

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Rapid Detection of *Monilinia fructicola* and *Monilinia laxa* on Peach and Nectarine using Loop-Mediated Isothermal Amplification**

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1724381> since 2021-01-31T11:36:23Z

*Published version:*

DOI:10.1094/PDIS-01-19-0035-RE

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **Rapid Detection of *Monilinia Fructicola* And *Monilinia Laxa* on Peaches and Nectarines Using**  
2 **Loop-Mediated Isothermal Amplification**

3

4 Sara Franco Ortega<sup>1</sup>, Maria del Pilar Bustos López<sup>1,2</sup>, Luca Nari<sup>3</sup>, Neil Boonham<sup>4</sup>, Maria Lodovica  
5 Gullino<sup>1,2</sup>, Davide Spadaro<sup>1,2</sup>

6

7 <sup>1</sup> Centre of Competence for the Innovation in the Agro-environmental Sector - AGROINNOVA,  
8 University of Turin, via Paolo Braccini 2, I-10095 Grugliasco, TO, Italy

9 <sup>2</sup> Department of Agricultural, Forestry and Food Sciences (DiSAFA), University of Torino, via Paolo  
10 Braccini 2, I-10095 Grugliasco, TO, Italy

11 <sup>3</sup> AGRION, Fondazione per la ricerca l'innovazione e lo sviluppo tecnologico dell'agricoltura  
12 piemontese, 12030 Manta (Cn), Italy

13 <sup>4</sup> FERA Sand Hutton, York, UK, Current address: IAFRI, Newcastle University, Newcastle upon  
14 Tyne, NE1 7RU, UK

15

16

17

18

19

20

21

22

23

24 Corresponding Author: Davide Spadaro, Email: [davide.spadaro@unito.it](mailto:davide.spadaro@unito.it)

25

## 26 **Abstract**

27 *Monilinia laxa* and *Monilinia fructicola* are two causal agents of brown rot, one of the most important  
28 diseases in stone fruit. Two species cause blight on blossoms and twigs, and brown rot on fruits in  
29 pre- and postharvest. Both species are worldwide distributed in North and South America, Australia  
30 and Japan. In Europe, *M. laxa* is endemic, while *M. fructicola* was introduced in 2001 and it is now  
31 widespread in several countries. Currently, both species are coexisting in European stone fruits  
32 orchards. *Monilinia* spp. overwinter in cankers and mummified fruits. Mummy monitoring during  
33 winter permits to understand which species of *Monilinia* will be prevalent in orchard during the  
34 following season, permitting to plan an appropriate crop protection. Traditionally, the identification  
35 has been carried out using morphological features and even with PCR-based assays that requires time  
36 and well-equipped laboratories. In this study, two isothermal-based methods were designed to  
37 identify these pathogens in a faster way than using traditional methods. The Loop-mediated  
38 AMPLification (LAMP) assays were validated on some isolates of *Monilinia* spp. coming from the  
39 mummy monitoring according to the international EPPO standard (PM7/98) taking into account the  
40 specificity, sensitivity, repeatability and reproducibility. The sensitivity of both assays was checked  
41 by monitoring at different time points two nectarines varieties artificially inoculated and stored at two  
42 different temperatures. The reliability of both LAMP assays against the quantification of the inoculum  
43 was compared with previously published qPCR assays. Both LAMP methods were able to detect low  
44 number of cells. These LAMP methods could be a useful tool for the monitoring brown rot causal  
45 agents in the field and during postharvest.

46

47 **Keywords:** LAMP, brown rot, *Prunus persica*, nectarine, peach, field, molecular diagnostics

48 *Monilinia laxa* (Aderhold and Ruhland), *Monilinia fructicola* (Winter) Honey, *Monilinia*  
49 *fructigena* (Adehold and Ruhland) and *Monilia polystroma* Leeuwen are the main agents of brown  
50 rot, one of the most important diseases in *Prunus*, *Malus* and *Pyrus* species (Batra 1991).

51 Brown rot is particularly serious in peach and nectarine production by causing blossom and twig  
52 blights and brown rot on the fruits at preharvest, harvest and postharvest (Hong et al. 1997). Brown  
53 rot losses can reach 90%, by considering the harvest and postharvest stages (Hong et al. 1997; Hong  
54 et al. 1998). However, the postharvest stage is the most critical one, due to the optimal environment  
55 for *Monilinia* growth (Harvey 1978; Eckert and Ogawa 1988). The most important and widespread  
56 species on peaches and nectarines are *M. laxa* and *M. fructicola*. *M. fructicola* was originally  
57 identified in North and South America, Australia and Japan (EPPO/CABI 1997) and was introduced  
58 in Europe (France) on peaches in 2001 (Lichou et al. 2002). This pathogen is now spread throughout  
59 Europe, with reports in Austria (OEPP/EPPO 2002), Hungary and Spain on peaches (De Cal et al.  
60 2009), Italy on nectarines (Pellegrino et al. 2009), Germany on blackberries and plums (Hinrichs-  
61 Berger and Müller 2010), Slovenia on peaches (Munda and Viršček Marn 2010), and Poland on  
62 apples, pears and plums (Poniatowska et al. 2013). In 2005, *M. fructicola* was included in the A2  
63 EPPO List of quarantine organisms due to the high yield losses reported on peaches, apricots and  
64 nectarines in Europe (EFSA 2011). On the other hand, *M. laxa* is a quarantine pathogen in China and  
65 in some parts of North America (Martini and Mari 2014) and was the most prevalent species in Europe  
66 until the introduction of *M. fructicola*. Currently, both species are coexisting in European stone fruits  
67 orchards (Villarino et al. 2013).

68 *M. fructigena*, instead, is more prevalent in pome fruits and it has a low incidence in stone  
69 fruits (Martini and Mari 2014). *M. polystroma*, morphologically similar to *M. fructigena*, was initially  
70 identified in Japan (Côté et al. 2004) and has been introduced in Europe with reports in apples in  
71 Hungary (Petróczy and Palkovics 2009) and Croatia (Di Francesco et al. 2015), in apricots in  
72 Switzerland (Hilber-Bodmer et al. 2012), and in peaches in Italy (Martini et al. 2014). However, this

73 species is less aggressive and less prevalent, similarly to other minor species, such as *M. numecola*  
74 and *M. tunnanensis* (Cox et al. 2018).

75 In the United States, the stone fruit market has an annual value of approximately 4.4 billion  
76 \$ (Cox et al. 2018). Brown rot is mainly caused by *M. fructicola*, the most predominant in stone  
77 fruits, while *M. laxa* is present in some areas of North America with lower incidence (Cox et al.  
78 2018). However, *M. laxa* causes important losses due to development of blossom and shoot blights  
79 on almonds, apricots, nectarines and cherries (Cox et al. 2011).

80 *Monilinia* spp. overwinter in cankers on branches and on the mummified fruits remaining  
81 from one year to the next one. Mummy monitoring during winter permits to understand which species  
82 of *Monilinia* will be prevalent in orchard during the following season, permitting to plan an  
83 appropriate crop protection. Ascospores produced on the apothecia of infected mummified fruits are  
84 an important source of inoculum that causes primary infections or blossom blights in the early spring  
85 (Tate and Wood 2000).

86 Due to the importance of brown rot caused by *Monilinia* spp., intensive disease management  
87 is key to reduce blossom blight during spring and to reduce the risk of fruit infection. Different  
88 sensitivity to fungicides, such as benzimidazoles and dicarboximides (Chen et al. 2013; Egüen et al.  
89 2016), has been reported for different species of *Monilinia*. The use of fungicides during preharvest  
90 is the most common measure used to control the incidence of pre and postharvest brown rot (Eckert  
91 and Ogawa 1988, Thomidis et al. 2009), however the decision about the optimal moment for chemical  
92 control is based on forecasting models. The request of reducing the use of fungicides is boosting the  
93 development of alternative crop protection strategies (Malavolta et al. 2003), including biocontrol  
94 agents (Larena et al. 2005; Zhang et al. 2010; Banani et al. 2015) or natural substances (Mari et al.  
95 2008; Lopez-Reyes et al. 2013; Santoro et al. 2018).

96 A correct identification of the species of *Monilinia* may improve crop protection. Fungal  
97 isolation and micromorphological analysis are necessary to identify the species of *Monilinia*, but  
98 morphological features may vary depending on the incubation media and conditions making difficult

99 an accurate species-specific identification (Byrde 1977, Lane 2002). In addition, micromorphological  
100 identification requires a long incubation period that is critical for decision making (OEPP/ EPPO  
101 2009). Different assays based on PCR amplification of rDNA internal transcribed spacer region (Ioos  
102 and Frey 2000), RAPD (Boehm et al. 2001; Côté et al. 2004), PCR with SCAR primers (Hughes et  
103 al. 2000), microsatellites using a nested-PCR (Ma et al. 2003; Boehm et al. 2001) or qPCR (Van  
104 Brouwershaven et al. 2009; Guinet et al. 2016, Wang et al. 2018) have been developed to differentiate  
105 the species of *Monilinia*. However, these methods are time-consuming and require transport to a  
106 diagnostics laboratory, which delays the decision making process.

107 Loop-mediated isothermal AMPlification (LAMP) assay demonstrated to be a specific,  
108 sensitive and reliable tool for fungal identification in routine diagnostics (Franco Ortega et al. 2018a).  
109 The LAMP reaction is an isothermal DNA amplification method with an enzyme copying the target  
110 region faster than other PCR based methods, whilst avoiding the use of thermal cycling (Notomi et  
111 al. 2000; Nagamine et al. 2002). The enzyme is also robust, thus complex DNA extraction is not  
112 required to perform the LAMP assay, which is not affected by inhibitors. The greatest advantage of  
113 the LAMP assay is the possibility of using battery-powered platforms, such as Genie II or Genie III  
114 (Optigene, Horsham, UK), which make the LAMP a suitable diagnostic tool for direct detection in  
115 field, packinghouse or during transport and storage stages.

116 The aim of the present study was to monitor the occurrence of the emerging pathogen *M.*  
117 *fructicola* and other species of *Monilinia* spp. in peach and nectarine orchards during the first four  
118 consecutive years of occurrence in Italy. Mummy monitoring during winter time permits to plan an  
119 appropriate crop protection strategy against brown rot in the following growing season. A collection  
120 of isolates of *Monilinia* spp. was created, by isolating from peach and nectarine mummies harvested  
121 at the end of the winter season. The strains were identified morphologically and with molecular tools.  
122 The collection constituted the basis for developing two LAMP assays, one for *M. fructicola* and the  
123 other one for *M. laxa*, the two main species of *Monilinia*. The molecular assays were validated on a  
124 selection of isolates from the mummy collection and on inoculated nectarines.

