	0.19/0.03
	$EC_{50\ mean}$	0.05	0.07
	RF max/min	4.13/0.81	6.30/0.87
Tebuconazole	EC_{50} (μg mL^{-1}) max/min	0.42/0.02	0.37/0.13
	$EC_{50\ mean}$	0.18	0.22
	RF max/min	11.43/0.63	9.95/3.61

g agar. PDA mycelial plugs were taken from margins of 10-d-old colonies with using a cork borer (0.6 cm diam.). The plugs were then each placed upside down at the centres (one plug per plate) of Petri plates (9 mm diam.) containing PDA + fungicide. Unamended PDA plates were used as experimental controls. The plates were then incubated at 25°C for 10 d in the dark. Each isolate was tested in triplicate for each fungicide and concentration. Mean colony diameters (minus the diameter of the inoculation plugs) were determined by measuring two diameters at right angles to each other in each plate, at after 7 and 10 d incubation. These data were expressed as daily mycelium growth rates and percentages of growth inhibition relative to the unamended controls.

Data analyses

The fungicide EC_{50} values (concentrations inhibiting mycelium growth to 50% of experimental controls) were determined by regressing percentages of relative mycelium growth inhibition against the $\log_{10}$ of fungicide concentrations. EC_{50} values for each isolate were calculated with the GraphPadPrism® software (version 9.1.1), using the log dose-response relation. EC_{50}s allowed calculation of Resistance Factors (RFs), which showed sensitivity levels of the different isolates (Schnabel *et al.*, 2004). Each RF is defined as the EC_{50} of the isolate divided by the mean EC_{50} value of sensitive isolates. As sensitive/standard reference isolates were not available for *Monilinia* spp. for the selected fungicides, and a baseline population was also not available, we defined sensitive/susceptible isolates based on Minimum Inhibitory Concentration (MIC) for each fungicide. MIC measures sensitivity to antifungal agents (Xie *et al.*, 2012), and is defined as the lowest concentration of fungicide that completely inhibits fungal growth. Average EC_{50} of sensitive isolates for each fungicide, defined as isolates for which growth was completely inhibited at the MIC concentration, were used to calculate RF values, and isolates were classified as resistant to each active ingredient when the RF was greater than 10 (Campia *et al.*, 2017).

RESULTS

Sampling, isolation and molecular identification of Monilinia *isolates*

A total of 50 isolates were obtained from peach, cherry or apricot fruits with typical brown rot symptoms. The ITS phylogeny consisted of 63 sequences, including *Botrytis cinerea* (BCE4) as outgroup. A total of 437 characters were included in the phylogenetic analysis, 20 characters were parsimony-informative, 6 were variable and parsimony-uninformative, and 411 were constant. A maximum of 1,000 equally MP trees were saved (Tree length = 32, CI = 0.875, RI = 0.969, RC = 0.848). Bootstrap support values from the MP analysis are included in Figure 1. The phylogenetic tree showed that the isolates clustered in two different lineages, with reference isolates of *M. fructicola* and *M. laxa*. Thirteen isolates (26% of the isolates collected) clustered with *M. fructicola*, and 37 (74%) clustered with *M. laxa*. *Monilinia fructicola* was identified from only two orchards: in Manta (Cuneo) with ten isolates and in Falicetto (Cuneo) with three isolates. *Monilinia laxa* was predominantly collected from cherry and peach fruits, while only one isolate (CVG 1536) was isolated from apricot. *Monilinia fructicola* was isolated only from cherry and peach fruits. None of the collected isolates were identified as *M. fructigena* or *M. polystroma*.

In vitro *sensitivity to fungicides*

The fungicides used for *in vitro* sensitivity tests inhibited growth of *M. laxa* and *M. fructicola* isolates at different levels. EC_{50} values for each fungicide for the fifty isolates were obtained from *in vitro* assays for mycelial growth (Figure 2).

Four *M. fructicola* isolates were resistant to azoxystrobin, with EC_{50} values, respectively, of 7.47, 3.44, 2.82

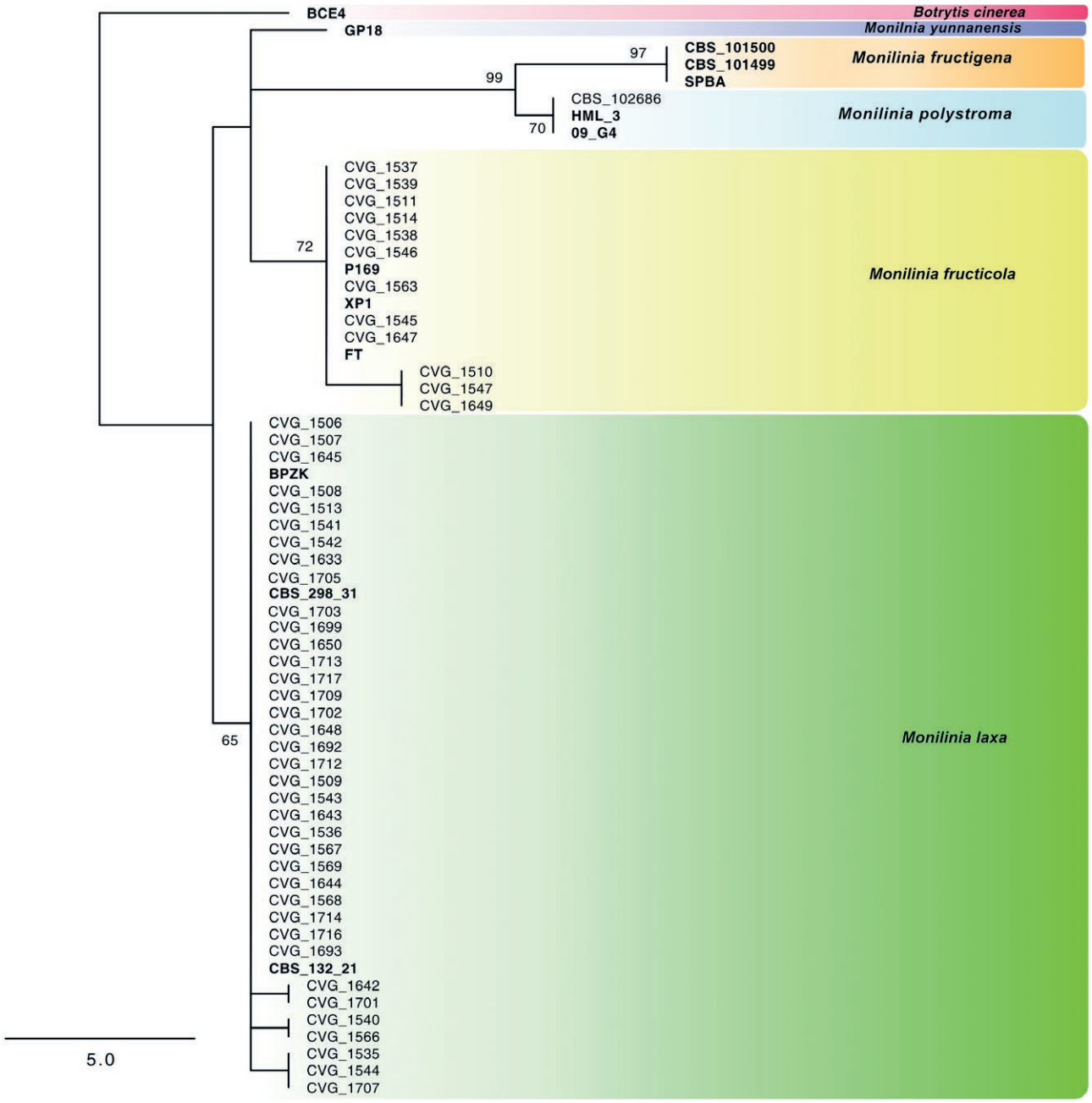

Figure 1. The most parsimonious tree obtained from a heuristic search of ITS sequence alignments of *Monilinia* spp. Bootstrap support values are shown at the nodes. The scale bar represents the number of changes. The tree was rooted to *Botrytits cinerea* (BCE4). GenBank isolates are indicated in bold font.

and 2.37 μg mL^{-1}, giving resistance factor (RF) values of, respectively, 48.96, 22.54, 18.48 and 15.52. Within the *M. laxa* isolates, CVG 1643 had the greatest sensitivity to azoxystrobin (EC_{50} = 0.05 μg mL^{-1}), and lowest sensitivity was recorded for CVG 1540 (EC_{50} = 3.44 μg mL^{-1}). Resistance factor values for azoxystrobin in *M. fructicola* isolates ranged from 48.96 to 0.19. The minimum inhibitory concentration (MIC) for azoxystrobin of sensitive isolates was 3 μg mL^{-1}, while some isolates grew at concentrations up to 100 μg mL^{-1}. Two isolates of *M. laxa* (CVG 1540, CVG 1566) and two isolates of *M. fructicola* (CVG 1514, CVG 1547) had RF values greater than 10.