125

## 126 **Materials and Methods**

127 **Pathogen isolation.** Forty-six peach and nectarine fields listed in Table S1 were monitored  
128 during the winter seasons from 2008-2009 to 2011-2012 to verify the occurrence of *Monilinia*  
129 *fruticola* and of other species of *Monilinia*. The monitoring was carried out on twenty cultivars of  
130 peach and nectarine: ‘Alitop’, ‘Amiga’, ‘Big Top’, ‘Caldesi’, ‘Diamond Ray’, ‘Elegant Lady’,  
131 ‘Firebrite’, ‘Fire Top’, ‘Fire Sweet Red’, ‘Maeba Top’, ‘Nectaross’, ‘Orion’, ‘Red Valley’, ‘Rome  
132 Star’, ‘Royal Glory’, ‘Sweet Lady’, ‘Stark Red Gold’, ‘Vista Rich’, ‘Venus’, and ‘V3’ (Table S1).  
133 Twenty-five mummified fruits, when present, were collected from each orchard, disinfected in a  
134 sodium hypochlorite (1%) solution, rinsed under tap water and dried. Small pieces of infected  
135 material were placed on Potato Dextrose Agar (PDA; Sigma-Aldrich, Saint Louis, MO, USA) to  
136 isolate the pathogen. The samples were grown at 23-25°C for four days. Single spore cultures of each  
137 *Monilinia* isolates were obtained and stored in 60% glycerol at -80°C. The isolates were stored at  
138 Agroinnova microorganism collection (University of Turin, Italy). For the development of the LAMP  
139 assays, besides the isolates of *Monilinia* spp. coming from the field monitoring, some *Monilinia* spp.  
140 from the FERA collection (York, UK) and two isolates of *M. polystroma* provided by the University  
141 of Bologna were used.

142 **Fungal isolates and DNA extraction.** Single spore isolates listed in Table 1 were grown on  
143 PDA for 10 days at room temperature. Mycelium was collected and the total genomic DNA was  
144 extracted with E.Z.N.A. Fungal DNA mini kit (OMEGA Bio-Tek, Norcross, GA, USA), according  
145 to the manufacturer’s instructions. The DNA concentration of each isolate was measured using a  
146 Nanodrop 1000 (ThermoFisher, Delaware, USA) and the concentration of DNA was adjusted at 1 to  
147 50 ng/μl. A multiplex PCR developed by Côté et al. (2004) with the primers listed in Table S2 was  
148 used to identify all the samples obtained during the monitoring. Fungal species identification was  
149 confirmed with the primers designed by Hughes et al. (2000), including the primer ITS1 for *M.*

150 *fructigena*, and the primers described by Gell et al. (2007). Amplified products were checked on 1.5%  
151 agarose gel stained with ethidium bromide.

152 **Crude extraction method from peaches.** DNA was extracted from artificially inoculated  
153 peaches using a crude extraction method to avoid the use of long and complex procedures. The crude  
154 extraction was based on the procedure described by Chomeczynski and Rymaszewski (2006) and  
155 Tomlinson et al. (2010a). Alkaline PEG buffer (1 ml) composed of 20 mM KOH at pH 13.5 with 50  
156 g L<sup>-1</sup> of PEG average of Mm: 4,600 a with 1 ball bearing (7/16” stainless steel 316 GD Spheric  
157 Trafalgar Ltd) in a 5 ml tube was used for the crude extraction by adding 1-2 g of the inoculated peach  
158 material (adding the skin and flesh of the inoculated point, cutting by a sterile scalpel). Samples were  
159 manually shaken for one minute. The solution was ten-fold diluted to use in the LAMP reaction. The  
160 peach genomic DNA was also obtained in parallel with E.Z.N.A. Plant DNA kit (OMEGA Bio-Tek)  
161 to compare the results from both DNA extraction methods.

162 **LAMP primer design.** Six LAMP primers including two external primers, F3 and B3; two  
163 internal primers, FIP (F1c+ F2) and BIP (B1c+B2); and two loop primers, Floop and Bloop, were  
164 designed from *M. fructicola* and *M. laxa* sequences according to the method described by Notomi et  
165 al. (2000). The LAMP primers for *M. fructicola* were designed on an intron in the cytochrome b,  
166 associated with the Qol fungicides resistance, present in *M. fructicola* (GenBank accession number  
167 GQ304941.1), but absent in other *Monilinia* species (Luo et al. 2010). The primers for *M. laxa* were  
168 designed on a genomic sequence identified as a SCAR marker by Gell et al. (2007) (GenBank  
169 accession number: EF207417.1). All the primers were manually designed taking into account the  
170 annealing temperature. Possible hairpin and secondary structures were checked using the OligoCalc  
171 program (<http://biotools.nubic.northwestern.edu/OligoCalc.html>), while possible interactions  
172 between primers was controlled using the Multiple Primer Analyzer (Thermo Scientific)  
173 (<https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>). HPLC-purified primers were synthesized by Eurofins (UK) and both LAMP  
174  
175 Davide Spadaro  
Plant Disease



176 assays are available in kit format from OptiGene Ltd (Horsham, UK: <http://www.optigene.co.uk>).  
177 BLASTn analysis of the F1c and B1c of both primers was performed to check possible cross-  
178 reactivity *in silico*.

179 **LAMP assay.** The LAMP reaction (25 µl) contained 200 nmol/l of each external primer (F3  
180 and B3), 2 µmol/l of each internal primers (FIP and BIP), 1 µmol/l of each loop primer, 1x Isothermal  
181 Mastermix ISO-004 (OptiGene Ltd) with 1 µl of the pure DNA or the crude extractions. A Genie II  
182 ® instrument (OptiGene Ltd) and a StepOne (Applied Biosystem, California, USA) was used to carry  
183 out the LAMP tests. The program conditions were 45 min at 65°C and a measure of the annealing  
184 temperature from 95°C to 70°C with a reduction of 0.05°C/s. The StepOne was programmed as  
185 described previously by Franco Ortega et al. (2018b). A negative control with water and a positive  
186 control with the pure DNA of the target were included in each assay. The COX assay described by  
187 Tomlinson et al. (2010b) was applied as internal control to verify if the plant DNA from the samples  
188 resulted negative with the LAMP assays for *M. fructicola* and *M. laxa* could be amplified.

189 **Validation of the LAMP assays.** The LAMP assays were validated according to the  
190 international standard EPPO PM 7/98 taking into consideration specificity, sensitivity, repeatability  
191 and reproducibility. The specificity of the assays were checked in triplicate using an inclusivity-  
192 exclusivity panel of the target and non-target *Monilinia* spp., as well as other common pathogens of  
193 stone fruits. The sensitivity was tested on 10-fold serial dilutions of the DNA from one isolate of *M.*  
194 *fructicola* (isolate MSR38) and one isolate of *M. laxa* (isolate 1406) with four replicates of each  
195 dilution. The extraction and the LAMP assays were performed on different days with different  
196 machines (Genie II and StepOne) by three members of the lab staff to evaluate the reproducibility.  
197 The reliability of both LAMP assays was confirmed in parallel with a qPCR assay on two cultivars  
198 of nectarines, using three biological replicates and three technical replicates for each assay.

199 **Artificial inoculation and LAMP assay on fruit.** Nectarines ‘Amiga’ and ‘Fire Top’ were  
200 inoculated with *M. fructicola* MSR2 and *M. laxa* 1506. Healthy nectarines were disinfected by  
201 submerging in 2% hypochlorite (vol: vol) solutions before being wiped with distilled water and air-

202 dried. The fruit inoculation was performed in artificial wounds (2 mm in diameter and 4 mm deep)  
203 using 10 µl of the spore suspension ( $10^5$  conidia/ml) prepared from 10-day old *Monilinia* cultures  
204 grown on PDA. The fruits were stored at 18°C or at 4°C. The DNA from the inoculation region of  
205 the fruit was used in the crude extraction method and the E.Z.N.A. Plant DNA kit. The quantity of  
206 the fruit tissue of each nectarine used in each extraction was measured to calculate the number of  
207 cells present in the sample. The LAMP assay was carried out with the crude extraction method at 2  
208 days post inoculation (dpi), 4/5 dpi, 7 dpi, 9 dpi, 11/12 dpi for the nectarines stored at 18°C and at 2  
209 dpi, 4/5 dpi, 7 dpi, 9 dpi, 11/12 dpi, 14 dpi and 16 dpi for the nectarines stored at 4°C. Sampling times  
210 were 4 and 11 dpi for 'Amiga' and 5 and 12 dpi for 'Fire Top'. The fruit symptoms were also scored  
211 using the following disease index: 0: no evident symptoms; 1: brown rot lower than 1 mm diameter;  
212 2: brown rot of 1-3 mm diameter; 3: brown rot of 1-2 cm diameter; 4: at least half of the fruit surface  
213 presented brown rot; 5: mummified fruit. The disease severity was calculated using the average of  
214 three replicates (the same 3 nectarines selected randomly from the inoculated batch and posteriorly  
215 used for both DNA extractions). Samples prior to inoculation were used in both LAMP and qPCR  
216 analysis as negative controls.

217 **Real-Time PCR.** The primers designed by Hughes et al. (2000), Mcf-F1 and Mfc-R1 specific  
218 for *M. fructicola* and MI-Mfg-F2 and MI-Mfc-R1 specific for *M. laxa*, were used to quantify the  
219 number of cells present in the samples. The 25 µl reactions were carried out using 1x Power SYBR  
220 Green PCR Master Mix (Applied Biosystems), 120 nM of each primer and 1 µl of the peach genomic  
221 DNA extracted using the commercial kit. The amplification was carried out using the following  
222 protocol: 95°C for 10 min, 40 cycles of 15 s at 95°C, 1 min at 60°C, and 45 s at 72°C in a OneStep  
223 Plus Real-Time PCR system (Applied Biosystems). A standard curve was performed using *M.*  
224 *fructicola* MSR2 and *M. laxa* 1506 ranging from 9.4 ng/µl to 9.4 fg/µl. A positive control with DNA  
225 extracted from cultured pathogen and a negative control of water were included in each run. The  
226 quantity of DNA present in the sample was calculated according to the standard curve measures,  
227 while the number of cells was estimated using the *Monilinia fructicola* genome weight (0.000048 ng;

228 <https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=NGKE01#contigs>) using the formula: number of  
 229 cells / $\mu\text{l}$  = DNA quantity/0.000048 (Amaral Carneiro et al. 2017). The amount of fruit used (ranging  
 230 from 1 g to 2 g) and the elution volume of the extracted DNA were used to calculate the total number  
 231 of cells.

232 **Analytical specificity and sensitivity assays.** The inoculated nectarines (24 samples) and  
 233 eight negative samples (four for either cultivar) were used to calculate some analytical parameters for  
 234 both LAMP assays, which were compared with the SYBR Green qPCR of Hughes et al. (2000).  
 235 Diagnostic sensitivity (DSe) or true-positive rate and analytical specificity (DSp) or true-negative  
 236 rate, were calculated using the formula as follows:  $Dse = \frac{\sum TP}{(\sum TP + FN)}$ ;  $Dpe = \frac{\sum TN}{(\sum TN + FP)}$ ;  
 237 where TP (True Positive) is the number of positive samples in the experiment, TN (True Negative) is  
 238 the number of real negative samples, FN (False Negatives) are the negative samples using the LAMP  
 239 assay but infected with *Monilinia* spp., and FP (False Positives) are the positive results using LAMP  
 240 assay and naturally free from pathogens (Altman and Bland, 1994). The likelihood positive ratio  
 241 (LR+) and the likelihood negative ratio (LR-) were calculated according to the ratio between DSe and  
 242 the FP-rate and the FP-rate and Dse, respectively, using the calculator tool Diagnostic Test Calculator  
 243 (<http://araw.mede.uic.edu/cgi-bin/testcalc.pl>)

244

## 245 Results

246 **Field monitoring.** During the monitoring, 1889 mummified fruits were harvested (281 in  
 247 2008/09, 480 in 2009/2010, 542 in 2010/2011, and 586 in 2011/2012). The number of isolates of the  
 248 different species of *Monilinia* were recorded to get the incidence in the orchards: 200 isolates from  
 249 the mummies of 2008/09, 417 from 2009/10, 396 from 2010/11, and 470 from 2011/12. This  
 250 monitoring permitted to identify the evolution over time of the species of *Monilinia* spp. from the  
 251 mummies, able to cause brown rot in peaches and nectarines.

252 An increase in the occurrence of *M. fructicola* was shown during the years, from a minimum of 6.2%  
253 during the 2008-2009 winter season to a maximum of 49.6% in the 2011-2012. On the other hand,  
254 the incidence of *M. laxa* decreased from 91.8% in 2008-2009 to 46.6% in 2011-2012. In the 2011-  
255 2012 winter season, the incidence of *M. fructicola* overtook the incidence of *M. laxa*. (Figure 1). The  
256 incidence of *M. fructigena* was constantly low and did not vary significantly during the study period:  
257 from 2.0% (2008-09), to 2.7% (2009-10), 2.1% (2010-11) and 3.8% (2011-12).

258 *M. fructicola* was more abundant in peaches than in nectarines during the winter season 2011-2012  
259 (Figure 2). Among the cultivars analyzed during the last winter season, 'Fire Top' (90%), 'Fire Sweet  
260 Red' (90%), 'Rome Star' (76.1%), 'Diamond Ray' (69.1%), 'Alitop' (63.35%), and 'Nectaross'  
261 (61.1%) showed the highest occurrence of *M. fructicola*, while the varieties with the highest incidence  
262 of *M. laxa* were 'Stark Red' (100%), 'Caldesi' (69.2%), 'Royal Glory' (66.7%) and 'Big Top'  
263 (56.9%). The highest incidence of *M. fructigena* was found in the variety 'Caldesi' (30%), while in  
264 the other varieties the incidence was just 7%.

265 Variation in the *Monilinia* species present was found in different areas. All samples from three  
266 locations (Dronero, Manta and Piasco; Piedmont, northern Italy) were identified as *M. laxa*, while *M.*  
267 *laxa* was not isolated from the samples from Castellar and Scarnafigi. The other areas showed  
268 different percentages of *M. laxa* and *M. fructicola*. *M. fructigena* was present in less than 8% of the  
269 samples tested (Figure S1).

270 A collection of isolates of *Monilinia* spp. was created, useful for the development of a new diagnostic  
271 method to distinguish the predominant species of *Monilinia*. A significant number of isolates from  
272 the monitoring were used in the specificity test of both LAMP assays.

273 **Design and validation of the LAMP assay using DNA from pure culture.** The primers for  
274 the identification of *M. fructicola* and *M. laxa* were designed on the cytochrome b sequence and on  
275 the SCAR marker region, identified by Gell et al. (2007), respectively. All primers were checked for  
276 lack of secondary structure, self-annealing or hairpin.

277 BLASTn analysis of the F1c and B1c (primer FIP and BIP) for the detection of *M. fructicola* showed  
278 100% sequence identity with *M. fructicola* (GenBank accession number KM610206.1), while the F1c  
279 and B1c regions of the FIP and BIP primers for the detection *M. laxa* had 100% identity to the  
280 sequence of this species (accession number EF207417.1).

281 Initially, identification of the *Monilinia* spp. was carried out using the primers designed by  
282 Hughes et al. (2000) and by Gell et al. (2007). Both PCR analyses confirmed the results of the LAMP  
283 assays. The isolates of *Monilinia* spp. from the monitoring, as well as other isolates, were used to  
284 validate the specificity of the LAMP assays, which is reported in Table 1. The LAMP primers  
285 amplified only the targets (*M. fructicola* and *M. laxa*, respectively), while untargeted amplifications  
286 were not obtained. The time to positive (Tp) for the *M. fructicola* LAMP assays ranged from 7 min  
287 35 s to 25 min, with an annealing temperature ranging from 84.06°C to 85.54°C. For the *M. laxa*  
288 LAMP assay the Tp ranged from 7 min 11 s to 24 min 26 s, with an annealing temperature ranging  
289 from 81.52°C to 85°C.

290 The sensitivity testing demonstrated that the *M. fructicola* LAMP assay was able to detect  
291 100-999 fg of DNA, while the *M. laxa* LAMP assay had a lower detection limit of 10-99 fg of DNA  
292 (Table 2). The assays consistently showed positive results in the three replicates tested, even by  
293 changing the machine, the operator, or the day of the test.

294 ***In vivo* testing.** Both LAMP assays were tested on two varieties of nectarines. Brown rot  
295 symptoms were visible earlier on ‘Amiga’ than on ‘Fire top’ nectarines at both temperatures. ‘Fire  
296 top’ nectarines inoculated with *M. fructicola* and stored at 18°C started to exhibit concentric lesions  
297 from the inoculation area with evident symptoms of brown rot at 7 dpi (Table 3). Instead, ‘Amiga’  
298 nectarines stored at 18°C exhibited symptoms at 4 dpi (Table 4). Nectarines inoculated with *M.*  
299 *fructicola* and stored at 4°C showed the first symptoms of brown rot at 14 dpi on ‘Fire Top and at 7  
300 dpi on ‘Amiga’. Brown rot symptoms were visible earlier by inoculating *M. laxa* than *M. fructicola*  
301 on both varieties.

302 The LAMP assay for *M. fructicola* was able to detect the presence of the pathogen in both nectarine  
303 varieties before the appearance of brown rot symptoms (Table 3 and 4). The Tp ranged from 4 min  
304 33 s to 15 min from the DNA of nectarines stored at 18°C, and ranged from 5 min 52 s to 36 min,  
305 with the majority of the positive results between 9-13 min, from the DNA of nectarines stored at 4°C  
306 (Tables 3 and 4). The annealing temperature ranged from 84.35 to 85.18°C (Tables 3 and 4).

307 The *M. laxa* LAMP assay gave positive results prior to symptom development in both nectarine  
308 varieties and at both storage temperatures (Tables 5 and 6). Anyway, there were differences in the  
309 LAMP assay for *M. laxa* between the varieties: ‘Fire Top’ nectarines were not positive until 7 dpi at  
310 both storage temperatures (Table 5), while in ‘Amiga’ nectarines the pathogen was detected at 2 dpi  
311 (Table 6). Using the DNA from *M. laxa*-inoculated ‘Amiga’ nectarines stored at 18°C, the LAMP  
312 assay produced a Tp ranging from 4 min 2 s to 24 min 15 s (Table 6). The Tp of the nectarines stored  
313 at 4°C ranged from 4 min 2 s to 28 min 12 s. The annealing temperature ranged from 83.15 to 83.55°C  
314 when all samples were compared (Tables 5 and 6).

315 **Comparison between qPCR and LAMP assay.** The number of cells present during the  
316 experiments were quantified using SYBR Green and the combination of primers obtained by Hughes  
317 et al. (2000) (Tables from 3 to 6). In order to check the sensitivity of the LAMP assays, the number  
318 of cells of three nectarines were compared with an average of the disease index of the same nectarines  
319 to obtain a real comparison between molecular data and symptoms. The number of cells increased  
320 over time reaching a maximum of 20,000,000 cells for the variety ‘Amiga’ stored at 18°C. The  
321 number of cells of the nectarines increased day after day, similarly with the disease index. This trend  
322 was observed in both varieties (‘Amiga’ nectarines inoculated with *M. fructicola* and *M. laxa* stored  
323 at 18°C and 4°C respectively, as well as ‘Fire Top’ inoculated with *M. laxa* and stored at 18°C),  
324 however the LAMP assay gave reliable detection.

325 Different results were obtained with the qPCR and the LAMP assays. In the case of *M.*  
326 *fructicola*-inoculated nectarines, both techniques showed positive results for both nectarine varieties  
327 at both storage temperatures, with only one exception in which the qPCR was not able to detect any

328 cells ('Fire Top' nectarines at 2 dpi). However, as observed in the Table 3 and 4, there were significant  
329 differences between the number of positives obtained with each technique, being the qPCR more  
330 repeatable.

331 On the other hand, there were bigger differences between the qPCR and the LAMP assays to  
332 detect *M. laxa*. In the case of 'Fire Top' nectarines, the nectarines stored at 18°C at 2 dpi and the  
333 nectarines stored at 4°C at 2 and 4 dpi, were only detected with the qPCR and not with the LAMP  
334 (Table 5). However, 'Amiga' nectarines stored at both temperatures were detected with the LAMP at  
335 all time points, but the qPCR produced negative results in two cases: 18°C stored nectarines at 2 dpi  
336 and 4°C stored nectarines after 9 dpi (Table 6). The LR+ and LR- of both assays were calculated to  
337 check the effectiveness of the LAMP assay in comparison with the qPCR with SYBR Green. In the  
338 case of the LAMP assay for *M. fructicola*, the value of Dsp and Dse were 1.0 (100%), while the Dsp  
339 and Dse values of the specific primers were 0.88 (88%) and 0.96 (96%) respectively. The *M. laxa*  
340 LAMP assay showed a DSp value of 0.83 (83%) and a DSe value of 0.67 (67%) compared with the  
341 0.88 (88%) and 0.73 (73%) of the DSp and Dse values of the specific primers.