For cyprodinil, one *M. laxa* isolate (CVG 1703) was resistant to the fungicide, with a high EC_{50} of 1.11 μg

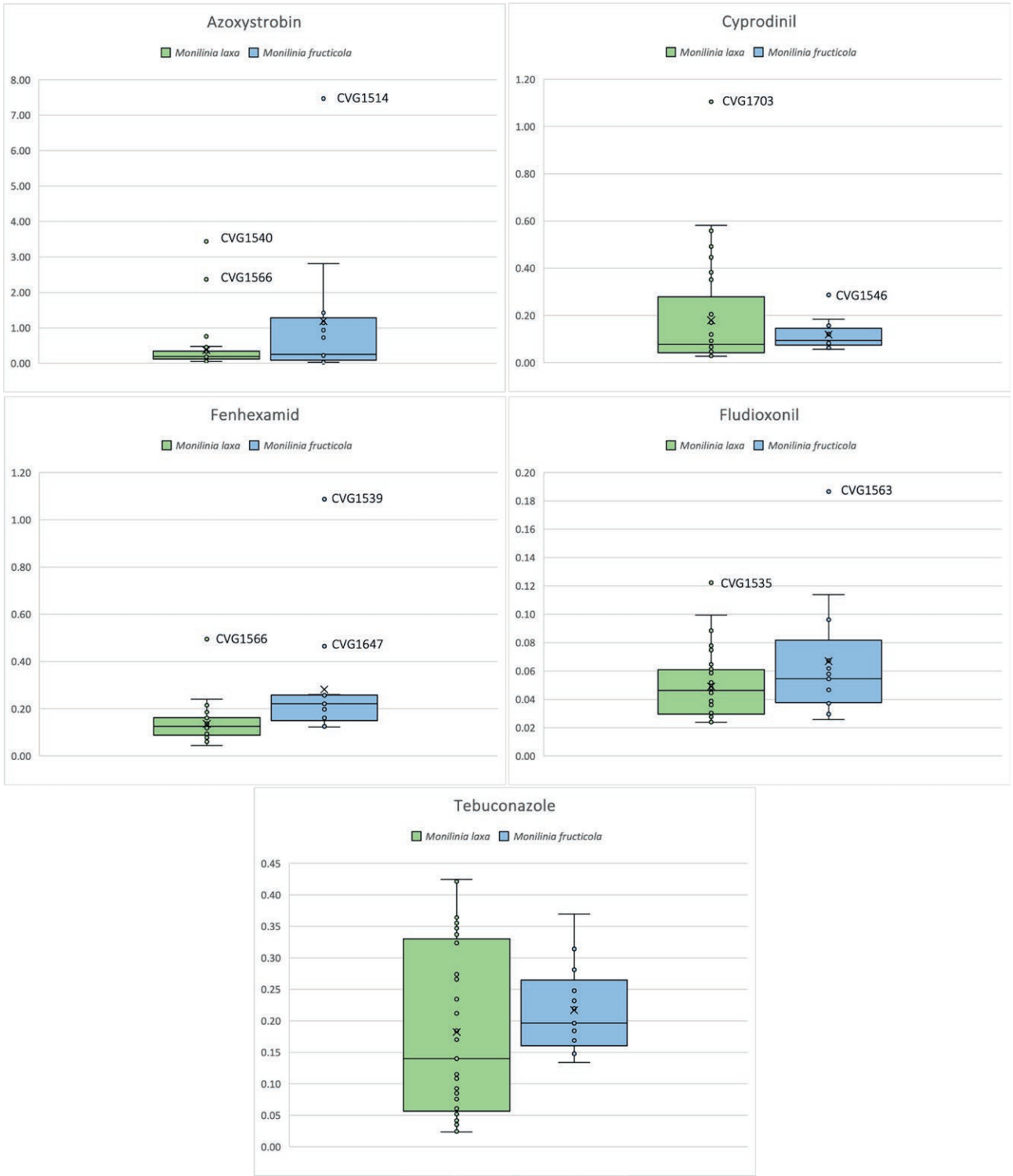

Figure 2. Boxplots of EC_{50s} (μg mL^{-1}) for activities of five fungicides against isolates of *Monilinia laxa* and *M. fructicola*.

mL^{-1} RF of 25.33. The lowest EC_{50} values for cyprodinil for both species were 0.03 μg mL^{-1}, whereas the greatest EC_{50} for *M. fructicola* was 0.29 μg mL^{-1}. Isolates defined as sensitive for cyprodinil had MICs of 0.3 μg mL^{-1}, while some isolates grew at concentrations up to 100 μg mL^{-1}. RF values greater than 10, were recorded for the

M. laxa isolates CVG 1509, CVG 1544, CVG 1703 and CVG 1709.

None of the assessed isolates showed resistance to fenhexamid. The lowest EC_{50} for this fungicide was for the *M. laxa* isolate CVG 1540 (EC_{50} = 0.04), while the *M. fructicola* isolate CVG 1539 was the least sensitive to fenhexamid (EC_{50} = 1.09 μg mL^{-1}. The greatest fenhexamid EC_{50} for *M. laxa* was 0.49 μg mL^{-1}. MIC of 1 μg mL^{-1} was recorded for the sensitive isolates, while other isolates grew at up to 10 μg mL^{-1} of this fungicide. No isolates had RFs greater than 10.

For fludioxonil, the most sensitive *M. laxa* isolate had an EC_{50} of 0.02 μg mL^{-1}, while the greatest EC_{50} was 0.12 μg mL^{-1}. For *M. fructicola*, isolate CVG 1537 was the most sensitive (EC_{50} = 0.03 μg mL^{-1}), while the least sensitive isolate CVG 1563 had an EC_{50} 0.19 μg mL^{-1}. No isolates had RF values greater than 10.

For tebuconazole, EC_{50} values for *M. fructicola* were from 0.13 to 0.37 μg mL^{-1}, and for *M. laxa* were from 0.02 to 0.42 μg mL^{-1}. Sensitive isolates had MIC values of 1 μg mL^{-1}, while some isolate grew at up to 10 μg mL^{-1} of this fungicide. Three isolates of *M. laxa* (CVG 1709, CVG 1713, CVG 1717) had RF values greater than 10.

DISCUSSION

Use of site-specific fungicides is widespread in Europe, and *Monilinia* spp. resistance to different fungicides has been reported in several countries (Malandrakis *et al.*, 2013; Egüen *et al.*, 2015; Hrustić *et al.*, 2018). Site-specific fungicides are commonly used by Italian stone fruit growers, and chemical control is the most effective strategy for control brown rot caused by *Monilinia* spp., which require a maximum of three field fungicide applications during each production season. In Italy, the predominant *Monilinia* species are *M. laxa*, *M. fructigena* and *M. fructicola* (Montuschi *et al.*, 2016).

Phylogenetic analyses based on isolate ITS sequences showed two divergent clusters. One cluster included *M. fructicola* and *M. laxa*, and the second contained *M. fructigena* and *M. polystroma*. *Monilia yunnanensis* and *Botrytis cinerea* formed two outgroup clusters. Based on these results, *M. laxa* (37 isolates) was the most common species found in the sampled orchards, while the other 13 isolates were *M. fructicola*. The coexistence of *M. fructicola* and *M. laxa* was previously reported in other countries, including Spain (Villarino *et al.*, 2013), Greece (Papavasileiou *et al.*, 2015) and the United States of America (Boehm *et al.*, 2001). The present study has shown that both *M. laxa* and *M. fructicola* are present, in the Cuneo province. As a larger number of *M. laxa* than *M. fructicola* isolates were collected, this prevalence could be due to low temperatures that have characterized past production seasons, as *M. laxa* grows more rapidly than *M. fructicola* at low temperatures (Papavasileiou *et al.*, 2015). These conditions may have promoted development and spread of *M. laxa* over *M. fructicola*. Further investigations are required with more extensive sampling over consecutive years, to confirm this trend and clarify effects of temperature and climate on prevalence and distribution *Monilinia* spp.