342 Taking into consideration the likelihood ratios, the LR+ and LR- of *M. fructicola* LAMP assay  
343 were infinite, while the LR- of the *M. laxa* LAMP assay was 0.17 and the LR+ was infinite. The same  
344 values calculated for the qPCR primers with SYBR Green for *M. fructicola* and *M. laxa* showed in  
345 both cases infinite LR+ and LR- of 0.04 and 0.12, respectively.

346

## 347 Discussion

348 The occurrence of *Monilinia* spp. was monitored during four winter seasons in Italian peach  
349 and nectarine orchards for a better understanding of the establishment of the introduced species *M.*  
350 *fructicola* and of the evolution of the species of *Monilinia* spp.. The results of the mummy monitoring  
351 over 4 years helped to understand the capacity of coexistence of *M. laxa* and *M. fructicola* in the same  
352 orchards. Since the first report of *M. fructicola* in Europe, the pathogen, listed as an EPPO A2

353 pathogen due to the damage caused (OEPP/EPPO 2005), has spread to different countries. Despite  
354 the importance of stone fruit production in Italy, an extensive monitoring activity has not not  
355 performed by other authors since the first occurrence (Pellegrino et al., 2009). The geographical  
356 region where *M. fructicola* was first identified in Italy, was the focus of this study. *M. laxa* and *M.*  
357 *fructicola* could produce fruit losses up to 90% (Larena et al. 2005; Hong et al. 1997), under favorable  
358 weather conditions. The losses caused by *M. fructigena* are by far lower, with a relatively low  
359 incidence in Italy. In this study, the incidence of *M. fructicola* increased year after year, overtaking  
360 the incidence of *M. laxa* in the 2011-12 survey. This suggested a higher fitness of *M. fructicola* and  
361 the higher adaptability to the weather conditions of northern Italy.

362         The estimation of the mummy inoculum and the optimal environmental conditions for the  
363 disease development should be considered to understand the seasonal variation of the disease and to  
364 ensure the timely implementation of a management strategy against brown rot (Luo et al. 2001a; Luo  
365 and Michailides 2001a). The monitoring of mummies during winter permits to plan an appropriate  
366 crop protection strategy, which depends on the species of *Monilinia* present in the orchard. An  
367 Integrated Pest Management (IPM) approach is suggested by EFSA (2011) in order to effectively  
368 control brown rot and blossom blight, focusing on reducing the inoculum of *Monilinia* in the field  
369 and therefore the risk of infection (Luo and Michailides 2001b). The importance of the mummies as  
370 an important inoculum source was confirmed during the survey. Therefore, removal and destruction  
371 of the fruit mummies is a key aspect of the sanitation process, especially given the diffusion speed of  
372 brown rot in orchards.

373         During postharvest, the incidence of brown rot is associated with the incidence of *Monilinia*  
374 spp. in orchard. Any delay on the application of control measures for *Monilinia* spp. can cause losses  
375 of over 50% particularly during postharvest, but yield losses can be reduced if correct control  
376 measures are timely applied (Margosan et al. 1997). However, some of the measures developed to  
377 reduce brown rot have been demonstrated to be species-dependent (Mari et al. 2012; Lopez-Reyes et  
378 al. 2013), making necessary a precise identification of the causal agent of brown rot. Until now, no



379 LAMP tests have been developed to directly check the presence of *Monilinia* on fruit, therefore the  
380 methods described in this study could help growers to control the presence of brown rot in peaches,  
381 by supporting the choice of the correct control measures before harvesting and during storage.

382 One of the biggest drawbacks of the PCR-based methods is the inhibition of the reaction by some  
383 components of plant tissue which results in false negative results (Wilson 1997), therefore most PCR-  
384 based methods involve long and complex DNA extraction methods. As an alternative, the LAMP  
385 assays for the detection of *M. fructicola* and *M. laxa* were validated using a crude extraction method,  
386 which is simpler, and showed to be reliable and effective in the detection of both pathogen species.  
387 The validation of the assays was carried out according to the international standard published by  
388 EPPO (PM7/98). A significant number of isolates from the monitoring were used to validate the  
389 specificity of both LAMP assays. To verify the parameters of both LAMP assays *in vivo*, we focused  
390 on fully ripe nectarines, which were stored at two temperatures reflecting the storage and shelf life  
391 conditions of stone fruit.

392       The LAMP assay for *M. fructicola* was less sensitive than the LAMP assay for *M. laxa*.  
393 Notwithstanding, on nectarines inoculated with different amounts of *M. laxa*, the pathogen  
394 detection was possible even without visible symptoms. The LAMP results were confirmed using  
395 qPCR. The lowest number of cells in all the ‘Fire Top’ nectarines inoculated with *M. fructicola* was  
396 259.7 cells (no symptoms visible) giving two positive replicates out of nine with the qPCR and six  
397 out of nine with the LAMP assay, which verified the reliability of the assay. In a similar situation,  
398 *M. laxa* (73.5 cells) produced less positive results with the LAMP assay (1/9 compared to 2/9 with  
399 the qPCR). In contrast, ‘Amiga’ nectarines were less susceptible to *M. fructicola* and *M. laxa* with  
400 less cells quantified and lower symptoms in all the experiments. The use of the LAMP assay on the  
401 ‘Amiga’ variety confirmed the reliability and effectiveness of the LAMP assays on the detection of  
402 few cells (22.8 cells and even 1.3 cells) independently from the nectarine variety, suggesting that  
403 the assay is potentially a powerful tool for pre-symptomatic detection.

404           According to the natural disease development, a consistent and constant increase in the  
405 number of pathogen cells was expected over time, however, in some cases, the pattern of the disease  
406 did not correspond with the number of cells shown. In particular, when the whole fruit showed brown  
407 rot or the nectarines were mummified, the number of cells were not the highest ones. This could be  
408 explained with the high amount of fungal DNA present in the sample, especially in the mummified  
409 fruits, which could inhibit the qPCR. However, the LAMP assay reliably detected the pathogen even  
410 in these cases.

411           On the other hand, the LAMP assays were able to detect the presence of the pathogen even  
412 without visible symptoms, when the qPCR did not produce positive results. In summary, both LAMP  
413 assays were validated and could be reliably used for the detection of *M. fructicola* and *M. laxa*.  
414 Despite the use of a crude extraction method instead of a commercial and complex DNA extraction  
415 used for the qPCR assay, the results of the LAMP assay were consistent, and no inhibition or loss of  
416 sensitivity were reported taking into consideration the likelihood ratios which were similar to those  
417 of the qPCR for both pathogens. The monitoring performed during four winter seasons suggested a  
418 higher adaptability of *M. fructicola*, compared to *M. laxa*, in the Italian orchards. The LAMP assays  
419 allow a faster identification in orchard of *Monilinia* spp., helping the growers to speed the decision  
420 making process about crop protection strategies, to be used in the following growing season.

421

## 422 **Acknowledgments**

423           The research leading to these results has received funding from the European Union's Horizon 2020  
424 research and innovation program under grant agreement No 634179 "Effective Management of Pests  
425 and Harmful Alien Species - Integrated Solutions" (EMPHASIS). The authors have no conflicts of  
426 interest to declare.

427 **Literature Cited**

- 428 Altman, D. G., and Bland, J. M. 1994. Diagnostic tests 2: Predictive values. *BMJ*. 309:102.
- 429 Amaral Carneiro G., Matic S., Ortu G., Garibaldi A., Spadaro D., Gullino M.L. 2017.
- 430 Development and validation of a TaqMan real time PCR assay for the specific detection and
- 431 quantification of *Fusarium fujikuroi* in rice plants and seeds. *Phytopathology*, 107:885-892.
- 432 Banani, H., Spadaro, D., Zhang, D., Matic, S., Garibaldi, A. and Gullino, M. L. 2015.
- 433 Postharvest application of a novel chitinase cloned from *Metschnikowia fructicola* and overexpressed
- 434 in *Pichia pastoris* to control brown rot of peaches. *Int. J. Food Microbiol.* 199:54-61.
- 435 Batra, L. R., 1991. World Species of *Monilinia* (fungi) Their Ecology, Biosystematics and
- 436 Control. In: *Mycologia Memoir No. 16*. J. Cramer, eds. Koeltz Botanical Books, Berlin, Germany.
- 437 Byrde, R. J. W. and Willetts, H. J. 1977. *The Brown Rot Fungi of Fruit*. Pergamon Press,
- 438 London, UK.
- 439 Boehm, E. W. A., Ma, Z., and Michailides, T. J. 2001. Species-specific detection of *Monilinia*
- 440 *fructicola* from California stone fruits and flowers. *Phytopathology*. 91:428-439.
- 441 De Cal, A., Gell, I., Usall, J., Viñas, I., and Melgarejo, P. 2009. First report of brown rot caused
- 442 by *Monilinia fructicola* in peach orchards in Ebro Valley, Spain. *Plant Dis.* 93:763-763.
- 443 Chen, F., Liu, X., and Schnabel, G. 2013. Field strains of *Monilinia fructicola* resistant to both
- 444 MBC and DMI fungicides isolates from stone fruit orchards in the Eastern United States. *Plant Dis.*
- 445 97:1063-1068.
- 446 Chomczynski, P., and Rymaszewski, M. 2006. Alkaline polyethylene glycol-based method for
- 447 direct PCR from bacteria, eukaryotic tissue samples, and whole blood. *Biotechniques*. 40:454-458.
- 448 Côté, M.-J., Tardif, M. C. C., Meldrum, A. J., Cote, M. J., Tardif, M. C. C., and Meldrum, A.
- 449 J. 2004. Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa* and *Monilia polystroma* on
- 450 inoculated and naturally infected fruit using multiplex PCR. *Plant Dis.* 88:1219-1225.