The use of site-specific fungicides increases risks of selection of fungicide-resistant pathogens, with gradual reductions in fungicide efficacy and disease control. *Monilinia* spp. have also been classified by the FRAC as pathogens of moderate risk for development of fungicide resistance. For these reasons, sensitivity was assessed of different *Monilinia* spp. isolates to five fungicides that represent chemical classes widely used in Italy for brown rot control.

Nine isolates of *M. laxa* and two of *M. fructicola* gave RF values greater than 10 for different fungicides. The greatest RF was recorded for azoxystrobin in one *M. fructicola* isolate. To define resistant isolates to azoxystrobin, Amiri *et al.* (2010) and Luo and Schnabel (2008) have suggested 3 μg mL^{-1} as a discriminatory concentration for resistance to this fungicide, so MIC of 3 μg mL^{-1} was set in the present study as the discriminatory dose for azoxystrobin resistance. Since it was not possible to compare different discriminatory doses obtained from different protocols, and as a baseline population was not included, classification of susceptible isolates in the present study was based only on MIC values. These results showed that two *M. fructicola* and two *M. laxa* isolates were resistant to QoIs. These results are similar to those of Hrustić *et al.* (2018), who reported presence of moderately resistant isolates of *M. laxa* and *M. fructicola*.

Several mechanisms of resistance to QoI fungicides have been proposed, but in most cases resistance is due to a single point mutation (G143A) in the mitochondrial *cytochrome b* (*Cytb*) gene that leads to an amino acid change in position 143 from glycine to alanine (Hrustić *et al.*, 2018). This mutation has been reported only in fungi without specific introns close to this amino acid position (Grasso *et al.*, 2006). This intron is present in *M. laxa* isolates after the position 143, as reported by Miessner and Stammler (2010). Similarly, in *M. fructicola* isolates the intron is also present but is located downstream of the codon for glycine at position 143, suggesting that this point mutation may not lead to QoI resistance in *M. fructicola* (Luo *et al.*, 2010). Further investigations are required to elucidate the resistance mechanism in *Monilinia* spp. isolates collected in the present study.

For fenhexamid, Malandrakis *et al.* (2013) reported *M. laxa* isolates with EC_{50}s from 0.02 to 1 μg mL^{-1}, while Förster *et al.* (2007), for *M. fructicola*, reported EC_{50}s ranging from 0.09 to 0.21 μg mL^{-1}. Based on EC_{50} measurements, the present study results showed that the assessed *M. fructicola* isolates were less sensitive to fenhexamid than the *M. laxa* isolates. However, calculations of RFs showed that neither the *M. fructicola* nor the *M. laxa* isolates were resistant to fenhexamid. This is probably because fenhexamid had been used only occasionally against brown rot in the sampled orchards.

Two fludioxonil + cyprodinil applications per year are authorized for control of brown rot of stone fruit in Italy. Fludioxonil EC_{50}s for *M. fructicola* have been reported as from 0.05 to 0.21 μg mL^{-1} (Förster *et al.*, 2007), while only Fazekas *et al.* (2014) have reported reduced sensitivity to cyprodinil for *M. laxa*. Results from the present study showed that most of the tested isolates were susceptible to fludioxonil and cyprodinil. Based on RFs greater than 10, only four *M. laxa* isolates had reduced sensitivity to cyprodinil. For fludioxonil, no isolates showed high RF values, suggesting absence of resistance to this fungicide.

Resistance to DMI fungicides has been detected in *M. fructicola* isolates from peach in the United States of America (Chen *et al.*, 2013; Pereira *et al.*, 2020) and Brazil (Lichtemberg *et al.*, 2016). For tebuconazole, a MIC of 1 μg mL^{-1} has been reported and used as the discriminatory dose. Results obtained for tebuconazole showed high EC_{50}s compared with values reported by May-De Mio *et al.* (2011) and by Pereira *et al.* (2020). Based on calculated RFs, only three isolates of *M. laxa* showed RF >10. In the sampled orchards, tebuconazole has been constantly used in past years (pers. comm. from orchard technicians).