- 451 Cox, K. D., Villani, S. M. Raes, J. J., Freier, J., Faubert, H., Cooley, D., and Clements, J. 2011.  
452 First reports of brown fruit rot on sweet cherry (*Prunus avium*) and plum (*Prunus domestica*), and  
453 shoot blight on apricot (*Prunus armenica*), kwanzan cherry (*Prunus serrulata*) and sweet cherry  
454 (*Prunus avium*) caused by *Monilinia laxa* in New York, Rhode Island and Massachussets. Plant Dis.  
455 95:1584.
- 456 Cox, K. D., Villani, S. M., Poniatowska, A., Schnabel, G., Holb, I., and Fajardo, J. 2018.  
457 Recovery plan for *Monilinia polystroma* causing Asiatic Brown rot of Stone fruits. Plant Health Prog.  
458 19:107-124.
- 459 Di Francesco, A., Fruk, M., Martini, C., Jemric, T., and Mari, M. 2015. First report of asiatic  
460 brown rot (*Monilinia polystroma*) on apple in Croatia. Plant Dis. 99:1181.
- 461 Eckert, J. W., and Ogawa, J. M. 1988. The Chemical Control of Postharvest Diseases:  
462 Deciduous Fruits, Berries, Vegetables and Root/Tuber Crops. Annu. Rev. Phytopathol. 26:433-469.
- 463 EFSA, E.F.S.A. 2011. Pest risk assessment of *Monilinia fructicola* for the EU territory and  
464 identification and evaluation of risk management options 1. EFSA journal. 4: 155.  
465 <http://www.efsa.europa.eu/en/efsajournal/doc/2119.pdf>.
- 466 Egüen, B., Melgarejo, P., and De Cal, A. 2016. The effect of fungicide resistance of the structure  
467 of *Monilinia laxa* populations in Spanish peach and nectarine orchards Eur. J. Plant Pathol. 145:815-  
468 827.
- 469 Elmer, P. A. G., and Gaunt, R. E. 1993. Effect of frequency of dicarboximide applications on  
470 resistant populations of *Monilinia fructicola* and brown rot in New Zealand orchards. Crop Prot.  
471 12:83-88.
- 472 EPPO/CABI. 1997. *Monilinia fructicola*. Pages 530-535. In Quarantine Pest for Europe, 2nd  
473 edition, eds. CAB International, Wallingford, UK.
- 474 Franco Ortega, S., Tomlinson, J., Hodgetts, J., Spadaro, D., Gullino, M. L., and Boonham N.  
475 (2018a). Development of loop-mediated isothermal amplification assays for the detection of

476 seedborne fungal pathogens, *Fusarium fujikuroi* and *Magnaporthe oryzae*, in rice seeds. Plant Dis.  
477 102:1549-1558.

478 Franco Ortega, S., Tomlinson, J., Gilardi, G., Spadaro, D., Gullino, M.L., Garibaldi, A.  
479 Boonham, N. (2018b) Rapid detection of *Fusarium oxysporum* f.sp. *lactucae* on soil, lettuce seeds  
480 and plants using loop-mediated isothermal amplification. Plant Pathol. 67:1462-1473.

481 Gell, I., Cubero, J., and Melgarejo, P. 2007. Two different PCR approaches for universal  
482 diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees. J. Appl. Microbiol.  
483 103:2629-2637.

484 Guinet, C., Fourrier-Jeandel, C., Cerf-Wendling, I., and Ioos, R. 2016. One-Step Detection of  
485 *Monilinia fructicola*, *M. fructigena*, and *M. laxa* on *Prunus* and *Malus* by a Multiplex Real-Time  
486 PCR Assay. Plant Dis. 100:2465-2474.

487 Harvey, J. M. 1978. Reduction of Losses in Fresh Market Fruits and Vegetables. Annu. Rev.  
488 Phytopathol. 16:321-341.

489 Hilber-Bodmer, M., Knorst, V., Smits T. H. M. and Patocchi, A. 2012. First report of Asian  
490 Brown Rot caused by *Monilinia polystroma* on apricot in Switzerland. Plant Dis. 96:146.

491 Hinrichs-Berger, J., and Müller, G. 2010. First record of *Monilia fructicola* on blackberry fruits.  
492 J. Plant Dis. Prot. 117:110-111.

493 Hong, C., Holtz, B. A, Morgan, D. P., and Michailides, T. J. 1997. Significance of thinned fruit  
494 as a source of the secondary inoculum of *Monilinia fructicola* in California nectarine orchards. Plant  
495 Dis. 81:519-524.

496 Hong, C., Michailides, T. J., and Holtz, B. A. 1998. Effects of wounding, inoculum density,  
497 and biological control agents on postharvest brown rot of stone fruits. Plant Dis. 82:11

498 Hughes, K. J. D., Fulton, C. E., McReynolds, D., and Lane, C. R. 2000. Development of new  
499 PCR primers for identification of *Monilinia* species. EPPO Bull. 30:507-511.

500 Ioos, R., and Frey, P. 2000. Genomic variation within *Monilinia laxa*, *M. fructigena* and *M.*  
501 *fructicola* and application to species identification by PCR. Eur J Plant Pathol. 106:373-378.

- 502 Lane, C. R. 2002. A synoptic key for differentiation of *Monilinia fructicola*, *M. fructigena* and  
503 *M. laxa*, based on examination of cultural characters. EPPO Bull. 32:489-449.
- 504 Larena, I., Torres, R., De Cal., A., Liñan, M., Melgarejo, P., Domenichini, P., Bellini, A.,  
505 Mandrin J. F., Lichou, J., Ochoa de Eribe, X., and Usall, J. 2005. Biological control of postharvest  
506 brown rot (*Monilinia* spp.) of peaches by field applications of *Epicoccum nigrum*. 2005. Biol. Control.  
507 32:305-310.
- 508 Lichou, J., Mandrin, J.F., Breniaux, D., Mercier, V., Giauque, P., Desbrus, D., Blanc, P. and  
509 Belluau, E. 2002. Une nouvelle moniliose. Phytoma. 547, 22-25.
- 510 Lopez-Reyes, J. G., Spadaro, D., Prella, A., Garibaldi, A. and Gullino, M. L. 2013. Efficacy of  
511 plant essential oils on postharvest control of rots caused by fungi on different stone fruits *in vivo*.  
512 Food Protection. 76:631-639.
- 513 Luo, Y., Michailides, T. J., 2001. Factors affecting latent infection of prune fruit by *Monilinia*  
514 *fructicola*. Phytopathology. 91:864–872.
- 515 Luo, Y., Michailides, T. J., 2001b. Risk analysis for latent infection of prune by *Monilinia*  
516 *fructicola* in California. Phytopathology. 91:1197–1208.
- 517 Luo, Y., Morgan, D. P., and Michailides, T. J. 2001. Risk analysis of brown rot blossom blight  
518 of prune caused by *Monilinia fructicola*. Phytopathology. 91:759-768.
- 519 Luo, C. X., Hu, M. J., Jin, X., Yin, L. F., Bryson, P. K., and Schnabel, G. 2010. An intron in  
520 the cytochrome b gene of *Monilinia fructicola* mitigates the risk of resistance development to QoI  
521 fungicides. Pest Manag. Sci. 66:1308-1315.
- 522 Ma, Z. Z., Luo, Y. Y. L., and Michailides, T. J. 2003. Nested PCR assays for detection of  
523 *Monilinia fructicola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in  
524 California. J Phytopathology. 151:312-322.
- 525 Malavolta, C., Cross, J. V., Cravedi, P., and Jorg, E. 2003. Guidelines for integrated production  
526 of stone fruits IOBC Technical Guideline III 2nd Edition. IOBC/WPRS Bulletin. 26.

- 527 Margosan, D. A., Smilanick, J. L., Simmons, G. F., and Henson, D. J. 1997. Combination of  
 528 hot water and ethanol to control postharvest decay of peaches and nectarines. *Plant Dis.* 81:1405-  
 529 1409.
- 530 Mari, M., Leoni, O., Bernardi, R., Neri, F., and Palmieri, S. 2008. Control of brown rot on stone  
 531 fruit by synthetic and glucosinolate-derived isothiocyanates. *Postharvest Biol Tec.* 47:61-67.
- 532 Mari, M., Martini, C., Guidarelli, M. N., and Neri, F. 2012. Postharvest biocontrol of *Monilinia*  
 533 *laxa*, *Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans*  
 534 strains. *Biol. Control.* 60:132-140.
- 535 Martini, C., Lantos, A., Di Francesco, A., Guidarelli, M., D'Aquino, S., and Baraldi, E. 2014.  
 536 First Report of Asiatic Brown Rot Caused by *Monilinia polystroma* on Peach in Italy. *Plant Dis.*  
 537 98:1585.
- 538 Martini, C., and Mari, M. 2014. *Monilinia fructicola*, *Monilinia laxa* (Monilinia rot, Brown  
 539 rot). in: Postharvest decay-control strategies. S. Bautista- Baños. ed. Academic Press, UK.
- 540 Munda, A., and Viršček Marn, M. 2010. First Report of Brown Rot Caused by *Monilinia*  
 541 *fructicola* affecting peach orchards in Slovenia. *Plant Dis.* 94:1166-1166.
- 542 Nagamine, K., Hase, T., and Notomi, T. 2002. Accelerated reaction by loop-mediated  
 543 isothermal amplification using loop primers. *Mol. Cell. Probes.* 16:223-229.
- 544 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase,  
 545 T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28:E63.
- 546 OEPP/EPPO. 2002. *Monilinia fructicola* found in Austria. *Bull OEPP.* 11:170.
- 547 OEPP/EPPO. 2009. Diagnostics: *Monilinia fructicola*. *Bull OEPP.* 39:337–343.
- 548 Pellegrino, C., Gullino, M. L., Garibaldi, A., and Spadaro, D. 2009. First Report of Brown Rot  
 549 of Stone Fruit Caused by *Monilinia fructicola* in Italy. *Plant Dis.* 93:668.
- 550 Petróczy, M., and Palkovics, L. 2009. First report of *Monilia polystroma* on apple in Hungary.  
 551 *E. J. Plant. Pathol.* 125:343-347.