Data obtained in the present study showed that *M. laxa* and *M. fructicola* coexist in stone fruit orchards in Cuneo province of Piedmont, with *M. laxa* being the predominant fungus associated with brown rot. Different levels of sensitivity to the tested fungicides were also recorded within the isolate sets of both of these fungi. However, due to the low number of tested isolates, it is not possible to determine if selection of resistant isolates is occurring in the investigated territory. Including a baseline population is also important for establishing a reference point that allows discrimination of sensitivity levels. In the present study, it was not possible to define appropriate resistance baselines due to the absence of orchards in the sampled area where fungi were not exposed to specific fungicide. Therefore, systematic and widespread sampling should be carried out to determine the resistance levels of *Monilinia* spp. populations in this major stone fruit production area. This should include large numbers of isolates, and appropriate reference isolates or baseline populations. Since chemical control in the field remains the most effective strategy for control of *Monilinia* spp., moderate use of these fungicides is recommended to prevent fungicide resistance and maintain their efficacy against these important pathogens.

ACKNOWLEDGEMENTS

Research reported in this paper was in the project "POSTFRUIT: Difesa post-raccolta dei prodotti ortofrutticoli" and was funded by Fondazioni Bancarie Cuneesi.

LITERATURE CITED

Abate D., Pastore C., Gerin D., De Miccolis Angelini R.M., Rotolo C., … Faretra F., 2018. Characterization of *Monilinia* spp. Populations on Stone Fruit in South Italy. *Plant Disease* 102: 1708–1717. https://doi.org/10.1094/PDIS-08-17-1314-RE

Amiri A., Brannen P.M., Schnabel G., 2010. Reduced Sensitivity in *Monilinia fructicola* Field Isolates from South Carolina and Georgia to Respiration Inhibitor Fungicides. *Plant Disease* 94: 737–743. https://doi.org/10.1094/PDIS-94-6-0737

Boehm E.W.A., Ma Z., Michailides T.J., 2001. Species-Specific Detection of *Monilinia fructicola* from California Stone Fruits and Flowers. *Phytopathology* 91: 428–439. https://doi.org/10.1094/PHYTO.2001.91.5.428

Bustos Lòpez M., Spadaro D., Garibaldi A., Gullino M.L., 2012. Sensibilità di isolati di Monilinia laxa e Monilia fructicola a fungicidi impiegati in pre-raccolta su drupacee. *Protezione delle colture* 5: 37.

Campia P., Venturini G., Moreno-Sanz P., Casati P., Toffolatti S.L., 2017. Genetic structure and fungicide sensitivity of *Botrytis cinerea* populations isolated from grapevine in northern Italy. *Plant Pathology* 66: 890–899. https://doi.org/10.1111/ppa.12643

Chen F., Liu X., Chen S., Schnabel E., Schnabel G., 2013. Characterization of *Monilinia fructicola* Strains Resistant to Both Propiconazole and Boscalid. *Plant Disease* 97: 645–651. https://doi.org/10.1094/PDIS-10-12-0924-RE

Egüen B., Melgarejo P., De Cal A., 2015. Sensitivity of Monilinia fructicola from Spanish peach orchards to thiophanate-methyl, iprodione, and cyproconazole: fitness analysis and competitiveness. *European Journal of Plant Pathology* 141: 789–801. https://doi.org/10.1007/s10658-014-0579-2

EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests, European and Mediterranean Plant Protection Organization 21 Boulevard Richard Lenoir, 75011 Paris, France September 2019

Fazekas M., Madar A., Sipiczki M., Miklós I., Holb I.J., 2014. Genetic diversity in Monilinia laxa populations in stone fruit species in Hungary. *World Journal of Microbiology and Biotechnology* 30: 1879–1892. https://doi.org/10.1007/s11274-014-1613-4

Förster H., Driever G.F., Thompson D.C., Adaskaveg J.E., 2007. Postharvest Decay Management for Stone Fruit Crops in California Using the "Reduced-Risk" Fungicides Fludioxonil and Fenhexamid. *Plant Disease* 91: 209–215. https://doi.org/10.1094/PDIS-91-2-0209

FRAC, 2020. List of first confirmed cases of plant pathogenic organisms resistant to disease control agents https://wwwfracinfo/ Accessed 08 July 2022.