- 552 Poniatowska, A., Michalecka, M., and Bielenin, A. 2013. Characteristic of *Monilinia* spp. fungi  
553 causing brown rot of pome and stone fruits in Poland. Eur. J. Plant Pathol. 135:855-886.
- 554 Sanoamuang, N., and Gaunt, R. E. 1995. Persistence and fitness of carbendazim- and  
555 dicarboximide-resistant isolates of *Monilinia fructicola* (Wint.) Honey in flowers, shoots and fruit of  
556 stone fruit. Plant Pathol. 44:448-457.
- 557 Santoro, K., Maghenzani, M., Chiabrando, V., Bosio, P., Gullino, M. L., Spadaro, D. and  
558 Giacalone, G. 2018. Thyme and savory essential oil vapor treatments control brown rot and improve  
559 the storage quality of peaches and nectarines, but could favor gray mold. Foods.7:E7.
- 560 Spadaro D., and Droby S. 2016. Development of biocontrol products for postharvest diseases  
561 of fruit: the importance of elucidating the mechanisms of action of yeast antagonists. Trends in Food  
562 Sci Tech. 47:39-49.
- 563 Tate, K. G., and Wood, P. N. 2000. Potential ascospore production and resulting blossom blight  
564 by *Monilinia fructicola* in unsprayed peach trees. N. Z. J. Crop Hortic. Sci. 28:219-224.
- 565 Thomidis, T., Michailides, T., and Exadaktylou, E. 2009. Contribution of pathogens to peach  
566 fruit rot in northern Greece and their sensitivity to iprodione, carbendazim, thiophanate-methyl and  
567 tebuconazole fungicides. J. Phytopathol. 157:194-200.
- 568 Tomlinson, J. A., Boonham, N., and Dickinson, M. 2010a. Development and evaluation of a  
569 one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of  
570 phytoplasmas. Plant Pathol. 59:465-471.
- 571 Tomlinson, J. A., Dickinson, M. J., and Boonham, N. 2010b. Rapid Detection of *Phytophthora*  
572 *ramorum* and *P. kernoviae* by two-minute dna extraction followed by isothermal amplification and  
573 amplicon detection by generic lateral flow device. Phytopathology. 100:143-149.
- 574 Van Brouwershaven, I. R., Bruil, M. L., Van Leeuwen, G. C. M., and Kox, L. F. F. 2009. A  
575 real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of  
576 fruit crops. Plant Pathol. 59:548-555.



- 577 Villarino, M., Egüen, B., Lamarca, N., Segarra, J., Usall, J., Melgarejo, P., and De Cal, A. 2013.  
578 Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards  
579 in Spain. *Eur. J. Plant Pathol.* 137. 835-845.
- 580 Wang, J-R., Guo, L-Y., Xiao, C-L., Zhi, X. 2018. Detection and Identification of Six *Monilinia*  
581 spp. causing brown rot using Taqman Real-time PCR from pure cultures and infected apple fruit.  
582 *Plant Dis.* 102:1527-1533.
- 583 White, T., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal  
584 ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and*  
585 *Applications.* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San  
586 Diego, CA.
- 587 Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ.*  
588 *Microbiol.* 63:3741-751.
- 589 Zhang D., Spadaro D., Garibaldi A., and Gullino M. L. 2010. Screening and efficacy evaluation  
590 of new antagonists for their efficacy against postharvest brown rot of peaches. *Postharvest Biol Tec.*  
591 55:174-181.
- 592

593 **Tables**

594 **Table 1.** Specificity testing results of the inclusion-exclusion panel of target and non-target species.

595

ISOLATE CODE	SPECIE	ORIGE N	<i>MONILINIA FRUCTICOLA</i> LAMP TEST		<i>MONILINIA LAXA</i> LAMP TEST	
			AVERAGE		AVERAGE	
			Tp (min:s)	Annel °C	Tp (min:s)	Annel °C
1326	<i>Monilinia fructicola</i>	Unknown	07:35±00:17	84.52±0.46	Negative	Negative
C3-29	<i>M.fructicola</i>	Italy	11:05±01:57	84.18±0.16	Negative	Negative
MSR2	<i>M.fructicola</i>	Italy	10:50±01:14	84.07±0.01	Negative	Negative
MSR38	<i>M.fructicola</i>	Italy	09:45±00:15	84.56±0.30	Negative	Negative
MSR47	<i>M.fructicola</i>	Italy	08:00±00:30	84.19±0.08	Negative	Negative
P164C13-12(1)	<i>M.fructicola</i>	Italy	09:25±01:15	84.64±0.74	Negative	Negative
C1C4	<i>M.fructicola</i>	Italy	10:05±00:48	84.76±0.79	Negative	Negative
C14-12 (5)	<i>M.fructicola</i>	Italy	10:15±01:24	84.27±0.1	Negative	Negative
2603	<i>M.fructicola</i>	Italy	10:20±00:23	84.55±0.41	Negative	Negative
MUS26	<i>M.fructicola</i>	Italy	09:15±01:34	84.2±0.12	Negative	Negative
S14CF	<i>M.fructicola</i>	Italy	09:55±01:36	84.14±0.06	Negative	Negative
953	<i>M.fructicola</i>	Unknown	18:35±07:09	84.36±0.21	Negative	Negative
866	<i>M.fructicola</i>	Unknown	14:20± 05:34	84.52 0.36	Negative	Negative
1371	<i>M.fructicola</i>	Australia	10:35±00:23	84.24±0.12	Negative	Negative
853	<i>M.fructicola</i>	Unknown	09:50±02:08	84.42±0.56	Negative	Negative
881	<i>Monilinia laxa</i>	France	Negative	Negative	07:30	82.88±0.45
1402	<i>M. laxa</i>	Italy	Negative	Negative	09:20±00:31	82.44±0.18
1406	<i>M. laxa</i>	Italy	Negative	Negative	10:15±00:40	82.57±0.26
1516	<i>M. laxa</i>	Italy	Negative	Negative	18:45±04:01	82.44±0.24
1757	<i>M. laxa</i>	Italy	Negative	Negative	09:10±01:02	82.54±0.25
1790	<i>M. laxa</i>	Italy	Negative	Negative	07:40±00:31	82.26±0.03

1331	<i>M. laxa</i>	Italy	Negative	Negative	<b>13:30±02:23</b>	<b>83.33±1.66</b>
1368	<i>M. laxa</i>	Australia	Negative	Negative	<b>10:35±00:09</b>	<b>82.62±0.25</b>
1281	<i>M. laxa</i>	Italy	Negative	Negative	<b>10:35±01:57</b>	<b>82.63±0.18</b>
ML1	<i>M. laxa</i>	Italy	Negative	Negative	<b>09:15±01:00</b>	<b>83.29±0.76</b>
887	<i>M. laxa</i>	UK	Negative	Negative	<b>17:50±04:48</b>	<b>82.68±0.74</b>
888	<i>M. laxa</i>	UK	Negative	Negative	<b>17:00±07:26</b>	<b>82.86±0.91</b>
890	<i>M. laxa</i>	UK	Negative	Negative	<b>10:20±01:08</b>	<b>83.08±0.81</b>
1369	<i>M. laxa</i>	Australia	Negative	Negative	<b>08:45±01:09</b>	<b>82.95±0.81</b>
1370	<i>M. laxa</i>	Australia	Negative	Negative	<b>08:30±01:09</b>	<b>82.91±0.61</b>
1767	<i>M. laxa</i>	Unknown	Negative	Negative	<b>09:50±01:17</b>	<b>81.97±0.45</b>
1791	<i>Monilinia fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1756	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1248	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1249	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1515	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1756	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
48	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1760	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1762	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1763	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1765	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1770	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1773	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
1718	<i>Monilinia polystroma</i>	Unknown	Negative	Negative	Negative	Negative
1719	<i>M. polystroma</i>	Unknown	Negative	Negative	Negative	Negative
1538	<i>Verticillium albo-atrum</i>	Unknown	Negative	Negative	Negative	Negative
1540	<i>Colletotrichum truncatum</i>	Unknown	Negative	Negative	Negative	Negative
1542	<i>Helminthosporium solani</i>	Unknown	Negative	Negative	Negative	Negative
1543	<i>Cylindrocarpon</i>	Unknown	Negative	Negative	Negative	Negative
1544	<i>Botryosphaeria berengeriana</i>	Unknown	Negative	Negative	Negative	Negative
1547	<i>Botryosphaeria laricina</i>	Unknown	Negative	Negative	Negative	Negative

<b>1548</b>	<i>B. laricina</i>	Unknown	Negative	Negative	Negative	Negative
<b>2692</b>	<i>Colletotrichum acutatum</i>	Unknown	Negative	Negative	Negative	Negative
<b>92</b>	<i>Zythia fragariae</i>	Unknown	Negative	Negative	Negative	Negative
<b>221</b>	<i>G. cingulata/C. acutatum</i>	Unknown	Negative	Negative	Negative	Negative
<b>256</b>	<i>Colletotrichum acutatum</i>	Unknown	Negative	Negative	Negative	Negative
<b>308</b>	<i>Eutypa lata</i>	Unknown	Negative	Negative	Negative	Negative
<b>332</b>	<i>Macrophoma japonica</i>	Unknown	Negative	Negative	Negative	Negative
<b>452</b>	<i>cylindrocarpon</i>	Unknown	Negative	Negative	Negative	Negative
<b>667</b>	<i>Zythia sp.</i>	Unknown	Negative	Negative	Negative	Negative
<b>668</b>	<i>Mycosphaerella pinodes</i>	Unknown	Negative	Negative	Negative	Negative
<b>677</b>	<i>Lambertella corni-maris</i>	Unknown	Negative	Negative	Negative	Negative
<b>931</b>	<i>Phytophthora fragariae</i>	Unknown	Negative	Negative	Negative	Negative
<b>1138</b>	<i>C.fragariae</i>	Unknown	Negative	Negative	Negative	Negative
<b>1725</b>	<i>Rhizopus spp</i>	Unknown	Negative	Negative	Negative	Negative
<b>2556</b>	<i>Phytophthora fragariae var fragariae</i>	Unknown	Negative	Negative	Negative	Negative
<b>1539</b>	<i>Alternaria spp.</i>	Unknown	Negative	Negative	Negative	Negative
<b>77</b>	<i>Penicillium citrinum</i>	Unknown	Negative	Negative	Negative	Negative
<b>MCAL</b>	<i>Aspergillus flavus</i>	Unknown	Negative	Negative	Negative	Negative
<b>X1</b>	<i>Penicillium expansum</i>	Unknown	Negative	Negative	Negative	Negative
<b>DPO1</b>	<i>Diaporthe spp</i>	Unknown	Negative	Negative	Negative	Negative
<b>BC</b>	<i>Botrytis cinerea</i>	Unknown	Negative	Negative	Negative	Negative
<b>GUN1</b>	<i>Guignardia</i>	Unknown	Negative	Negative	Negative	Negative
<b>PS</b>	<i>Plasmopora</i>	Unknown	Negative	Negative	Negative	Negative
<b>ALTALTER</b>	<i>Alternaria alternata</i>	Unknown	Negative	Negative	Negative	Negative

596

597

598 **Table 2.** Sensitivity testing of the *M. fructicola* and *M. laxa* LAMP assay reporting the number of  
 599 replicates amplified out the total replicates tested. In parenthesis the time to positive average of the  
 600 positive results. The sensitivity testing was carried out using two different isolates.  
 601

	<i>M. fructicola</i> isolates		<i>M. laxa</i> isolates	
	MSR38	c14-12	1406	1790
<b>1-10 ng</b>	5/5(07:09)	4/4(09:35)	5/5(11:49)	4/4(08:26)
<b>100-999 pg</b>	5/5(08:00)	4/4(10:35)	5/5(10:41)	4/4(09:37)
<b>10-99 pg</b>	5/5(09:21)	4/4(11:45)	3/5(17:55)	3/4(16:45)
<b>1-9.9pg</b>	3/5(09:56)	1/4(14:30)	1/5(14:15)	0/4
<b>100-999 fg</b>	0/5	0/4	1/5(29:15)	0/4
<b>10-99 fg</b>	0/5	0/4	0/5	0/4
<b>1-9.9fg</b>	0/5	0/4	0/5	0/5

611 **Table 3.** Results of the *M. fructicola* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from the ‘Fire  
 612 Top’ nectarines inoculated with *M. fructicola* and stored at 18°C and 4°C. The LAMP assay was performed on the crude extracted DNA while the  
 613 qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).