FRAC, 2022. Fungal Control Agents Sorted by Cross Resistance Pattern and Mode of Action (Including FRAC Code Numbering) https://wwwfracinfo/ Accessed 08 July 2022.

Grasso V., Palermo S., Sierotzki H., Garibaldi A., Gisi U., 2006. Cytochromeb gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Management Science* 62: 465–472. https://doi.org/10.1002/ps.1236

Holb I.J., 2008. Brown rot blossom blight of pome and stone fruits: symptom, disease cycle, host resistance, and biological control. *International Journal of Horticultural Science* 14. https://doi.org/10.31421/IJHS/14/3/796

Holb I.J., Schnabel G., 2007. Differential effect of triazoles on mycelial growth and disease measurements of Monilinia fructicola isolates with reduced sensitivity to DMI fungicides. *Crop Protection* 26: 753–759. https://doi.org/10.1016/j.cropro.2006.07.001

Hrustić J., Mihajlović M., Grahovac M., Delibašić G., Tanović B., 2018. Fungicide sensitivity, growth rate, aggressiveness and frost hardiness of Monilinia fructicola and Monilinia laxa isolates. *European Journal of Plant Pathology* 151: 389–400. https://doi.org/10.1007/s10658-017-1380-9

Katoh K., Standley D.M., 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution* 30: 772–780. https://doi.org/10.1093/molbev/mst010

Kumar S., Stecher G., Tamura K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 33: 1870–1874. https://doi.org/10.1093/molbev/msw054

Landi L., Feliziani E., Romanazzi G., 2016. Surveys for *Monilinia* spp. on stone fruit in central-eastern Italy. *Acta Horticulturae* 225–230. https://doi.org/10.17660/ActaHortic.2016.1144.33

Lichtemberg P.S.F., Zeviani W.M., Michailides T.J., May de Mio L.L., 2016. Comparison of the sensitivity of *Monilinia fructicola* isolates to tebuconazole in Brazil using three methods. *Canadian Journal of Plant Pathology* 38: 55–63. https://doi.org/10.1080/07060661.2016.1147496

Luo C.-X., Hu M.-J., Jin X., Yin L.-F., Bryson P.K., Schnabel G., 2010. An intron in the cytochrome b gene of Monilinia fructicola mitigates the risk of resistance development to QoI fungicides. *Pest Management Science* 66: 1308–1315. https://doi.org/10.1002/ps.2016

Luo C.-X., Schnabel G., 2008. Adaptation to Fungicides in *Monilinia fructicola* Isolates with Different Fungicide Resistance Phenotypes. *Phytopathology* 98: 230–238. https://doi.org/10.1094/PHYTO-98-2-0230

Malandrakis A., Koukiasas N., Veloukas T., Karaoglanidis G., Markoglou A., 2013. Baseline sensitivity of *Monilinia laxa* from Greece to fenhexamid and analysis of fenhexamid-resistant mutants. *Crop Protection* 46: 13–17. https://doi.org/10.1016/j.cropro.2012.12.009

Mancini V., Makau S., Landi L., Romanazzi G., 2021. Identification of *Monilinia* spp. from stone fruits in the Marche region of Italy. *Acta Horticulturae* 91–96. https://doi.org/10.17660/ActaHortic.2021.1325.15

Martinelli E., Vitale S., Valente M., Riccioni L., 2013. Segnalate in Lazio infezioni di Monilinia fructicola su drupacee. *Informatore Agrario* 2: 55–57.

Martini C., Lantos A., Di Francesco A., Guidarelli M., D'Aquino S., Baraldi E., 2014. First Report of Asiatic Brown Rot Caused by *Monilinia polystroma* on Peach in Italy. *Plant Disease* 98: 1585–1585. https://doi.org/10.1094/PDIS-05-14-0551-PDN

Martini C., Guidarelli M., Di Francesco A., Ceredi G., Mari M., 2016. Characterization of thiophanate methyl resistance in italian *Monilinia fructicola isolates*. *Journal of Plant Pathology* 98. https://doi.org/10.4454/JPP.V98I3.003

May-De Mio L.L., Luo Y., Michailides T.J., 2011. Sensitivity of *Monilinia fructicola* from Brazil to Tebuconazole, Azoxystrobin, and Thiophanate-Methyl and Implications for Disease Management. *Plant Disease* 95: 821–827. https://doi.org/10.1094/PDIS-07-10-0511

Miessner S., Stammler G., 2010. *Monilinia laxa, M. fructigena* and *M. fructicola*: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome b gene sequences *Journal of Plant Diseases and Protection*: 162–167.