614

	DPI	Disease Index	LAMP assay			qPCR assay		
			Tp (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	11:31±01:55 (09:02-14:41)	5/9	84.85±0.07	32.22±1.03 (30.34-33.08)	9/9	3,143.8 (8,477.9-2,000)
	5	0.00	10:03±03:02 (04:33-12:50)	6/9	84.54±0.1	36.39±0.58 (35.69-37.10)	4/9	995.8 (1,439-685.3)
	7	0.67	10:56±00:10 (10:50-11:04)	2/9	85.18±0.01	36.06±2.87 (33.24-39.25)	4/9	759.7 (3,352.9-141.9)
	9	1.00	07:06±04:25 (06:33-07:47)	3/9	84.81±0.14	21.43±8.96 (13.26-36.83)	5/9	5.4+06 (3.98E+08-1,636)
	12	4.00	09:02±01:32 (07:05-11:17)	9/9	85.09±0.1	21.25±6.84 (12.59-30.73)	9/9	6.3+06 (6E+08-42,931)
	14		Not tested					
	16		Not tested					
4°C	2	0.00	18:52±14:48 (09:37-35:57)	3/9	84.43±0.54	33.69±1.09 (32.31-35.30)	7/9	1,559.4 (3,223-668.4)
	5	0.00	12:13±08:21 (07:01-30:59)	8/9	84.56±0.14	Negative		
	7	0.00	12:11±01:25 (11:02-15:00)	6/9	85.17±0.11	38.89±0.78 (38.34-39.44)	2/9	259.7 (346.5-194.2)
	9	0.00	10:28±23 (10:12-10:54)	3/9	84.69±0.17	27.94±5.23 (20.52-33.72)	9/9	2.0+05 (1E+07-9,696)
	12	0.00	19:50±08:54 (13:-36:56)	4/9	84.62±0.83	33.91±1.68 (32.20-36.51)	9/9	6,428.6 (1.6+04 -1,638.9)
	14	1.33	10:23±02:00 (09:08-13:57)	5/9	84.98±0.09	18.61±1.71 (16-20.49)	6/9	7.7E+06 (3E+07-2.8E+06)
	16	0.00	13:49±02:00 (11:11-17:22)	9/9	84.83±0.08	33.82±7.03 (23.49-39.23)	4/9	2,573.7 (5.9E+06-149.6)

615

616 **Table 4.** Results of the *M. fructicola* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from ‘Amiga’  
 617 nectarines inoculated with *M. fructicola* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA  
 618 while the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
 619

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	12:11±01:25 (11:02-15:00)	6/9	85.17±0.11	36.11±1.46 (34.58-39.06)	8/9	28 (62.7-5.9)
	4	1.00	08:18±02:02 (06:35-10:55)	6/9	84.67±0.08	34.23±2.15 (31.99-37)	4/9	66.6 (216.3-15.5)
	7	3.00	08:22±01:38 (07:05-11:46)	9/9	84.95±0.15	22.99±6.47 (16.68-30.98)	6/9	2.2E+04 (6.2 E+05-333.9)
	9	4.00	08:09±00:50 (06:52-09:23)	9/9	84.67±0.13	18.61±1.71 (16-20.49)	6/9	3.9E+05 (1.6E+06-1.46E+06)
	12	5.00	09:51±02:00 (09:57-12:40)	9/9	84.35±0.28	29.88±3.24 (26.75-33.17)	9/9	1,048.5 (5,435.4-185.4)
	14		Not tested					
	16		Not tested					
4°C	2	0.00	14:20±04:19 (11:44-25:31)	9/9	85.0±0.15	36.85±1.85 (33.93-38.33)	8/9	22.8 (106.1-10.5)
	4	1.33	12:14±03:52 (09:50-12:28)	6/9	84.64±0.15	30.33±1.6 (28.26-33.51)	9/9	497.9 (1,482.5-93.6)
	7	0.33	12±01:18 (11:34-15:14)	7/9	85.00±0.12	30.19±2.17 (27.81-33.29)	9/9	626.7 (2,186.9-122.3)
	9	0.00	10:26±00:53 (09:22-11:30)	6/9	84.9±0.08	28.91±2.8 (24.44-32.62)	9/9	1844.3 (1.9E+04-261.5)
	12	1.00	07:13±00:02 06:43-07:426	6/9	84.73±0.14	34.04±4.03 (28.15-39.05)	8/9	123.8 (2,747.3-8.9)
	14	0.67	08±02:03 06:17-11:29	9/9	84.7±0.15	28.15±5.98 (18.6-33.39)	9/9	2754 (4.2E+05-174.4)
	16	1.67	07:25±01:52 (05:52-11:15)	9/9	84.72±0.19	23.7±4.18 (19.43-30.80)	9	2.8E+04 (2.7E+05-681.3)

620

622 **Table 5.** Results of the *M. laxa* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from the ‘Fire Top’  
 623 nectarines inoculated with *M. laxa* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA while  
 624 the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
 625

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	Negative			36.36±2.36 (34.69-38.02)	2/9	104.0 (317.4-71.1)
	4	1.33	Negative			Negative		
	7	2.33	12:02±01:39 (10:34-14:23)	4/9	83.55±0.12	36.52±0.84 (35.55-37.03)	3/9	186.9 (358.4-137.7)
	9	4.00	07:52±00:56 (06:45-09:44)	9/9	83.47±0.24	20.66±1.15 (19-22.09)	9/9	2.3E+07 (6E+07-9.9E+06)
	11	5.00	11:13±03:27 (08:29-12:39)	6/9	83.15±0.4	29.37±3.52 (27.17-37.11)	9/9	15E+05 (6,7E+05-418)
	14		Not tested					
	16		Not tested					
4°C	2	0.00	Negative			36.11±1.08 (34.91-37.01)	3/9	126.3 (282.6-139.6)
	4	1.33	Negative			34.45±1.38 (33.47-35.42)	2/9	671.0 (1,289.5-403.9)
	7	0.00	12:46	1/9	83.55	38.32±0.89 (37.69-38.95)	2/9	73.5 (112.1-38.2)
	9	0.00	13:40±08:25 (08:04-28:12)	5/9	83.26±0.15	31.38±1.3 (29.47-32.55)	9/9	1.7E+04 (6.1E+04-9,168.9)
	11	1.00	09:27±00:22 (09:04-09:55)	6/9	83.29±0.16	24.31±6.66 (19.38-34.18)	6/9	2.3E+06 (6E+07-3084.4)
	14	1.33	06:59±04:11 (04:02-11:47)	4/9	83.44±0.07	27.88±2.23 (27.25-30.26)	8/9	5.3 E+04 (2.7E+05-1,2E+04)
	16	1.33	08:07±00:21 (07:46-08:28)	3/9	83.44±0.08	26.6±9.99 (17.52-38.18)	7/9	1.3E+05 (1.8 E+08-54.3)

626



627 **Table 6.** Results of the *M. laxa* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from ‘Amiga’  
 628 nectarines inoculated with *M. laxa* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA while  
 629 the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
 630

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	12:29±05:09 (09:00-24:15)	8/9	83.43±0.14	Negative		
	4	1.33	08:44±01:44 (07:16-10:41)	9/9	83.35±0.2	32.4502±3.832 (29.19-39.92)	7/9	81.2 (717.9-0.6)
	7	3.67	08:48±02:44 (08:02-13:20)	9/9	83.25±0.21	28.5379±4.06 (22.81-32.04)	9/9	997.1 (4.6E+04-96)
	9	4.00	07:16±02:28 (04:02-10:00)	9/9	83.35±0.11	29.3679±0.41 (28.97-29.79)	3/9	666.9 (870-502.9)
	12	5.00	08:06±01:13 (06:40-09:50)	9/9	83.27±0.18	25.62±4.09 (20.21-31.58)	9/9	8,151.5 (3E+05-152)
	14	Not tested						
	16	Not tested						
4°C	2	0.00	11:12±01:46 (09:41-13:58)	2/9	83.48±0.1	38.7275±1.07 (37.11-39.93)	5/9	1.3 (3.8-0.6)
	4	0.33	11:30±03:17 (06:59-20:20)	5/9	83.28±0.16	30.9794±2.08 (27.77-33.81)	9/9	188 (1,606.1-28.3)
	7	0.67	10:54±02:17 (08:23-11:55)	5/9	83.28±0.17	25.9199±6.02 (17.79-31.35)	9/9	7,481.7 (1.7E+06-198.5)
	9	1.00	08:55±03:21 (07:38-16:29)	9/9	82.89±1.66	Negative		
	12	1.33	10:04± 06:31 (07:38-16:29)	7/9	83.26±0.13	24.96±1.57 (22.51-26.72)	9/9	1.4 E+04 (7.3E+04-4,382.9)
	14	2.67	07:01±00:17 (05:56-23:09)	9/9	83.47±0.11	24.113±5.9738 (15.28-31.16)	9/9	2.5E+04 (9E+06-225.4)
	16	3.33	0:0:14:01±58 (06:38-07:37)	9/9	83.47±0.11	35.185±2.9199 (30.38-38.35)	6/9	15.3 (379.6-1.8)

632        **FIGURE LEGENDS**

633

634        **Figure 1.** Incidence of *Monilinia* spp. (%) during the monitoring of four winter seasons (2008-2009  
635 to 2011-2012). *Monilinia laxa* (solid line), *Monilinia fructicola* (dashed line), *Monilinia fructigena*  
636 (dotted line).

637

638        **Figure 2.** Percentage of *Monilinia* spp. isolated from A) nectarine and B) peaches during the winter  
639 season 2011-2012.

640

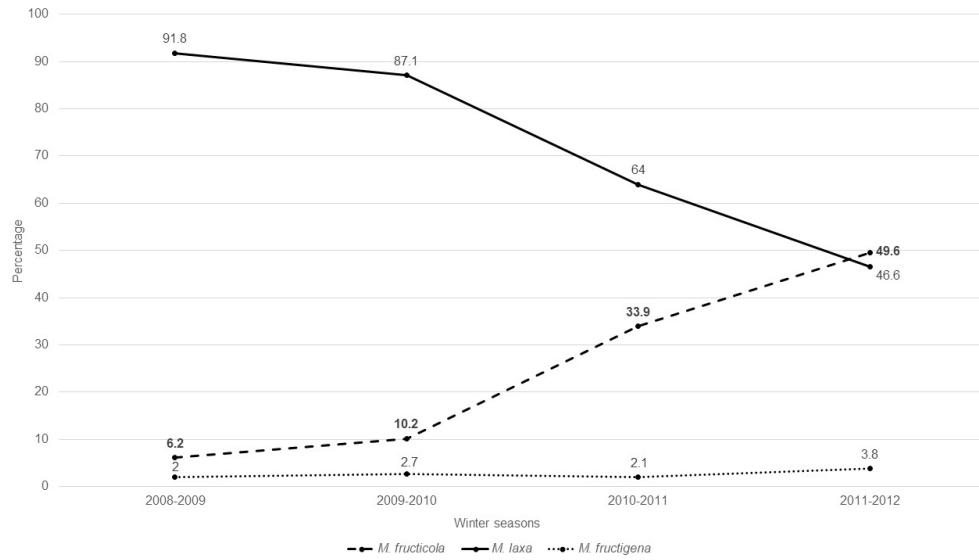


Figure 1. Incidence of Monilinia spp. (%) during the monitoring of four winter seasons (2008-2009 to 2011-2012). *Monilinia laxa* (solid line), *Monilinia fructicola* (dashed line), *Monilinia fructigena* (dotted line).

338x190mm (96 x 96 DPI)

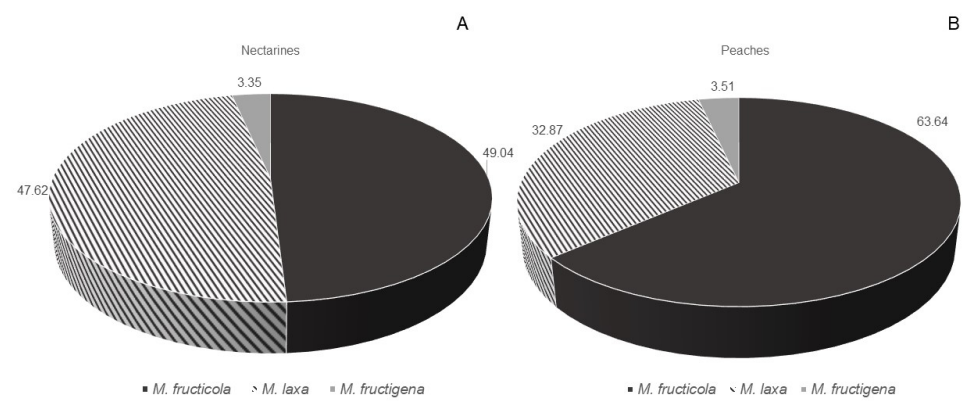


Figure 2. Percentage of Monilinia spp. isolated from A) nectarine and B) peaches during the winter season 2011-2012.

338x190mm (96 x 96 DPI)

## Supplementary material

**Table S1.** Orchards monitored during the 2011-2012 winter season with the geographical area, the host, cultivar and percentage of *Monilinia* spp. isolated.

Orchard number	Location	Host	Cultivar	Occurrence of <i>M. fructicola</i> during			Percentage of different species of <i>Monilinia</i> during 2011-2012		
				2008-2009	2009-2010	2010-2011	<i>M. fructicola</i> (%)	<i>M. laxa</i> (%)	<i>M. fructigena</i> (%)
1	CASTELLAR	Nectarine	Fire Top				90	0	10
2	COSTIGLIOLE	Nectarine	Big Top	N	N	N	62.5	37.5	0
3	DRONERO	Nectarine	Big Top	N	N	N	0	100	0
4	FOSSANO	Nectarine	Diamond Ray	N	N	N	75	25	0
5	FOSSANO	Nectarine	Diamond Ray	N	N	N	81.25	18.75	0
6	FOSSANO	Nectarine	Big Top	N	N	N	0	100	0
7	LAGNASCO	Nectarine	Big Top	N	N	Y	57.1	42.9	0
8	LAGNASCO	Peach	Vista Rich	N	N	Y	45.55	54.55	0
9	LAGNASCO	Nectarine	Amiga	N	N	N	40	40	20
10	LAGNASCO	Nectarine	Orion	Y	Y	Y	90	10	0
11	LAGNASCO	Nectarine	Big Top	N	N	Y	66.7	33.3	0
12	LAGNASCO	Nectarine	Diamond Ray	N	Y	Y	81.8	18.2	0
13	LAGNASCO	Peach	Red Valley	N	N	Y	54.6	45.5	0
14	LAGNASCO	Nectarine	Big Top	N	Y	Y	88.9	11.1	0
15	LAGNASCO	Nectarine	Diamond Ray	N	N	N	0	76.9	23.1
16	LAGNASCO	Peach	Royal Glory	N	N	N	26.7	66.7	6.6
17	LAGNASCO	Nectarine	Caldesi	N	N	N	0	69.2	30.8
18	LAGNASCO	Nectarine	Fire Sweet Red	Y	Y	Y	90	0	10
19	MANTA	Nectarine	Big Top	N	N	N	0	100	0
20	PIASCO	Nectarine	Big Top	N	N	N	0	100	0
21	REVELLO	Nectarine	Diamond Ray	N	N	Y	100	0	0
22	REVELLO	Nectarine	Venus	N	N	N	25	58.3	16.7
23	REVELLO	Nectarine	Alitop	N	N	Y	100	0	0
24	REVELLO	Nectarine	Venus	N	N	N	46.15	53.85	34

25	SALUZZO	Peach	Elegant Lady	N	N	N	75	12.5	12.5
26	SALUZZO	Nectarine	Nectaross	N	N	N	62.5	37.5	0
27	SALUZZO	Nectarine	Big Top	N	N	Y	100	0	0
28	SALUZZO	Nectarine	Big Top	N	N	N	40	60	0
29	SALUZZO	Nectarine	Venus	N	N	N	62.5	37.5	0
30	SCARNAFIGI	Peach	Rome Star	N	N	N	87.5	0	12.5
31	SCARNAFIGI	Nectarine	Big Top	N	N	N	100	0	0
32	SCARNAFIGI	Peach	Rome Star	N	N	N	100	0	0
33	SAVIGLIANO	Nectarine	V3	N	N	N	0	100	0
34	SAVIGLIANO	Nectarine	Big Top	N	N	N	25	75	0
35	SAVIGLIANO	Peach	Rome Star	N	N	N	16.7	83.3	0
36	SAVIGLIANO	Nectarine	Diamond Ray	N	N	Y	76.5	23.5	0
37	SAVIGLIANO	Peach	Vista Rich	N	N	N	66.7	33.3	0
38	SAVIGLIANO	Nectarine	Amiga	N	N	N	60	40	0
39	VERZUOLO	Nectarine	Stark Red Gold	N	N	N	0	100	0
40	VERZUOLO	Nectarine	Big Top	N	N	N	20	80	0
41	VERZUOLO	Nectarine	Big Top	N	N	N	66.7	33.3	0
42	VERZUOLO	Nectarine	Amiga	N	N	N	0	100	0
43	VERZUOLO	Peach	Rome Star	N	N	Y	100	0	0
44	VERZUOLO	Nectarine	Big Top	N	N	N	20	80	0
45	VERZUOLO	Nectarine	Nectaross	N	N	Y	60	40	0
46	VERZUOLO	Nectarine	Alitop	N	N	N	26.7	60	13.3

**Table S2.** Primers used in this study.

<b>PRIMER</b>	<b>SEQUENCE</b>	<b>TAXA DETECTED</b>	<b>REFERENCE</b>
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. fructigena</i> and <i>M. polystroma</i>	Côté et al. (2004)
<b>MO368-8R</b>	5'-AGA TCA AAC ATC GTC CAT CT-3'		
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. fructicola</i>	Côté et al. (2004)
<b>MO368-10R</b>	5'-AAG ATT GTC ACC ATG GTT GA-3'		
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. laxa</i>	Côté et al. (2004)
<b>LAXA-R2</b>	5'-TGC ACA TCA TAT CCC TCG AC-3'		
<b>ITS1</b>	5'-TCC GTA GGT GAA CCT GCG G-3'	<i>M. fructigena</i>	White et al. (1990)
<b>MFG-R2</b>	5'-GGT CAA CCA TAG AAA ATT GGT-3'		Hughes et al., (2000)
<b>MCF-F1</b>	5'-TAT GCT CGC CAG AGG ATA ATT A-3'	<i>M. fructicola</i>	Hughes et al. (2000)
<b>MFC-R1</b>	5'-GAT TTT AGA GCC TGC CAT TA-3'		
<b>MI-MFG-F2</b>	5'-GCT CGC CAG AGA ATA ATC-3'	<i>M. laxa</i>	Hughes et al. (2000)
<b>MI-MFC-R1</b>	5'-GAT TIT AGA GCC TGC CAT TG-3'		
<b>IGENAS</b>	5'-TGCTCTGCCCCGTACCCAG-3'	<i>M. fructigena</i>	Gell et al. (2007)
<b>IGENAAS</b>	5'-GGATTTATTGTGATGTAGTTTCG-3'		
<b>ICOLAS</b>	5'-GAGACGCACACAGAGTCAG-3'	<i>M. fructicola</i>	Gell et al. (2007)
<b>ICOLAAS</b>	5'-GAGACGCACATAGCATTGG-3'		
<b>ILAXAS</b>	5'-TGAGCACGAGTGAATGTATAG-3'	<i>M. laxa</i>	Gell et al. (2007)
<b>ILAXAAS</b>	5'-TGAGCACGAGGGGCATATC-3'		

**Figure S1.** A. Percentage of *M. fructicola*, *M. laxa* and *M. fructigena* in function of the *A. Prunus persica* cultivars during the 2011-2012 winter season. B. A. Geographical origin during the 2011-2012 winter season.