Montuschi C., Baschieri T., Rimondi S., Rossi R., Antoniacci L., Bugiani R., 2016. Monilinia fructicola in Emilia-Romagna: Indagini condotte sul territorio regionale dal 2010 al 2015. *ATTI Giornate Fitopatologiche* 2: 395–402.

Mustafa M.H., Bassi D., Corre M.-N., Lino L.O., Signoret V., … Cirilli M., 2021. Phenotyping Brown Rot Susceptibility in Stone Fruit: A Literature Review with Emphasis on Peach. *Horticulturae* 7: 115. https://doi.org/10.3390/horticulturae7050115

Myresiotis C.K., Karaoglanidis G.S., Tzavella-Klonari K., 2007. Resistance of *Botrytis cinerea* Isolates from Vegetable Crops to Anilinopyrimidine, Phenylpyrrole, Hydroxyanilide, Benzimidazole, and Dicarboximide Fungicides. *Plant Disease* 91: 407–413. https://doi.org/10.1094/PDIS-91-4-0407

Papavasileiou A., Testempasis S., Michailides T.J., Karaoglanidis G.S., 2015. Frequency of brown rot fungi on blossoms and fruit in stone fruit orchards in Greece. *Plant Pathology* 64: 416–424. https://doi.org/10.1111/ppa.12264

Pellegrino C., Gullino M.L., Garibaldi A., Spadaro D., 2009. First Report of Brown Rot of Stone Fruit Caused by *Monilinia fructicola* in Italy. *Plant Disease* 93: 668–668. https://doi.org/10.1094/PDIS-93-6-0668A

Pereira W.V., Primiano I.V., Morales R.G.F., Peres N.A., Amorim L., May De Mio L.L., 2017. Reduced Sensitivity to Azoxystrobin of *Monilinia fructicola* Isolates From Brazilian Stone Fruits is Not Associated With Previously Described Mutations in the Cytochrome *b* Gene. *Plant Disease* 101: 766–773. https://doi.org/10.1094/PDIS-09-16-1247-RE

Pereira W.V., Morales R.G.F., Bauer A.I.G., Kudlawiec K., May-De-Mio L.L., 2020. Discontinuance of tebuconazole in the field restores sensitivity of *Monilinia fructicola* in stone fruit orchards. *Plant Pathology* 69: 68–76. https://doi.org/10.1111/ppa.13101

Pratella G.C., 1996. La fase post-raccolta: la screpolatura delle ciliegie. *Frutticoltura* 10: 71–74.

Schnabel G., Bryson P.K., Bridges W.C., Brannen P.M., 2004. Reduced Sensitivity in *Monilinia fructicola* to Propiconazole in Georgia and Implications for Disease Management. *Plant Disease* 88: 1000–1004. https://doi.org/10.1094/PDIS.2004.88.9.1000

Swofford D.L., 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods) v. 4.0b10. Sunderland; MS, USA: Sinauer Associates.

Villarino M., Egüen B., Lamarca N., Segarra J., Usall J., … De Cal A., 2013. Occurrence of Monilinia laxa and M. fructigena after introduction of M. fructicola in peach orchards in Spain. *European Journal of Plant Pathology* 137: 835–845. https://doi.org/10.1007/s10658-013-0292-6

White T.J., Bruns T.D., Lee S.B., Taylor J.W., 1990. *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*. pp. 315–322 In: *PCR Protocols: A Guide to Methods and Applications*, (Innis, M. A., D. H. Gelfand, J. J. Sninsky, T. J. White, ed.). Academic Press, Inc., New York.

Xie J., Singh-Babak S., Cowen L., 2012. Minimum Inhibitory Concentration (MIC) Assay for Antifungal Drugs. *BIO-PROTOCOL* 2. https://doi.org/10.21769/BioProtoc.252